

Detection of Phosphotyrosine with Labeled SH2 Domains

Important Information: This protocol is designed for use with SH2-Domains supplied by Marligen Biosciences and is intended for research use only. As with all Western blotting protocols, reagent concentrations and conditions may need optimization depending upon the sample type, sample amount, blotting method, membrane type and the sensitivity desired

Intended Use: This protocol, in conjunction with the recommended reagents, can be used to detect specific phosphotyrosine motifs in protein cell extracts.

Materials Required from Marligen:

Component	SH2 Domains	Glutathione-Peroxidase Conjugate
Catalog Number	Varies with domain	11933-005
Quantity	50 µg	5 µg
Concentration	1.0 µg/ µl	0.1 µg/ µl
Volume	50 µl	50 µl

When the SH2 domains are used at a working concentration of 1 µg/ml, the above reagents will be sufficient for probing:

- 50 membrane strips (2.5 x 7 cm)
- 15 Novex-type gels (8.5 cm x 6.5 cm)
- 10 Bio-Rad Criterion gels (13 cm x 7 cm)

Materials Needed but not Supplied:

PBS (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4)
 PBS-glycerol (PBS + 10% glycerol)
 TBST Buffer (150 mM NaCl, 10 mM Tris (pH 8.0), 0.05% Tween 20)
 Chemiluminescent Detection Substrate (ECL – Amersham)
 Nitrocellulose membrane
 Nonfat dry milk
 100 mM Orthovanadate solution
 0.5 M EDTA solution
 Film for chemiluminescent detection

Advanced preparations:

- Dilute the supplied SH2 Domain to 0.1 µg/µl by adding 450 µl of PBS (pH 7.4) + 10% glycerol to the tube (a 1:10 dilution). In order to avoid repeat freeze-thaws, divide the diluted SH2 domain into 50 µl aliquots and store at -80°C until needed.

Protocol:

Preparation of labeled SH2 domain

Add 5 µl (0.5 µg) of GSH-HRP to 50 µl (5 µg) SH2 domain. Mix gently but thoroughly. Incubate for 30 minutes at room temperature. Store at 2-8°C. (This amount will ultimately provide a 5 ml working solution of 1 µg/mL – sufficient material for 5 blots when using 1 ml per blot.)

Labeled SH2 domains are stable for at least 1 month when stored at 4°C and protected from light. Labeled probes should not be frozen.

Prepare cell extracts

Before preparing extracts, wash adherent or pelleted cells with cold 1 X PBS + 1 mM sodium orthovanadate.

Extract proteins from cells using a cell extraction buffer containing phosphatase and protease inhibitors. Quantitate cell extracts using a Bradford assay or similar method.

Blotting

Load 10-50 µg of cell extract per lane and separate proteins by SDS-PAGE on 10% acrylamide, a 4-15% gradient gel, or other suitable gel.

Transfer the proteins from the gel to the nitrocellulose membrane using standard Western blotting techniques.

Prepare the following blocking solution: 10% nonfat dry milk in TBST plus 1 mM orthovanadate and 5 mM EDTA.

Block the membranes in blocking solution for 1 hour at room temperature with gentle shaking or overnight at 4°C with gentle agitation.

Detection of phosphotyrosine

Immediately before use, prepare the Detection Reagent by adding 10 µl of labeled SH2 domain to 1 ml of blocking solution. The amount of Detection Reagent added to the blocking solution should be varied to achieve optimal results in the same manner that an antibody concentration would be optimized in a traditional Western blotting protocol.

Transfer the blocked membrane to a hybridization bag. It is not necessary or recommended to wash the blot before incubation with the Detection Reagent. Add the Detection Reagent to the



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Detection of phosphotyrosine (continued)

hybridization bag, ensuring that the membrane is fully covered. Before sealing the membrane in the bag remove as many air bubbles as possible. Incubate the blocked membranes with the labeled SH2 domain for 1 hour at room temperature with gentle shaking or agitation. Keep the membrane protected from light.

Remove the membrane from the bag, and rinse the membrane briefly with TBST buffer. Wash the membrane for 20 minutes with TBST using gentle agitation. Change the TBST two times during this 20-minute wash. Use enough TBST to cover the membrane.

Following the 20-minute wash, remove the membrane from the wash buffer and detect with Chemiluminescent Detection Substrate (ECL – Amersham or equivalent). Expose to film according to the substrate product insert. Higher sensitivity may be obtained by using a more sensitive substrate such as SuperSignal West Dura or SuperSignal West Femto Substrates from Pierce.

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