

Cycloheximide-decay assay

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INTRODUCTION

This protocol is used by the Sommer-lab to determine semi-quantitative protein-turnover in yeast cells.

MATERIALS

- 4 x 2ml Eppendorf tubes per yeast strain with 500 μ l 30 mM Na-Azide on ice; label the tubes with strain name plus time point (e.g. 0, 30, 60 and 90 min.)
- Glass-beads (0.8 mm diameter)
- Water bath at 30 °C

YPD

- 2 % Yeast extract
- 1 % peptone
- 2 % glucose in water
- autoclave without glucose and add the glucose afterwards from a 20 % filter-sterilized stock

Solutions and Buffers

- 100 mM PMSF in ethanol

Lysis buffer

- 50 mM Tris-Cl
- 1 % SDS
- 1 mM EDTA
- add 1 mM PMSF before use

3 x Sample buffer

- 50 mM Tris-Cl pH 6.8
- 3 % SDS
- 30 % Glycerol
- add 100 mM DTT before use

METHODS

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 10 - 15 OD_{600} cells by centrifugation in a 50 ml Falcon-tube using a table-centrifuge at room temperature.
4. Suspend the pellet in 4.5 ml fresh YPD and place in pre-warmed 30 °C water bath.

5. Add 100 ul of 100 mM Cycloheximide and mix carefully; remove 1 ml of cell suspension and pipette it into the tube labeled with "0" (time point 0); Mix and place on ice. Start the timer and continue to incubate the rest of the cell suspension at 30 °C
6. Collect further 1 ml aliquots at the given time points and mix them with the Na-Azide. Depending on the half life of the protein of interest this time will vary (usually between 10 min and 1 hour).
7. When all samples are collected, pellet the cells in the Eppendorf tubes by centrifugation (2 min., 1,000 x g).
8. Remove supernatant and suspend the pellet in 150 ul ice-cold Lysis buffer supplemented with 1 mM PMSF and then add 100 ul glass beads.
9. Break the cells by vigorous shaking on a vortex or IKA-vibrax VXR shaker for 2 min at maximum speed.
10. Add 100 ul 3 x Sample buffer and incubate the samples 10 min at 65 °C.
11. Spin down insoluble material and load 5 ul of the supernatant onto a SDS-PAAG.
12. Transfer proteins onto a PVDF-membrane, block the membrane and incubate with antibody of your choice.
13. Analysis may be done by classical ECL-Western or by fluorescence-labeled antibodies.

BIBLIOGRAPHY

Gauss, R., Sommer, T. & Jarosch, E. The Hrd1p ligase complex forms a linchpin between ER-luminal substrate selection and Cdc48p recruitment. 2006, *EMBO J.* 25, 1827-1835.