Immunoprecipitation Protocol

Note: If using a pre-existing cell lysate, begin protocol at step 5.

1. Wash adherent cells twice in the dish or flask with ice-cold PBS and drain off PBS. Wash non-adherent cells in PBS and centrifuge at 800 to 1000 rpm in a table-top centrifuge for 5 minutes to pellet the cells.

2. Add ice-cold modified RIPA buffer to cells (1 ml per 10 7 cells/100 mm dish/150 cm2 flask; 0.5 ml per 5 x 106 cells/60 mm dish/75 cm2 flask).

3. Scrape adherent cells off the dish or flask with either a rubber policeman or a plastic cell scraper that has been cooled in ice-cold distilled water. Transfer the cell suspension into a centrifuge tube.

4. Gently rock the suspension on either a rocker or an orbital shaker at 4 degrees Celsius for 15 minutes to lyse cells.

5. Centrifuge the lysate at 14,000 x g in a precooled centrifuge for 15 minutes. Immediately transfer the supernatant to a fresh centrifuge tube and discard the pellet.

6. To prepare protein A or G agarose/sepharose, wash the beads twice with PBS and restore to a 50% slurry with PBS. It is recommended to cut the tip off of the pipette when manipulating agarose beads to avoid disruption of the beads.

7. Pre-clear the cell lysate by adding 100 ul of either protein A or G agarose/sepharose bead slurry (50%) per 1 ml of cell lysate and incubating at 4 degrees Celsius for 10 minutes on a rocker or orbital shaker. Pre-clearing the lysate will reduce non- specific binding of proteins to the agarose or sepharose when it is used later on in the assay.

8. Remove the protein A or G beads by centrifugation at 14,000 x g at 4 degrees Celsius for 10 minutes. Transfer the supernatant to a fresh centrifuge tube.

9. Determine the protein concentration of the cell lysate, e.g. by performing a Bradford assay. Dilute the cell lysate at least 1:10 before determining the protein concentration because of the interference of the detergents in the lysis buffer with the Coomassie-based reagent.

10. Dilute the cell lysate to approximately 1 ug/ul total cell protein with PBS to reduce the concentration of the detergents in the buffer. A more concentrated cell lysate (i.e., 10 ug/ul) may be necessary to immunoprecipitate a protein, which is found in low levels in a cell model. Add the recommended volume of the immunoprecipitating antibody (see datasheet for detailed information) to 500 ul (i.e., 500 ug) of cell lysate. The optimal amount of antibody that will quantitatively immunoprecipitate the protein of interest should be empirically determined for each cell model.

11. Gently rock the cell lysate/antibody mixture for either 2 hours or overnight at 4 degrees Celsius on a rocker or an orbital shaker. A 2 hour incubation time is recommended for the immunopecipitation of active enzymes for kinase or phosphatase assays.

12. Capture the immunocomplex by adding 100 ul protein A or G agarose/sepharose bead slurry (50 ul packed beads) and gently rocking on either a rocker or orbital shaker for either 1 hour or overnight at 4 degrees Celsius. In many instances, immunocomplex capture can be enhanced by adding 2 ug of a bridging antibody (e.g., rabbit-anti-mouse IgG). This is especially important with antibodies, which bind poorly to protein A, such as mouse IgG1 or antibodies generated in chicken.

13. Collect the agarose/sepharose beads by pulse centrifugation (i.e., 5 seconds in the microcentrifuge at 14,000 rpm). Discard the supernatant and wash the beads 3 times with 800 ul ice-cold modified RIPA buffer. Occasionally, washing with modified RIPA buffer will strip the immunocomplex off of the agarose/sepharose beads. In these cases, washing with the milder PBS is recommended.

14. Resuspend the agarose/sepharose beads in 60 ul 2x sample buffer and mix gently. This will allow for sufficient volume to run three lanes. The agarose/sepharose beads are boiled for 5 minutes to dissociate the immunocomplexes from the beads. The beads are collected by centrifugation and SDS-PAGE is performed with the supernatant. Alternatively, the supernatant can be transferred to a fresh microcentrifuge tube and stored frozen at –20 degrees Celsius for later use. Frozen supernatant should be reboiled for 5 minutes directly prior to loading on a gel.