Small Scale Native Affinity Purifications of Solubilized Membrane Proteins from Yeast

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[Abstract] In this protocol, we show how to purify membrane proteins from yeast using affinity purification under native conditions at a small scale.

Materials and Reagents

1. PBS
2. HEPES
3. KOAc*
4. $Mg(OAc)_2$
5. $CaCl_2$
6. Sorbitol
7. Wash buffer
8. EDTA free protease inhibitors (Roche Diagnostics)
9. Digitonin (EMD Chemicals)
10. Protease Inhibitors (DMSO, leupeptin, pepstatin) (Sigma-Aldrich)
11. ANTI-Flag M2 affinity gel (Sigma-Aldrich)
12. 3x FLAG peptide (Sigma-Aldrich)
13. $NaF^{\#}$ (Ser/Thr phosphatase inhibitor)
14. Na_3VO_4 (Tyr phosphatase inhibitor)
15. Lysis buffer (see Recipes)
16. Immunoprecipitation buffer (see Recipes)
17. Elution buffer (see Recipes)

Equipment

1. Beckman centrifuge and TLA-55 rotor (Beckman Coulter)
2. Acid washed 425-600 glass beads (Sigma-Aldrich)
3. Beckman centrifuge tubes (Beckman Coulter)

Procedure

A. Preparation of cell lysate

1. Collect 25 OD of cells. Wash cells once with 1 ml H_2O or PBS at 4 °C. Store at -80 °C if necessary.
2. Resuspend cells in 150 µl ice-cold immunoprecipitation buffer with 0.1% digitonin and supplemented with protease inhibitors (no DTT) and phosphatase inhibitors.
3. Add glass beads below the meniscus. Break cells by vortexing for 10 min in the cold room.

   Note: Digitonin is a less harsh nonionic detergent that permeablizes membranes and can be used to extract membrane proteins in intact forms.
4. Add 850 µl of immunoprecipitation buffer with 1.16% digitonin plus protease inhibitor (no DTT) to cell lysate, bringing the total volume to 1 ml. This raises the final digitonin concentration to 1%. Membrane proteins are solubilized by nutating lysate at 4 °C for 40 min. Pipette liquid into fresh Beckman centrifuge tubes.
5. Centrifuge cell lysate at 100,000 *x g* for 10 min to clear up unlysed cells, cell nuclei, and membranes. Membranes proteins are extracted from the membranes, hence in the supernatant. Collect supernatant for immunoprecipitation (use TLA-55 rotor, 47,246 rpm). Save 80 µl of the supernatant as input.

B. FLAG fusion protein immunoprecipitation

1. Use 50 µl of gel suspension per reaction (~25 µl of packed gel volume). Smaller amounts of resin (~10 µl of packed gel volume, which binds > 1 µg FLAG-tagged protein) can be used.
2. Thoroughly suspend the ANTI-FLAG M2 affinity gel in the vial. The ratio of suspension to packaged gel volume should be 2:1.
3. Immediately transfer 50 µl of the resin in its suspension buffer to a fresh test tube. For resin transfer use a plastic pipette tip with the end cut off.
4. Centrifuge the resin for 1 min at 400 *x g*. Wait for 1-2 min before handling the samples.
5. Remove the supernatant with a gel-loading pipette tip.
6. Wash the packed gel 4x with 1 ml of immunoprecipitation buffer with 0.1% digitonin. Be sure that most of the wash buffer is removed and no resin is discarded. This will ensure all of the glycerol is removed before the protein is bound to the gel.
7. In case of numerous immunoprecipitation samples, wash the resin needed for all samples together.

8. After washing, divide the resin according to the number of samples tested. Each wash should be performed with a 1x wash buffer at a volume equal to 20 times the total packed gel volume (*e.g.,* 25 µl packed gel volume, >500 µl wash buffer).
9. Add 800 µl of cell lysate to the washed resin. If necessary, bring the final volume to 1 ml by adding immunoprecipitation buffer. The volume of cell lysate to be used depends on the expression level of FLAG-tagged protein in the cells.
10. For negative control, add only 1 ml of lysis buffer with no protein.
11. Agitate samples and control gently on a roller shaker for 2.5 h.
12. Centrifuge the resin for 1 min at 400 *x g*. Remove the supernatants with a gel loading pipette tip. Save 80 µl of the supernatant as unbound.
13. Wash the resin 4x with 1 ml immunoprecipitation buffer.

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 30 µl of 1x wash buffer with 0.25% digitonin and 2 µg/µl 3x FLAG peptide.
2. Add 30 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. Be careful not to transfer any resin.

Recipes

1. Immunoprecipitation buffer

Immunoprecipitation 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 MgOAc
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

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 0.5 M PMSF in DMSO, leupeptin, pepstatin.

2. To make 1 ml 1x lysis buffer with 0.1% digitonin
   500 µl 2x lysis buffer
   10 µl 10% digitonin
   Protease inhibitors
   490 µl H_2O
3. To make 3.6 ml 1x lysis buffer with 1.16% digitonin
   1.8 ml 2x lysis buffer
   418 µl 10% digitonin
   Protease inhibitors
   1.38 ml H_2O
4. To make 22 ml 1x lysis buffer with 0.1% digitonin
   11 ml 2x lysis buffer
   220 µl 10% digitonin
   Protease inhibitors
   10.7 ml H_2O
5. To make 120 µl elution buffer for 4 samples
   60 µl of 2x lysis buffer
   48 µl of 5 µg/µl 3x FLAG peptide
   3 µl of 10% digitonin
   9 µl of H_2O

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References

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