IMMUNOPRECIPITATION: PROTEIN A SEPHAROSE

Buffers & Beads:

HNM buffer in a 1L filter flask (may already be made)

- 11.9g HEPES in 500mL RNase/DNase Free dH2O
- pH 7.5
- Add 8.77g NaCl
- Add 1 ml $MgCl_2$ (1M stock soln)
- Q.S. to 1L and vacuum filter

HNTM buffer in a 1L filter flask (may already be made)

- Prepared same as above
- Add 10ml of Triton-x 100

Resuspended Protein A sepharose beads in 10ml HNTM buffer incubate 4°C over night (probably already made)

Samples: For each IP use 75ug cytosolic and/or 37.5ug nuclear lysate: need aliquots of each lysate for the antibody of interest and the control antibody.
i.e, 2 aliquots of each lysate (test Ab + ctrl)➔ **2IPs/ sample**

N.B: If you plan on isolating RNA: Then you need 7 tubes per antibody (6 tubes/ lysate/ Ab for RNA isolation and 1 tube /lysate/ Ab for western = 14 tubes total).

Example:

Sample	conc. (ug/ul)	Amt (ul) for 75ug	amt (ul) for 2 IP*	HNM**
PA cyto	1.8	42	84	808
30 min cyto	1.2	62.5	125	769

*volume of protein lysate needed for 2 IP's
**850 ul – volume of protein lysate. Ex: 850 – 42 = 808 ul HNM buffer

Protocol:

1. Prepare (1) 1.5mL microfuge tube for each IP:
   - HNM buffer from chart above
   - 10ul Halt Protease inhibitor
   - 10ul VRC (add only if want to isolate RNA; don't add for protein only)
   - 10ul RNase Inhibitor (add only if want to isolate RNA; don't add for protein only)
   - 1ul 0.1M DTT
   - 33ul 0.5M EDTA
2. Added 10ul of antibody of interest or 4 ul anti-hamster IgG (neg. control)
3. Added 37.5ug nuc lysate or 75 ug cyto lysate per IP to appropriate tubes
4. Incubate on rotator for 3 hours @ RT (5S-11)
5. Beads: don't invert or mix!
   - Make a 1:1 bead to buffer slurry:
     - Gently remove suspension buffer leaving beads in tube
     - Ex: if 4ml of beads remain → add 4ml of HNM buffer
     - Gently swirl beads and stir with pipette tip to resuspend (note: don't want to get beads on side of tube so don't invert or vortex)
   - Add 100ul of bead slurry to sufficient number microfuge tubes (50ul beads) = 1 aliquot of beads per IP

- Spin at 13.2K rpm for 3min @ 4°
- Make sure there is 50ul beads. If not, add more.
- Aspirate off excess buffer. Wash 2x w/ 1 ml of HNM buffer, aspirate last wash and store beads at 4°.

6. After the 3 hour incubation of Lysate and Antibody, transfer lysate/antibody complexes to bead tubes

7. Rotate for 2hrs at RT

8. Meanwhile, make Urea Wash in a 50mL conical tube
   - 3g Urea
   - 50ml HNTM buffer
9. Spin down beads and lysate @ 13.2K for 3 min @4°
10. Aspirate off supernatant (note: aspirate off buffer down to the 100ul mark)
11. Wash 3x with 1M Urea/HNTM buffer
    - washes = 1ml/tube, vortex, and 2min spin down 13.2K rpm
    - (note: aspirate off buffer down to the 100ul mark)
12. Wash 2x with HNTM buffer
13. Last Wash: Aspirate off all buffer. Use pipette tip to get last remnant.

IP for western

Add 50ul of WSB and Boil 5min
- Store -20° until gel prepared

Follow Western Protocol
- Boiled Samples again
- Loaded 25ul of sample on to gel

IP RNA Isolation (alternatively, use TriZol protocol for Isolation of RNA)

1. To each 50ul IP sample, add the following:
   - 5µl 10mg/ml Proteinase K Stock
   - 100µl HNTM Buffer
   - 1µl 10% SDS
2. Vortex the samples, briefly spin down and put in the water bath at 55°C for 20 minutes.
3. Extract with an equal volume of Acid Phenol/ Chloroform/ Isoamyl Alcohol (pH =4.5).
4. Vortex and spin at 4°C at 13,200rpm for 3 minutes.
5. Remove top aqueous layer to a sterile 1.5ml RNAse/ DNAse free microfuge tube (note: thick interface layer contains beads)
6. At this time you can combine the aqueous layers of same samples
7. Precipitate the RNA with 1/10 volume of 3M Sodium Acetate (pH = 5.5) and 2.5 volumes of 100% cold ethanol.

   N.B: if volume of all recommended reagents don't fit into one tube, split the aqueous layer into several tubes then add 1/10 of that vol. of NaAcetate and 2.5x 100% cold EtOH

8. Store at -80°C overnight.
9. The next day spin the samples down at 13,200rpm for 1 hour. Aspirate the supernatant off RNA pellet, allow EtOH to evaporate off pellet for 5 minutes @ RT (don't over dry)
10. Resuspend each pellet in nuclease free water (note: for 6 combined sampes add 60ul dH_2O, if separated samples 10ul/IP)
11. Vortex, and briefly spin down

12. Allow the pellets to sit at RT for 30 minutes
13. Vortex the samples, briefly spin down and incubate at 65°C for 15 minutes.
14. Again, vortex and briefly spin down.
15. Combine replicate samples in to one tube if you haven't already done it.
16. Treat RNA with DNAse:

DNAse Treatment of RNA (alternatively, use Ambion's DNA free kit)

1. For 60ul of RNA, add
   6µl DNAse I Reaction Buffer
   6µl DNAse I
2. Vortex, briefly spin down and incubate 15 minutes at RT.
3. Add 6µl 25µM EDTA.
4. Vortex and briefly spin down.
5. Incubate 10 minutes at 65°C.
6. Vortex and briefly spin down.
7. Spec at 260-280nm using 3ul of RNA in 800ul of RNase/ DNase free water.
8. Calculate RNA conc: OD x 40 x .8/ 3 = ug/ ul RNA