Western blot protocol

Lysis buffers

Nonidet-P40 (NP40) buffer
150 mM NaCl
1.0% NP-40 (possible to substitute with 0.1% Triton X-100)
50 mM Tris-HCl pH 8.0
Protease Inhibitors

RIPA buffer
150 mM NaCl
1.0% NP-40 or 0.1% Triton X-100
0.5% sodium deoxycholate
0.1% SDS (sodium dodecyl sulphate)
50 mM Tris-HCl pH 8.0
Protease Inhibitors

Tris-HCl buffer
20 mM Tris-HCl pH 7.5
Protease Inhibitors

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 pH to 6.8.

Running buffer (Tris-Glycine/SDS)

25 mM Tris base
190 mM glycine
0.1% SDS
Check the pH, which should be about pH 8.3. Adjust if necessary.

Transfer buffer (Wet)
25 mM Tris base
190 mM glycine
20% methanol
Check the pH, which should be about pH 8.3. Adjust if necessary.

For proteins larger than 80 kDa, we recommend that SDS is included at a final concentration of 0.1%.

Transfer buffer (Semi-dry)
48 mM Tris
39 mM glycine
20% methanol
0.04% SDS

Blocking buffer:

5% milk or BSA or dry milk powder. Add to TBST buffer. Mix well and filter. Failure to filter can lead to "spotting" where tiny dark grains will contaminate the blot during colour development.

Procedure:

1. Sample preparation

1. Use a small volume (50 µl) of lysate for a protein assay.
2. To the remaining volume of cell lysate, add an equal volume of 2X Laemmli Sample Buffer.
3. Boil cell lysate in sample buffer at 100°C for 5 minutes and aliquot. Store lysates at -20°C. Aliquot cell lysates (50- 100 µl) to avoid repeat freeze/thaw cycles.
4. Defrost tubes containing cell lysate at 37°C. Centrifuge at 16,000 x g in a microcentrifuge for 5 minutes.

2. Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight markers. Load 20- 30 µg of total protein from cell lysate or tissue homogenate, or 10 -100 ng of purified protein.
2. Run the gel for 1 to 2 hours at 100 V. Use Tricine-SDS-PAGE for small peptides. Use this link for protocol on how to detect small peptide by SDS-PAGE: http://www.lifetein.com/Detect_Small_peptide.html

Gel percentage will depend on the size of the protein:

4 - 40 kDa	20%
12 - 45 kDa	15%
10 - 70 kDa	12.5%
15 - 100 kDa	10%
25 - 200 kDa	8%

3. Transferring the protein from the gel to the membrane

1. Prepare the transfer stack. Activate PVDF with methanol for one minute and rinse with transfer buffer before preparing the stack.

4. Antibody staining

1. Block the membrane for 1 hour at room temperature or overnight at 4°C using 5% blocking solution.
2. Incubate membrane with appropriate dilutions of primary antibody in 5% or 2% blocking solution overnight at 4°C or for 2 hours at room temperature.
3. Wash the membrane in three washes of TBST, 5 minutes each.
4. Incubate the membrane with the recommended dilution of labeled secondary antibody in 5% blocking buffer in TBST at room temperature for 1 hour.
5. Wash the membrane in three washes of TBST, 5 minutes each, then rinse in TBS.
6. For signal development, follow the kit manufacturer's recommendations.
7. Remove excess reagent and cover the membrane in transparent plastic wrap.
8. Acquire image using darkroom development techniques for chemiluminesence, or normal image scanning methods for colorimetric detection.

http://lifetein.com/chat/778818-Antibody

Western Blot: Technique, Theory, and Trouble Shooting

Western blotting is an important technique used in cell and molecular biology. Researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells using Western Blot.

A mixture of proteins is separated based on molecular weight through gel electrophoresis. The gels are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The bound antibodies are then detected by developing the film.

The multi-tag positive loading control protein is used to demonstrate that your Western Blot protocol is efficient and correct and that the antibody recognizes the target protein which may not be present in the experimental samples. Loading such protein into your positive control lane results in a reliably detectable protein sample. It means all the steps of your Western blot functioned properly, including gel electrophoresis, protein transfer to blotting membrane, membrane blocking and antibody labelling. It also gives you greater confidence that the results in the other lanes are real rather than artifactual.

We strongly recommend the use of a positive control protein when setting up a new experiment; this will give you immediate confidence in your Western Blot protocol.

When trying to detect low-abundance proteins, it is especially important to know that your Western blot is functioning as expected. If you detect your protein of interest in the control lane, then an absence of the protein in other lanes is probably directly related to its low abundance rather than a faulty step in the blotting protocol.

Highlights:

- Demonstrate that your protocol is efficient and correct.
- Perfect positive control protein for your Western blotting.
- Save time and resources for all your protein research projects.
- Give you confidence in your Western blot protocol.
- Can be used for immuno-precipitation, affinity purification, Western blot and dot blot.

Order this positive loading control protein now: http://lifetein.com/peptide-product/multitag-protein-p-1.html

This paper provides the theoretical explanation of the Western Blotting procedure, troubleshooting tips for common problems: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3456489/

Troubleshooting: (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3456489/)

Even though the procedure for western blot is simple, there could be many unexpected results:
(1) Unusual or unexpected bands
(2) No bands
(3) Faint bands or weak signal
(4) High background on the blot
(5) Patchy or uneven spots on the blot

(1) Unusual or unexpected bands: These can be due to protease degradation, which produces bands at unexpected positions. It is advisable to use a fresh sample which had been kept on ice or alter the antibody. If the protein seems to be in too high of a position, then reheating the sample can help to break the quaternary protein structure. Similarly, blurry bands are often caused by high voltage or air bubbles present during transfer. In this case, it should be ensured that the gel is run at a lower voltage, and that the transfer sandwich is prepared properly. In addition, changing the running buffer can also help the problem. Nonflat bands can be the result of too fast of a travel through the gel, due to low resistance. To fix this the gel should be optimized to fit the sample. Finally, white (negative) bands on the film are due to too much protein or antibody.

(2) No bands: This is due to many reasons related to antibody, antigen, or buffer used. If an improper antibody is used, either primary or secondary, the band will not show. In addition, the concentration of the antibody should be appropriate as well; if the concentration is too low, the signal may not be visible. It is important to remember that some antibodies are not to be used for western blot. Another reason for no visible bands is the lowest concentration or absence of the antigen. In this case, antigen from another source can be used to confirm whether the problem lies with the sample or with other elements, such as the antibody. Moreover, prolonged washing can also decrease the signal. Buffers can also contribute to the problem. It should be ensured that buffers like the transfer buffer, TBST, running buffer and ECL are all new and noncontaminated. If the buffers are contaminated with sodium azide, it can inactivate HRP.

(3) Faint bands or weak signal: It can be caused by low concentration of antibody or antigen. Increasing exposure time can also help to make the band clearer. Another reason could be nonfat dry milk masking the antigen. In this case use BSA or decrease the amount of milk used.

(4) High background on the blot: It is often caused by too high concentration of the antibody, which can bind to PVDF membranes. Another problem could be the buffers, which may be too old. Increasing the washing time can also help to decrease the background. Additionally, too high of an exposure can also lead to this problem. Therefore, it is advisable to check different exposure times to achieve an optimum time.

(5) Patchy or uneven spots on the blot: They are usually caused by improper transfer. If there are air bubbles trapped between the gel and the membrane, it will appear darker on the film. It is also important to use a shaker for all incubation, so that there is no uneven agitation during the incubation. Once again, washing is of utmost importance as well to wash the background. This problem can also be caused by antibodies binding to the blocking agents; in this case another blocking agent should be tried. Filtering the blocking agent can also help to remove some contaminants. Finally, this problem can also be caused by aggregation of the secondary antibody; in this case, the secondary antibody should be centrifuged and filtered to remove the aggregated.