PROT_17v1

Small scale IP - Marta Forn july 2016

This protocol is written working with 30-40 ODs (100ml OD_{600}: 0.6-0.8)

Everything (Buffers, tubes, incubations…) at 4ºC and bead washes in the cold room

Buffers and reagents

Stop Buffer (at 4ºC, TOXIC)
15mL NaCl 5M
10mL EDTA 0,5M pH7,4
1g Sodium Fluoride
32,5mg Sodium Azide
475mL H_2O

100mL UBA Buffer (at 4ºC)

60mL	H_2O
2.5mL	HEPES 1M pH7,5
15mL	KoAc 1M
1mL	$MgCl_2$ 1M
0,5mL	$CaCl_2$ 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 4ºC
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 4ºC
6. Wash 2 times with 200µl UBA buffer for 4min in the nutator at 4ºC
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)

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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 H_2O)
    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