Western Blot analysis

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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 deionized 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

Primary and Secondary 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 Primary and Secondary Antibody Dilution Buffer:

1X TBS-T with 4% Bovine Serum Albumin (BSA)

Blotting Membrane:

Nitrocellulose or PVDF membrane

Protocol

cell preparation

Step 1.

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

Sample preparation

Step 2.

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% NP-40, 1:100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). Transfer the supernatant to a new tube and discard the pellet□centrifugate 14000rpm 30min at 4°C. Test the concentration of supernatant, and prepare the protein denaturation.

Sample preparation

Step 3.

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

NOTES

Kelsey Knight 31 May 2016

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 4.

Transfer the proteins to PVDF membranes with a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions.

Membrane Blocking and antibody Incubation

Step 5.

The membranes were blotted with 5% dehydrated milk for 1 h and then incubated with primary antibodies overnight at 4°C with agitation.

washing membranes

Step 6.

Wash for 10 minutes with TBST three times .

Antibody Incubation

Step 7.

Incubate the membrane for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted to 1:5000 in 5% nonfat dry milk/TBST.

washing mebranes

Step 8.

Wash for 10 minutes with TBST three times.

Protein Detection

Step 9.

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