Immunoprecipitation with Magnetic Dynabeads-Protein A/G

Day 1: Binding of antibody to beads and pre-clearing of extract

1. Extract 700µg mitochondrial protein in 200µL extraction buffer for 45 minutes at 4°C with occasional vortexing. This amount is sufficient for two reactions: the IP of interest and the control IP.
2. Centrifuge for 40 min at 25,000g and collect supernatant.
3. Mix the 200µL mitochondrial extract with 20µL 0.1M Na-phosphate/0.08% Tween 20/0.05% tDOC pH 8 buffer and save 10µL ("pre-clear" fraction).
4. Prepare antibody solution by mixing serum and Na-phosphate pH 8 buffer to final volume of 100µL and final Na-phosphate concentration of 0.1M (80µL serum and 20µL 0.5 M Na-phosphate pH 8 buffer).
5. Resuspend magnetic beads by vortexing for1-2 minutes.
6. To two Eppendorf tubes add100µL beads/tube, and to a third tube add 200µL beads (room temperature). Place tubes on magnet and pipette off supernatant. The three tubes are required for the following reactions: binding of the antibody, pre-clearing of the extract and the control reaction.
7. Wash beads 3X with 0.1M Na-phosphate pH 8 buffer (0.5mL/100µL beads).
8. Resuspend washed beads in one of each of the following solutions:
    a. 100µL antibody solution (prepared in step 4)/100µL beads
    b. 210µL mitochondrial extract solution (prepared in step 3)/200µL beads
    c. 100µL 0.1M Na-phosphate/0.08% Tween 20 pH 8 buffer (control)/100µL beads
9. Incubate tubes at 4°C overnight with rotational mixing.

Day 2: Cross-linking of antibody to beads and immunoprecipitation

1. Place tube containing the antibody on magnet and pipette solution off beads (save solution if antibody is limited: it can be re-used for immunoblotting or for additional IP-s). Let the other two tubes rotate until later (step 10).
2. Wash beads 3X with 0.1M Na-phosphate/0.08% Tween 20 pH 8 buffer (0.5 ml/wash).
3. Wash beads 2X with 0.2M TEA/0.08% Tween 20 pH 8 (1mL/wash).
4. Prepare (FRESH) the cross-linker: 20mM DMP (dimethyl pimelimidate dihydrochloride) in 0.2M TEA/0.08% Tween 20 pH 8 (5.4mg/mL).
5. Incubate beads in 1ml of the 20mM DMP solution with rotational mixing for 30 minutes, at room temperature, in the dark.
6. Discard cross-linker and stop reaction by adding 1ml 50mM Tris/0.08% Tween 20 pH 7.5 and incubate for 15 minutes, at room temperature, with rotational mixing.
7. Wash 3X with PBS/0.08% Tween 20 pH 8 (1 ml/wash).

8. Remove antibody not cross-linked to the beads by eluting twice with 100µl 0.1M glycine/0.08% Tween 20 pH 2.5 and rotational mixing, at room temperature, for 10 minutes each time.
9. Wash 3X with PBS/0.08% Tween 20 pH 8 (1 ml/wash).
10. Place tubes containing the pre-cleared mitochondrial extract and control solution (see step 1) on magnet. Discard control solution and divide pre-cleared extract as follows: use100µL to resuspend antibody cross-linked to the beads, 100µL to resuspend "control" beads and save remaining 10µL ("input" fraction).
11. Incubate the two tubes (actual IP and control IP) at 4^{O}C overnight, with rotational mixing.

Day 3: Elution of immunoprecipitate

1. Place tubes on magnet and pipette solutions off beads (save: "unbound" fractions).
2. Wash beads in each tube with 200µL of 0.1M Na-phosphate /0.08% Tween 20 /0.05% tDOC pH 8 buffer and save wash solutions ("wash" fractions).
3. Wash 5X with 0.1M Na-phosphate /0.08% Tween 20/0.05% tDOC pH 8 buffer (1mL/wash).
4. Elute twice, each time with 100µL of 0.1M glycine/0.05% DDM pH 2.5, at room temperature, with rotational mixing, for15 minutes.
5. Immediately restore physiological pH of each elution fraction by adding 20µL 1M Tris pH 7.5.
6. For both the control and the IP beads combine the two elution fractions (total volume 240µL) and add 36µL cold trichloroacetic acid (TCA) to precipitate protein.
7. Wash beads 2X with 0.1M Na-phosphate /0.08% Tween 20/0.05% tDOC pH 8 buffer (1ml/wash).
8. Elute any material still present on the beads with 50µL 1X Laemmli sample buffer (with or without ß-mercaptoethanol, depending on the size of the protein of interest as compared to the sizes of the heavy and light chains of the antibody), for 10 min, at 55^{O}C.
9. Analyze fractions (pre-clear, input, unbound, wash and elutions under different conditions) by SDS-PAGE followed by immunoblotting.

SOLUTIONS:

Extraction buffer (50mM HEPES, 150mM NaCl, 1% tDOC)
50µL 1M HEPES pH 7.6
30µL 5M NaCl
100µL 10% tDOC
100µL 10X complete mini protease inhibitor
720µL Milli-Q H_2O

0.1M and 0.5M Na-phosphate buffers, pH 8
Mix 93.2mL 1M Na_2HPO_4 with 6.8ml 1M NaH_2PO_4 and add either 900mL Milli-Q H_2O (to make 0.1M buffer) or 100mL Milli-Q H_2O (to make 0.5M buffer).
For stock solutions:
1M Na_2HPO_4: 71g anhydrous salt in 500mL Milli-Q H_2O
1M NaH_2PO_4: 69g monohydrate salt in 500mL Milli-Q H_2O

0.2M triethanolamine, pH 8
A triethanolamine buffer of this concentration and pH is available commercially

1M Tris-HCl, pH 7.5
60.6g Tris in 500mL Mili-Q H_2O, pH with concentrated HCl

0.1M glycine, pH 2.5
3.76g glycine in 500mL Milli-Q H_2O, pH with HCl

For solutions containing **0.08% Tween 20 and/or 0.05% DDM or tDOC** add:
-0.8µL/ml Tween 20
-5µL/mL of 10% DDM or tDOC