Westernblot

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 Na_2HPO_4
0.018 M KH_2PO_4

- Resolve in 800 ml ddH_2O.
- Adjust pH to 7.4 using HCl.
- Fill it up to 1l.
- Autoclave it.
- 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)

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Procedure

Sample preparation

Preparation of lysate from cell culture:

- Place the cell culture dish on ice and wash the cells with ice-cold PBS.
- Aspirate the PBS and add ice-cold lysis buffer (1 ml per 10 cm dish).
- Scrape adherent cells off the dish using a cold plastic cell scraper and gently transfer the cell suspension into a precooled microcentrifuge tube.
- If required, cells can be harvested by trypsinization and washed with PBS prior to resuspension in lysis buffer.
- Incubate at 4°C for 30 min with constant agitation.
- Centrifuge at 16,000 x g for 20 min at 4°C.
- Transfer the supernatant to a fresh tube on ice, and discard the pellet.
- Remove a small volume (10-20 µl) of lysate for analysis by a protein assay. Determine the protein concentration for each cell lysate.
- 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.
- Add ½ volume of 3x Laemmli sample buffer.
- Boil each cell lysate in sample buffer at 95°C for 5 min.
- 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%

- 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.
- 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

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

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