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METHODS PAGE

Method for demonstrating Src activation by Western Blotting

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The activation state of Src kinase and homologous kinase family members can be determined by immunoprecepitation and either a kinase assay or Western blotting for active Src (pY416). The method used in our lab follows.

1. Apparatus

Apart from appropriately treated cells or tissue, routine lab glassware, volumetric apparatus, a setup to complete routine western blotting, and preparation of lysates, you will need a sample rotator for small tubes (e.g. Labquake rotator from Thermo Scientifc), a 2ml Potter-Elvehjem Tissue Homogenizer, and a sonic dismembrator with a tip (from Fisher Scientific or other suppliers).

2. Reagents

1. Src antibody. (For Src family, SC-18, Santa Cruz biotechnology,(use 1:500 for WB)
2. pTyr416 antibody (Cell Signal Cat. #2101) (use 1:1000 for WB)
3. Protein A sepharose beads (Sigma Aldrich)
4. Lysis buffer. This is prepared as described previously (14, 30, 39).

   50 mM Tris}HCl (pH 7.5),

   150 mM NaCl,

   1% (w/v) Triton X-100,

   0.1%(w}v) NaN3,

   1 mMEGTA,

   0.4 mM EDTA,

   0.2 mM Na vanadate

   This may be prepared as a 10x stock with phosphatase and protease inhibitors added to the diluted 1x buffer before use. Make 2 ml working buffer per sample, and keep on ice.
5. Binding buffer, Wash buffer: the detergents can be omitted for this. Keep the phosphatase and protease inhibitors in these.
6. Protease inhibitor (e.g. cocktail from Sigma or Complete tablets from Roche). Use these as a 1x final concentration.

3. Procedure

1. Lyse samples in lysis buffer. Usually 15-20 mg of tissue in 1 ml is adequate.
2. Homogenize in the Potter-Elvehjem Tissue Homogenizer at 30 rpm. 5-7 strokes are usually adequate.
3. Sonicate immediately for 5 seconds at a

setting of 5.

4. Spin down samples at 10,000 g for 10 min at 4°C.
5. Separate the supernatant and quantify proteins.
6. Add 1mg lysate protein (about 1/10 final volume) to 800 microliters of binding buffer, and to this add 5 µg/ml primary antibody for 2 h at 4°C.
7. Meanwhile preweigh 4 mg Protein A beads per sample, swell them in wash buffer, and presoak these in wash buffer by making a slurry such that the final volume is 100 microliters for every 4 mg.
8. Add 4 mg of protein A beads for 1 h to the tube containing the lysate-antibody mix.
9. Touch spin down the beads at 10,000g and wash three times in wash buffer.
10. Boil the beads in 60 microliters of 2 x Laemmli sample buffer, spin them down and analyze the supernatant by Western blot.
11. For western blot analysis the blocking should be done in 5% BSA for the phosphoblot, This should be done before stripping and blotting for total Src as a loading control.
12. For storage the supernatant may be transferred to a new tube and frozen at –20°C. This should be boiled for 5 min before loading.
13. The band intensity of the phopho bands is normalized to that of the total Src bands and can be depicted as in comparison to that of basal or control conditions.