PRC2 purification from insect cell

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[Cell lysis]

1. Prepare buffers

BC 0 and BC1000

Stock	Final concentration	BC 0	BC 1000
1M Hepes-NaCl (pH 7.8)	20mM	20	20
5M NaCl	0 or 1 M	0	200
50% Glycerol	10%	200	200
10% NP-40	0.02%	2	2
500mM EDTA	1mM	2	2
DW		upto 1000ml	Upto 1000ml
		1000ml	1000ml

BC350 (separately make it as below or combine BC 0 and BC1000)

Stock	Final concentration		
1M Hepes-NaCl (pH 7.8)	20mM	10	20
5M NaCl	350mM	35	70
50% Glycerol	10%	100	200
10% NP-40	0.02%	1	2
500mM EDTA	1mM	1	2
DW		upto 500ml	Upto 1000ml
		500ml	1000ml

Protease Inhibitors, Phosphatase inhibitors freshly

Protease inhibitors : PMSF, Benzamidine, Aproprotin, Pepstatin, Leupeptine

Phosphatase inhibitors : NaF, NaVO3

While making whole cell extract, increase the concentration of NP-40 to 0.1%. Reduce the concentration of NP-40 to 0.02% when you elute the protein from the first column.

2. For 200ml cultured insect cells, add 20ml of BC350 (0.1% NP-40). Depending on the pellet amount you may change the volume. Resuspend thoroughly before sonication.

3. Sonication. Duty cycle 4 (40% intensity)/ pulse on 1s/pulse off 1s/ total time 40s x 4 times (20s interval)

4. Incubate on ice for 10 min. Mix it well every 2 mins by inverting the tube.

5. Centrifuge the sample at 15000rpm (JA 25.5 rotor) for 20 mins. Check whether the pellet has dark spots (This indicates that the cell lysis and sonication were successful.)

6. Carefully collect supernatant (WCE). **Keep 50ul of WCE for the analysis**.

In each step, always keep at least 30-50ul of samples for the analysis.

- When incubating extracts with beads, use the rotator to prevent beads to precipitate at the bottom. Make sure not to rotate fast. If you see bubbles, that is a sign of fast rotation.

[Ni-NTA column] if one of proteins has 6x-HIS tag

1. Use 1.5ml of Ni-NTA column and equilibrate the column in BC350 (0.1% NP-40). For centrifuge, don't excess 500xg.

2. Add final 5mM Imidazole (pH 8) to the whole cell extract (WCE). Be extra careful with concentration. Some 6x HIS tagged proteins could start eluting from 20mM Imidazole. Add WCE to Ni beads. Incubate 2 hours in 4 degrees (Batch). From here to the elution step, all buffers need at least 5mM imidazole (IDZ).

4. Centrifuge at 500g for 3 mins. Save the supernatant (Ni Flow-through) for analysis.

5. Wash the beads with BC350 (7.5mM IDZ, 0.1% NP-40) three times for 5 mins / BC150 (7.5mM IDZ, 0.02% NP-40) twice for 5 mins.

6. Elute with BC150 (150mM IDZ, 0,02% NP-40). Use 1-1.5 column vol of buffer each time and incubate for 5mins. Centrifuge and take out the elution. Repeat until the concentration of protein decreased significantly. 4-5 times is enough.

7. Run SDS-PAGE gel to see the result.

[anti-FLAG agarose bead]

1. Equilibrate the bead (350ul bead volume-depend on the abundance of the protein) with BC350 or BC150 (depends on the previous eluate buffer condition). The binding capacity of FLAG beads is approximately 1mg/ml.

2. Incubate the sample with anti-FLAG agarose bead for at least 3 hours in 4 degrees.

4. Wash with BC350 three times (Batch) 10 mins in 4 degrees. Depending on the protein you can wash with BC500 in the middle.

5. Elution. Wash beads with BC180. Add 1 column vol of BC180 then add 3x FLAG peptide (final concentration of 3x FLAG peptide is app 100ug/ml). Incubate at 4 degrees for at least 1 hour. Elute three more times. You may proceed more if protein still comes out.

[Streptactin bead] Proceed this step if one of the subunits has STREP-tag.

1. Equilibrate Streptactin beads with BC180 or BC buffers with previous eluate condition. If you are purifying PRC2 accessory proteins alone, DO NOT use BC buffer that contains lower than 300mM NaCl. The binding capacity of Streptacin beads is 3 mg/ml. Try to use a minimal amount of beads.

2. Incubate the sample with Streptactin beads for at least 2 hours. Do not incubate overnight. The efficiency of elution may go down.

3. Wash with corresponding BC buffers 3 times (5 min incubation). Wash once more with BC buffers including 0.1% NP-40 and 0.1 % Triton X-100 (1 min incubation).

4. Make elution buffer [BC buffer (0.1% NP-40 and 0.1% Triton X-100) + 30mM Biotin or desthiobiotin (d-biotin]. If you use desthiobiotin, you can regenerate the column. If you use biotin, you cannot regeneration the column. Most of the protocols recommended using of 2.5mM Biotin (or D-biotin). But the efficiency of elution is not good. When melting biotin or d-biotin powder, you might see the precipitation. The buffer pH should be close to pH 8 so that biotin(d-biotin) melts in the buffer. This will increase the efficiency of the elution.

5. Elute with the elution buffer (Use 0.5 column volume). Incubate for 15 mins at 4 degrees. Repeat 4-6 times. Do not leave eluate overnight, instead, go to the next column or dialyze against BC180 (0.02% NP-40, no Triton X-100).

[Q-sepharose column]

This step is to remove all possible nucleic acid contaminants. Nucleic acids do not bind to Q column.

1. Equilibrate Q-sepharose beads with BC200. The capacity of this bead is about 3-5mg/ml. Determine the amount of beads based on the total protein in FLAG eluates.

2. Add FLAG eluates and incubate for 1hour at 4 degrees.

3. Wash with BC200. x3

4. Elute with BC550. Normally I use 1 column vol for elution and elute until the concentration of protein reduces significantly. You can manage the volume to concentrate the protein.

5. Dialyze the protein to BC100.

[Glycerol gradient- if subunits are not stoichiometric: optional]

1. Use BC100 (final 15% glycerol) and BC100 (final 35% glycerol) and make 15-35% glycerol gradient (gradient maker).

2. Add concentrated sample (150ul maximum) from the previous purification step. This sample contains 10% glycerol therefore the sample will be at the top.

3. Ultracentrifuge 37000 rpm for 20 hours.

4. take each 200ul from the top and label the column fraction.

5. take samples and run SDS-PAGE gel to check the result.

6. Gather the fraction that is in the right size.