Large Scale Native Affinity Purifications of Solubilized Membrane Proteins from Yeast

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[Abstract] This protocol can be used to purify membrane proteins from yeast samples under native conditions at a large scale. This protocol has been developed primarily for FLAG-tagged proteins. This protocol can however be slightly modified and applied to other tags, such as GST or HA.

Materials and Reagents

1. EDTA free protease inhibitors (Roche Diagnostics)
2. Digitonin (EMD Chemicals)
3. Protease Inhibitors (DMSO, leupeptin, pepstatin) (Sigma-Aldrich)
4. ANTI-Flag M2 affinity gel (Sigma-Aldrich)
5. 3x FLAG peptide (Sigma-Aldrich)
6. $NaF^{\#}$ (Ser/Thr phosphatase inhibitor)
7. Na_3VO_4 (Tyr phosphatase inhibitor)
8. HEPES
9. KOAc
10. $Mg(OAc)_2$
11. $CaCl_2$
12. Sorbitol
13. Glycerol
14. KOH
15. PMSF
16. DMSO
17. Lysis buffer (see Recipes)
18. Immunoprecipitation buffer (see Recipes)
19. 50 ml lysis buffer (see Recipes)
20. 14 ml IP buffer with 2% digitonin (see Recipes)
21. 20 ml IP buffer with 0.1% digitonin (see Recipes)
22. 40 ml IP buffer with 0.1% digitonin (see Recipes)

23. 3x FLAG elution buffer (see Recipes)

Equipment

1. Avestin Emulsiflex C3 homogenizer (Avestin®)
2. Beckman centrifuge and Type 70 Ti rotor (Beckman Coulter)
3. Beckman polycarbonate centrifuge tubes (Beckman Coulter, catalog number: 355631)
4. 1 L centrifuge bottles
5. 50 ml conical tubes

Procedure

A. Preparation of cell lysate

1. Collect 4,000 OD of cells collected at OD_{600}~1.4. Split to 4x 1 L centrifuge bottles. Spin at 5,000 rpm for 20 min using Rotor JLA 8.100.
2. Wash samples in each bottle with 100 ml H_2O. Transfer to 50 ml conical tubes. Store at -80 °C if necessary.
3. Resuspend cells in 50 ml ice-cold lysis buffer supplemented with protease inhibitors (no DTT) and phosphatase inhibitors (add 50 ml buffer to first bottle, vortex, transfer all to the next bottle and so on. Eventually you will have two 50 ml conical tubes of sample).
4. Use Avestin Emulsiflex C3 homogenizer to disrupt cells.
5. Clear unlysed cells by centrifugation at 1,000 *x g* twice or three times.
6. Transfer supernatant to Beckman polycarbonate tubes (the filling level is 16.5 ml in Type 70 Ti rotor). Use a Type 70 Ti rotor for high-speed spin at 44,000 *x g* (24,453 rpm) for 30 min to pellet microsomes.
7. Microsomes are resuspended in 0.5 ml lysis buffer and then diluted with 14 ml immunoprecipitation buffer with 2% digitonin supplemented with protease inhibitors.
8. Membrane proteins can be solubilized by nutating lysate at 4 °C for 1.5 h. Remove unsolubilized material by centrifugation at 44,000 *x g* for 30 min (24,453 rpm in a Type 70 Ti rotor).

B. FLAG Fusion Protein Immunoprecipitation

1. Use 300 µl of gel suspension per reaction (~150 µl of packed gel volume).
2. Thoroughly suspend the ANTI-FLAG M2 affinity gel in the vial and transfer them to a 15 ml conical tube.
3. Centrifuge the resin for 1 min at 400 *x g*. Wait for 1-2 min before handling the samples. Remove the supernatant with a gel-loading pipette tip.

4. Wash the packed gel 4x with 5 ml of immunoprecipitation buffer with 0.1% digitonin.
5. Add the ~15ml cell lysate to the ANTI-FLAG resin. Immuoprecipitations are rotated for 3 h or overnight at 4 °C.
6. Wash the resin 4x with 10 ml immunoprecipitation buffer with 0.1% digitonin. Transfer the beads into an Eppendorf tube.

C. Elution of the FLAG –fusion protein

Elution with 3x FLAG peptide. This elution efficiency is very high using this method.

1. Prepare 3x FLAG Elution Buffer. Add 150 µl of 1x immunoprecipitation buffer with 0.25% digitonin and 1 µg/µl 3x FLAG peptide.
2. Add 150 µl of 3x FLAG elution buffer to the resin in the test tube.
3. Incubate the samples with gentle shaking for 30 min at 4 °C.
4. Centrifuge the resin for 1 min at 400~1000 *x g*. Transfer the supernatant to fresh test tubes.
5. Repeat eluation with another 150 µl of 3x FLAG elution buffer with 0.25% digitonin and 0.1 µg/µl 3x FLAG peptide. Combine the eluates.

Recipes

1. Lysis buffer

Lysis buffer	1 L	2x buffer without detergent
50 mM HEPES (FW 238.3)		23.83 g
50 mM KOAc* (FW 98.14)		9.814 g
2 mM $Mg(OAc)_2$		4 ml of 1 M $Mg(OAc)_2$
1 mM $CaCl_2$		20 ml of 0.1 M $CaCl_2$
200 mM sorbitol (FW 182.17)		72.87 g
1 mM $NaF^{\#}$ (Ser/Thr phosphatase inhibitor)		0.084 g
0.3 mM Na_3VO_4 (Tyr phosphatase inhibitor)		0.1103 g

2. Immunoprecipitation buffer

Immunoprecipitation buffer	1 L	2x buffer without detergent
50 mM HEPES-KOH (pH 6.8)		23.83 g
50 mM KOAc* (FW 98.14)		9.814 g
2 mM $Mg(OAc)_2$		4 ml of 1 M $Mg(OAc)_2$
1 mM $CaCl_2$		20 ml of 0.1 M $CaCl_2$
15% glycerol (v/v)		300 ml of 100% glycerol

Add Mili-Q H_2O to final volume, adjust to pH=6.8 using 0.5 M KOH.

Add the following protease inhibitors per 50 ml immunoprecipitation buffer before use: 1 complete tablet, EDTA free protease inhibitors (crush tablet first in weigh paper).

Add 100 µl of 0.5 M PMSF in DMSO.

3. 50 ml lysis buffer

   25 ml 2x lysis buffer

   25 ml H_2O

   1 EDTA free protease inhibitor tablet

   100 µl of 0.5 M PMSF in DMSO

4. 14 ml of IP buffer with 2% digitonin

   7 ml of 2x IP buffer

   2.8 ml of 10% digitonin

   1ml EDTA free protease inhibitor tablet

   28 µl of 0.5 M PMSF in DMSO

   4.2 ml of H_2O

5. 20 ml of IP buffer with 0.1% digitonin

   10 ml of 2x IP buffer

   0.2 ml of 10% digitonin

   9.8 ml of H_2O

   Protease inhibitors (20 µl of PMSF, leupeptin, and pepstatin)

6. 40 ml of IP buffer with 0.1% digitonin

   20 ml of 2x IP buffer

   0.4 ml of 10% digitonin

   19.6 ml of H_2O

   Protease inhibitors (40 µl of PMSF, leupeptin, and pepstatin)

7. 150 µl of elution buffer with 1µg/µl 3x FLAG peptide

   75 µl of 2x IP buffer

   3.75 µl of 10% digitonin

   30 µl of 5 mg/ml 3x FLAG peptide

   41.25 µl of H_2O

8. To make 150 µl of elution buffer with 0.1 µg/µl 3x FLAG peptide

   75 µl of 2x IP buffer

   3.75 µl of 10% digitonin

   3 µl of 5 mg/ml 3x FLAG peptide

   68.25 µl of H_2O

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References

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