Purification of the yeast HRD-ligase complex by immunoprecipitation from solubilized microsomes

Thomas Sommer

Max-Delbrük-Center for Molecular Medicine, Berlin, Germany

INTRODUCTION

This protocol is used by the Sommer-lab to purify the HRD-ligase complex from yeast microsomes for subsequent analysis by immunoblotting. It may be up-scaled and the samples may then be analyzed by mass-spectroscopy. You need a yeast strain expressing myc- or HA-epitope tagged variants of subunits of the HRD-ligase.

MATERIALS

IP15-LB

- 200 mM Kac
- 50 mM Tris-Cl pH 7.5
- 0.1 mM EDTA

IP15

- 200 mM KAc
- 50 mM Tris-Cl pH 7.5
- 0.1 mM EDTA
- 10 % Glycerol
- 0.5 % NonidetP 40 (we also use 1 % Digitonin in some instances; Digitonin should be added from a 5 % stock in H_2O immediately before use since it tends to precipitate from aqueous solutions over time; store the 5 % Digitonin stock at -20 °C).

3 x Sample buffer

- 50 mM Tris-Cl pH 6.8
- 3 % SDS
- 30 % Glycerol
- add 100 mM DTT when required

METHODS

Preparation of the microsomes

1. Grow yeast cells expressing myc-tagged the myc- or HA-epitope tagged HRD-ligase subunits in Erlenmeyer-flasks over night at 30 °C in YPD.
2. Dilute the cells in the morning in fresh YPD to an OD_{600} of 0.3 and continue shaking at 30 °C until the culture has an OD_{600} of 1 – 1.2.
3. Harvest 50 – 70 OD_{600} cells by centrifugation in a 50 ml Falcon-tube using a table-centrifuge.
4. Wash the cell-pellet once with ice-cold sterile water containing 1 mM PMSF and suspend the cells in ice-cold 0.8 ml IP15-LB buffer containing 1 mM PMSF. Keep the cells on ice from now on.

5. Transfer the cells suspension into a 15 ml Falcon-tube and add 0.8 ml glass-beads
6. Break the cells by vigorous shaking on a vortex or IKA-Vibrax VXR shaker for 2 min at maximum speed.
7. Add 2 ml IP15-LB, mix and remove cell-debris by low speed centrifugation (950 x g for 3 min.) in a table-centrifuge at 4 °C.
8. Transfer the supernatant carefully to a 2 ml Eppendorf tube and pellet the microsomes by centrifugation in a cooled Eppendorf-centrifuge at 20,800 x g for 20 min. Remove the supernatant and keep the pellet.

Solubilization of the microsomes

1. Add 500 ul IP15 buffer to the pellet from step 1.8 and suspend carefully by pipetting up and down with a Gilson-pipette. Incubate for at least 30 min on a rotating wheel at 4 °C.
2. Remove insoluble material by centrifugation at 20,800 x g for 15 min. Transfer the supernatant into a fresh 1.5 ml Eppendorf tube.

Immunoprecipitation

1. Remove 50 ul from the solubilized microsomes into a fresh Eppendorf tube (serves later as “Input” control).
2. Add 15 ul ProteinA-Sepharose and 0.5 ul monoclonal anti-HA or anti myc antibody to the rest
3. Incubate on a rotating wheel at 4 °C over night.
4. Spin down the beads in a Eppendorf-centrifuge and carefully remove the supernatant.
5. Wash the beads twice with IP-15 buffer. After the second wash remove all liquid with a Hamilton syringe.
6. Add 50 ul sample buffer without DTT and incubate for 20 min. at 42 °C.
7. Transfer the soluble material into a fresh tube using a Hamilton syringe and add 5 ul 1 M DTT.
8. Incubate at 65 °C for 10 min. Also add 40 ul 3x sample buffer with DTT to the 50 ul input material (see step 3.1) and incubate at 65 °C.
9. Load 5 ul of the IP-reaction and 10 ul of the input material onto a SDS-PAA minigel. Separate the proteins at 120 V (takes roughly 120 min.).
10. Transfer onto a PVDF-membrane using your preferred protocol, block the membrane and incubate with appropriate antibodies.

BIBLIOGRAPHY

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