

IMMUNOPRECIPITATIONS

The conditions to be used for IPs depend upon the nature of the epitope(s) recognized by the antibodies or antisera. Our anti-hsp70 monoclonal antibodies (2A4; 3A3; 4G4 and 5A5) only work under "native", or relatively gentle conditions, whereas some antibodies, such as C92, will still work in the presence of significant concentrations of SDS. Below are several different methods; the antibodies that we currently use in the lab are listed in parentheses beside the techniques in which they work. If you are testing a new antibody/antisera, it is advisable to test several of these methods to see which, if any, works best.

Denaturing precipitations (C92):

1. Label 60 mm dish of subconfluent cells with ^{35}S -methionine or other radioactive amino acid (rule of thumb; 2 hours with 45 μCi).
2. Wash cells 2x with ice cold PBS, scrape off in PBS, and pellet.
3. Solubilize pellet with 150 μl 1x Laemmli buffer without dyes, boil for 10 minutes, and sonicate to shear DNA.
4. Pre-clear lysate by spinning in microfuge for 10 min.
5. Dilute 50-100 μl of sample 1/10 in RIPA buffer so that the final SDS concentration is 0.1% (final volume 500 μl - 1 ml). Add primary antibody to sample (amount varies depending upon the antibody; for C92, 1 μl of ascites is sufficient). Incubate for 2hrs. at 4°C on shaker.
6. If you are using a monoclonal antibody, add 5 μl goat anti-mouse IgG secondary antibody and incubate for 30 min at 4°C on shaker.
**NOTE This is not necessary for all monoclonals or for most polyclonal sera; this step is included when using antibodies that do not bind protein-A agarose. If you are not sure about your antibody, include this step.
7. Add 40 μl of 50% Protein-A agarose in RIPA/SDS. Incubate 30 min at 4°C on shaker (prior to adding this, pellet Protein-A agarose and wash 2x with RIPA).
8. Pellet Protein A beads. Wash 5x with 500 μl RIPA/SDS; resuspend gently. After washes, pellet beads.
9. Add 35 μl 2x Laemmli buffer (with dyes) to beads, resuspend and boil 10 min. Vortex, then remove and save supernatant. Add 35 μl H₂O to beads, resuspend, and boil for 10 min. Vortex, remove supe and add to original supernatant with dyes. Run on standard SDS-PAGE gel and fluorography.

1x Laemmli buffer:

40 mM tris
1% SDS
1% BME
100mM DTT

RIPA/SDS buffer:

150 mM NaCl
1% sodium deoxycholate
1% triton X-100
10 mM tris pH 7.4
0.1% SDS
1 mM PMSF (can also use pepstatin and aprotinin)

Nondenaturing precipitations:

There are multiple different buffers that can be considered "nondenaturing" IP buffers. Listed below are the conditions used for our hsp70 monoclonals (3A3, 4G4, 5A5). The procedure is essentially the same as above but the lysate is prepared differently.

1. Label cells, wash with PBS and scrape off plate as described above.
2. Add 500 μ l lysis buffer to cell pellet; incubate on ice 5 min and clarify lysate by spinning in microfuge for 10 min.
3. Split lysate into fractions for IP; dilute each fraction with lysis buffer to 500 μ l-1 ml.
4. Perform antibody and protein A incubations as described above (substitute protein G agarose in the case of 3A3; it is an IgG3 and binds protein G much better than A).
5. Wash 5x with lysis buffer, followed by 1-2x with RIPA/SDS.
6. After washes add Laemmli and boil as described above.