RNA-Protein complex Immunoprecipitation

Preparation of mRNP lysate from culture cells

All magnetic pull down steps should put the tube with magnetic stand on ice

1. Grow and harvest tissue culture cells by washing two times with ice-cold PBS and pellet by centrifugation at 4°C /2000rpm/ 5 min. We need 2 big dishes (150mm) of cells per sample.
2. Loosen the final cell pellet by gently flicking the bottom of the tube and add an approximately equal volume of ice-cold PLB buffer supplemented with RNase inhibitors and protease inhibitors.
3. Mix cells by pumping several times with a hand pipettor (no vortex!) and place on ice for 10 min.
4. Spin 15 min at 12,000 rpm (16,000 g) /4°C. Transfer supernatant to the fresh Epepndorf tubes.
5. Freeze and store at -80oC. (Its better to use lysate directly for IP).
6. Pre-clear the supernatant with 15 µg (30 µl from stock 0.5µg/µl) of IgGl control, for 30 min/4°C. Add 50 µl Dynal protein G magnetic beads non-coated with Ab, incubate 30 min/4°C with rotation. (Note that pre-clearing is not required for IP followed by RT-PCR. Its required only for IP followed by microarray)
7. Put on magnetic stand for 2 min. Save supernatant. This is your pre-cleared lysate.
8. Do Bradford to measure protein concentration (measure 2 µl of a 1:100 dilution). We routinely get 15-30 µg/µl concentration of lysates and you will need anywhere from 50-100 µl per IP of lysate.

Prepare antibody coated Dynal protein G beads

1. Use Dynal protein G magnetic beads from Invitrogen.
2. Take 100 µl of Dynal beads and leave on magnetic stand for 2 min then take supernatant out.
3. Wash twice with 1 ml NT2 buffer and resuspend beads in 100 µl NT2.
4. Add 20 µg antibody to beads and rotate at 4 °C overnight.
5. Next morning, gently wash beads with 1 ml ice-cold of NT2 buffer 2 times and resuspend in 100 µl NT2. The beads are now ready for use.

Immunoprecipitation of mRNPs

1. Use 1.5 ml Eppendorf tubes. Add all the additives 10 µl 0.1 M DTT (do not add the DTT to the pellet directly, as this will reduce you antibody and the IP will not work!), 10 µl RNAseout, and proteinase inhibitor in 800 µl PLB. Add 100 µl lysate (even if concentration of protein is lower than 3Oug/ul) and 100 µl antibody coated protein G beads. Rotate 4 hrs at 4°C, end-over-end. Wash pellet 5 times with 1 ml aliquots of ice-cold NT-2 buffer.
2. After last wash, add 100u1 NT2 buffer having 5ul DNase I (2Ulul). Keep at 37°C for 5-10 mins. Add 1 ml NT2 buffer, put on magnetic stand for 2 min, discard supernatant.

3. Then, add the following to the PAS pellet: 5 µl of Proteinase K (l0mg/ml), 1 µl 10% SDS and 100 µl NT2. If you have several samples, its good to make a mastermix of NT2 buffer (Proteinase K and SDS). Incubate at 55^{o}C for 15-30 min, with mixing.
4. Put on magnetic stand and collect supernatant (~ 100 µl).
5. To beads add 200 µl NT2 buffer, pippet several times and put on magnetic stand, collect supernatant (~200 µl). Discard beads.
6. Combine supernatants (100 µl and 200 µl) and add 300 µl lower layer of acid phenol-CHCI3 (Ambion). vortex, 1min RT (or 37^{o}C in shaker), short spin at RT (imp) /1 min/ max speed.
7. Collect 250 µl of upper layer, add 25 µl 3M sodium acetate, pH 5.2, 625 µl 100% ETOH and 1 µl glycogen, mixwell, keep O/N -20^{o}C.
8. Next day, mix the tubes by inversion 3-5 times, spin 14,000 rpm/ 4 ^{o}C /30 min and discard supernatant.
9. To the pellet add 1ml of 70% ETOH and mix by inversion or vortexing, spin 14,000rpm/4 ^{o}C /2 min.
10. Discard supernatant, spin pellet 14,000 rpm/ 4 ^{o}C /1 min. Pipette any 70% ETOH, air dry pellet at RT for 5 min. resuspend in10-20 µl of RNase free H_2O.Use RNA as planned. Do not measure OD this will probably waste most of your sample.

Buffer

Polysome lysis buffer PLB:
100 mM KCl
5 mM $MgCl_2$
10 mM Hepes, pH 7.0
0.5% NP-40

To be added at the time of use:
1 mM Dithiothrectol (DTT)
100 unit/ml RNase OUT
1X Complete Protease Inhibitor Cocktail (Roche cat#1697498)

NT2 Buffer:	100 mL NT2
50 mM Tris, pH 7.4	5 ml (1M stock)
150 mM NaCl	3 ml (5M stock)
1 mM $MgCl_2$	0.1 ml (1M stock)
0.05% NP-40	500 µl (10% stock)