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Direct-Blot™ Western Blotting Protocol V.4

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[1]BioLegend

1 Works for me dx.doi.org/10.17504/protocols.io.98rh9v6

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EXTERNAL LINK

https://www.biolegend.com/protocols/direct-blot-western-blotting-protocol/4247/

MATERIALS TEXT

- **1X Cell Lysis Buffer:** 20mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40,2 mM EDTA, 1µg/ml leupeptin, 1µg/ml aprotinin ,1mM Na_3PO_4, 1mM PMSF, 5mM NaF, 3mM $Na_4P_2O_4$
- **5X SDS Sample Buffer:** 312.5mM Tris-HCl (pH 6.8) , 10% SDS (w/v), 250mM DTT, 50% Glycerol, 0.05% Bromophenol Blue (w/v) Use at 1X, 80.0g NaCl, 4.4g Na_2HPO_4, 2.4g KH_2PO_4, 2.0g KCl. Add ddH_2O up to 10L, pH to 7.2 with HCl
- **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 distilled water to 1.0L
- **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).
- **Blocking Buffer:** 1X TBS-T with 5% nonfat dry milk
- **Wash Buffer**: 1X TBS-T
- **Direct-Blot™ Antibody Dilution Buffer:** 1X TBS-T with 5% nonfat dry milk. **If phosphorylation-specific antibodies are used, the membrane blocking buffer and antibody dilution buffer should not contain milk.
- **Alternate Blocking Buffer:** 1X TBS-T with 4% Bovine Serum Albumin (BSA)
- **Alternate Direct-Blot™ Antibody Dilution Buffer:** 1X TBS-T with 4% Bovine Serum Albumin (BSA)
- **Blotting Membrane:** Nitrocellulose or PVDF membrane

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 20 µl 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.Note: 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.

Membrane Blocking:

8 Remove the blotted membrane from the transfer apparatus and immediately place in blocking buffer consisting of 5% nonfat dry milk/TBS-T.Note: If phosphorylation-specific antibodies are used, the membrane blocking buffer and antibody dilution buffer should not contain milk.

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

Antibody Incubation:

10 Dilute the Direct-Blot™ antibody to the recommended concentration/dilution in 5% nonfat dry milk/TBS-T (usually at a 1:1000-1:2000 dilution). Place the membrane in the Direct-Blot™ antibody solution and incubate for 2 hours at room temperature, or overnight at 4°C with agitation.Note: If phosphorylation-specific antibodies are used, the membrane blocking buffer and antibody dilution buffer should not contain milk.

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

Protein Detection:

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

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

14 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.