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NB600-1049 Protocol

Western Blot Protocol for S6K Antibody (NB600-1049)

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Western Blot Protocol

Solutions and Reagents:

Transfer Buffer:
25 mM Tris-base (pH 8.5), 0.2 M Glycin, 20% methanol

Cell Extract Buffer:
50 mM Pipes/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, 20 ug/ml Leupeptin, 10 ug/ml Pepstatin, 10 ug/ml aprotinin, and 1 mM PMSF.

SDS-PAGE Loading Buffer:
62.5 mM Tris-HCl, (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromphenol blue

10X TBS (Tris-Buffered Saline):
To prepare 1 liter of 10X TBS: 24.2 g Tris-base, 80 g NaCl, adjust pH to 7.6 with HCl (use at 1X).

TBS/T Washing Buffer:
1X TBS, 0.1% Tween-20

Blocking Buffer:
1X TBS/T with 5% BSA

Primary Antibody Dilution Buffer:
1X TBS/T with 5% BSA

Western Blot Detection:
Protein marker, secondary anti-rabbit antibody conjugated to HRP, chemiluminescent reagent, peroxide.

Protein Blotting:
1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures, wash cells with 1X PBS, aspirate. Scrape cells into PBS and spin down to pellet.
3. Lyse cells by adding Cell Extract Buffer (one volume of cell pellet, or 100 ul per well of 6-well plate or 500 ul per plate of 10 cm2 plate). Freeze and thaw 3 times. Centrifuge lysate at microcentrifuge using top speed. (~14000 rpm). Keep the supernatant and discard the pelleted cell debris.
4. Add SDS Loading Buffer and heat to 95-100oC for 5 minutes, cool on ice.
5. Microcentrifuge for 5 minutes.
6. Load 5-20 ul onto SDS-PAGE gel (10 cm x 10 cm).
Note: We recommend loading prestained molecular weight markers to verify electrotransfer.
7. Electrotransfer to nitrocellulose membrane.

Membrane Blocking & Antibody Incubations:

Note: Volumes are for 10 cm x 10 cm of membrane. For different sized membranes, adjust volumes accordingly
1. Incubate membrane in 25 ml of Blocking Buffer for 1 hour at room temperature.
2. Wash 3 times for 5 min each with 15 ml of TBS/T.
3. Incubate membrane and NB600-1049 1-2 ug/ml in 10 ml Primary Antibody Dilution Buffer with gentle agitation overnight at 4C.
4. Wash 3 times for 5 minutes each with 15 ml of TBS/T.
5. Incubate membrane with HRP-conjugated secondary antibody in 10 ml of Blocking Buffer with gentle agitation for 1 hour at room temperature.
6. Wash membrane as in step 4.

7. Proceed with detection.

Detection of Proteins:
1. Remove the wash buffer and place the blot in a plastic bag or clean tray containing chemiluminescent working solution (0.125 ml/cm2) and peroxide (ECL detection method).
2. Rotate the bag or tray to allow the solution to cover the surface of the membrane for 1-5 minutes.
3. Remove blot from bag or tray and place it between two pieces of write-on acetate transparency film. Smooth over covered blot to remove air bubbles and excess substrate.
Expose to X-ray film. An initial exposure of 10-60 seconds is recommended for film.