Direct-Blot™ Western Blotting Protocol V.4

Sam Li

BioLegend

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

BioLegend

Tech. support email: tech@biolegend.com

Sam Li

BioLegend

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**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 10^6 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.
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:

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.

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

Antibody Incubation:

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.

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

Protein Detection:

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

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

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.