

WESTERN IMMUNOBLOTTING PROTOCOL

A. Preparation of lysate

From cells

1. Place the cell culture dish in ice and wash the cells with ice-cold PBS.
2. Drain the PBS, then add ice-cold lysis buffer (1 ml per 10⁷ cells/100mm dish/150cm² flask; 0.5ml per 5x10⁶ cells/60mm dish/75cm² flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microfuge tube.
4. Maintain constant agitation for 30 minutes 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 minutes at 12,000 rpm but this must be determined by the end-user (e.g. leukocytes need a 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.

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 microfuge 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 lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2x300 µl lysis buffer, then maintain constant agitation for 2 hours at 4°C (e.g. 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 diluted 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 12000 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.

B. Electrophoresis

1. Preparation of PAGE gels. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide.
2. Preparation of samples for loading into gels. To denature, use a loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS), and boil the mixture at 95-100°C for 5 minutes.

3. Load 20-40 μg total protein per mini-gel well. Note: Take care not to poke the well bottom with the tip as this will create a distorted band. Never overfill wells. This could lead to poor data if samples spill into adjacent wells, and poorly resolved bands.
4. When the dye molecule reaches the bottom of the gel, the power is turned off. Proteins will slowly elute from the gel at this point, so do not store the gel; proceed immediately to transfer.

C. Transfer of proteins

1. Two types of membranes are available: nitrocellulose and PVDF.
2. Transfer can be done in wet or semi-dry conditions.
 - a) In wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring no air bubbles have formed between the gel and membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on.
 - b) In semi-dry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode respectively). As for wet transfer, it is important that the membrane is closest to the positive electrode and the gel closest to the negative electrode. The proportion of Tris and glycine in the transfer buffer is not necessarily the same as for wet transfer.

D. Blocking the membrane

1. Two blocking solutions are traditionally used: non-fat milk or BSA. Some antibodies give a stronger signal on membranes blocked with BSA as opposed to milk for unknown reasons.
2. To prepare a 5% milk or BSA solution, weigh 5 g per 100 ml of Tris Buffer Saline Tween20 (TBST) buffer. Mix well and filter.
3. Incubate for 1 hour at room temperature or overnight at 4°C agitation. Rinse for 5 seconds in TBST after the incubation.

E. Incubation with the primary antibody

1. Dilute primary antibody in TBST at the suggested dilution.
2. Incubate the membrane with diluted primary antibody for 1 hour at 37°C, or 2 hours at room temperature, or overnight at 4°C with agitation.
3. Remove antibody solution. Wash the membrane 3 times for 5-10 minutes each time at room temperature in TBST (50mM Tris, 100mM NaCl, 0.05% Tween-20, pH 7.6) with agitation. Note: Increase the concentration of Tween-20 to 0.1% reduces the background and increases the specificity, but it will reduce the sensitivity.

F. Incubation with secondary antibody

1. Incubate membrane with secondary HRP-conjugated diluted (according to manufacturer's instructions) in TBST for 1 hour at room temperature with shaking.
2. Remove antibody solution. Wash the membrane 3 times for 5-10 minutes each time at room temperature in TBST (50mM Tris, 100mM NaCl, 0.05% Tween-20, pH 7.6) with agitation.

G. Chemiluminescent Reaction

1. Prepare and use the Chemiluminescent substrate according to the manufacturer's instructions.
2. Immediately wrap the membrane and expose to X-ray films for 10 second to 1 hour period. The exposure time may vary according to the mount of antibody and antigen.