Mass Spectrometry

Immunoprecipitation (IP) protocol

1. Equilibrating the beads

Except for section 3, all steps should be performed on ice or at 4°C.

Protein A beads: Affi-Prep Protein A (Bio-Rad) beads have a binding capacity of 5-10 mg/ml rabbit IgG.

Protein G beads: GammaBind Plus Sepharose (GE Healthcare) beads have a binding capacity of 25 mg/ml mouse IgG.

In both cases, for initial binding tests we recommend the use of 1 mg of antibody per ml beads.

Wash beads 2× with 10 bed-volumes (bv) TBST (1×TBS + 0.04% Triton X-100) in 1.5 ml Eppendorf tubes: resuspend beads by flicking. Pellet beads by spinning for 10 sec in picofuge. Remove supernatant (SN) using suction line fitted with syringe and 27G needle.

2. Binding antibodies to beads

Add 10 bv TBST to the washed beads, then add purified antibody to give desired concentration. Mix beads with rotation for 1 h at RT or o/n at 4°C.

Wash beads 3× briefly with 10 bv TBST.

3. Cross-linking of antibodies to beads *(this section only: at room temperature!)*

Wash the beads 3× briefly with 10 bv 0.2 M Na-borate, pH 9.2.

(NB: the Na-borate solution forms crystals; microwave to re-dissolve, then cool to RT.)

Prepare a ***fresh*** solution of 20 mM DMP (i.e. 5.2 mg/ml) in 0.2 M Na-borate pH 9.2.

DMP = dimethyl pimelimidate dihydrochloride (Sigma D 8388. NB: DMP is highly moisture-sensitive; take DMP pot out of –20˚C freezer 30 min before use, to equilibrate to RT).

Add 20 bv of this solution to the beads and mix at RT for **30 min: no longer !!!**

Stop cross-linking reaction by washing beads 2× 10 min with 10 bv 0.2 M Tris-HCl, pH 8.0.

Wash beads 2× briefly with 10 bv TBST to re-equilibrate.

4. <u>Pre-elution with glycine</u> (removes non-crosslinked antibodies)

Wash beads 2× briefly with 10 bv 0.1 M glycine, pH 2.0, then wash 3× briefly with 10 bv TBST to re-equilibrate. Ab-coupled beads can be stored at 4˚C in TBST + 0.05% NaN_3.

5. <u>Cell extract preparation</u>

Clarify whole-cell extracts by centrifugation in 1.5 ml epp(s) at 14,000 rpm, 4˚C for 10 min (S14). Transfer the SN to a fresh tube and spin again. The SN from this spin is the clarified extract.

6. <u>Extract pre-clearing</u>

Transfer clarified extract to a 15 ml Falcon containing 1/10th volume protein-A/G beads, pre-loaded and cross-linked to non-specific IgG, and equilibrated with TBST. Incubate with rotation at 4˚C for 30 min. Pellet beads and transfer SN (pre-cleared extract) to a fresh tube.

7. <u>Immunoprecipitation</u>

Transfer pre-cleared extract to a 15 ml Falcon tube containing specific antibody-coupled beads. Aim to saturate the beads with extract (e.g. 20 mg protein per 100 µl beads). Incubate with rotation at 4˚C for 30 min. Pellet the beads, remove the SN (keep for analysis if desired).

8. <u>Washing the beads</u>

(parameters in step 1 may vary according to Ab and complex, but washes indicated in step 2 and 3 have to be carried out!)

Step 1:

5 standard washes: to pelleted beads, add 20-100 bv ice-cold IP-buffer plus 0.1% NP-40, 1×PIM, 0.1 mM PMSF. Resuspend beads by flicking, incubate with rotation at 4°C for 5 min. Pellet beads, aspirate as much SN as possible using needle.

Optional: 3 higher-stringency washes: as above, but with higher [salt] and [detergent], (e.g. 0.45 M NaCl), according to the desired stringency.

Step 2:

10 no-detergent washes: *(detergent must be removed: it interferes with MS/MS analysis!)*

Resuspend beads in 10 bv ice-cold wash-buffer. Spin, completely remove SN.

For on bead digest, please stop here at this step. Both the elution and the tryptic digest will be performed by the protein/MS facility.
If you continue with a glycine elution, please continue with step 3.

Step 3:

2 washes to remove pH-buffering agents: Wash beads twice briefly with 20 bv of 150 mM NaCl to remove pH-buffering agents. Resuspend beads in NaCl, transfer to 0.5 ml epp. Pellet beads and remove SN.

9. Elution of immunoprecipitated protein from the beads ('glycine elution')

Under the hood: add one or two bv of 0.1 M glycine (pH 2.0) to the beads, mix by flicking for 3 min. Pellet beads, transfer SN into a fresh Axygen low-retention 0.2 ml PCR tube, and repeat elution twice. Check for low pH of the combined eluates. Neutralize pH by adding 1 M Tris-HCl pH 8.0 (approx 15 ul for 100 ul eluate, check with pH paper).
Neutralise eluted beads by washing 3× briefly with 10 bv TBST. Transfer 1/10th vol eluate to fresh 0.5 ml Eppi, add 4× sample buffer for SDS-PAGE silver analysis. Use remaining eluate directly for in-solution digest, or freeze in liquid nitrogen and store at -80˚C.

10. IP bead recovery

Add 10 bv 0.1 M glycine (pH 2.0) to the beads, mix by flicking for 1 min. Pellet beads and aspirate SN. Repeat once. Neutralise beads by washing 3× briefly with 10 bv TBST.
Store beads at 4˚C in TBST + 0.05% NaN_3 for future use.

IP-buffer:	**stock conc**	**per 200ml**	**per 500ml**
20 mM Tris-HCl, pH 7.5	1 M	4 ml	10 ml
150 mM NaCl	5 M	6 ml	15 ml
10% glycerol	87%	23 ml	57.5 ml
2 mM EDTA	0.5 M	0.8 ml	2 ml

PIM (Protease inhibitor mix):

Leupeptin, Pepstatin, Chymostatin, each prepared at 10 mg/ml in DMSO. Dilute 1/1000 to generate 1x PIM in extract or wash buffer.

Wash-buffer:
20 mM Tris-HCl pH 7.5, 150 mM NaCl