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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

10 x PBS:

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

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

blocking buffer:

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

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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, centrifuge at 16,000 x g for 20 min at 4°C.
6. Transfer the supernatant to a fresh tube on ice, and discard the pellet.
7. Remove a small volume (10-20 µl) of lysate for analysis by a protein assay. Determine the protein concentration for each cell lysate.
8. 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.
9. Add $^1/_2$ volume of 3x Laemmli sample buffer.
10. Boil each cell lysate in sample buffer at 95°C for 5 min.
11. 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.

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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.