Immunoprecipitation protocol

Note: Listed is my standard protocol for immunoprecipitation from detergent extracts of metabolically labelled cells. Generally, I use between 10^6 and $2x10^7$ cell equivalents for each precipitation, depending upon the expected expression level. You will probably have to play around with cell number and volumes, etc. I would use 1ml of lysis buffer for $1x10^7$ cells, but up to a maximum of $4x10^7$ cells; this also varies from cell line to cell line, but is a good general rule.

The other thing you will have to play around with is in terms of the extract. The protocol below provides an extract that is generally considered a post-nuclear lysate. If you are concerned with proteins present in the nuclear fraction, you may prefer to first fractionate the cells into nuclear and cytoplasmic fractions prior to performing the immunoprecipitation. A protocol for doing this is listed at the end; I would then simply adjust the buffer conditions for each of the lysates to be similar to that described below. Furthermore, it is generally believed that both of these protocols exclude the cytoskeleton from the extracts, i.e. the cytoskeleton generally remains insoluble during these procedures and is discarded.

Reagents

Lysis buffer

50mM Tris pH 7.4
150-300mM NaCl (we generally use 300mM to reduce non-specific binding, but of course some interactions may be sensitive to this amount of salt)
1% (w/v) Triton X-100 (or NP-40)
0.02% (w/v) NaN_3
Protease inhibitor cocktail (add just before use)
If using low # of cells, add bovin serum albumin to 0.1% (w/v)

Wash buffer

Same as lysis buffer, but with 0.05% (w/v) Triton X-100 and no protease inhibitors.

Protein A-Sepharose or Gammabind G-Sepharose

This depends on your antibody; if you have a rabbit polyclonal serum or a mouse IgG2a or IgG2b monoclonal, use protein A-sepharose - it's cheaper and more efficient. If you have anything else (including IgG1 or IgG3 mouse monoclonals, rat monoclonals or serum, or human monoclonals), use Gammabind G-Sepharose - it has a broader spectrum of immunoglobulin species binding capacity. I buy both from

Pharmacia. For other species, check the reactivity charts in Harlowe and Lane.

Antibody

Protease Inhibitor Cocktails
My cocktail is rather extensive and anal:

*Phenyl methyl sulfonyl fluoride (PMSF) or derivative (such as **AEBSF**, from ICN) - 0.25mM final concentration from 50mM stock (PMSF in EtOH; AEBSF in H_2O)
*E-64 (from Sigma or Boehringer Mannheim) - 2.5μg/ml final concentration from 0.5mg/ml stock in DMSO
*iodoacetamide - 10mM final concentration (this will kill some enzymes and antibody reactivities, so be careful)
*aprotinin (from Sigma, ICN or BM) - 33μg/ml final concentration from 6.6mg/ml stock in H_2O
leupeptin (from Sigma, ICN or BM) - 10μg/ml final from 2mg/ml stock in H_2O
tosyl-lysyine chloromethyl ketone (TLCK) - 0.1mM final from 20mM stock in EtOH
tosyl-phenylalanin chloromethyl ketone (TPCK) - same as above
pepstatin A (from Sigam, ICN or BM) - 5μg/ml final from 1mg/ml stock in DMSO

Protocol

1. Lyse up to $4x10^7$ cells in 1ml lysis buffer on ice, 30 minutes (see labeling and lysis protocol).

2. **FOR LYMPHOID CELL LINES ONLY:** Spin out nuclear pellet 30 seconds at half speed in a microfuge at 4˚C

3. Clarify lysate by centrifugation for 10-15 minutes at full speed in a microfuge at 4˚C

4. In meantime, incubate 50μl of protein A-sepharose or gammabind G-sepharose with 500μl-1ml of hybridoma supernatant, or 1-3μl of serum or ascites; turn end-over-end for at least 30 minutes at room temp or 1 hr at 4˚C (Note: for serum or ascites, can replace this step by adding stuff directly to the sample; to keep everything similar, however, doing it this way is advised).
   - after incubation, pellet beads (30 seconds, half-speed in microfuge) and wash 2X with Wash buffer; remove all supernatant

5. Add lysate (fraction or all of it) to pellet of antibody-coated beads. Incubate end over-end at 4˚C for at least 1 hr; 2 hrs is best; some antibodies are better

overnight; in some cases, incubation at room temp is fine (you should play around and figure out which is best for your system) - if you don't know better, do it in the cold

6. At end of incubation, pellet beads (30 seconds, half-speed in microfuge); if you want to do a second antibody incubation, save supe and add it to a new set of beads
   - wash 4X with cold wash buffer, vortexing at each wash
   - wash 1X with cold PBS
   - analyze pellet however you want (SDS-PAGE, fluorometry, etc.)

Controls:

- I always do a non-specific antibody in addition to the specific one; if it is a serum, I use normal rabbit serum (pre-immune); if it is a monoclonal antibody, I use one of a similar isotype but with no reactivity to the cells used
- it is often helpful to do one or two pre-clearings as well. In this case, you can use either protein A (or gammabind G) beads alone or pre-bound to non-specific antibody (normal rabbit serum or non-specific monoclonal), and incubate the beads for 1hr at 4˚C, prior to spinning out and transfering supe to tube with desired antibody. You'll have to see how dirty the gels are to know if this is necessary. **NOTE: THIS IS ABSOLUTELY NECESSARY FOR B-LYMPHOID LINES OR SPLEEN CELLS TO CLEAR OUT ENDOGENOUS IMMUNOGLOBULIN: YOU MAY NEED TO PRE-CLEAR SEVERAL TIMES, AND YOU MAY NEED TO USE ANTI-IG ANTIBODIES DURING THESE STEPS!!!!!!!**

Simple Nuclear/Cytoplasmic fractionation for small number of cells (<10^7)

1. Wash cells with cold PBS and collect by centrifugation

2. Resuspend cells in 1ml cold PBS and transfer to microfuge tube;
   - spin 15 seconds at 4˚C, full speed or 1 minute at 1/3 speed

3. Resuspend cells gently in 0.4ml of ice cold buffer A (10mM Hepes pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, protease inhibitor cocktail)
   - incubate on ice for 15 minutes

4. Add 25ml of 10% (w/v) NP-40 (or triton X-100) and vortex for 10 seconds

5. Spin 30 seconds at half-speed in the microfuge at 4˚C

6. Remove supernatant; add NaCl to 0.15M and save as “cytoplasmic extract”

7. Pellet; resuspend in 200ml of ice cold buffer C (20mM Hepes pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, protease inhibitor cocktail)
- rotate end-over-end for 15 minutes at 4˚C

8. Spin 5 minutes at full speed in microfuge at 4˚C; collect supernatant and save as “nuclear extract”; pellet may contain DNA, cytoskeleton and other insoluble crap

9. Dilute nuclear extract and add stuff to it and to cytoplasmic extract to make buffer conditions similar to immunoprecipitation protocol