Western blot protocol

Sample preparation

Lysis buffers:

To prepare samples for running on a gel, cells and tissues need to be lysed to release the proteins of interest. This solubilizes the proteins so they can migrate individually through a separating gel. We use RIPA buffer (beyotime P0013B) for whole cell extracts and membrane-bound proteins.

Protease and phosphatase inhibitors:

As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer. Pmsf (Phenylmethanesulfonyl fluoride) is the protease inhibitors we always use.

Preparation of lysate from tissues:

Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases. The RIPA (with 1mM pmsf) should be ice-cold prior to homogenization.

Cut the tissue into small pieces, For a ~20 mg piece of tissue, add ~250 µl lysis buffer rapidly to the tube, homogenize with an electric homogenizer (or a glass homogenizer) until fully lysed.

Centrifuge for 5 min at 10000~14000g 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.

Determination of protein concentration

Use PAGE gels, a Bradford assay, a Lowry assay or a BCA assay. Bovine serum albumin (BSA) is a frequently-used protein standard.

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.

Electrophoresis

1). Clean the glass and set up the electrophoresis system.

2). Prepare PAGE gels, choose different concentrations of separating gel depending on the molecular weight of the protein:

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12
30-100	10
50-200	8

3). 4% Stacking gel, select pre-stained protein Marker Proteins in size, to help distinguish the size and protein electrophoresis tracer.

4). Load samples and run the gel, each hole sample volume 20-40μg protein, pay attention when spotting the sample, do not overflow into adjacent wells which will cause gum poked holes and cross-contamination.

5). Subject to electrophoresis according to the instructions recommended by electrophoresis method, Terminate the gel electrophoresis when bromophenol blue runs out the gel.

Transfer of proteins and staining (Western blotting)

1). Transfer the protein to the membrane from the gel. (according to the instructions recommended by trans-blot system.)

2). View proteins in membranes: Ponceau Red. After the transfer, use Ponceau dye staining about 2 ~ 5min to check the transfer is successful or not. Then rinse off the dye with distilled water.

3). Block the membrane generally with 5% non-fat milk, or 3%~5% BSA. Blocking at 4 ℃ with shaker shaking overnight, or 37 ℃ blocking for 2 hours. TBS wash 5-10 seconds after blocking.

4). Incubate with the primary antibody. According to the recommended antibody dilution, dilute antibody with TBST (or 1% to 3% non-fat milk) . Then load the membrane with a ziplock bag and fixed lamination machine, add the antibody solution to the ziplock, and make sure the membranes are incubated with sufficient volume of antibody.

5) 37℃ shaking incubation 1 ~ 3 h (according to the antibody titer and affinity), or 4 ℃ overnight incubation, TBST wash on the shaker at room temperature for 3 times, each time 5 ~ 10 min.

6). Incubate with the secondary antibody, the same as step (4) , dilution with TBST, 37℃ shaking incubation 1 ~ 3 h.

Chemiluminescence, developing and fixing, for HRP-conjugated antibodies: ECL are the traditional kits used.

In darkroom, load the developer and fixative respectively into plastic trays mixing the two reagents, volume of A and B, in a centrifuge tube. Ensure the membrane surface proteins have sufficient contact with the mixture. Remove residues after 1 ~ 2 min.

Take out the film at a red light, open the film clip, put the film on the membrane, remove the film clip, adjust the exposure time based on the strength of the signal, remember that an over-exposed film is not suitable for analysis.

After the exposure is complete, remove the film, quickly immerse in developing solution, terminate development. After development, the film should be immediately immersed in the fixing solution to a transparent film. After washing with tap water to remove residual fixative, dry at room temperature.

WB of phosphorylated protein

Carrying out western blots on phosphorylated proteins can require further optimization and a few unique conditions. Please see the suggestions below for tips on optimizing your WB on phosphorylated proteins.

1. Phosphorylated proteins are more easily degraded and dephosphorylated, so sample treatment should be quicker and always on ice, phosphatase inhibitors and protease inhibitors are both needed in lysing. And make sure to select protease inhibitors that do not inhibit phosphorylase.

2. Low expression of phosphorylated proteins may require larger loading quantity of sample, try 50ug~100ug. Also increase the concentration of primary antibody and extend the exposure time to help to make the target band more obvious.

3. BSA is the better blocking buffer in phosphorylated protein WB for preventing high background. Do NOT use milk. Casein in milk will result in high background. It is suggested to extend the blocking time if some non-specific bands are observed.