

Western Blot Protocol

Solutions and reagents

lysis buffers:

RIPA buffer (radioimmunoprecipitation assay buffer):

- 50 mM Tris-HCl, pH 8.0
- 150 mM NaCl
- 1% NP-40 or 0.1% Triton X-100
- 0.5% sodium deoxycholate
- 0.1% SDS
- protease inhibitors

NP-40 buffer:

- 50 mM Tris-HCl, pH 8.0
- 150 mM NaCl
- 1.0% NP-40 (or 0.1% Triton X-100)
- protease inhibitors

3 x Laemmli buffer/ sample buffer:

- 150 mM Tris-HCl, pH 6.8
- 300 mM DTT
- 6% SDS
- 0.3% bromophenol blue
- 30% glycerol

10x PBS:

- 1.37 M NaCl
- 0.027 M KCl
- 0.1 M Na₂HPO₄
- 0.018 M KH₂PO₄

- Resolve in 800 ml ddH₂O.
- Adjust pH to 7.4 using HCl.
- Fill it up to 1l.
- Autoclave it and store at room temperature.
- Dilute 1:10 before use.

blocking buffer:

- 3-5% non-fat dry milk or BSA
- in PBST (PBS + 0.1% Tween 20)

Procedure

Sample preparation – lysate from cell culture

1. Place the cell culture dish on ice and wash the cells with ice-cold PBS.
2. Aspirate the PBS and add ice-cold lysis buffer (1 ml per 10 cm 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. If required, cells can be harvested by trypsinization and washed with PBS prior to resuspension in lysis buffer.
5. Incubate at 4°C for 30 min with constant agitation.
6. Centrifuge at 16,000 x g for 20 min at 4°C.
7. Transfer the supernatant to a fresh tube on ice, and discard the pellet.
8. Remove a small volume (10-20 µl) of lysate for analysis by a protein assay. Determine the protein concentration for each cell lysate.
9. 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.
10. Add ½ volume of 3x 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 SDS-PAGE

Polyacrylamid percentage of SDS-gel for best resolution of proteins based on their molecular weight:

Protein size	Gel percentage
4-40 kDa	20%
12-45 kDa	15%
10-70 kDa	12,5
15-100 kDa	10%
25-200 kDa	8%

1. Load equal amounts of protein into the wells of the SDS-PAGE (10-50 µg/lane protein of cell lysate or 10-100 ng/lane purified protein). Add molecular weight marker in one of the lanes.
2. Run the gel according to manufacturer's instructions (e.g. 1-2 h at 200 V).

Protein transfer from gel to membrane

Use either nitrocellulose or PVDF membrane. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. Follow the manufacturer's instructions for blotting.

Antibody incubation

1. After transfer briefly rinse the membrane in distilled water or PBST.
2. Block the membrane 1 h at RT with blocking buffer.
3. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer ON at 4°C or 1-2 h at RT.
4. Wash the membrane with PBST for 15 min/ 3x 5 min.
5. Incubate the membrane with recommended dilution of conjugated secondary antibody in blocking buffer for 1 h at RT.
6. Wash the membrane with PBST for 15 min/ 3x 5 min.
7. For signal development follow the kit manufactures instructions of the detection kit used.