General Protocol for Western Blotting

Key Solutions and Reagents

Lysis buffer: Radioimmunoprecipitation assay buffer (RIPA buffer)

50 mM Tris-HCl, pH 8.0

150 mM NaCl

1% Nonidet P-40 (NP-40) or 0.1% Triton X-100

0.5% sodium deoxycholate

0.1% sodium dodecyl sulphate (SDS)

1 mM sodium orthovanadate

1 mM NaF

Protease inhibitors tablet (Roche)

Loading buffer: 2x Laemmli buffer

4% SDS

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris-HCl

Check the pH and adjust to pH 6.8 if necessary.

Running buffer: Tris/Glycine/SDS

25 mM Tris

190 mM glycine

0.1% SDS

Transfer buffer

25 mM Tris

190 mM glycine

20% methanol

For proteins larger than 80 kD, we recommend that SDS be included at a final concentration of 0.1%.

Ponceau S staining buffer

0.2% (w/v) Ponceau S

5% glacial acetic acid

Tris-buffered saline with Tween 20 (TBST) buffer

20 mM Tris, pH 7.5

150 mM NaCl

0.1% Tween 20

Blocking buffer

3% bovine serum albumin (BSA) in TBST

Stripping buffer

20 ml 10% SDS

12.5 ml 0.5 M Tris HCl, pH 6.8

67.5 ml ultrapure water

0.8 ml 2-mercaptoethanol

Procedure

Sample prep (based on a typical cell culture scenario)

1. Place the cell culture dish in ice and wash the cells with ice-cold Tris-buffered saline (TBS).
2. Aspirate the TBS, then add ice-cold RIPA buffer (1 ml per 100 mm dish).
3. Scrape adherent cells off the dish using a cold plastic cell scraper and gently transfer the cell suspension into a precooled microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. If necessary, sonicate 3 times for 10–15 sec to complete cell lysis and shear DNA to reduce sample viscosity.
6. Spin at 16,000 x g for 20 min in a 4°C precooled centrifuge.
7. Gently remove the centrifuge tube and place it on ice.
   Transfer the supernatant to a fresh tube, also kept on ice, and discard the pellet.
8. Remove a small volume (10–20 µl) of lysate to perform a protein assay. Determine the protein concentration foreach cell lysate.
9. If necessary, aliquot the protein samples for long-term storage at –20oC. Repeated freeze and thaw cycles cause protein degradation and should be avoided.
10. Take 20 µg of each sample and add an equal volume of 2x Laemmli sample buffer.
11. Boil each cell lysate in sample buffer at 95°C for 5 min.
12. Centrifuge at 16,000 x g in a microcentrifuge for 1 min.

Protein separation by gel electrophoresis

1. Load equal amounts of protein (20 µg) into the wells of a mini (8.6 x 6.7 cm) or midi (13.3 x 8.7 cm) format SDSPAGE gel, along with molecular weight markers.
2. Run the gel for 5 min at 50 V.
3. Increase the voltage to 100–150 V to finish the run in about 1 hr.

Gel percentage selection depends on the size of the protein of interest. A 4–20% gradient gel separates proteins of all sizes very well.

For details, please refer to the *Protein Blotting Guide*, bulletin 2895.

Transferring the protein from the gel to the membrane

1. Place the gel in 1x transfer buffer for 10–15 min.
2. Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.
3. Place the cassette in the transfer tank and place an ice block in the tank.
4. Transfer overnight in a coldroom at a constant current of 10 mA.

***Note**: Transfer can also be done at 100 V for 30 min–2 hr, but the method needs to be optimized for proteins of different sizes.*

Antibody incubation

1. Briefly rinse the blot in water and stain it with Ponceau S solution to check the transfer quality.
2. Rinse off the Ponceau S stain with three washes with TBST.
3. Block in 3% BSA in TBST at room temperature for 1 hr.
4. Incubate overnight in the primary antibody solution against the target protein at 4°C.

***Note**: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature depending on antibody quality and performance.*

5. Rinse the blot 3–5 times for 5 min with TBST.
6. Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.

***Note**: The antibody can be diluted using 5% skim milk in TBST.*

7. Rinse the blot 3–5 times for 5 min with TBST.

Imaging and data analysis

1. Apply the chemiluminescent substrate to the blot according to the manufacturer's recommendation.
2. Capture the chemiluminescent signals using a CCD camera-based imager.

***Note**: The use of film is not recommended in this step because of its limited dynamic range*.

3. Use image analysis software to read the band intensity of the target proteins.

Stripping and reprobing

1. Warm the buffer to 50°C.
2. Add the buffer to the membrane in a container designated for stripping. Incubate at 50°C for up to 45 min with some agitation.
3. Rinse the blot under running water for 1 hr.
4. Transfer the membrane to a clean container, wash
4. 5 times for 5 min with TBST.
5. Block in 3% BSA in TBST at room temperature for 1 hr.
6. Incubate overnight in the primary antibody solution (against the loading control protein) at 4°C.

***Note**: The antibody should be diluted in the blocking buffer at the manufacturer's recommended ratio.*

7. Rinse the blot 3–5 times for 5 min with TBST.
8. Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.

***Note**: The antibody can be diluted using 5% skim milk in TBST.*

9. Rinse the blot 3–5 times for 5 min with TBST.

Imaging and data analysis

1. Apply the chemiluminescent substrate to the blot following the manufacturer's suggestions.
2. Capture the chemiluminescent signals using a CCD camera-based imager.

***Note**: The use of film is not recommended in this step because of its limited dynamic range.*

3. Use image analysis software to read the band intensity of the loading control proteins.
4. Use the loading control protein levels to normalize the target protein levels.