

***In vitro* deUbiquitination assay with purified GST-DUBs**

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INTRODUCTION

This protocol is designated to detect the activity of a purified GST-deubiquitinating enzyme with a pool of poli-ubiquitinated conjugated proteins in sepharose beads.

MATERIALS

Reagents

- Lipofectamin 2000, (Invitrogen)
- HA monoclonal antibodies (SIGMA)
- Sepharose gamma bind G (GE)

IP buffer

- 50 mM Tris-Cl pH 7.6
- 150 mM NaCl
- 5mM EDTA pH 8.00
- 2mM MgCl₂
- 0.5% NP40
- 0.1% SDS
- Supplied with P.I. and NEM 10mM

Reaction buffer

- Tris-Cl 50mM pH 7.5
- NaCl 150 mM
- EDTA 2mM pH 8.00
- DTT 2 mM

METHODS

A. Immunoprecipitation of UbHA-proteins

1. 100 mm Petri dish of COS7 cells were transfected with Lipofectamin 2000, with pCDNA3 plasmid codify for Ubiquitin tagged HA.
2. After 30 hours from transfection, cells were lysed with 1 ml of IP buffer and centrifuged for 10 min at 4°C at 13000 RPM.
3. Clear supernatant was precleaned under gently rotation at 4°C with 20 µl sepharose gamma bind G.
4. HA antibodies were added (1:100) to the precleaned cell lysate, and incubate for 1 h at 4°C under gently rotation.
5. 60 µl of sepharose gamma bind G were added in to the precleaned cell lysate and incubate O/N at 4°C under rotation.
6. The resin was rescued with centrifugation at 1000 RPM for 5 min at 4°C and wash 1 time with lyse buffer and 2 times with PBS 1x.

7. The sepharose UbHA-proteins beads were storage in presence of 10% glycerol at -20°C.

B. DUB assay, time kinetic

1. 10 μ l of sepharose beads UbHA-proteins are incubated with 20 nM of purified GST-protein at 37°C in 30 μ l of reaction buffer for 1, 2, 4, 8, 16 h. 10 mM NEM is added in a 16h sample as control of DUB inactivation.
2. To block the reactions, denaturation loading buffer (Invitrogen) was added in all the samples, denaturated 10 min at 95 °C and loaded SDS-PAGE gel (Invitrogen).
3. Anti mouse HA antibodies were used for Western blot.

C. DUB assay, titration.

- The pool of sepharose beads UbHA-proteins can be used to measure the activity of a titrated purified GST-protein in a prefix time of incubation.

BIBLIOGRAPHY

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