

***In vitro* Protein Ubiquitination Assays**

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[Abstract] Ubiquitin can be added to substrate protein as a protein tag by the concerted actions of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin protein ligase (E3). At the present of E1 and ubiquitin, E2 activity can be determined by the thio-ester formation. The E3 activity of a putative protein as well as the E2/E3 or E3/substrate specificities also can be explored by *in vitro* ubiquitination assay. The result can be detected by western blot with certain antibody. Purified proteins expressed from bacterial system are always used in this assay.

Materials and Reagents

1. Cell crude extract
2. Purified protein or crude extract of ubiquitin activating enzyme (E1)
3. Purified recombinant ubiquitin conjugating enzyme (E2) fused with a protein tag (such as 6x His tag)
4. Purified recombinant ubiquitin ligase (E3)
5. Purified ubiquitin or recombinant ubiquitin protein fused with a tag (Kraft *et al.*, 2005; Liu *et al.*, 2010)
6. ATP (Sigma-Aldrich, catalog number: A7699)
7. MgCl₂ (Sigma-Aldrich, catalog number: V900020)
8. Anti-ubiquitin antibody or antibody of a certain protein tag
For example:
Anti-Ub, raised in our laboratory
Anti-His (Santa Cruz, catalog number: sc-0836)
Nickel-HRP (KPL, Kirkegaard & Perry Laboratories, catalog number: 24-01-01)
Anti-GST (Beijing Protein Innovation, catalog number: AbM59001-2H5-PU)
Anti-MBP (New England Biolabs, catalog number: E8030S)
9. Amylose resin (New England Biolabs, catalog number: E8021)
10. DTT
11. SDS-PAGE gel

12. Tris
13. PMSF
14. Skimmed milk powder or BSA
15. Chemiluminescent HRP substrate kit (EMD Millipore, catalog number: WBKLS0100)
16. Glycerol
17. Bromophenol blue
18. MBP (maltose binding protein) Column buffer (see Recipes)
19. 20x reaction buffer (see Recipes)
20. 4x SDS sample buffer with or without DTT (or β - mercaptoethanol) (see Recipes)
21. 1x PBS (see Recipes)

Equipment

1. Centrifuge
2. Thermo-mixer (Eppendorf Thermomixer comfort)
3. Protein electrophoresis apparatus
4. Western blot apparatus

Procedure

- A. DTT sensitive thio-ester assay of E2 protein
 1. The reaction is performed in total 30 μ l, including 1.5 μ l of 20x buffer, 50 ng of E1, 200-500 ng E2, and 2 μ g ubiquitin.
 2. Incubate the reactions at 37 °C for 5 min.
 3. Split the reactions by adding 10 μ l 4x SDS sample buffer with or without DTT (or β -mercaptoethanol).
 4. Boil the samples at 100 °C for 5 min.
 5. The reaction products are separated with 12% SDS-PAGE gel and detected by Western blotting with anti-ub antibody or antibody for certain tag fused with the E2 protein to detect the formation of DTT-sensitive thio-ester bonds.
 - a. The sample proteins separated by 12% SDS-PAGE gel are electroblotted to nitrocellulose membrane at 100 V for 75 min.
 - b. The membrane is blocked with 1x PBS containing 5% skimmed milk powder for 1 h at room temperature.
 - c. The membrane was then incubated first with primary antibody (suggested dilution ratio: 1:5,000 for anti-Ub, 1:500 for anti-His, 1:500 for anti-MBP antiserum) then with secondary antibody diluted in 1x PBS containing 3%

skimmed milk for 1 h at room temperature. Wash the membrane with 1x PBS for two times (15 min each) after it was incubated with the primary and secondary antibody. If detect His tagged protein with Nickel-HRP, just incubate the membrane with 1:15,000 dilution of Nickel-HRP in 1x PBS containing 1% BSA for 1 h, wash the membrane two times and then bands can be detected.

- d. Bands were detected with the Millipore chemiluminescent HRP substrate kit (Figure 1).

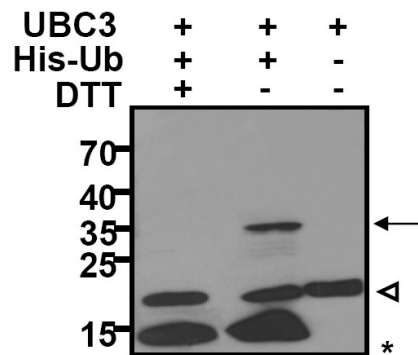


Figure 1. DTT sensitive thio-ester assay of His-UBC3 (Kraft *et al.*, 2005). The reaction samples were detected with anti-His antibody. The arrows indicate the DTT-sensitive thioester linkage. The open triangles indicate bands of E2 protein itself that was not attached to Ub. Asterisks indicate His-tagged Ub.

B. Autoubiquitination assay of E3 protein

The E3 proteins can be purified in a 1.5-ml Eppendorf tube from cell crude extract just before use (recombinant MBP (Maltose binding protein)-E3 protein is used as an example below).

1. Vortex and thoroughly suspend the amylose beads.
2. Aliquot 100 μ l of bead suspension to a sterile microcentrifuge tube.
3. Add 1 ml of MBP column buffer and resuspend the beads.
4. Centrifuge at 400 x g for 2 min and decant supernatant. Repeat wash.
5. Add 0.5-1 ml of crude extract (the total amount of the E3 protein should be 0.5-1 μ g) to the tube containing the prewashed beads.
6. Rotate at room temperature for 1 h (or 4 $^{\circ}$ C for 2 h).
7. Wash the beads with 1 ml of 50 mM Tris-HCl (pH 7.5) for three times (like steps B3-4) and remove all the liquid of the final wash using very thin tips.

8. Prepare the reactions in total 30 μ l, including 1.5 μ l of 20x reaction buffer, 50 ng of E1, 200–500 ng of E2, and 5 μ g of ubiquitin. Add the reaction system to the tubes containing the amylose resin beads binding with MBP-E3 proteins.
9. The reactions minus E1 and minus E2 respectively should be performed at the same time as control.
 - a. Incubate the reactions at 30 °C for 1.5 h with agitation (900 rpm) in a thermomixer.
 - b. Split the reactions by adding 10 μ l 4x SDS sample buffer (with DTT or β -mercaptoethanol) and boil the samples at 100 °C for 5 min.
 - c. The reaction products are separated with 8–12% SDS-PAGE gel and detected with anti-ubiquitin antibody or antibody for certain tag fused with ubiquitin or anti-MBP antibody by Western blotting. (see steps A5a-d) (Figure 2)

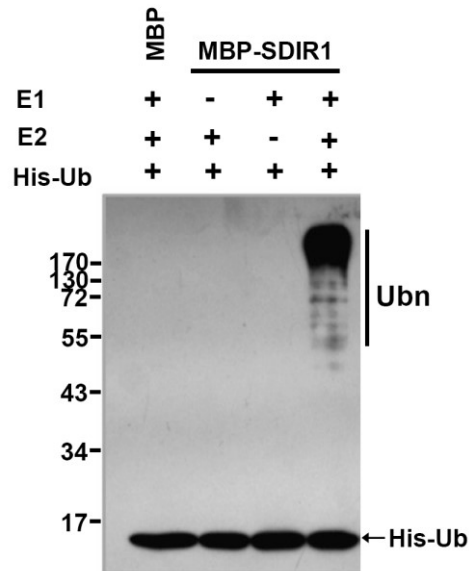


Figure 2. E3 ligase autoubiquitination activity of MBP-SDIR1 (Zhang *et al.*, 2007). MBP-SDIR1 was assayed for E3 activity in the presence of E1 (from wheat), E2 (UBCH5b) and 6x His tagged ubiquitin. Samples were resolved by 8% SDS-PAGE. The nickel–horseradish peroxidase (Nickel-HRP) was used to detect His tag ubiquitin. *Note: E2/E3 specificities could be explored using different E2 proteins combined with the same E3 in this assay.*

C. E3/substrate ubiquitination assay

The E3 and substrate protein should be fused with different tag (and the tag also should be different with the tag fused with E1, E2 and ubiquitin) and the recombinant proteins

should be expressed and purified before use. The proteins also can be prepared via *in vivo* expression such as agroinfiltration in *Nicotiana benthamiana*.

1. Prepare the reactions in total 30 μ l, including 1.5 μ l of 20x reaction buffer, 50 ng of E1, 200 ng of E2, 200-500 ng E3, 500 ng substrate proteins and 5 μ g of ubiquitin. The reactions minus E1, minus E2 (and minus E3) respectively should be performed at the same time as control.
2. Incubate the reactions at 30 °C for 1.5 h.
3. Split the reactions by adding 10 μ l 4x SDS sample buffer (with DTT or β -mercaptoethanol) and boil the samples at 100 °C for 5 min.
4. The reaction products are separated with 8–12% SDS-PAGE gel and detected with anti-ubiquitin antibody or antibody for certain tag fused with substrate protein by Western blotting (see steps A5a-d) (Figure 3).

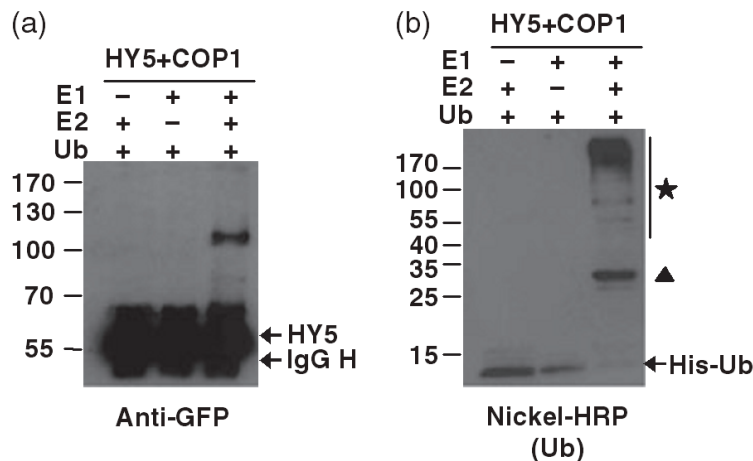


Figure 3. HY5-GFP (substrate protein) was ubiquitinated by Myc-COP1 (E3 ligase) (Zhao *et al.*, 2013). HY5-GFP and Myc-COP1 were all expressed via agroinfiltration. Then, the cell lysates were mixed and immunoprecipitated with anti-Myc antibody. The immunoprecipitated product was applied for a further *in vitro* ubiquitination assay. E1 (from wheat), E2 (UBCH5b) and 6x His tagged Ubiquitin (Ub) were added to the reaction. ★ represents the mixture of poly-ubiquitinated HY5-GFP and Myc-COP1. ▲ means mono-ubiquitinated E2.

(a) The *in vitro* ubiquitination samples were detected by immunoblot with anti-GFP antibody.

(b) The *in vitro* ubiquitination samples were detected by immunoblot with Nickel-HRP (or anti-His antibody) to detect His-Ubiquitin.

Note: The E3 proteins can be purified in a 1.5-ml Eppendorf tube from cell crude extract just before use as steps B1-5. And then the following E3-substrate

ubiquitination reactions should be performed with agitation (900 rpm) in a thermomixer.

Recipes

1. MBP column buffer

20 mM Tris-HCl (pH 7.4)

0.2 M NaCl

1 mM EDTA

Add 1 mM DTT and 1 mM PMSF before use

2. 20x reaction buffer

1 M Tris pH 7.5

40 mM ATP

100 mM MgCl₂

40 mM DTT

Note: Aliquoted and stored at -20 °C. Take out an aliquot from -20 °C just before use and each aliquot can be used only once.

3. 4x SDS sample buffer

0.2 M Tris pH 6.8

8% SDS

40% glycerol

0.004% bromophenol blue

0.4 M DTT (or 20% β-mercaptoethanol)

The buffer without DTT or β-mercaptoethanol can be prepared according to this recipe

4. 1x PBS

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

2 mM KH₂PO₄

Adjust to pH 7.4

Acknowledgments

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