

Western Blot Analysis—Phosphoprotein-Specific Antibody Mixture
AfCS Procedure Protocol ID PP0000000700
Version 1, 01/29/02

The following procedure is the final step in a 3-step process of Western immunoblotting for detection of particular sites of phosphorylation on specific proteins recognized by modification-sensitive antibodies. The first step is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to resolve a mixture of proteins by size (AfCS Protocol *SDS-PAGE—Invitrogen Mini-Cell*, PP0000000200 or *SDS-PAGE—Bio-Rad Criterion Cell*, PP0000000300). The second step is the transfer of resolved proteins from the gel to a membrane support (i.e., nitrocellulose or polyvinylidene difluoride [PVDF]) via electroelution (AfCS Protocol *Protein Transfer—Invitrogen XCell SureLock Mini-Cell*, PP0000000400, or *Protein Transfer—Bio-Rad Criterion Blotter*, PP0000000500). The product of the second step is referred to as the blot. This final step involves processing of the blot for detection of specific phosphoproteins with a mixture of antibodies, the product of which is the final Western immunoblot.

Unless otherwise stated, all of the following incubations and washes are conducted at room temperature with gentle shaking using an oscillating platform shaker. Volumes are specified for 8 x 8 cm Invitrogen gel blots (should be doubled for 8.7 x 13.3 cm Bio-Rad Criterion gels).

Procedures

1. Wash blot twice, each time for 5 min, with 25 ml Tris-buffered saline with 0.1% Tween 20 (0.1% TBST)..
2. Incubate blot for 1 hr in 25 ml of 5% dry milk/0.1% TBST.
3. Quickly rinse blot twice with 15 ml of 0.1% TBST.
4. Add a mixture of phosphospecific antibodies diluted in bovine serum albumin, 5%, in Tris-buffered saline Tween, 0.1% (5% BSA/0.1% TBST) to the blot and incubate overnight at 4 °C. The exact composition of the cocktail is specified in each experimental protocol (an example is provided below in *Reagents and Materials*).
5. Quickly rinse the blot twice with 15 ml 0.1% TBST.
6. Incubate the blot for 1 hr with secondary antibody solution (antibody composition can vary depending on the species of the source of the primary antibodies; see example below in *Reagents and Materials*).
7. Wash blot 3 times with 0.1% TBST, for 10 min each time.
8. Prepare fresh solution of ECL Plus Western Blotting Detection Reagents (ECL Plus).
9. Incubate the blot in ECL Plus for 5 min, shaking vigorously.
10. Lift blot with blunt forceps and drain briefly by touching one end on paper towel.
11. Place damp blot in transparent sheet protector, being careful not to trap air bubbles.
12. Create image with Molecular Dynamics Storm 860 gel and blot analysis system.
13. For normalization of lane-to-lane or sample variability, incubate the blot at room temperature for one hr with a standard antibody solution. This antibody, such as one to G protein β subunits, is not phosphorylation sensitive (see example below

in *Reagents and Materials*). Note: It is possible that the standard antibody can be included in the cocktail of phosphospecific antibodies. If so, this step and the following two steps may be eliminated.

14. Wash blot with 0.1% TBST 3 times, each time for 5 min.
15. Repeat steps 6 through 12.

Reagents and Materials

Tris-buffered saline with 0.1% Tween 20 (0.1% TBST), 4L: AfCS Solution Protocol ID PS0000006400

Dry milk, 5%, in Tris-buffered saline Tween, 0.1% (5% dry milk/0.1% TBST): AfCS Solution Protocol ID PS0000002400

Bovine Serum Albumin, 5%/Tris-buffered Saline Tween, 0.1% (5% BSA/0.1 % TBST), 500 ml: AfCS Solution Protocol ID PS0000002000

Molecular Dynamics Storm 860 gel and blot analysis system: Amersham Pharmacia Biotech; catalog no. 860PC (includes 520 nm long pass filter for blue-excited fluorescence to detect chemifluorescence emitted by ECL Plus)

Examples:

Cocktail of phosphospecific antibodies

Mix following antibodies diluted (as specified below) in 5% BSA/0.1% TBST:

Phospho-Stat6 (Tyr641): Cell Signaling Technology (CST); catalog no. 9361	1:3000
Phospho-p90RSK (Ser380): CST; catalog no. 9341	1:1000
Phospho-Akt (Ser473): CST; catalog no. 9271	1:500
Anti-MAP kinase activated (diphosphorylated ERK-1&2) unconjugated: Sigma-Aldrich; catalog no. M8159	1:5000

Secondary antibody solution, antibodies diluted in 5% dry milk/0.1% TBST

Anti-rabbit Ig, horseradish peroxidase-linked F(ab')₂ fragment (from donkey):

Amersham Pharmacia Biotech; catalog no. NA9340

Anti-mouse Ig, horseradish peroxidase-linked F(ab')₂ fragment (from sheep):

Amersham Pharmacia Biotech; catalog no. NA9310

Standard antibody solution: G Protein β subunit antibody solution

B600 (PNAS 90: 3675, 1993) diluted 1:100,000 in 5% BSA/0.1% TBST or a comparable antibody (named T20): Santa Cruz Biotechnology; catalog no. sc-378

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