Low throughput protocol for immunoprecipitation followed by mass spectrometry of cells stably expressing an HA-tagged protein

Analysis of protein complexes by mass spectrometry provides a powerful approach for identifying proteins that associate with other proteins. Frequently, this can be done by expressing the protein of interest with an epitope tag, such as a Hemagglutinin-A (HA) epitope, using either a stably expressed lentivirus or by gene editing the HA epitope into the gene of interest. The protocol has been used extensively to create the Bioplex protein interaction network [Huttlin et al **Nature**. 545:505-509 (2017); Huttlin et al **Cell**, 162: 425-440 (2015)].

Reagents

1x MCLB stock:

50 mM Tris, pH 7.5
150 mM NaCl
0.5 % NP40
(Made in batches of 0.5L @ 1x, filtered and stored at 4°C)

1X MCLB (add Roche protease inhibitor tablet-Catalog number 4693116001 + 1mM (final conc.) DTT):

-Make 30mL MCLB with inhibitors and DTT for first wash or diluting samples if necessary (30μL of 1M DTT, 3 mini protease inhibitor tablets)

50% Bead slurry:

Prepare slurry of anti-HA beads (mouse monoclonal 12CA5, Sigma) in a 1.5 mL tube. Spin beads gently at 3000rpm x 30 seconds to pellet. Remove buffer and wash with 3x 1mL MCLB (no inhibitors). Can store at 4°C for several days.

HA elution buffer: Can use either 50mM Tris pH 7.5/150mM NaCl or use PBS. PBS has been used for high throughput purposes.

HA peptide for elution: 250μg/mL HA peptide dissolved in HA elution buffer. Crude HA peptide from Bio-Synthesis Inc: Sequence: H_2N-YPYDVPDYA-CO_2H

Protocol – this protocol is for 293T cells but has been used broadly for many cell types. Typically, cells used have the proteins to be immunoprecipitated have stably expressed HA-tagged proteins via lentivirus vectors, or proteins fused with an HA epitope using gene editing.

*Cell Harvest: 2x15cm plate or 5x10cm plates per IP. Include HA-tagged GFP bait as a control.

Harvest cell pellets: Wash plates 2x with cold PBS, then add 5mL PBS per plate. Gently pipette up and down to dislodge cells and homogenize or gently scrape. Transfer to 15mL conical tube and pellet cells 3000rpm x 5 min, discard sup. Add 1mL PBS and

transfer to 2mL tube. Spin, aspirate PBS, and snap freeze pellet. Store at -80 or use immediately.

1. Quick thaw frozen 293T pellets in 2mL tubes in 37°C water bath—(approximately 3 seconds). Transfer to ice (metal block) and add ~1.2mL MCLB.
2. Once pellet is thawed, pipette up and down to resuspend and tumble tubes for 20 min in the cold room (4°C) to lyse cells.
3. Spin at 16.1rcf for 20 min in pre-chilled bench-top centrifuges to clear lysate.
4. Reserve 25-50uL of lysate for QC protein assay and western blot of input (if desired) Carefully transfer remaining supernatant to fresh 1.5mL tube containing 40μL of washed HA bead slurry. Use 1.5mL tubes, not 2mL for the IP, as the conical shape is more ideal for pelleting the beads/washing in subsequent steps.
5. Incubate cleared lysate with beads for 3 hours with tumbling in the cold room (4°C).
6. Spin samples 1 min x 3000rpm to pellet beads in 4°C centrifuge.
7. Carefully decant supernatant using aspirator or 1mL pipette (be careful not to aspirate beads!)
8. Add 1mL MCLB to each tube, and gently resuspend beads by shaking.
9. Repeat spin/wash step 3 more times for a total of 4 x 1mL washes with detergent present.
10. Perform 3x1mL washes in the absence of detergent 50mM Tris/150mM NaCl, without NP-40.
11. Carefully aspirate remaining wash buffer. Use gel loading tip and pipettor to remove as close to beads as possible. Can use P3111 capillary tips which are smaller than the agarose resin, but not entirely necessary...beads do not have to be dry.
12. Add 100μL elution buffer (HA peptide elution buffer + 250μg/mL HA peptide)
13. Incubate in shaker at 37°C (gentle shaking), 30 min.
14. Collect bead eluate by centrifuging 1 min at 3000 rpm
15. Repeat elution with equal volume of HA peptide; incubate second elution for 15 mins.
16. Transfer eluate to labeled 1.5mL tubes; freeze at -80°C.
17. Proceed to TCA precipitation
18. **The following steps constitute the TCA precipitation/acetone wash and trypsinization in preparation for analysis by mass spectrometry:** Can perform TCA precipitation overnight at 4 degrees or for 45 mins on ice.
19. Add 55μL neat TCA to samples (assuming 2x100μL elution), vortex to mix, then gently spin to ensure TCA is not in tube caps.
20. Spin max speed at 4°C for 30 mins (13,000+ rpm); carefully aspirate all but ~30μL of sample
21. Wash pellet with 1mL cold 10% TCA made in HPLC grade water
22. Spin max speed 15 min, vacuum as in Step 2
23. Wash with 1mL cold Acetone
24. Spin max speed 10 min, vacuum
25. Repeat Acetone wash 2 more times (3 acetone washes total; NOTE: do not reduce to 2

washes. TCA tracks along and samples do not reach basic pH in 200mM EPPS digest buffer)

26. Air dry or use speedvac to dry pellet for digest—must be completely dry, as acetone can cause peptide modifications (DO NOT HEAT).
27. Trypsin digestion (6hr):
28. Resuspend dried pellet in 40μL of 200mM EPPS (pH 8.5)/10% Acetonitrile (digest buffer).
29. Spot check ~0.2μL of sample for a couple of samples to ensure pH is ~8.5.
30. Add 100ng trypsin per sample (Thermo). Stock is 20μg in ~20μL (measure to confirm 20μL for each tube). This is 1μg/μL, make a master mix of trypsin digest buffer and add 40μL to each sample. Do not vortex, as this can dislodge the pellet.
31. Incubate at 37°C for 6 hours (warm room or thermomixer, can shake gently).
32. Acidify with 2 digest volumes of 5% formic acid in HPLC grade water. For 40μL digest,add 80μL 5% formic acid. Spot check pH for a couple of samples to ensure ~pH 2.
33. Proceed to stage tip followed by analysis by mass spectrometry.