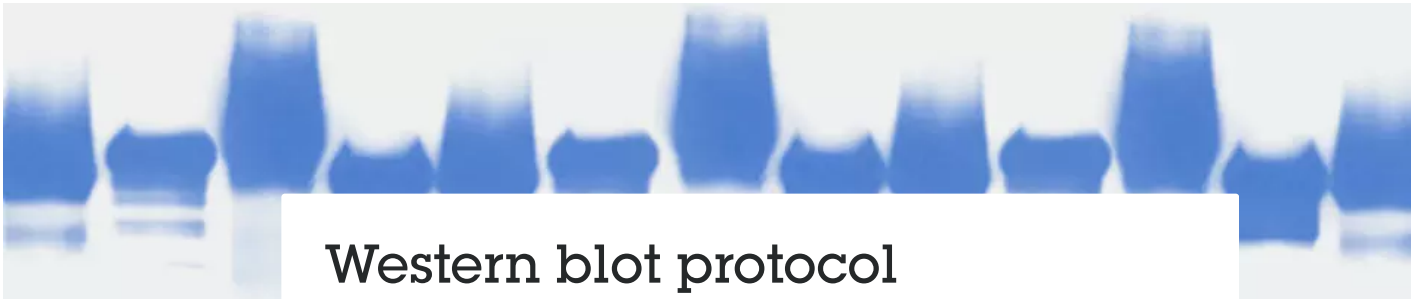


Western blot protocol

Our western blot protocol includes solutions and reagents, procedure, and useful links to guide you through your experiment.

RELATED

Western blotting uses **specific antibodies** to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

View our western blot protocol video below.

For other video protocols please visit our video protocols library here.

>> Print the full western blot protocol

Anti-Mouse IgG (HRP) (ab205719)

IRDye® secondary antibodies

Primary antibodies for western blot

Find the right HRP secondary antibodies

Guide for fluorescent WB

>> View out western blot protocol diagram

Contents

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

Solutions and reagents: 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 cm^2 flask; 0.5 mL per $5x10^6$ cells/60 mm dish/75 cm^2 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

3.1 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.

3.2 Run the gel for 1–2 h at 100 V.

T*he 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

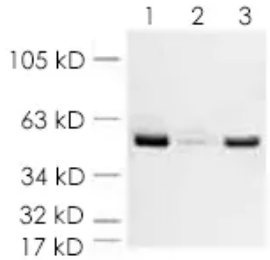
1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of 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.

Useful links

View more western blot protocols

View all Abcam loading controls.

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, blocking peptides and positive control proteins.

View AbExcel secondary antibodies for exceptional western blots.

Watch our easy-to-follow video protocols.

Protocols are provided by Abcam “AS-IS” based on experimentation in Abcam’s labs using Abcam’s reagents and products; your results from using protocols outside of these conditions may vary.

Webinar transcript

The purpose of western blotting is to separate proteins on a gel according to the molecular weight. The proteins are then transferred onto a membrane where they can be detected using antibodies. Heat the samples and 95 degrees C for five to 10 minutes in a sample buffer containing a reducing agent such as beta mercaptoethanol. This results in linearized proteins with a negative charge proportional to their size.

Place a gel into the electrophoresis tank and ad in buffer, ensuring the tops of the wells are covered. Acrylamide percentage of the gel being used depends on the molecular weight of the target protein. Node a molecular weight market into the first lane then load the samples into adjacent wells. All the samples which contained equal amounts of protein. Once all the samples are loaded, ad running buffer, place the lid onto the electrophoresis tank. Turn on the power supply and set the voltage recommended by the manufacturer of the gels in the gel tank. You should be able to see bubbles rising through the tank. Run the gel until the die front has moved sufficiently down the gel.

The next stage is to transfer the proteins from the gel onto a membrane. Membranes are usually made from nitrocellulose or PVDF. Remove the gel from the tank and carefully release it from its plastic case. Cut up the wells and the gel foot and place the gel into transfer buffer. Prepare the transfer stack by sandwiching the membrane and gel between filter paper and sponges. The membrane should be traced to the positive electrode and the gel closest to the negative electrode. Use a small roller to remove any bubbles between the gel and the membrane. Cap the transfer case closed and submerge into a transfer tank containing transfer buffer. Add water to the outer chamber to keep the system cool and put on the lid. Turn on the power supply to begin protein transfer. Time and voltage require optimization, so check the manufacturer's instructions for guidance.

Now that the proteins have migrated from the gel onto the nitro cellulose membrane, the protein of interest can be detected as an antibody. The membrane can be removed from the cassette and the molecular weight market should now be visible. If required, the transfer of proteins can be confirmed by staining the membrane with [inaudible 00:04:40] solution. To prevent nonspecific binding of the antibody, the membrane needs to be blocked. Pour blocking buffer onto the membrane and agitate gently on a rocker. Typically, this is done using a solution of five percent milk or bovine serum albumin, BSA, for two hours at room temperature or overnight at four degrees. The time and type of blocking buffer should be optimized, so check the data sheet of the primary antibody you intend to use for details.

After the membrane is blocked, remove the blocking buffer and add the diluted primary antibody in the same solution. Incubate on the rocker as before. Typically primary antibody incubations are for one hour at room temperature or overnight at four degrees C. Antibody concentration and incubation time will need to be optimized. Refer to the antibody datasheet for guidance. Pour off the primary antibody and rinse the membrane twice in wash buffer. Follow with one 15 minute wash and three 10 minute washes on a rocker. The wash buffer is usually Trys buffered saline, TBS, or phosphate buffered, saline, PBS, with 0.1 percent tween 20.

Pour off the wash buffer and incubate the membrane in conjugating secondary antibody which has been diluted in blocking buffer. Usually this is done for one hour at room temperature, but antibody concentration and incubation time will need to be optimized. Pull off the secondary antibody and wash the membrane has shown previously.

There are several different systems for detection. If the secondary antibodies conjugate into an enzyme, incubate the membrane in the appropriate substrate before imaging. If the secondary antibodies are fluorescent counjugates then you can move directly onto the imaging step. Imaging can be carried out with x Ray film or with a digital imaging system. Place the membrane into an imaging tray. Place the imaging tray into imaging system. Exposure times will most likely need to be optimized in order to clearly detect the bands relating to the proteins of interest.