

General western blot protocol

Guidance for running an efficient and accurate experiment

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Introduction

Western blotting is used to visualize proteins that have been separated by gel electrophoresis. The gel is placed next to a nitrocellulose or PVDF (polyvinylidene fluoride) membrane and an electrical current causes the proteins to migrate from the gel to the membrane. The membrane can then be probed by antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

Solutions and reagents

Lysis buffers

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

NP-40 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 (radioimmunoprecipitation 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

- 20 mM Tris-HCl
- 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 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

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

3–5% 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.

Sample lysis

Preparation of lysate from cell culture

1. Place the cell culture dish on ice and wash the cells with ice-cold PBS.
2. Aspirate the PBS, then add ice-cold lysis buffer (1 mL per 10^7 cells/100 mm dish/150 cm² flask; 0.5 mL per 5×10^6 cells/60 mm dish/75 cm² flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Alternatively cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer in a microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. Centrifuge in a microcentrifuge at 4°C. You may have to vary the centrifugation force and time depending on the cell type; a guideline is 20 min at 12,000 rpm but this must be determined for your experiment (leukocytes need very light centrifugation).
6. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

Preparation of lysate from tissues

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round-bottom microcentrifuge tubes or Eppendorf tubes and immerse in liquid nitrogen to snap freeze. Store samples at -80°C for later use or keep on ice for immediate homogenization. For a ~5 mg piece of tissue, add ~300 µL of ice cold lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2 x 200 µL lysis buffer, then maintain constant agitation for 2 h at 4°C (eg place on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present; protein extract should not be too dilute to avoid loss of protein and large volumes of samples to be loaded onto gels. The minimum concentration is 0.1 mg/mL, optimal concentration is 1–5 mg/mL.
3. Centrifuge for 20 min at 12,000 rpm at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice; discard the pellet.

Sample preparation

1. Remove a small volume of lysate to perform a protein quantification assay. Determine the protein concentration for each cell lysate.
2. Determine how much protein to load and add an equal volume 2X Laemmli sample buffer.

We recommend reducing and denaturing the samples using the following method unless the online antibody datasheet indicates that non-reducing and non-denaturing conditions should be used.

3. To reduce and denature your samples, boil each cell lysate in sample buffer at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use.

Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. 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 1–2 h at 100 V.

The time and voltage may 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.

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

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%

Gradient gels can also be used.

Transferring the protein from the gel to the membrane

The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. We recommend following the manufacturer's instructions. Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step.

Prepare the stack as follows:

Figure 1. Example of prepared stack.



Antibody staining

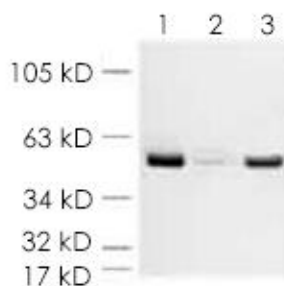
1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 min each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.
5. Wash the membrane in three washes of TBST, 5 min each.
6. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

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Useful links

View all Abcam [loading controls](#).

Figure 2. Example loading control: ab8227 beta actin.



All lanes: beta Actin antibody – loading control (ab8227) at 1/5000 dilution

Lane 1: HeLa whole cell extract

Lane 2: Yeast cell extract

Lane 3: Mouse brain tissue lysate

View our list of available [positive control lysates](#) and [blocking peptides](#).

View [AbExcel secondary antibodies](#) for exceptional western blots.