

***In vitro* ubiquitination assay**

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INTRODUCTION

Modification with ubiquitin (Ub) has been reconstituted *in vitro* for many known Ub targets. Assays usually involve incubation of bacterially expressed targets with recombinant or purified enzymes, Ub and ATP. Ub modification results in a target mobility shift of 8kDa, and is detected by anti-Ub immunoblot. The efficiency of *in vitro* ubiquitination assay depends on the quality of the enzymes involved. Here is described the assay developed in our laboratory.

MATERIALS

Ubiquitination buffer

- 25 mM Tris-Hcl pH7.6
- 5 mM MgCl₂
- 100 mM NaCl
- 1 mM DTT
- 2 mM ATP

1x YY buffer

- 50 mM Hepes pH 7.5
- 10% Glycerol, 150 mM NaCl
- 1% Triton X-100
- 1 mM EDTA
- 1 mM EGTA

Inhibitors for phosphatases and protease were freshly added to the lysis and washing buffers

- 20 mM Na pyrophosphate pH 7.5
- 50 mM NaF
- 2 mM PMSF in ethanol
- 10mM Na vanadate in HEPES pH 7.5
- Protease Inhibitor Cocktail 1:500 (Sigma)

METHODS

1. Nedd4 (E3), Ub, are produced as GST-fusion proteins, cleaved from the tag and purified.
2. Reaction mixtures (50 μ l) containing purified enzymes:
 - 100 ng E1 (BostonBiochem)
 - 150 ng of purified HIS-tagged Ubch5B
 - 0.5 μ g Nedd4 (E3)
 - 2.5 μ g of the substrate (either full-length eps15 or mutants, or γ -ENAC, or N4WBP5A, all produced as GST fusion proteins)

- 5 μ g of Ub in ubiquitination buffer
- 3. Reactions are incubated at 30°C for 1h, followed by four washes in YY binding buffer, and detected in immunoblot.

BIBLIOGRAPHY

Woelk T, Oldrini B, Maspero E, Confalonieri S, Cavallaro E, Di Fiore PP, Polo S. (2006) Molecular mechanisms of coupled monoubiquitination. *Nat Cell Biol.* 2006 Nov;8(11):1246-54.