Dynabead G IP on bead digest protocol for Mass Spectrometry

Harvest cell lysates

Re-suspend cell pellet in chilled CHAPS lysis buffer supplemented with Complete EDTA-free protease inhibitor (1 tablet/10mL; Roche/Sigma, cat# 4693159001)*

CHAPS lysis buffer:

Vol	Reagent	Final concentration
0.5 mL	1M HEPES	10 mM
2 mL	5M NaCl	200mM
5 mL	10% CHAPS	1%
0.5 mL	1M $MgCl_2$	10mM
50 uL	1M DTT	1mM
Up to 50mL	Nuclease-free water	-

*Complete EDTA-free Protease inhibitor cocktail tablets added fresh (1 tablet/10mL lysis buffer). Also add RNase inhibitor for any affinity purifications (prior to addition of RNA) or for protein complexes that might be dependent on RNA (add before lysis of cells)

1. Briefly vortex sample to mix
2. While on ice, pass lysates through a 26.5g needle 5x (once up and down = 1x)
3. Incubate lysates on ice for 20min
4. Centrifuged lysates at 14,000 x g for 20min at 4°C
5. Transfer supernatant to a fresh tube, supplemented with RNase inhibitor (NEB, cat# M0314L) and store at -80°C

IFIT2 Immunoprecipitation

1. Wash 50uL (per sample) of Dynabeads protein G (thermo, cat # 10003D) 2x with 500 µL of lysis buffer
2. Resuspend beads in 200 µL of lysis buffer
3. Add 12 µg of the following antibodies to 50 µL of beads (binding capacity = 8 µg IgG/mg beads):

Antibody	Isotype	Manufacturer
Ms anti-target protein	IgG 2c	……
Ms IgG	IgG	Sigma (cat# I5381)

4. Incubate beads on a rotator for 30 min at RT
5. Collected beads and keep unbound fraction for later analysis of antibody binding efficiency.
6. Wash beads 3x in 500 µL of lysis buffer
7. Add 2 mg of target lysate to each sample, and incubate at 4°C on a rotator for 2 hrs 15 min.
8. Collect beads and unbound lysate for later analysis
9. Wash beads 3x with 500 µL of lysis buffer. Prior to removing last wash, remove 100 µL of beads (1/5th total sample) to a fresh tube for SDS-PAGE analysis
10. Transferred sample to new microfuge tube
11. Wash remaining beads 3x with 500 µL of PBS (do **not** supplement with RNase or protease inhibitors), then process for MS analysis (below) *Note, after first and second PBS wash, transfer the sample to new microfuge tube again to eliminate any detergent that may have adhered to the plastic

Trypsinize the protein off of beads:

1. Further wash beads 3x with freshly prepared 20 mM Ammonium Bicarbonate (remove as much wash buffer as possible during these washes!). This can be supplemented with salt, but CANNOT contain any glycerol, detergents, protease inhibitors, or anything else that will interfere with downstream analysis. After last wash, centrifuge 3,000 x g for 2min to remove supernatant
2. Remove as much wash buffer as possible during each wash. There should be no liquid above the bead bed. You can store the beads at -80°C after this step
3. Add just enough wash buffer (ammonium bicarbonate) to cover beads (30µL beads + 20µL for sepharose beads; for magnetic beads the entire supernatant can be removed effectively, so add 20µL of buffer to 50µL of starting bead volume)
4. add 10 µl 10 ng/µL sequencing grade trypsin (Promega, cat# V5111)
5. Incubate @ 37C for 3-6 hours (I incubated 6 hours at 1500 rpm)
    a. Noticable antibody degradation begins at ~8hrs (Native antibodies are more resistant to proteolysis that cytosolic proteins)
6. Harvest the supernatant being careful not to remove any of the beads. Wash the beads 2x with 30-50 µL of ammonium bicarbonate, and pool all fractions (the trypsin digest and the two washes). The supernatant can be reduced and alkylated at this time, another aliquot of trypsin can be added and the digestion continued, or the supernatant can be acidified by the addition of acetic acid or the sample can be stored at -80 after acidification

Reduction and alkylation:

7. Add TCEP to final concentration of 1mM. Incubate 37C for 1 hour.
8. Add iodoacetamide (Thermo, cat# 90034; make up fresh each time with ammonium bicarbonate; one use only) to final concentration of 10mM. Incubate RT for 30 min in the dark.
9. Quench with N-AcetylCysteine at final concentration of 12mM (this needs to be in excess of iodoacetamide).
10. Proceed with the C18 column protocol (Thermo-Pierce, cat# 89870) to clean up and concentrate peptides.

C18 column purification and concentration (following manufacturer's protocol):

- Activation Solution: 50% Methanol; 400µL per sample
  Note: ACN can be substituted for methanol.
- Equilibration Solution: 0.5% TFA in 5% ACN; 400µL per sample
- Sample Buffer: 2% TFA in 20% ACN; 1µL for every 3µL of sample
- Wash Solution: 0.5% TFA in 5% ACN; 400-800µL per sample; wash volume will be dependent upon amount and type of contaminants present in sample
- Elution Buffer: 70% ACN; 40µL per sample

11. Tap column to settle resin. Remove top and bottom cap. Place column into a receiver tube.
12. Add 200µL of Activation Solution to rinse walls of the spin column and to wet resin.
13. Centrifuge at 1500 × g for 1 minute. Discard flow-through.
14. Repeat steps 12-13
15. Add 200µL Equilibration Solution. Centrifuge at 1500 × g for 1 minute. Discard flow-through.
16. Repeat step 15.

17. Load sample on top of resin bed.
18. Place column into a receiver tube. Centrifuge at 1500 × g for 1 minute.
19. To ensure complete binding, recover flow-through and repeat steps 17-18. Keep flow-through for confirmation of sample binding.
20. Place column into a receiver tube. Add 200µL Wash Solution to column and centrifuge at 1500 × g for 1 minute. Discard flow-through.
21. Repeat step 20 (total of 2 washes; can perform a third or fourth wash if sample contains high levels of contaminants (i.e., 2M urea or ≥100mM ammonium bicarbonate)).

22. Place column in a new receiver tube. Add 20µL of Elution Buffer to top of the resin bed. Centrifuge at 1500 × g for 1 minute.
23. Repeat step 22 with another volume of 20uL.
24. Dry eluate in speed vac and store at -80°C
25. Add 0.1% formic acid in water just prior to MS analysis.