

Small scale IP - Marta Forn july 2016

This protocol is written working with 30-40 ODs (100ml OD₆₀₀: 0.6-0.8)

Everything (Buffers, tubes, incubations...) at 4°C and bead washes in the cold room

Buffers and reagents**Stop Buffer** (at 4°C, TOXIC)

15mL NaCl 5M
10mL EDTA 0,5M pH7,4
1g Sodium Fluoride
32,5mg Sodium Azide
475mL H₂O

100mL UBA Buffer (at 4°C)

60mL H₂O
2.5mL HEPES 1M pH7,5
15mL KoAc 1M
1mL MgCl₂ 1M
0,5mL CaCl₂ 1M
1mL NP40%
20mL Glycerol

UBA Buffer + Protease inhibitors. In 10mL add:

- 1 pill of cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail
- 1mM of AESB (100x stock: 10mg/100ul)

Laemmli Buffer 4x (room temperature)

200mM Tris-HCl (pH6,8)
8% SDS
0,4% Bromophenol blue
40% Glycerol
Add DTT before use

DTT 1M

Dissolve 1.5 g of DTT in 8 mL of H₂O. Adjust the total volume to 10 mL, dispense into 1-mL aliquots, and store them in the dark (wrapped in aluminum foil) at -20°C

Magnetic Beads+Ab. For each sample and IP:

Process similarly the beads+Ab than the beads that you will use as negative control

1. Wash 20 µl Dynabeads Prot G (Invitrogen, 10009D) with 200ul PBS-T0,1% for 3min in the nutator at 4°C
2. Wash 2 times more
3. Resuspend in 20µl of PBS-T0,1% and add the Antibody
 - i.e. 2 µl anti-Flag M2 mouse, Sigma F1804
 - i.e. 1µl anti-HA rabbit, Sigma H6908
4. Incubate **4-6h** or **O/N** in the wheel at 4°C
5. Wash 2 times with 200µl PBS-T0,1% for 4min in the nutator at 4°C
6. Wash 2 times with 200µl UBA buffer for 4min in the nutator at 4°C
7. Resuspend in 20µl UBA buffer

When I beads are concentrated (first, last step) is better to use commercial tips (you loose less, less binding to the wall of the tip)

1. Collect the cultures in exponential phase
 - a. Centrifugate at 700g for 4min at 4°C.
 - b. Resuspend the pellet in 1mL of STOP buffer and transfer it in a screw tube.
(If you had a 100ml cultures don't combine the tubes, you will have 2 tubes/sample)
 - c. Discard the SN and snap freeze the pellet in liquid nitrogen.
 - d. Keep it at -80°C or continue the protocol.
2. Cell Lysis
 - a. Add the inhibitors to the UBA Buffer.
Magdalena's Lysis Buffer could also been used: 40mM HEPES, 100mM NaCl, 0,1% NP40, 10% Glycerol and PIs.
 - b. Resuspend the pellet in 500µl of UBA Buffer+PIs
 - c. Add 1scoop of glass beads.
 - d. Break it 4 times with the Precellis 24's soft program (2: 5000-1x10-005).
 - e. Invert tubes and puncture bottoms with a hot (gas burner) 22-gauge (grey) needle and place tubes in 2.0 ml tubes, spin them at max speed for 10 sec.
 - f. Transfer samples to a 1.5ml tube. If you have more than 1tube for sample, combine all of them.
3. Solubilize proteins and clarify the lysate
 - a. Add 1µl Benzonase (from MPI, 750U/µl) per 1ml of lysate.
 - b. Incubate it in the wheel for 1h at 4°C.
 - c. Centrifugate at 20.627g for 15min at 4°C.

* Remember to wash the beads coupled to the antibody (for next step)*

 - d. Transfer the SN in a new tube.
4. Immunoprecipitation
 - a. Take 50µ for the input.
 - b. 450-500µl will be used for each1 IP (i.e. 450µl for HA Ab and 450µl for control w/o Ab).
 - c. Add 20µl of beads+antibody.
 - d. Nutate for 3-16h at 4°C.
 - e. Wash the beads with 500µl of the UBA Buffer nutating for 4 min at 4°C.
 - f. Wash the beads with 5000µl of the PBS-T0,1% nutating for 4 min at 4°C.
 - g. Repeat the last step
 - h. Resuspend the beads in 5000µl of the PBS-T0,1%, transfer it to a new tube and nutate it for 4 min at 4°C
 - i. Discard the SN and add 50µl of Laemmli Buffer 2x + 0.1M DTT
(i.e. 25µl Laemmli Buffer 4x + 5µl DTT 1M + 20µl H₂O)
 - j. Boil it for 5-10 min at 95°C in the Thermoblock at max rpm.
 - k. Transfer the SN to a new tube. You can directly load it to the an acrylamide gel or keep it at -20°C