IMMUNOPRECIPITATION PROTOCOL

A. Preparing Lysates

Lysates from cell

1. Aspirate media. Treat cells by adding fresh media containing modulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer to each plate (10 cm) and incubate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate on ice three times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 4°C, 14,000 rpm and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

Lysates from tissue

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round bottom microfuge tubes and immerse in liquid nitrogen to "snap freeze". Store samples at -80°C for later use or keep on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, add ~300 µl lysis buffer rapidly to the tube, homogenize with an electric homogenizer.
4. Rinse the blade twice with another 300 µl lysis buffer for each rinse, then maintain constant agitation for 2 hours at 4°C (e.g place on an orbital shaker in the refrigerator).
5. Centrifuge for 20 minutes at 4°C, 12,000 rpm in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, discard the pellet.

B. Immunoprecipitation

1. On ice, in a tube add 10-500 µg cell lysate plus the recommended amount of antibody. These amounts will be chosen depending on the abundance of the protein and the affinity of the antibody for the protein, typically in a pilot experiment where a fixed amount of protein is precipitated by increasing amounts of antibody.
2. Incubate the sample with the antibody between 1 hour to overnight, at 4°C, preferably under agitation.
3. Meanwhile prepare the Sepharose beads. If using a monoclonal antibody choose protein G-coupled Sepharose beads, if using a polyclonal antibody protein A-coupled Sepharose beads are usually suitable. If the beads come as a powder incubate 100 mg of beads in 1 ml PBS 0.1M, wash for one hour so they swell

up, then centrifuge, remove the supernatant and discard. Add 1ml PBS-BSA 1% w/v, mix for one hour and rinse in PBS twice. Remove the supernatant and add 400 μl of buffer made with protease inhibitors. The slurry is now ready for use. It can be stored at 4°C for a few days; for longer periods keep the beads in PBS with 0.02% azide. You can also buy pre-swollen beads as slurry ready for use.

4. Mix the slurry well and add 70-100 μl of the beads to each sample. Always keep samples on ice.
5. Incubate the lysate-beads mixture at 4°C under rotary agitation for 4 hours.
6. When the incubation time is over, centrifuge the tubes, remove the supernatant and wash the beads in lysis buffer three times (each time centrifuging at 4°C and removing the supernatant).
7. Finally, remove the last supernatant and add 25-50 μl of 2x loading buffer. Boil at 95-100°C for 5 minutes to denature the protein and separate it from the protein-A/G beads, then centrifuge and keep the supernatant where the protein is now.
8. You can then freeze the samples or run them on a SDS-PAGE. Analyze sample by western blot.