IRS Immunoprecipitation

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Protein Isolation from Tissue Samples

1. Start with frozen tissue (keep tissue frozen until sonication-may want to pulverize tissue before sonication)
2. In a microfuge tube, add 500 µl RIPA + protease/phosphatase inhibitors (see recipe at end of protocol) to the frozen tissue chunk or pulverized tissue.
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. When all samples have been sonicated, rotate at 4° C for 30-60 min.
4. Quantify protein using BCA assay (see BCA protocol). Proceed to "Immunoprecipitation".

Immunoprecipitation

1. Move 1-2 milligrams of total protein into a fresh microfuge tube and dilute to 4 µg/µl with RIPA + inhibitors (500 µl total volume if you have sufficient protein)
2. To "pre-clear" the sample, add 50 µl protein A-conjugated agarose beads (Pierce catalog #0020333) to each sample
3. Rotate on tube rotator 60 min at 4° C
4. Spin 4000 rpm for 10 min at 4° C in the microfuge. Transfer supernatant to new tube.
5. To the supernatant, add 5 µl of appropriate antibody (IRS-1).
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 at 4° C for 60 min.
8. Spin 2500 rpm in the microfuge 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 in the microfuge 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 SDS polyacrylamide 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 in case multiple (or unexpected) bands appear on Western blots.

- 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. (can purchase through Fisher)

SOLUTIONS

RIPA (the easy way)
98.5 mls PBS (non-sterile)
1 ml NP-40
0.5 ml 20% SDS
filter sterilize

RIPA + Protease 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
} phosphatase inhibitors*
250 µl PMSF (10 mg/ml)
1 ml 25X complete (Roche)
} protease inhibitors*
QS to 25 mls with RIPA
CHILL

6X Reducing Buffer
0.35 M Tris-HCl (pH 6.8)
10% (w/v) SDS
36% (v/v) glycerol
5% β-mercaptoethanol)
0.012% (w/v) bromophenol blue
and 0.3% (v/v) Tween-20
QS with 1X PBS

*Protease and Phosphatase inhibitors should be added fresh on the day you will use them.