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Western Blotting Protocol V.2

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[1]BioLegend

1 Works for me dx.doi.org/10.17504/protocols.io.98mh9u6

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EXTERNAL LINK

https://www.biolegend.com/protocols/western-blotting-protocol/4269/

GUIDELINES

Tips:

High background

1. Transfer buffers may have become contaminated. Contamination can be transferred to the blots from electrophoresis and related equipment used in blot preparation.
2. Post-antibody washes may not have been performed for a sufficient period of time or were not performed in a high enough volume.
3. The blocking and incubation agents used were not freshly prepared or were too dilute.

WB for phosphorylated proteins:

1. Add phosphatase inhibitors and keep samples on ice at all times to preserve the phosphorylated state of the proteins.
2. If high background is observed, replace milk with 5% (w/v) BSA in TBS-T for blocking and antibody dilution as phosphor-specific antibody detects casein in the milk
3. If low signal or no signal is detected, make sure that the phosphorylation state of the protein was appropriately induced. Run the required positive control with your samples to confirm.

No Signal or Poor Signal:

1. Transfer efficiency may have been poor. Check protein transfer by staining the gel and/or membrane.
2. Incorrect storage of antibodies or ECL western blotting detection reagents may result in a loss of signal.
3. Insufficient protein may have been loaded on the gel. Depending on the location of the target protein, membrane or nuclear preparations may be required (instead of whole cell lysates).
4. Film exposure time may have been too short.

MATERIALS TEXT

- **1X Cell Lysis Buffer:** 20 mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 2 mM EDTA, 1µg/ml leupeptin, 1µg/ml aprotinin, 1 mM Na_3PO_4, 1 mM PMSF, 5 mM NaF, 3 mM $Na_4P_2O_4$.
- **5X SDS Sample Buffer:** 312.5 mM Tris-HCl (pH 6.8), 10% SDS (w/v), 250 mM DTT, 50% Glycerol, 0.05% Bromophenol Blue (w/v), Use at 1X.
- **10X SDS Running Buffer:** Dissolve 144g of Glycine, 30g of Tris base and 10g SDS in 800ml of distilled H_2O. Add distilled H_2O to 1 liter. Use at 1X.
- **Transfer Buffer:** 3.0g Tris base, 14.4g Glycine, 200ml Methanol. Add deionized water to 1.0L.
- **Blocking Buffer:** 1X TBS-T with 5% nonfat dry milk.
- **Wash Buffer:** 1X TBS-T.
- **Primary and Secondary Antibody Dilution Buffer**: 1X TBS-T with 5% nonfat dry milk.
- **Alternate Blocking Buffer:** 1X TBS-T with 4% Bovine Serum Albumin (BSA).
- **Alternate Primary and Secondary Antibody Dilution Buffer:** 1X TBS-T with 4% Bovine Serum Albumin (BSA).
- **Blotting Membrane:** Nitrocellulose or PVDF membrane.
- **10X TBS-T (Tris-buffered saline containing Tween-20):** Dissolve 80g of NaCl, 2g of KCl, 30g of Tris base and 10ml Tween-20 in 800ml of distilled H_2O. Adjust the pH to 7.4 with HCl. Add distilled H_2O to 1 liter. Use at 1X (containing 0.1% Tween-20).

Sample Preparation:

1 Place cells in a microcentrifuge tube and centrifuge to collect the cell pellet.

2 Lyse the cell pellet with 100µl of lysis buffer on ice for 30 min (For 1 X 106 cells, lyse with 100µl of lysis buffer).

3 Centrifuge at 14,000 rpm (16,000xg) for 10 minutes at 4°C.

4 Transfer the supernatant to a new tube and discard the pellet. Remove 20µl of supernatant and mix with 20µl of 2x sample buffer.

5 Boil for 5 min. Cool at room temperature for 5 minutes. Microcentrifuge for 5 minutes.

6 Load up to 40µl of sample to each well of a 1.5mm thick gel*.*Guidelines for choosing gel percentages are based on protein size to be detected: 4-5% gel, > 200 kD; 7.5% gel, 120-200 kD; 8-10% gel, 40-120 kD; 13% gel, 15-40 kD; 15% gel, < 20 kD.

7 Set gel running conditions according to the manufacturer's instructions. Transfer the proteins to a nitrocellulose or PVDF membrane with variable power settings according to the manufacturer's instructions.

For Amyloid Beta Detection, Boiling Method:

8 Immediately after transferring the gel onto the membrane, submerge the membrane in boiling PBS for 5 minutes. After boiling, continue as normal to the membrane blocking step of the protocol.

Membrane Blocking:

9 Remove the blotted membrane from the transfer apparatus and immediately place in blocking buffer consisting of 5% nonfat dry milk/TBS-T**.

10 Incubate the blot for 1 hour at room temperature, or overnight at 4°C with agitation.

Antibody Incubation:

11 Dilute the primary antibody to the recommended concentration/dilution in 5% nonfat dry milk/TBS-T. Place the membrane in the primary antibody solution and incubate for 2 hours at room temperature, or overnight at 4°C with agitation.

12 Wash three times for 5 minutes each with Wash Buffer (TBS containing 0.1% Tween-20).

13 Incubate the membrane for 30 minutes at room temperature with horseradish peroxidase (HRP)- conjugated secondary antibody, diluted to 1:1000 - 1:5000 in 5% nonfat dry milk/ TBS-T.

14 Wash 4 times for 10 minutes each with TBS containing 0.1% Tween-20 and once for 2 minutes with PBS.

Protein Detection:

15 Incubate membrane (protein side up) with 10ml of ECL (enhanced chemiluminescence substrate) for 1-2 minutes. The final volume required is 0.125ml/cm2.

16 Drain off the excess detection reagent, wrap up the blots, and gently smooth out any air bubbles.

17 Place the wrapped blots, protein side up, in an X-ray film cassette and expose to x-ray film. Exposures can vary from 5 seconds to 60 minutes.