abcam

Immunocytochemistry and immunofluorescence protocol

Procedure for staining of cell cultures using immunofluorescence

Preparing the slide

1. Coat coverslips with polyethylineimine or poly-L-lysine for 1 h at room temperature.
2. Rinse coverslips well with sterile H_2O (three times 1 h each).
3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 h.
4. Grow cells on glass coverslips or prepare cytospin or smear preparation.
5. Rinse briefly in phosphate-buffered saline (PBS).

   For wash buffer we recommend 1x PBS 0.1% Tween 20.

Fixation

The cells may be fixed using one of two methods:

1. Incubating the cells in 100% methanol (chilled at -20°C) at room temperature for 5 min
2. Using 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature

The cells should be washed three times with ice-cold PBS.

Antigen retrieval (optional step)

Certain antibodies work best when cells are heated in antigen retrieval buffer. Check the product information for recommendations for each primary antibody being used.

1. Preheat the antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) to 95°C. This can be done by heating the buffer in a coverglass staining jar which is placed in a water bath at 95°C.
2. Using a small pair of broad-tipped forceps, place the coverslips carefully in the antigen retrieval buffer in the cover glass staining jar, making note of which side of the coverslips the cells are on.
3. Heat the coverslips at 95°C for 10 min.

4. Remove the coverslips from the antigen retrieval buffer and immerse them, with the side containing the cells facing up, in PBS, in the 6-well tissue culture plates.

5. Wash cells in PBS three times for 5 min.

Permeabilization

If the target protein is intracellular, it is very important to permeabilize the cells. Acetone fixed samples do not require permeabilization.

1. Incubate the samples for 10 min with PBS containing either 0.1–0.25% Triton X-100 (or 100 µM digitonin or 0.5% saponin). Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for membrane-associated antigens since it destroys membranes.

2. The optimal percentage of Triton X-100 should be determined for each protein of interest.

3. Wash cells in PBS three times for 5 min.

Blocking and immunostaining

1. Incubate cells with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species the secondary antibody was raised in: see antibody datasheet for recommendations).

2. Incubate cells in the diluted antibody in 1% BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C.

3. Decant the solution and wash the cells three times in PBS, 5 min each wash.

4. Incubate cells with the secondary antibody in 1% BSA for 1 h at room temperature in the dark.

5. Decant the secondary antibody solution and wash three times with PBS for 5 min each in the dark.

Multicolor immunostaining (optional step)

To examine the co-distribution of two (or more) different antigens in the same sample, use a double immunofluorescence procedure. This can be performed either simultaneously (in a mixture) or sequentially (one antigen after another).

Ensure you have antibodies for different species and their corresponding secondary antibodies. For example, rabbit antibody against antigen A, mouse antibody against antigen B. Alternatively, you can use directly conjugated primary antibodies conjugated to different fluorophores.

Simultaneous incubation

1. Incubate cells with blocking solution for 30 min.
2. Incubate cells with both primary antibodies in 1% BSA in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C.
3. Decant the solution and wash the cells three times in PBS, 5 min each wash.
4. Incubate cells with both secondary antibodies in 1% BSA for 1 h at room temperature in the dark.
5. Decant the secondary antibody solution and wash three times with PBS for 5 mins each in the dark.

Sequential incubation

1. First blocking step: incubate cells with the first blocking solution (10% serum from the species that the secondary antibody was raised in) for 30 min at room temperature.
2. Incubate cells with the first primary antibody in 1% BSA or 1% serum in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C, 1% gelatin or 1% BSA.
3. Decant the first primary antibody solution and wash the cells three times in PBS, 5 min each wash.
4. Incubate cells with first secondary antibody in 1% BSA in PBST for 1 h at room temperature in the dark.
5. Decant the first secondary antibody solution and wash three times with PBS for 5 min each in the dark.
6. Second blocking step: incubate cells with the second serum, (10% serum from the species that the secondary antibody was raised in) for 30 min at room temperature in the dark.

7. Incubate cells with the second primary antibody in 1% BSA or 1% serum in PBST in a humidified chamber in the dark for 1 h at room temperature, or overnight at 4°C.

8. Decant the second primary antibody solution and wash the cells three times in PBS, 5 min each wash in the dark.

9. Incubate cells with second secondary antibody in 1% BSA for 1 h at room temperature in the dark.

10. Decant the second secondary antibody solution and wash three times with PBS for 5 min each in the dark.

    If you have to detect more than two antigens, continue following steps 1–5 for the rest of the antibodies.

Counter staining

1. Incubate cells with 0.1–1 µg/mL Hoechst stain or DAPI (DNA stain) for 1 min.

2. Rinse with PBS.

Mounting

1. Mount coverslip with a drop of mounting medium.

2. Seal coverslip with nail polish to prevent drying and movement under microscope.

3. Store in dark at -20°C or +4°C.

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General western blot protocol

Guidance for running an efficient and accurate experiment

Contents

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 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

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.

General western blot protocol

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.

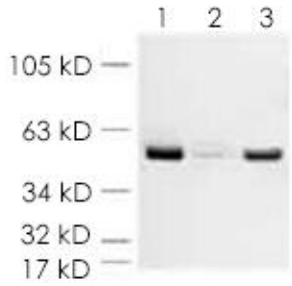
Antibody staining

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 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.