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Immunoprecipitation

Immunoprecipitation (IP) studies can be performed using protein isolated from cultured cells or tissues collected from animals. Use whichever protein isolation step fits your needs. Note that RIPA may not work for all proteins. Integral membrane proteins may require different lysis buffers.

Protein Isolation from Cultured Cells

1. Prepare cells in 6 well dishes as necessary for the experiment.
2. Aspirate media and wash with 1 ml cold PBS.
3. Aspirate wash and add 500 µl RIPA + inhibitors
4. Scrape cells and transfer to a microfuge tube.
5. Rotate at 4° C for 30-60 min
6. Shear DNA by drawing through a 21 gauge needle. Avoid making “foam”.
7. Quantify protein using BCA assay. Proceed to “Immunoprecipitation”.

Protein Isolation from Tissue Samples

1. Start with approximately 15-25 mg frozen tissue (keep tissue frozen until sonication)
2. In a microfuge tube, add 500 µl RIPA + inhibitors to the frozen tissue chunk
3. Using the tip sonifier, sonicate at output 3 and 50% duty cycle for 10-30 pulses (only until tissue is completely obliterated). If tissue remains after 30 pulses, return to ice to cool and rest. Avoid getting the tip too close to the surface of the liquid as this can cause foaming which should be avoided.
4. When all samples have been sonicated, rotate at 4° C for 30-60 min
5. Shear DNA by drawing through a 21 gauge needle. Avoid making “foam”.
6. Quantify protein using BCA assay. Proceed to “Immunoprecipitation”.

Immunoprecipitation

1. Move 500 µg of protein into a fresh tube and dilute to 1 µg/µl with RIPA + inhibitors (500 µl total volume)
2. To “Pre-clear” the sample, add 50 µl protein A-conjugated agarose beads (Pierce) to each sample
3. Rotate on tube rotator 60 min at 4° C
4. Spin 4000 rpm for 10 min at 4° C. Transfer supernatant to new tube.
5. To the supernatant, add 4 µl of appropriate antibody. (This antibody amount will vary depending upon the antibody you are using and will need to be determined empirically.)
6. Rotate 4° C overnight (overnight works best and is convenient, shorter incubations may work for your experiment).
7. Add 50 µl protein A-conjugated agarose beads. Rock 4° C 60 min.
8. Spin 2500 rpm for 1 min at 4° C.
9. Aspirate supernatant (careful not to disturb beads) and discard.
10. Add 500 µl of ICE COLD RIPA + inhibitors. Very briefly vortex. Spin 2500 rpm for 1 min at 4° C to pellet beads.
11. Repeat step #9 twice more (a total of three washes; if you are getting high background, do more washes)

12. Aspirate last RIPA wash supernatant
13. Add 7 µl of 6X protein reducing buffer. Briefly vortex. Boil at 100° C for 5 minutes. Spin and put tubes on ice to cool. Load the supernatant onto an acrylamide gel. Proceed to Western blotting analyses.

Notes:

- Be sure to include experimental and antibody controls (irrelevant isotype-matched antibodies) to your experimental design. Positive controls (overexpressed protein), when available, are also very helpful. Including these will facilitate troubleshooting and data interpretation should multiple (or unexpected) bands appear on Western blots.
- The number of cells needed to get a sufficient amount of protein may vary depending on cell type and confluency. The same can be said for the type of tissue that you are using. Scale up or down as needed.
- If doing Co-IP studies, some protein-protein interactions may not occur in RIPA buffer. You may have to try altering the detergent concentrations.
- Be sure that protein A will work for your Ig isoform – protein A will work for IgG – you may need to use protein L or G coupled beads.
- It is important to keep the sample cold during the incubation and washes as this will reduce non-specific interactions with the beads.
- If high background is an issue, try adding some pre-immune serum (irrelevant IgG) to the "Pre-clear" step.
- $NaVO_4$ and NaF are phosphatase inhibitors and may be excluded if protein phosphorylation is not an endpoint to your experiment.
- Pierce brand agarose beads have worked best in my experiments.

RIPA + Inhibitors (make only as much as you will need for 1 experiment)

20 mls RIPA
25 µl $NaV0_4$ (200 mM)
52.5 mg NaF
250 µl PMSF (10 mg/ml)
1 ml 25X complete
QS to 25 mls with RIPA
CHILL