Immunopurification and Immunoprecipitation

1. Plan conditions for dissociation of sample. Some standard conditions are as follows:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Detergents</th>
<th>DTT</th>
<th>Dissociation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild IP buffer</td>
<td>1% TX-100</td>
<td>(0.5 mM)</td>
<td>4 - 25°C for 5-60 min.</td>
</tr>
<tr>
<td>Standard IP buffer</td>
<td>1% TX-100, 0.2% SDS</td>
<td>(1 mM)</td>
<td>4 - 50°C for 5-60 min.</td>
</tr>
<tr>
<td>Harsh IP buffer</td>
<td>1% SDS</td>
<td>5 mM</td>
<td>37 - 100°C for 2-5 min.</td>
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</table>

IP buffer: 50 mM Tris-HCl, 150 mM NaCl, pH 8. Protease inhibitors should be freshly added.

IP buffer can also include 1 mM EDTA (to dissociate proteins from RNA) or MgCl₂ (to stabilize protein-RNA interactions).

NP-40 can be used in place of TX-100.

DTT in “mild” and “standard” is optional (0.5-1 mM is okay for most antibodies). Other conditions may be better for your application. Conditions for IPs must be empirically determined in each case. The conditions above are intended as initial ones to be tried first. Other conditions to consider are: urea at concentrations below 0.5 M; NaCl or KCl at concentrations up to 1 M; pH of 6 to 9; addition of 0.5% sodium deoxycholate (for membrane-bound antigens); addition of 5 mM iodoacetamide instead of DTT (this sulfhydryl blocker may inactivate some enzymatic activities); addition of BSA or other blocking agents.

2. Prepare antibody-sepharose conjugate ahead of time. This may be done by:

A. Binding antibody to protein A-sepharose or protein G-sepharose.
B. Cross-linking antibody to protein A-sepharose or protein G-sepharose.
C. Using a bridge antibody to link protein A to a mouse monoclonal antibody.

To prepare enough for analytical purposes (i.e., a lane on a gel), plan for 15 µl of packed antibody-bead conjugate per IP. If more than one IP will be done with a single antibody conjugate, prepare the conjugate in batch for all the IPs, and distribute to microfuge tubes for each IP.

- Controls: prepare a preimmune, non-immune, or irrelevant-immune control.
- Some investigators prefer to incubate the antibody with the dissociated sample, and do a second incubation with protein A or G conjugate (or S. aureus cells).

3. Dilute sample in buffer, vortex, and place at desired temperature for desired time to dissociate proteins in sample. If sample was dissociated in SDS (“Harsh”), dilute with 4 volumes of IP buffer + 1.25% TX-100 + 2 mM iodoacetamide. This will bring the final concentrations of SDS and TX-100 to 0.2% and 1.0%, respectively. Iodoacetamide will oxidize free sulfhydryl groups.

- For complex mixtures and/or crude lysates, a pre-clearing step may reduce the non-specific adsorption of certain proteins to protein A or G. Do this at this step. Spin down (15 seconds in microfuge) 50 µl of IgGSorb suspension (S. aureus cells), and wash in the appropriate buffer 3 times. IgGSorb forms a tight pellet, and is conveniently resuspended by re-pipetting. Transfer lysate to IgGSorb pellet and incubate for 30 minutes at the desired temperature. Then do spins in step 4. A tight IgGSorb pellet will also trap particulates.

4. Spin in microfuge at top speed at 4°C for 10 minutes. Transfer supernatant to a new tube and repeat the spin. Two spins generate a “cleared lysate” for doing the IP.
5. Adjust the volume of the cleared lysate, if needed, depending on the number of IPs planned. Plan for 0.5 to 1.0 ml per IP. Dilute with buffer identical to that present in the supernatant.

6. Add cleared lysate to packed antibody-beads in microfuge tube. Mix using end-over-end rotator. Avoid bubble formation (do not vortex).

   • A range of incubation conditions may be used. High affinity antibodies will bind antigen at room temperature in 1 hour. Low affinity antibodies will require an overnight incubation at 4°C. Optimal conditions may lie in-between, and will be influenced by problems such as proteolysis.

7. Collect immunoprecipitate by spin in microfuge 15 seconds at top speed (temperature in step 6). The supernatant may be saved to evaluate the depletion of the antigen during the IP.

8. Wash IPs. Add at least 10 bed volumes of wash buffer, vortex, and wait 1-5 minutes (temperature in step 6). Do at least 3 washes.

   • To reduce “background” try adding to wash buffer urea at 2 M, or NaCl at 0.5 M.

9. If IPs will be run on SDS gels, do 2 washes with IP buffer alone. These washes will remove TX-100 which will interfere with SDS-PAGE.

   • A water wash may be done with exactly 10 volumes of ddH₂O, but some antigens are released from the antibody by low ionic strength solutions.

10. For SDS-PAGE, add to the beads an equal volume of 2X sample buffer. Boil 5 minutes. Spin at top speed for 2 minutes. Load gel. A few beads in sample well will not interfere with electrophoresis.