Oestreich Lab Co-Immunoprecipitation (Co-IP) Protocol

BUFFERS:

1X PBS, kept at 4C

Whole Cell Lysis Buffer (un-supplemented), store at 4C
20mM HEPES pH 7.4
1% Triton x-100
100mM NaCl

RIPA I wash buffer, store at 4C
50mM HEPES pH 7.5
150mM NaCl
1% NP40
0.5% sodium deoxycholate

RIPA I.V. wash buffer, store at 4C
50mM HEPES pH 7.5
325mM NaCl
0.1% NP-40
0.1% sodium deoxycholate

2X CoIP loading dye, aliquot and store at 4C
100mM Tris pH 6.8
200mM DTT
4% SDS
0.001% bromophenol blue
20% glycerol

Additional reagents to supplement the buffers above:
100mM PMSF
1M DTT
0.5M NaF
10mg/mL Aprotinin
10mg/mL Leupeptin

DAY 1

1. Thaw fresh: 100mM PMSF, 1M DTT, 0.5M NaF, 10mg/mL Aprotinin, and 10mg/mL Leupeptin
2. Supplement Whole Cell Lysis Buffer (WCLB) as follows (per 2 IPs):
   4.435mL WCLB
   50uL 100mM PMSF
   5uL 1M DTT

500uL 0.5M NaF
5uL 10mg/mL Aprotinin
5uL 10mg/mL Leupeptin
…invert to mix and keep on ice.

3. Use between 10-20 million cells per IP, depending on the experiment. Harvest required number of cells and pool into a single 50mL conical tube. Resuspend well to ensure a homogenous mixture.
4. Portion cells out into 15mL conical tubes (1 per IP) using an equivalent volume for each tube.
5. Centrifuge at 1800rpm for 5 minutes at 4C.
6. Resuspend each cell pellet in 2mL of 1X ice-cold, IP-specific PBS (wash) and centrifuge again as in #4.
7. Aspirate off the supernatant and resuspend each cell pellet in 500uL ice-cold whole cell lysis buffer, supplemented with protease inhibitors (see buffer page for details).
8. Incubate on ice for 15 minutes; vortex every 5 minutes.
9. Pass each sample through a fresh, sterile 27 gauge needle (on 3mL syringe) 5 times.
10. Centrifuge at 13,200rpm in the refrigerated benchtop microcentrifuge for 15 minutes at 4C. After spin, proceed to step #12.
11. During the above spin, prepare the sepharose beads (Protein A or Protein G depending on the host species for your specific antibodies) as follows:
    a. Swirl beads well to mix; you want a slurry-type consistency
    b. Using a p1000, transfer 25uL of beads to labeled 1.5mL eppendorf tubes (1 per IP)
    c. Add 500uL of supplemented whole cell lysis buffer to each tube of beads
    d. Centrifuge at 3000rpm for 3 minutes at 4C
    e. Use a p1000 to remove the supernatant (leave the beads)
    f. Repeat “c” through “e” 2 more times. *Do not remove the final supernatant until the cell lysate has finished its centrifugation step (#10).*
12. Following centrifugation of cell lysate, set 15uL aside in a labeled 1.5mL eppendorf tube from the experimental (not control) IP tube. This is “input”. Store at 4C overnight.
13. Transfer the entire volume of whole cell extract for each IP to each of the appropriate bead-containing tubes.
14. Rotate in cold room for 20-30 minutes to “pre-clear” (remove any proteins interacting with the beads themselves) the whole cell lysate.
15. Centrifuge at 3000rpm for 3 minutes to pellet the beads. **For Day 1, the supernatant is the sample.** Transfer the entire supernatant to clean, labeled, 1.5mL screw-cap tubes. Discard the beads.
16. Add 5-10ug of antibody of interest and associated control antibody to each tube as appropriate and rotate overnight at 4C.

NOTE: Protease/phosphatase inhibitors can be stored overnight at 4C so you don't have to thaw them again on Day 2.

DAY 2

1. Supplement Whole Cell Lysis Buffer (WCLB) as follows (per 2 IPs):
   4.445mL WCLB
   50uL 100mM PMSF
   5uL 1M DTT
   500uL 0.5M NaF
   (no aprotinin/leupeptin on Day 2)
   …invert to mix and keep on ice.
2. Wash 30uL of Protein G/A (same as Day 1) beads as on day 1, using the supplemented WCLB above.
3. During the third spin for the beads, retrieve cell lysate + antibodies from cold room and keep on ice.
4. Quick spin cell lysate from Day 1 and add to the beads following the last wash/supernatant removal.
5. Rotate in the cold room for 1-2 hours.
6. During rotation, supplement:
   2.97mL of RIPA 1 buffer with 30uL 100mM PMSF (per 2 IPs) and
   1.98mL of RIPA 1.5 buffer with 20uL 100mM PMSF (per 2 IPs)
   …invert to mix and keep on ice.
7. Centrifuge the samples at 3000rpm for 3 minutes at 4C. **For Day 2, the beads are the sample.** Discard the supernatant.
8. Wash as follows:
   a. Add 500uL RIPA 1 buffer to the beads. Rotate in the cold room for 3-5 minutes.
   b. Centrifuge for 3 minutes at 3000rpm at 4C. Discard the supernatant.
   c. Add 500uL RIPA 1.5 buffer to the beads. Rotate in the cold room for 3-5 minutes.
   d. Centrifuge for 3 minutes at 3000rpm at 4C.
   e. Add 500uL RIPA 1 buffer to the beads. Rotate in the cold room for 3-5 minutes.
   f. Centrifuge for 3 minutes at 3000rpm at 4C. Discard the supernatant. During the last centrifugation step, retrieve the Input sample and place on ice.
9. Following washes:
   a. add 15uL of 2X SDS PAGE buffer to the 15uL of input sample.
   b. add 30uL of 2X SDS PAGE buffer to each of the bead samples.
10. Do not mix roughly, as the beads will settle on the walls of the tube (and not in the buffer). Boil for 5 minutes.
11. Quick spin and load for Western blot (usually 10-25uL for input depending on specific protein signal; 25-30uL for each IP. Do NOT load any beads).