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.
8. Transfer the supernatant to a fresh tube, also kept on ice, and discard the pellet.
9. Remove a small volume (10–20 μl) of lysate to perform a protein assay. Determine the protein concentration for each cell lysate.
10. If necessary, aliquot the protein samples for long-term storage at –20°C. Repeated freeze and thaw cycles cause protein degradation and should be avoided.
11. Take 20 μg of each sample and add an equal volume of 2x Laemmli sample buffer.
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 SDS-PAGE 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 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.

Life Science Group

Web site bio-rad.com  USA 1 800 424 6723 Australia 61 2 9914 2800 Austria 43 1 877 89 01 177 Belgium 32 (0)3 710 53 00 Brazil 55 11 3065 7550 Canada 1 905 364 3435 China 86 21 6169 8500 Czech Republic 420 241 430 532 Denmark 45 44 52 10 00 Finland 358 09 804 22 00 France 33 01 47 95 69 65 Germany 49 89 31 884 0 Hong Kong 852 2879 3200 Hungary 36 1 459 6100 India 91 124 4029300 Israel 972 03 963 6050 Italy 39 02 216091 Japan 81 3 6361 7000 Korea 82 2 3473 4460 Mexico 52 555 488 7670 The Netherlands 31 (0)38 540 666 New Zealand 64 9 415 2280 Norway 47 23 38 41 30 Poland 48 22 331 99 99 Portugal 351 21 472 7700 Russia 7 495 721 14 04 Singapore 65 6415 3188 South Africa 27 0 861 246 723 Spain 34 91 590 5200 Sweden 46 08 555 3270 Switzerland 41 026 674 55 05 Taiwan 886 2 2578 7189 Thailand 66 2 651 8311 United Arab Emirates 971 4 8187300 United Kingdom 44 020 8328 2000

BIO-RAD Laboratories, Inc.