

Video Article

# Detection of Protein Ubiquitination

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## Abstract

Ubiquitination, the covalent attachment of the polypeptide ubiquitin to target proteins, is a key posttranslational modification carried out by a set of three enzymes. They include ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3. Unlike to E1 and E2, E3 ubiquitin ligases display substrate specificity. On the other hand, numerous deubiquitylating enzymes have roles in processing polyubiquitinated proteins. Ubiquitination can result in change of protein stability, cellular localization, and biological activity. Mutations of genes involved in the ubiquitination/deubiquitination pathway or altered ubiquitin system function are associated with many different human diseases such as various types of cancer, neurodegeneration, and metabolic disorders. The detection of altered or normal ubiquitination of target proteins may provide a better understanding on the pathogenesis of these diseases. Here, we describe protocols to detect protein ubiquitination in cultured cells *in vivo* and test tubes *in vitro*. These protocols are also useful to detect other ubiquitin-like small molecule modification such as sumoylation and neddylation.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/1293/>

## Protocol

### Detection of protein ubiquitination in cultured cells

1. Transfect cultured cells with plasmids expressing the protein of interest and (epitope-tagged version of) ubiquitin.
2. Make complete cell lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) with 2mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitors.
3. Lyse cells with 100  $\mu$ l cell lysis buffer per plate (6 cm dish). If a larger dish is used, adjust volume accordingly. Swirl the dish carefully to let the lysis buffer cover the entire area of grown cells.
4. Collect the cells with a cell scraper and transfer the cell lysates into a 1.5 ml eppendorf tube. Place the tube onto a hot plate immediately to boil for 10 min.
5. Shear the cells with a sonication device.
6. Add 900ul of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton). Incubate samples at 4°C for 30-60 min with rotation.
7. Spin the diluted samples at 20,000 x g for 30 min. Transfer the resulting supernatant to a new eppendorf tube. Be careful not to disturb the pellet.
8. Measure the protein concentration.
9. Prepare Protein A- or G-agarose bead-conjugated antibody against the target protein in a compatible buffer (50% slurry). Cut the narrow end of a P-200 pipette tip and transfer 14-20  $\mu$ l of resin to 500-1,500  $\mu$ g of prepared cell lysates for immunoprecipitation. For cells with high transfection efficiency, 500  $\mu$ g will be enough. For cells with low transfection efficiency, more protein may be needed.
10. Incubate the cell lysate-bead mixture at 4°C overnight with rotation.
11. Spin down the beads at 5000 x g for 5 min. Aspirate the supernatant. Wash the resin with the washing buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% NP-40) twice.
12. Spin the beads for a final time at 20,000 x g for 30 sec. Aspirate the residual washing buffer and boil the resin with 2X SDS loading buffer.
13. Load samples onto a SDS-PAGE gel for immunoblotting analysis.
14. Detect ubiquitin and the target protein with respective antibodies. For immunoblotting, we generally detect ubiquitin first. The membrane will then be used to detect the protein precipitated.

### Detection of ubiquitination on target protein through *In vitro* ubiquitination assay

1. Make 5X ubiquitination buffer (100 mM Tris-HCl, pH 7.5, 25 mM MgCl<sub>2</sub>, 2.5 mM DTT, 10 mM ATP). Store them in small aliquots at -20 °C for up to 6 month. Some protocols also add creatine phosphate and creatine kinase into the buffer for ATP regeneration <sup>1,2</sup>.

2. For each reaction, prepare a mixture containing the following:

8 $\mu$ l	5X ubiquitination buffer
250 ng	ubiquitination E1
500 ng	ubiquitination E2
0.5 $\mu$ g	ubiquitin
0.5 $\mu$ g	protein of interest
	water to 40 $\mu$ l total volume

As controls, prepare similar reactions in the absence of either E1, E2, or ubiquitin.

- Incubate the mixture at 37°C for 1 hour or longer.
- Stop the reaction by adding SDS-PAGE sample buffer and boil the sample for 10 min.
- Load samples onto SDS-PAGE gel for immunoblotting analysis.
- Detect ubiquitin and the target protein with respective antibodies.

## Discussion

In this presentation, we first described steps that permit detecting ubiquitin modification on a protein of interest in cultured mammalian cells. In order to detect ubiquitination specifically on the protein of interest, not on non-covalently interacting proteins, we used a stringent condition for cell lysis, immunoprecipitation and washing<sup>1,3</sup>. Ubiquitination, detected by immunoprecipitation of target protein in such a harsh condition followed by anti-ubiquitin immunoblotting, is therefore likely to be specific to the target protein. To prevent protein deubiquitination during the experimental procedures, deubiquitinating enzyme inhibitors such as *N*-ethylmaleimide and ubiquitin aldehyde may be added to all buffers<sup>4</sup>. However, it is unlikely that the deubiquitination enzymes remain active after 10 min boiling procedure. Strong smears or ladders of high molecular species are typically the result of ubiquitination. The degree of ubiquitination of the protein of interest can be assessed by comparing the ratio of ubiquitinated/unmodified target protein in several experiment conditions. Meanwhile, free ubiquitin molecules are generally reduced when ubiquitination of target protein is increased. This protocol is also useful to detect other ubiquitin-like small molecule modification such as sumoylation and neddylation.

We also described an *in vitro* ubiquitination assay using purified or recombinant proteins. Various epitope-tagged ubiquitination components are commercially available. Ubiquitination E3 ligase is not necessary for ubiquitin conjugation *in vitro*.

## Disclosures

The authors have nothing to disclose.

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