Western blot protocol

A General Procedure for WB

Solutions and reagents:

Lysis buffers

These buffers may be stored at 4°C for several weeks or for up to a year aliquoted and stored at -20°C.

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 (Radio Immuno Precipitation Assay 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

Running, Transfer, and Blocking buffers

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

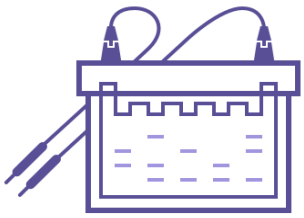
Blocking buffer:
5% milk or BSA (bovine serum albumin)
Add to TBST buffer. Mix well and filter. Failure to filter can lead to “spotting” where tiny dark grains will contaminate the blot during color development.

Procedure:

Step 1. Sample lysis

1. Treat cells by adding fresh media containing regulator for desired time. Aspirate media from cultures; wash cells with 1X PBS;
2. Aspirate lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate).
3. Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).

Step 2. Isolate protein



1. Heat a 20 µl lysis sample to 95–100°C for 5 min; cool on ice.
2. Microcentrifuge for 5 min.
3. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).
4. Run the gel for 1 to 2 hours at 100 V.

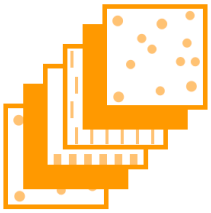
This time and voltage may require some optimization.

We recommend following the manufacturer’s instructions. A reducing gel should be used unless non reducing conditions are recommended on the antibody datasheet.

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%

Step 3. Transfer proteins from gel to a nitrocellulose or PVDF membrane



Prepare the transfer stack as follows:

The membrane can be either nitrocellulose or PVDF; each has its advantages. “Activate” PVDF with methanol for one minute and rinse with transfer buffer before preparing the stack. The time and voltage may require some optimization. We recommend following the manufacturer’s instructions. Transfer to the membrane can be checked using Ponceau Red staining before the blocking step.

The membrane is ready for antibody staining.

Step 4. Membrane Blocking

Minimize background signal from non-specific binding of antibody to the membrane

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

Step 5. Primary Antibody Incubation

Proceed to one of the following specific set of steps depending on the primary antibody used.

For Unconjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody and anti-biotin, HRP-linked Antibodyto detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.

For HRP Conjugated Primary Antibodies

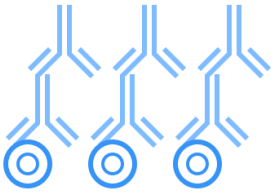
1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate with Anti-biotin, HRP-linked Antibody, to detect biotinylated protein markers, in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.

For Biotinylated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.

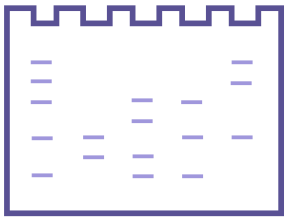
Do not add Anti-biotin, HRP-linked Antibody for detection of biotinylated protein markers. There is no need. The Streptavidin-HRP will also visualize the biotinylated

Step 6. Secondary Antibody Incubation



1. Incubate the membrane with the recommended dilution of labeled secondary antibody in 5% blocking buffer in TBST at room temperature for 1 hour.
2. Wash the membrane in three washes of TBST, 5 minutes each, then rinse in TBS.

Step 7. Detection of Proteins



For signal development, follow the kit manufacturer's recommendations.

1. Remove excess reagent and cover the membrane in transparent plastic wrap.
2. Acquire image using darkroom development techniques for chemiluminesence, or normal image scanning methods for colorimetric detection.

Good Luck and Enjoy Your Western Blot!