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Immunoprecipitation protocol:

Resin for IP: Immobilized protein-G from Pierce (cat #20398, 2ml), 50% w/v slurry

Buffer 1: 50 mM TrisHCl, 150 mM NaCl, pH 7.4, 0.1 % CHAPS
(add 1 mM $CaCl_2$ to buffer 1 if necessary to detect Ca-dependent protein interactions)

Buffer 2: 50 mM TrisHCl, 300 mM NaCl, pH 7.4, 0.1% CHAPS
(add 1 mM $CaCl_2$ if needed, as above)

1X reducing Laemmli buffer: 50 mM TrisHCl pH 6.8
(add bromophenol blue 10 % v/v glycerol
to give blue color) 2 % w/v SDS
1.2 M beta-mercaptoethanol.

a) Preparation of resin (may be done one day in advance):
- Resuspend well the resin slurry by rotation of the bottle. Try to avoid vortexing.
- Centrifuge ~ 500 µl slurry (equals to 250 µl final resin) at ~3000g x 1 min
- Carefully remove the supernatant.
- Resuspend the resin in 1 ml of buffer 1, invert several times to make an homogeneous suspension
- Centrifuge at 3000g x 1 min
- Repeat the resuspension + centrifugation an additional 2 times
- Resuspend the final pellet in 500 µl buffer 1 --> this is the resin ready for IP

b) Preparation of sample:
<u>- Conditioned medium:</u> centrifuge medium at 5,000g x 10min to eliminate all solid debris. Try to use serum-free medium to avoid non-specific capture of serum immunoglobulins. Supplement the medium with protease inhibitors but without EDTA if you are trying to detect Ca/Mg-dependent protein interactions.

<u>- Tissues or cell pellets:</u> extract the solid material in a suitable amount of lysis/extraction buffer. A mild extraction buffer is 50mM TrisHCl, 100 mM NaCl, 1% CHAPS, pH 7.6. Resuspend the material in extraction buffer (approx 1 ml buffer / 50 mg solids), vortex and let rotating at 4c for ~30min. Sonicate briefly (2 x 30sec pulses) if necessary. Add protease inhibitors if necessary. Extraction conditions must be as mild as possible when the objective is to detect protein interaction. If the objective is to achieve solubilization and recovery of the desired protein, increase the strength of the extraction conditions (e.g., instead of CHAPS use 0.1 % sodium deoxycholate + 1 % Nonidet P-40, or extract tissues with ethanolamine pH=8 or with chaotropic solutions)

After preparation of the sample, measure protein concentration and adjust to ~ 1-2 mg/ml with buffer 1.

<u>c) IP protocol (see flowchart at the end):</u>

1. Pipette 200 µl of resin slurry (~100 µl resin) into one eppendorf tube (tube 1). Pipette 100 µl of resin slurry into three additional eppendorf tubes (tubes 2, 3 and 4). Centrifuge them at 3000g x 1 min and remove the supernatants. Label the tubes 1 to 4
2. Add 600 µl of sample (~300 µg protein) to the resin in tube 1. Mix well and incubate 30 min with rotation at room temp. Centrifuge resin and carefully recover the supernatant (--> "pre-cleared sample"). Leave the precleared sample on ice. Discard tube 1 with resin (--> "non-specifically bound sample") or keep it for later analysis by PAGE.
3. Add 300 µl of buffer 1 to the resin in tube 2. Mix well.
4. Add 300 µl of buffer 1 containing 10 µg of primary antibody to tubes 3 and 4. Mix well.
5. Incubate tubes 2-3-4 for 30-60 min with rotation, at room temp. Centrifuge resins after incubation and discard supernatants. Carefully wash each tube 2 times with 600 µl of buffer 1. Resuspend the final pellets in 100 µl of buffer 1.
6. Add 300 µl of precleared sample to the tubes 2 and 3 (tube 4 has antibody but will not have sample). Add 300 µl of buffer 1 to tube 4. Mix tubes well and leave at 4c with rotation for 2h to overnight.

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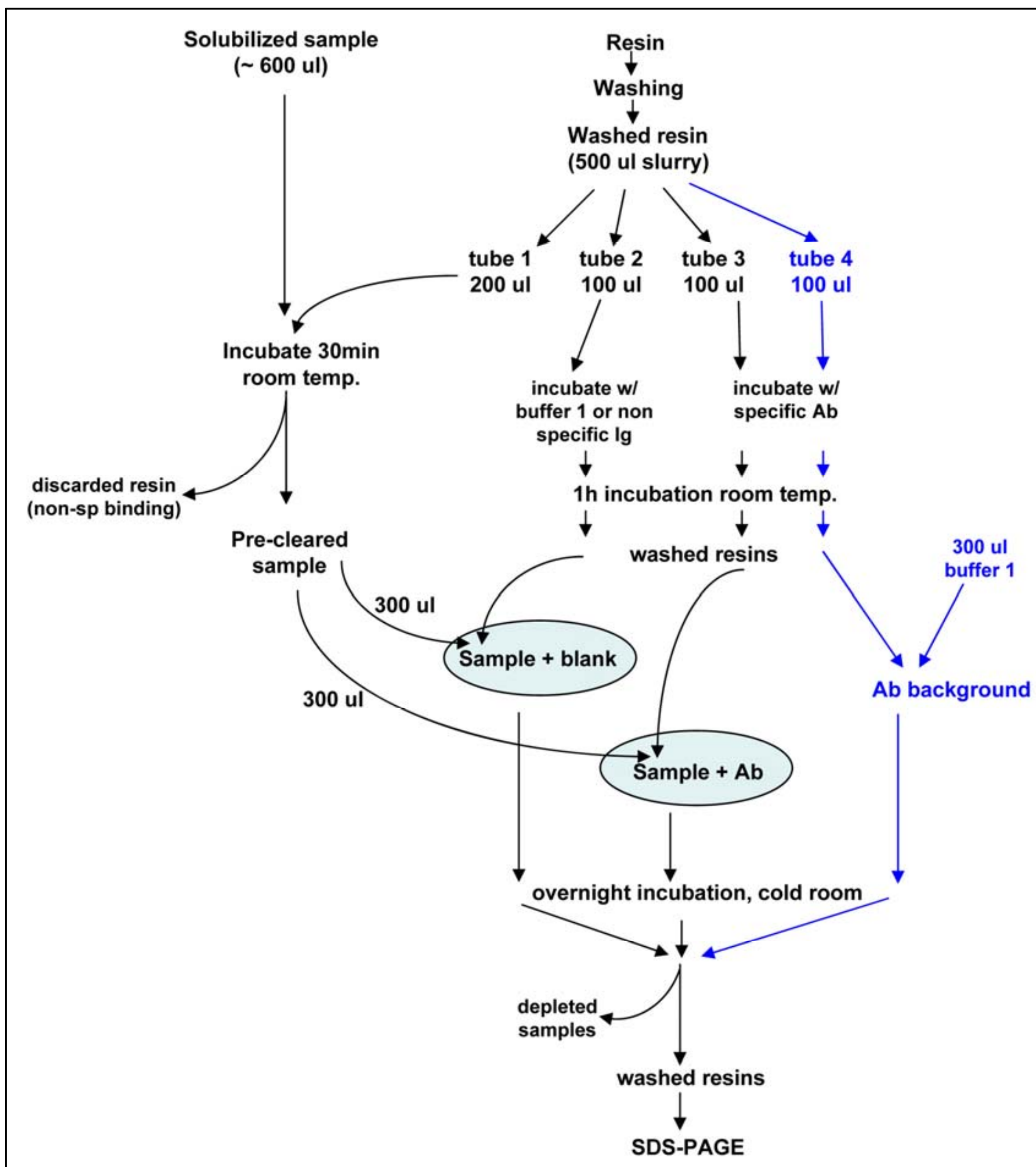
7. Centrifuge all tubes and carefully recover supernatants (-->"depleted samples")
8. Wash all tubes three times with 1 ml of buffer 2 each time.
9. After washing, resuspend the resins in a final volume of 100 µl of buffer 2 and check that the final volume of resuspended slurry is similar in all tubes. Transfer the samples to small eppendorf tubes that can fit in a PCR machine (do not use thin-wall tubes)
10. Centrifuge the small eppendorfs at 6000 g x 1 min and remove as much as possible of the supernatants. You should have ideally 50 µl of solid resin per tube.
11. Add to each pellet 50 µl of 1X Laemmli buffer and boil samples in the PCR machine at 95c to 100c (depending on the maximum temp allowed by the machine) for 5 minutes.
12. Quickly after boiling let tubes, cool for 30 seconds, vortex (tubes must be well-sealed) and centrifuge at 6000g x 1 min. Remove supernatants and transfer to new eppendorfs.
13. Apply the samples directly for SDS-PAGE (10 to 30 µl per lane, usually starting with 20 µl for pilot tests) or freeze them for 1-2 days before using. You can also apply ~10 µg of "pre-cleared" sample, "depleted" sample and "non-specifically captured" sample to test the efficiency of the IP procedure.



Tube 4 (blue lines) is optional and used to determine which bands in the blot correspond to primary Ab.