Protein/Protein and Protein/RNA Co-Immunoprecipitations

(Kenny Watkins and Alice Barkan , updated 12/09)

Part 1 is a simple coIP method for directed coIP assays, to determine whether two known proteins (or a protein and RNA) are found in a complex.
Part 2 is an elaboration of this method for the purification of complexes for identification of unknown components by mass spectrometry.

The protocol for protein/RNA coIPs ("RIP-chip") is described in detail in: A Barkan, Genome-Wide Analysis of RNA-Protein Interactions in Plants. Methods Mol Biol. 2009;553:13-37.

Part 1: Directed coIP assays to determine whether two known proteins (or a known protein and RNA) coimmunoprecipitate.

Immunoprecipitations involve incubating a lysate with an antibody specific for a molecule of interest, then incubating with beads/cells coated with protein A (which bind all IgGs) and pelleting the cells/beads. Traditionally, people used formalin-fixed StaphA cells, which have Protein A on their cell wall, for the second precipitation step. Nowadays, Protein A Sepharose/agarose beads are often used instead. But, we have found that the cells often work the best for routine assays, and are also inexpensive. The protocol below uses Staph A cells; modifications for other types of protein A matrices are described at the end of the procedure.

Reagents

co-IP Buffer (prepare 100 ml without the aprotinin and store at room temp)
20 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
0.5% NP-40
5 μg/ml aprotinin (add fresh to aliquot used for each experiment)

1.5X SDS sample buffer (prepare 1 ml. Stored at –20˚, and use for several months)
15% glycerol
60 mM Tris-HCl pH 6.8
3% SDS
7.5% ß-mercaptoethanol

Formalin-Fixed Staph A cells Sold as a 10% suspension . (We use IgG Sorb (Cat# IGSL 10) from The Enzyme Center.) (or use Protein A Dynabeads or Protein A Sepharose- methods included below)

Antibodies to the target proteins.
Antibodies that have been affinity purified against the antigen are ideal, because the majority of the IgGs will be directed against the protein of interest, reducing background, and decreasing the amount of IgG bands in the gel. However, crude serum can be used if it has a high specificity and affinity for the antigen. You will need more Protein A to quantitatively precipitate the IgGs if you use crude serum.

Cell/Organelle lysate (as concentrated and as fresh as practical. For chloroplast stroma: we routinely use extract at a concentration of ~5 mg/ml protein, stored in aliquots at –80˚. This can be stored for up to ~6 months without apparent loss of quality.)

All steps should be done on ice or at 4 ˚C unless otherwise indicated.
Use filtered solutions, gloves and filter tips when planning to analyze RNA from the Co IP's.
Avoid bubbles throughout this procedure, as this denatures proteins.

1. Lysate preparation. Details will differ for particular experiment, but should always avoid conditions that could disrupt complexes (i.e. no ionic detergents, salt no higher than 150 mM) and should maintain the protein concentration as high as possible (to minimize complex dissociation). Store the extract **in aliquots** at -80°.

For analysis of stromal protein complexes, and detection of coIPing proteins by western, 0.5 mg of stromal protein per IP is a good starting point. This will contain ~50 ng of your typical "regulatory" protein (e.g. CRP1, splicing factors, etc). A good antibody can detect less than 10 ng of protein on a Western.

For analysis of coIP'd RNAs, you could get away with less stroma than this.
For Mass Spec analysis of coIPd proteins, you may want to start with more stroma than this; in this case, it is best to do an additional enrichment step prior to the coIP, to reduce the level of irrelevant, abundant contaminants. (more on this below).

2. Wash Staph A cells/beads. This is critical because it washes away unbound Protein A, which would otherwise titrate out the antibody.

Shake the bottle of cells to give a homogeneous suspension. Remove enough to cover needs for ~2 days days. Divide between several microfuge tubes, no more than 0.8 ml per tube. Mark their volume on the tube. Spin them down ~1 minute in microfuge at top speed. Resuspend to original volume in co-IP buffer (first wash).

Pellet again and resuspend in coIP buffer to initial volume (second wash).
Pellet cells again and resuspend in coIP buffer to initial volume (third and final wash). Combine the aliquots. Store at 4° for up to 2 days.
(Staph A pellets are difficult to resuspend, so it is convenient to split the cells between a number of tubes. Pipet up and down vigorously to resuspend the cells during the washes.)

3. Pre-Clear Lysate. ***This is critical to reduce background.***
Bring the volume of lysate to be used for the coIP to 100-200 μl by adding cold coIP buffer.

(i) Spin the extract/lysate for 10 min at top speed in microfuge, to remove any insolubles.
THIS STEP IS VERY IMPORTANT TO REDUCE BACKGROUND CAUSED BY AGGREGATED PROTEIN and MUST be done immediately prior to the IP.

(ii) To reduce background further (highly recommended), further preclear the supernatant as follows: Pellet 100 μl of the washed StaphA cell suspension. Discard the sup.
Resuspend the pellet in the cleared lysate. Place on ice for 10 minutes.
Spin cells at top speed in microfuge. Use the sup for the IP.

4. Antibody Binding.

Add antiserum to lysate. Mix gently. Store on ice for 1 hr, with occasional gentle mixing.

The amount of antiserum will vary with the serum and protein sample. 10 μl of a typical affinity purified antibody is a good starting point. 20 μl of crude serum would be a good starting point.

The more IgGs, the greater the amount of StaphA cells required to recover all of those antibodies, and the larger the IgG bands in the gel. Resuspending large staphA pellets is difficult, and the IgG bands can obscure and distort other bands in their vicinity. So, crude serum or total IgGs are best avoided, if possible.

5. Staph A binding.

Add 100 μl of washed Staph cell suspension to each tube, if using antigen affinity purified antibody. Use 250 μl of washed Staph A cell suspension to each tube if using crude serum or total IgGs. (you'll need more StaphA to bind up all of the IgGs in crude serum).

Mix gently. Store on ice for 30 min-1 hr, with occasional gentle mixing.

Pellet cells in microfuge 1-2 min. Discard supernatant. (Sometimes it is useful to save the sup for Western analysis to examine the degree to which the antigen was depleted from the extract. For RNA coIPs, definitely save the sup for RNA extraction).

6. Wash cells/beads to remove material that isn't tightly bound.

Resuspend cells thoroughly in approximately 0.5 ml of coIP buffer. (it can be difficult to break up that cell pellet- be sure you do!)

Pellet cells for 1-2 minutes in microfuge. Discard supernatant.

Repeat, for a total of 3 washes. (The sups from the second and third washes can be discarded).

Note: If pilot experiments show that there is a lot of background with this method, the first StaphA wash after antibody binding can be done with higher salt: e.g. supplement the coIP buffer to 500 mM NaCl. However, this may also disrupt your complex.

7. Analyzing the results

For coIP of other proteins:

Resuspend final pellet in a minimal volume of 1.5X SDS sample buffer: use 30 μl if you had used 200 μl of StaphA cells. Use 20 μl if you had used 100 μl of StaphA cells.

Heat cells to 70^{O} for 10 minutes to denature proteins. Pellet cells for 5 minutes in microfuge. Load sup onto SDS-PAGE gel. (If using crude serum, avoid the 15 slot minigels because they are easily overloaded by the large amount of IgG.)

Do duplicate westerns: probe one to detect the "bait" antigen and the other to detect the putative partner. (Or, one western can be probed sequentially for each of the two proteins.) ***Beware- the large IgG band (~50 kDa) will bind the secondary antibody used to probe the western and will light up with most detection systems.*** So, if the proteins you are trying to

detect are in that size range, you should use the **ONE-STEP WESTERN BLOT KIT (GENSCRIPT)**. This reduces signal from the IgG bound to the membrane.

For coIP of RNAs: (see also chapter in Methods in Molecular Biology cited above**)**

Resuspend final pellet in 100 μl of Co-IP Buffer and take 10 μl for Western to check that the antigen indeed precipitated (i.e. 1/10th of IP). Pellet cells and resuspend in 1.5X SDS SB.

Extract RNA from the remaining 9/10th of the pellet fraction by adding an additional 80 μl of Co IP Buffer and 20 μl 10% SDS, 1 μl 10 μg/μl yeast tRNA, and 2 μl 500 mM EDTA. (Alternatively or in addition, you can add 1 μl of GlycoBlue (AMBION) to the pellet sample. However, it is best to apply a similar mass of pellet and sup RNA to the filter for slot-blotting to reduce artifacts associated with saturating the binding capacity of the filter; adding tRNA to the pellet sample is a good way to achieve this).

Do one phenol-chloroform isoamyl extraction and precipitate the RNA with 3 volumes of Ethanol. Store on ice for at least 20 minutes or place in freezer overnight.

Extract RNA from the supernatent fraction by adding 20 μl 10% SDS and 2 μl 500 mM EDTA (yeast tRNA is not needed). Do one phenol-chloroform isoamyl extraction and precipitate the RNA with 3 volumes of Ethanol. Store on ice for at least 20 minutes or place in freezer overnight.

Pellet RNA (15 min, top speed in microfuge), air dry and resuspend in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

SLOT BLOT ANALYSIS OF coIP'd RNA:

Resuspend RNA:
Immunoprecipitation Pellets in 300 μl TE and 100 μl 5XSSPE
Immunoprecipitation Supernatents in 600 μl TE and 200 μl 5XSSPE

Set up slot-blot manifold, using nylon membrane, 0.45μ pore size. (We use Magna nylon)
Use 100 μl of each RNA sample/slot. .
Vacuum filter RNA onto membrane.
Store the remaining sample for additional slot blots.

The following samples should be included on the slot blot:
Pellet AND sup RNAs for the experimental antibody
Pellet AND sup RNAs for a control antibody to a different protein
Total RNA extracted from an amount of stroma that is equivalent to that from which the pellet and sup RNAs were derived. The sum of the pellet and sup signals should be more or less equivalent to the stromal signal.

Remove membrane from slot blot apparatus and place RNA side-up on Whatman filter paper soaked with 5XSSPE to keep the membrane damp.
UV cross link in Stratalinker (optimal X-link button) and air dry membrane. Membrane is now ready for hybridization, using standard Northern blot hybridization conditions.

Part 2: Coimmunoprecipiation of known proteins to detect UNKNOWN partners by mass spectrometry.

1. Enrich complex-of-interest prior to the coIP.

Abundant proteins in the starting lysate will inevitably contaminate the immunoprecipitation due to physical trapping and non-specific antibody binding. When dealing with low-abundance bait proteins, the contaminants can be more abundant than the bait, and it can be difficult to distinguish true interactors from false positives. Therefore, it is time well-spent to enrich the bait complex from the crude extract prior to the immunoprecipitation. It is often convenient to do this by organelle fractionation and/or size fractionation (e.g. gel filtration or sucrose gradients). For example, the material we used for immunopurifying chloroplast splicing complexes was prepared by fractionating crude stroma on sucrose gradients, and immunoprecipitating from those fractions harboring particles of ~600-800 kDa, which contained the splicing complexes but lacked the most abundant stromal proteins. Even then, many contaminants were apparent in the mass spec data.

2. Crosslink affinity-purified antibodies to beads.

It is important to crosslink affinity-purified antibodies to the beads in order to keep the IgGs from being the predominant proteins recovered and possibly masking the detection of coIP'd proteins. Either Protein A/G agarose beads or magnetic beads (Dynabeads) can be used for this. The Dynabead protocol for crosslinking and coIP is provided after this section.

For ProteinA/G agarose:

Wash Protein A/G agarose beads (250 μl, 25% suspension, Santa Cruz Biotech) in PBS plus 0.1%TWEEN-20 (PBST). Repeat two times, for a total of 3 washes.
NOTE: Protein A/G agarose or Sepharose beads are FRAGILE! They should not be vortexed, and they should be pelleted by gentle centrifugation (3000 xg for 1 min).

Incubate with ~100-150 μg of affinity-purifed antibodies for 3 hr at 4˚C.
Remove unbound antibodies by several batch washes with PBST, followed by three washes with 0.2 M sodium borate pH 9.

Crosslink the antibodies to the beads by suspending the beads in 500 μl 0.2 M sodium borate containing 8 $mgml^{-1}$ dimethyl pimelimidate (DMP, Pierce).
Rotate the beads at 25 ˚C for one hour.
Stop the reaction by washing 3x with 0.2 M ethanolamine pH 8 and incubating the beads for 1 hour in the same buffer.

Wash the beads 3x with sterile PBS and store at 4 ˚C.

Aliquots of the beads should be taken before and after DMP treatment and analyzed by SDS-PAGE to assay for crosslinking efficiency. To do this, boil the beads in SDS sample buffer to remove antibodies that are not crosslinked, pellet beads, and run the supernatant on the gel. The stained gel will indicate whether there is abundant leaching of uncrosslinked IgGs.

If uncrosslinked antibody is detected following DMP treatment, wash the beads 3X with 0.1 M glycine pH 2.4. Then, re-equilibrated in PBS before use. This will remove some of the unbound IgGs, and reduce IgG contamination in the final prep.

3A. Coimmunoprecipitation with protein A/G agarose (or Sepharose).

The coIP method for analysis by mass spectrometry is similar to that described above for targeted coIPs, except that pre-enriched lysate is used as the starting material, and beads with crosslinked IgGs are used for the IP. Also, it is useful to do pilot experiments to determine the minimal amount of antibody beads that quantitatively immunoprecipitate the bait protein. Minimizing the amount of beads will reduce the background introduced by IgG leaching.

Remember: Protein A/G agarose or Sepharose beads are FRAGILE! They should not be vortexed, and they should be pelleted by gentle centrifugation (3000 xg for 1 min).

Pre-clear lysate: First, microcentrifuge the lysate for 10 minutes at ~12,000 x g to pellet insolubles. Transfer sup to new tube. Then, further pre-clear the lysate by incubating with 0.2 ml Protein A/G bead suspension LACKING bound antibody and equilibrated in coIP buffer. Incubate 5 minutes, 4˚C. Pellet beads GENTLY- 3000xg for 1 minute.

Rinse the antibody beads:
Pellet a sufficient quantity of antibody-coupled beads to quantitatively IP the bait protein (as determined in the pilot experiments). Resuspend in 1 ml coIP buffer. Pellet gently. Pipet off the supernatant. Repeat for a total of 3 washes.

Immunoprecipitate:
Resuspend the final rinsed antibody-bead pellet in the enriched lysate.
Rotate for 90 min at 4 ˚C.
Pellet beads gently. Save supernatant to check that the bait protein was depleted.

Wash: Wash bead pellet 5X in 1 ml coIP buffer, resuspending thoroughly each time. Mix gently (e.g. by flicking tube. Do NOT Vortex to avoid breaking the beads.

Elute bound proteins: Extract the proteins bound to the beads by resuspending them in minimal volume (~50-70 µl depending upon pellet size) 1.5X SDS loading buffer lacking DTT/ß-ME to minimize IgG elution. Heat to 70-95˚C 3-5 minutes. Pellet beads, and remove sup to new tube. **Repeat, and pool the two eluates**.

If the eluates need to be concentrated prior to running on a gel, you can use the methanol/chloroform method (Wessel and Flugge, 1984, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 1984 Apr;138(1):141-3.)

Samples can then be fractionated by SDS-PAGE (e.g. 4-15% SDS gradient gels, Biorad), stained using a method compatible with mass spectrometry, and bands excised for analysis. It is best to analyze the entire gel lane (e.g. proteins between 10 and 150 kDa) rather than just focus on bands that are visibly stained, as real interactors may not stain sufficiently to be visible.

3B. Using Protein A Dynabeads (Invitrogen) for immunoprecipitation.

Note: Protein-A Dynabeads come in a 1 ml aliquot in a glass vial. The beads seem to stick to glass *and* plastic unless a little nonionic detergent is present. When receiving a new batch, transfer the beads to a 1.5 ml eppendorf tube as follows:

- Suspend Dynabeads using a 1 ml pipetman and transfer the beads to a 1.5 ml eppendorf tube.
- Add NP-40 from a 10% filtered stock to the beads to a final concentration of 0.1%.
- Spin the beads a few seconds in the microfuge to pellet Dynabeads.
- Use the supernatant to wash the original glass container and cap to collect as much of the remaining beads as possible. Repeat a few times (spin, use sup to rinse glass etc.). You may need to use a small rubber-policeman to rub the vial's walls to get the last of the beads adhered to the glass into solution.
- Store the Dynabeads at 4 ˚C with parafilm wrapped around the top.

Using Dynabeads to collect antibody/antigen complexes: This is for coIPs in which the Dynabeads substitute for the StaphA cells and are not crosslinked to antibody. The protocol is virtually identical to that used for StaphA cells, except that the beads are collected with a magnet and they do not need to be vortexed as vigorously as StaphA cells. Instead of spinning down beads, the beads are pulled to the side of the tube by placing against the magnet for > 2 minutes.

Pre-wash Dynabeads:
3X with ~1 ml coIP buffer. Collect the beads with the magnet between each wash. Resuspend to their original volume in coIP buffer.

Note: In using the magnet to collect Dynabeads, the manufacturer suggests that it is important to leave the beads on the magnet during prewashes and washes for a consistent time **(>2 min**). Although the collection might appear to be almost instantaneous, smaller beads may take a bit longer to fully clear from solution and these beads may be disproportionately loaded with your complex (because of the kinetics of mixing).

We have found that, in general, 1 μl of affinity-purified antibody is collected with reasonable efficiency using 1-5 μl of the suspended beads at the concentration supplied by Invitrogen, after 45-60 min of rotating incubation at 4˚ C. As for all coIPs, the lysate plus antibody mixture should be kept as concentrated as possible to maximize kinetics. Invitrogen recommends that at least 1/10th of the total volume of the IP should be the packed beads to achieve maximal efficiency.

Keep in mind that the fraction of antibody-antigen complexes that are collected will be reduced if you do not include sufficient beads to bind all of the antibody in the solution. It is not beneficial to increase the amount of antibody unless you are sure that you have included sufficient beads to bind those antibodies. Pilot experiments can be performed to determining how much antibody is required to quantitatively IP the bait, and how many beads are needed to pull out a given amount of antibody. Use of affinity-purified antibody will be much more cost-effective with Dynabeads than use of crude serum, since the IgG quantity will be much lower.

Preclear lysate: Microcentrifuge for ~10 minutes at ~12,000 rpm. Carefully transfer the sup to a new tube.
Add antibody. Incubate at 4 ˚C for 1-2 hours, with occasional mixing (or rotation).

Collect antibody with Dynabeads: Add Dynabead suspension (amount determined as described above). Rotate for 1 hour at 4˚C.

Rinse beads 3X by resuspension in 1 ml coIP buffer, followed by bead collection.
Prior to the last wash, transfer the bead suspension to a fresh tube to remove proteins adhering to plastic.

Elute bound proteins prior to SDS-PAGE/Western: Elute by suspending in a minimal volume of 1.5X SDS loading buffer and heating at 95˚C for 3 min. Pull beads to side with magnet (>2 min) and transfer elute to a fresh tube. Add fresh 1.5X SDS loading buffer to beads and repeat heating step. Pull beads with magnet and combine 2nd elute with first.

Using Dynabeads with cross-linked antibodies for coIPs prior to mass spectrometry.

For subsequent analysis by mass spectrometry, Dynabeads are pre-bound with antibody (affinity-purified), crosslinked, and then used to directly IP the target antigen from lysate.

5 μl Dynabead suspension should be sufficient for each 1 μl of affinity-purified antibody.

The volumes below assume you are using 10 μl of antibody for each IP:

Wash beads:
Wash sufficient beads for your experiment (e.g. 50 μl beads for the target antibody: a negative control using a different antibody, and perhaps a no antibody control).
In this example, you will need 150 μl of beads. Therefore wash ~170 μl of bead suspension 3X with: 0.1M $NaPO_4$ pH 8 / 0.1% NP-40 and suspend to original volume in same buffer. Note: the chemistry of the cross-linker depends on not having primary amines in buffers **so Tris etc.** are avoided.

Add antibody to beads: 50 μl bead suspension is added to 10 μl affinity-purified antibodies. Rotate or mix at 4˚C for 60 min. Note: for efficient binding, continuous mixing and maintaining the highest concentration is ideal. You may find that you will need to increase the volume a bit (~150 μl) to overcome surface tension in 1.5 ml tubes in order to get good mixing.

Wash away unbound antibody. Wash beads a few times with 0.1 M $NaPO_4$ pH 8/ 0.1% NP-40. Wash beads 3X with 0.2M Triethanloamine pH 8.2/0.1%NP-40.

Crosslink antibody to beads: Make 25 mM DMP in 0.2 M Triethanloamine pH 8.2 /0.1%NP-40. Suspend each antibody-bead sample in 0.5 ml of this solution, and incubate for 45 min at room temp, with rotation.
Note: DMP is very sensitive to moisture and should be stored with parafilm wrapped around the lid and with fresh dessicant at 4˚C. The bottle should be put at room temp. for ~30 min before opening to remove sample and promptly reclosed, parafilmed and returned to dessicant at 4˚C.

Wash away unreacted DMP: wash 2X with 0.2M Triethanloamine pH 8.2 /0.1%NP-40.

Inactivate any remaining DMP: wash 2X with 0.1M Ethanolamine pH 8.2 /0.1% NP-40.

Suspend beads in 0.5 ml of 0.1 M Ethanolamine pH 8.2 /0.1% NP-40 and incubate 45 min at room temp with rotation.

Final wash: Wash beads 2-3X in coIP buffer.

Carry out the coIP. Add precleared lysate to beads. Incubate with rotation at 4˚C for 60 min.

Wash beads **SEVEN** times, including at least one transfer of the bead suspension to a fresh tube during a final wash to remove proteins adhering to plastic. (Three washes are sufficient for targeted coIP/Westerns).

Elute proteins: Elute by suspending in a minimal volume of 1.5X SDS sample buffer and heating at 95˚C for 3 min. Pull beads to side with magnet (>2min) and transfer elute to a fresh tube. Add fresh 1.5X SDS sample buffer to beads and repeat heating step. Pull beads with magnet and combine 2nd elute with first.