Co-Immunoprecipitation of germline proteins from adult *C. elegans*
August 2014 Christian's Method

Part 1: Grow worms & make popcorn (Dustin's protocol w/ modifications)

1. Make worm popcorn from synchronized adults
1.1. Day 1 Grow up worms to synchronize
1.1.1. Add 25mL of HB101 bacteria to 1 liter of complete S media.
1.1.2. Wash worms off of 40 recently starved plates and add to the liter of S media and bacteria.
1.1.3. Divide S media + bacteria + worms into 4x 250mL glass flasks. Shake at 220rpm at 20C for 3 days.

1.2. Day 4 late: Harvest embryos and hatch on unseeded plates
1.2.1. Pour worms into 1 liter pear funnel and let settle.
1.2.2. Drip into 50ml conical tube.
1.2.3. Wash with water 3X
1.2.4. Throw worms into bleach solution. (~3 minutes then rinse 3X with M9)
1.2.4.1. 3 parts water, 1 part bleach, 0.1 part 10M NaOH (50ml – 36mL water, 12.6mL bleach, 1.4mL 10N NaOH)
1.2.5. Let embryos hatch on about 20 unseeded (no food) plates.

1.3. Day 5 mid-day: Feed L1's
1.3.1. Add 40mL of HB101 bacteria to 1 liter of complete S media.
1.3.2. Wash hatched L1s and add to the liter of S media and bacteria
1.3.3. Divide S media + bacteria + worms into 4x 250mL glass flasks. Shake at 220rpm at 20C for 24 hours.

1.4. Day 6 early: Reduce temp
1.4.1 Reduce temp from 20C to 18C.
1.4.2 Otherwise worms will be too old by harvesting time on Day 8.

1.5. Day 8 early Harvest adults and make popcorn
1.5.1. Inspect worms on dissection scope to make sure most are adults (around 68 hours after feeding L1s).
1.5.2. Pour worms into 1 liter pear funnel and let settle
1.5.3. Pre-chill 50ml conical tubes in dry ice
1.5.4. Collect and wash 2X in water.
1.5.5. Wash 1X in Buffer H (lysis buffer) with NO detergent!! (see part 3 for recipe)
1.5.6. Pour liquid nitrogen into 50ml pre-chilled tube
1.5.7. Put pelleted worms in syringe and drip worms into liquid nitrogen
1.5.8. Put worm popcorn in 50ml conical tubes and freeze in -80C

This protocol usually nets somewhere around 10-20mL of packed popcorn depending on how healthy your strain is and the # of adults you have at bleaching stage. I've had terrible luck using worms from NGM plates and ALWAYS do liquid cultures now.

Part 2: Make worm powder

2.1. Pulverize worms in Retsch Mixer Mill MM-400 under cryogenic conditions
2.1.1. Chill 2x 25mL grinding jars w/ balls inside in liquid nitrogen until bubbling stops
2.1.2. Transfer around ~15mL of popcorn to each tube, leaving room for ball to move around. DO NOT OVERFILL.
2.1.3. NOTES: You must ALWAYS use both jars, similar to balancing centrifuge. Both jars MUST have popcorn in them, ball will damage an empty jar.
2.1.4. After filled with popcorn, return ball to jar, seal, and immerse in liquid nitrogen.
2.1.5. Keep jars in liquid nitrogen when not milling.
2.1.6. Pulverize worms for 2min @ 25Hz, 3 times. Chill on liquid nitrogen between milling sessions until bubbling stops.
2.1.7. Carefully remove powder from jars into small box or conical tube.
2.1.8. Store at -80C until ready to perform IP (can be stored indefinitely).

This usually produces somewhere in the ~5g range of powder, enough for a few IPs.

Part 3: Conjugate dynabeads to desired antibody (Bungo's protocol)

I always cross-link my antibodies to Invitrogen Dynabeads. This allows you to use the same antibody for Western blot without having to worry about heavy & light chains showing up. For a single Co-IP, I make 100uL of beads (50uL each for sample/negative control) w/ 25ug of antibody (12.5ug/IP).

Use Protein A Dynabeads for rabbit antibodies and Protein G for mouse antibodies.

Working IP antibodies:
mouse GFP (Roche, 11814460001)
mouse FLAG M2 (Sigma, F3165)
rabbit A-14 Myc (Santa Cruz Biotech, SC-789)

3.1. Bind 25ug of antibody to 100uL of Dynabeads.
3.1.1. Resuspend 100uL slurry of Dynabeads.
3.1.2. Concentrate in magnet, remove sup.
3.1.3. Wash twice in 1mL of 0.1M Na-phosphate buffer pH 7.0
3.1.3.2. 500mL 0.1M Na phosphate pH 7.0 (58mL 0.5M Na2HPO4, 21mL 1M NaH2PO4, 421mL ddH2O)
3.1.4. Add 25ug of antibodies and same volume of 0.1M Na phosphate buffer.
3.1.4.2. e.g. if 25ug of FLAG M2 is 25uL, add 25uL of 0.1M Na phosphate
3.1.5. Shake with gentle agitation on vortex machine for 25min.

3.2. Crosslink antibody to beads
3.2.1. Concentrate beads, remove sup.
3.2.2 Wash twice in 1ml of 0.1M Na-phosphate buffer pH 7.0 with 0.01% Tween-20 (Tween helps with protein stability in storage of Dynabeads)
3.2.3. Wash twice with 1ml of 0.2M triethanolamine, pH 8.2 (Dynabeads now have immobilized antibody)
3.2.4. Resuspend Dynabeads in 1ml of 20mM DMP (in common –20^{o}C freezer, Dimethyl Pimelimidate, D8388, Sigma) in 0.2M triethanolamine, pH 8.2 (note that this crosslinking solution must be prepared immediately before use)
3.2.4.2. Use 5.18mg DMP in 1mL 0.2M triethanolamine
3.2.5. Incubuate on rotation mixer for 30 minutes at RT (DO NOT OVER CROSSLINK)

3.3. Stop the crosslinking reaction
3.3.1. Concentrate beads, remove sup.
3.3.2. Resuspend beads in 1mL of 50mM Tris-HCl pH=7.5
3.3.3. Incubate with rotation mixing for 15min at RT.
3.3.4. Wash beads 3X in 1mL PBST (0.1% Tween-20)
3.3.4 Store beads in original volume but w/ PBST (e.g. 100uL PBST) at 4C. Do not freeze.
3.3.5 Beads can be stored for around ~1 month at 4C without any reduction in efficiency. No need to equilibrate prior to use.

<u>Part 4: Lyse Worms & perform IP</u>

Lysis buffer (Buffer H): 50mM Hepes pH 8.0, 150mM KCl, 2mM MgCl2, 15% Glycerol, 0.1mM EDTA pH 8.0, 0.5mM EGTA-KOH pH 8.0, 0.1% NP-40

Protease inhibitors (working concentrations): 0.1 mM AEBSF, 5 mM benzamidine, 1:200 aprotinin, Roche® Complete Mini tablets w/o EGTA

Phosphatase inhibitors (working concentrations): 1mM $Na_4P_2O_7$, 2mM Na-β-glcyerophosphate, 0.1mM Na_3VO_4, 5mM NaF

2X Sample Buffer (2XSB): 4% SDS, 20% Glycerol, 120mM Tric-HCl pH 6.8, 0.02% bromophenol blue (add 2-mercaptoethanol [B-Me] to 5% prior to use)

4.1. Lyse worms
4.1.1. Weigh 1g of worm powder for each strain you plan to use into 15mL conical tube (always do untagged negative control).
4.1.2. Resuspend in 5mL ice cold Buffer H w/ protease + phosphatase inhibitors.
4.1.3. Rotate 3min @ 4C or until re-suspended well.
4.1.4. Sonicate 2x30sec at medium amplitude with 1min rest on ice.
4.1.5. Spin @ ~50,000xg (max speed) @4C for 20' in JA-20 rotor.
4.1.5.2. Can do optional ~15min ultracentrifuge spin @ 4C. This helps get rid of lipids and seems to help with IP, but not necessary.
4.1.6. Take sup, move to new 15mL conical avoiding top lipid layer.

4.2. Perform IP
4.2.1. Take 50uL 'input' sample for each strain, add 50uL 2X SB+B-Me, boil 3 min, store at -20C.
4.2.2. Add 50uL of Dynabeads with conjugated antibody.
4.2.2.2. Note this is usually not enough to immuno-deplete, could increase to get more protein.
4.2.3. Rotate at 4C for 2-3hours.
4.2.4. Concentrate beads in magnet.
4.2.5. Take 50uL of sup as 'unbound' sample for each strain, add 50uL 2X SB+B-Me, boil 3 min, store at -20C.
4.2.6. Remove sup, wash beads 4X with 1mL ice cold Buffer H.
4.2.7. Remove as much sup as possible.
4.2.8. Boil in 50uL 2X SB+B-Me to elute IP'd proteins.
4.2.9. Concentrate in magnet, take sup (eluate) to new tube (removing beads).

<u>Part 5: Analyze IP'd proteins by Western blot</u>

See Western protocol.

I usually run 10% of IP sample (5uL) to analyze for the presence of IP'd protein (e.g. protein with GFP/FLAG tag)

To analyze for presence of Co-IP'd proteins, I run significantly more, up to 50% (25uL) of IP sample, depending on stoichiometry of complex.

For inputs/unbound samples, I run 10uL each for Western.