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Western Blotting Protocol

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Abstract

This protocol describes culturing adherent cells, harvesting whole-cell protein lysates by RIPA lysis after drug or transfection treatments, quantifying protein by BCA, and performing SDS-PAGE and membrane transfer for Western blot detection. Membranes are blocked and probed with primary and secondary antibodies, followed by chemiluminescent (or fluorescent) visualization and optional re-probing.

Expected results are consistent, comparable protein inputs across conditions (normalized to concentration) and clear, well-resolved target bands at the expected molecular weight with minimal background. Treatment-dependent changes (e.g., phosphorylation shifts after short serum-starve/drug exposure or expression changes after longer treatments) should be detectable as reproducible differences in band intensity or mobility between conditions.

Materials

- 0.25% Trypsin (Fisher, #MT25053CI)
- 10% Fetal Bovine Serum/FBS (Fisher, #SH3007203)
- 10% Charcoal Stripped Serum/CSS (Fisher, #SH3006803)
- Trypan Blue Solution (Fisher, #MT-25-900-CI)
- 1X PBS (Fisher, #4054-1012)
- RIPA (Fisher, #89901)
- Phosphatase inhibitor (Fisher, #78426)
- Protease inhibitor (Fisher, #78439)
- 1.5mL Eppendorf tube
- p200 pipette
- BCA analysis kit (Fisher, #PI23225)
- 4X Laemmli buffer (BioRad, #1610747)
- β-Mercaptoethanol
- Gel apparatus
- Running buffer (10x Tris/Glycine/SDS, Bio-Rad, #1610772)
- Gel loading tip (USA Scientific, #1022-0600)
- Pre-cast 4-20% 15-Well gradient gels (BioRad, #4561093EDU)
- Precision Plus Dual Color Ladder (BioRad, #1610394)
- Trans-Blot Turbo Transfer system (BioRad)
- Trans-Blot Turbo RTA Mini 0-2 um Nitrocellulose Transfer Kit (#1704270)
- 5% milk (Fisher, #NC9121673)
- 1X Tris Buffered Saline (BioRad, #1706435)
- Tween20 (Sigma-Aldrich, P1379-500ML)
- TBS-T
- 5% BSA (Fisher, AM2616)
- Kimwipe (Fisher, 06666A)
- Chemiluminescence reagents (Fisher, #509049325)
- Film (Research Products International Corp, #248300)
- Electronic imagers (BioRad/Licor)

Safety warnings

- Under/over seeded cells will result in aberrant results.
- Serum starve and treat for 30 minutes for phosphorylation targets.
- If not moving immediately to analysis, lysates can be stored at -20°C.
- Debris pellet can be removed with p20 pipette tip or the supernatant can be transferred to another tube to avoid contamination of protein lysate with residual debris.
- Use loading buffer and β-Mercaptoethanol to top up any wells with discordant volumes.
- In lieu of overnight antibody incubation, membranes may be probed for one hour at room temperature with a loss in band quality/definition and an increase in background.
- Do not blot the protein side, or allow the membrane to fully dry.
- Do not stack membranes in container, or allow them to contact the protein side of any other membrane(s).

Cell Culture and Harvest

1 Dissociate adherent cells with 0.25% Trypsin (Fisher, #MT25053CI) for 5-10 minutes.

2 Deactivated Trypsin in 10% Fetal Bovine Serum/FBS (Fisher, #SH3007203) or 10% Charcoal Stripped Serum/CSS (Fisher, #SH3006803) media, and pellet cells at 1500 RPM.

3 Re-suspend cells in culture medium, and count employing Trypan Blue Solution (Fisher, #MT-25-900-CI) and manual or electronic Hemacytometer.

4 Seed cells into individual wells of a 6-Well plate for all conditions equally and allow appropriate time for attachment (eg: 24-48 hours).
- Note: Under/over seeded cells will result in aberrant results, and seeding densities should be determined ahead of time to prevent harvest of over-confluent, clumpy or detached cells.

5 Treat cells with drug, or transfection reagents according to manufacturer's instructions. Note: Serum starve and treat for 30 minutes for phosphorylation targets, and treat for 24-96 hours with our without starvation for other targets.

6 Wash cells gently with 1X PBS (Fisher, #4054-1012) and conduct protein harvest in 120µL of RIPA (Fisher, #89901) supplemented with phosphatase inhibitor (Fisher, #78426) and protease inhibitor (Fisher, #78439), by scraping cells and transferring to a 1.5mL Eppendorf tube with a p200 pipette.

7 Incubate samples for 30 minutes at 4°C to allow lysis to proceed.
- Note: If not moving immediately to analysis, lysates can be stored at -20°C.

8 After 30 minute incubation, or thaw from freezing storage, spin down samples at max RPM at 4°C for 10 minutes to remove cell debris before protein quantification.
- Note: Debris pellet can be removed with p20 pipette tip or the supernatant can be transferred to another tube to avoid contamination of protein lysate with residual debris.

Western Blot

9 Conduct Bicinchoninic Acid (BCA) analysis, to determine protein lysate concentrations according to manufacturer's instructions (Fisher, #PI23225).

10 Dilute all samples in RIPA and 4X Laemmli buffer (BioRad, #1610747) to a total volume of 100µL at 1µg/µL.

- Note: Be sure to add β-Mercaptoethanol to Laemmli right before addition to working samples.

11 Heat samples for 5 minutes at 95°C to denature most proteins. Alternatively, for heat-sensitive proteins, membrane proteins, or when you need to preserve protein interactions, heat samples for 10 minutes at 65°C to denature.

12 Prepare gel apparatus, and employ appropriate running buffer (10x Tris/Glycine/SDS, Bio-Rad, #1610772, recommended).

13 Use a gel loading tip (USA Scientific, #1022-0600) to wash wells of gel with running buffer to remove storage buffer.

14 Load samples onto gel (BioRad pre-cast 4-20% 15-Well gradient gels [#4561093EDU] preferred), again using a gel loading tip.

15 Ensure all wells have the same volume to prevent band malformations.
- Note: Use loading buffer and β-Mercaptoethanol to top up any wells with discordant volumes. eg: 6µL of Loading Buffer and 4µL of Precision Plus Dual Color Ladder (BioRad, #1610394), or 10µL of Loading Buffer in an empty well, to make all wells 10µL.

16 Run gel(s) at 5 milliamps constant per gel for 30 minutes to allow samples to enter gel, then run at 10-50 milliamps constant until dye front leaves gel, or until desired separation occurs.

17 Transfer gel to membrane using BioRad Trans-Blot Turbo Transfer system along with the Trans-Blot Turbo RTA Mini 0-2 um Nitrocellulose Transfer Kit (#1704270) according to manufacturer's instructions (typically 1.3amps and 25 volts for 7 minutes for mixed molecular weight targets).

18 Block membranes in 5% milk (Fisher, #NC9121673) in 1X Tris Buffered Saline (BioRad, #1706435) plus 0.1% Tween20 (Sigma-Aldrich, P1379-500ML) (TBS-T) for 60 minutes on a shaker immediately after transfer completes.
- Note: For detection of phosphorylated-proteins blocking in 5% BSA TBS-T is recommended.

Probing Western Membranes

19 Wash whole or sectioned membranes with TBS-T to remove Milk.

20 Incubate membranes with appropriate primary antibody dilution in 5% BSA (Fisher, AM2616) in TBS-T overnight on a shaker at 4°C.

- Note: In lieu of overnight antibody incubation, membranes may be probed for one hour at room temperature with a loss in band quality/definition and an increase in background.

21 Wash membranes with TBS-T three times for a total of 15 minutes.

22 Incubate membranes with appropriate secondary antibody dilution in 5% Milk/BSA TBS-T for one hour on a shaker at 4°C.
- Note: If using fluorescent secondary antibodies, cover membranes to protect from light during incubation and washes.

23 Wash membranes with TBS-T three times for a total of 15 minutes.
- Note: If using fluorescent secondary antibodies go directly to imaging in a Li-Cor Odyssey or other imager.

24 Place membranes protein side up on a Kimwipe (Fisher, 06666A) to blot excess buffer, and allow to dry for 1-3 minutes.
- Note: Do not blot the protein side, or allow the membrane to fully dry.

25 Prepare chemiluminescence reagents (Fisher, #509049325) in a container large enough to fit membrane(s).

26 Incubate the membranes in chemiluminescence reagents for 1 minute with gentle agitation.
- Note: Do not stack membranes in container, or allow them to contact the protein side of any other membrane(s).

27 Expose membranes using film (Research Products International Corp, #248300) or electronic imagers (BioRad/Licor).

28 Wash membranes with TBS-T two times to remove chemiluminescence reagents.

29 Membranes can be stored at 4°C in TBS (not TBS-T), or re-probed for other targets immediately.