Direct-Blot™ Western Blotting Protocol Version 2

BioLegend, Inc.

Abstract

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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.

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.

Solutions and Reagents:

1X Cell Lysis Buffer:

20 mM Tris-HCl, pH 7.5

150 mM NaCl

1% NP-40

2 mM EDTA

1 µg/ml leupeptin

1 µg/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 144 g of Glycine, 30 g of Tris base and 10 g SDS in 800 ml of distilled H2O.

Add distilled H2O to 1 liter

Use at 1X

Transfer Buffer:

3.0 g Tris base

14.4 g Glycine

200 ml Methanol

Add distilled water to 1.0 L

10X TBS-T (Tris-buffered saline containing Tween-20):

Dissolve 80 g of NaCl, 2 g of KCl, 30 g of Tris base and 10 ml Tween-20 in 800 ml of distilled H_2O.

Adjust the pH to 7.4 with HCl. Add distilled H2O 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

Protocol

Sample preparation

Step 1.

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

Sample preparation

Step 2.

Lyse the cell pellet with 100 µl of lysis buffer on ice for 30 min (For 1 X 10^6 cells, lyse with 100 µlof lysis buffer).

DURATION

00:30:00

Sample preparation

Step 3.

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

DURATION

00:10:00

Sample preparation

Step 4.

Transfer the supernatant to a new tube and discard the pellet.

Sample preparation

Step 5.

Remove 20 µl of supernatant and mix with 20 µl of 2x sample buffer.

Sample preparation

Step 6.

Boil for 5 min.

DURATION

00:05:00

Sample preparation

Step 7.

Cool at room temperature for 5 minutes.

DURATION

00:05:00

Sample preparation

Step 8.

Microcentrifuge for 5 minutes.

DURATION
00:05:00

Sample preparation

Step 9.

Load up to 40 µl of sample to each well of a 1.5 mm thick gel.

NOTES
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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

Sample preparation

Step 10.

Set gel running conditions according to the manufacturer's instructions.

Sample preparation

Step 11.

Transfer the proteins to anitrocellulose or PVDF membrane with variable power settings according to the manufacturer's instructions.

Membrane Blocking

Step 12.

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

NOTES
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usually at a 1:1000-1:2000 dilution

Membrane Blocking

Step 13.

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

Antibody Incubation

Step 14.

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).

NOTES

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If phosphorylation-specific antibodies are used, the membrane blocking buffer and antibody dilution buffer should not contain milk.

Antibody Incubation

Step 15.

Place the membrane in the Direct-Blot™ antibody solution and incubate for 2 hours at room temperature, or overnight at 4°C with agitation.

Antibody Incubation

Step 16.

Wash for 5 minutes with Wash Buffer (TBS containing 0.1% Tween-20). [wash 1/3]

DURATION

00:05:00

Antibody Incubation

Step 17.

Wash for 5 minutes with Wash Buffer (TBS containing 0.1% Tween-20). [wash 2/3]

DURATION

00:05:00

Antibody Incubation

Step 18.

Wash for 5 minutes with Wash Buffer (TBS containing 0.1% Tween-20). [wash 3/3]

DURATION

00:05:00

Protein Detection

Step 19.

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

DURATION

00:01:00

Protein Detection

Step 20.

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

Protein Detection

Step 21.

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.