MANUAL

TUBE 1 (FLAG)

Catalog Number:
UM601

BACKGROUND

Ubiquitin and Polyubiquitylation

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitylation. The two most well characterized forms of polyubiquitylation occur via linkage at lysine 48 (K48) or lysine 63 (K63). The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein. Polyubiquitination is a reversible process, however, as these chains are degraded and/or removed by proteases known as deubiquitylates (DUBs). The dynamic nature of this signaling represents a major obstacle to the isolation and functional characterization of polyubiquitylated proteins. For this reason, the ubiquitylation state of many proteins is unknown or poorly characterized.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

Traditional strategies for characterization of ubiquitylated proteins often require immunoprecipitation of overexpressed ubiquitin with an epitope tag or the use of ubiquitin antibodies (expensive for large scale studies). Alternatively, isolation of polyubiquitylated proteins can be achieved with certain ubiquitin binding associated domains (UBAs), but these proteins display a low affinity for ubiquitin. Additionally, these strategies require the inclusion of inhibitors of both DUB and proteasome activity to protect the integrity of polyubiquitylated proteins. These conditions could alter cell physiology, which in turn may negatively impact the result or introduce experimental artifact. To overcome these problems, Dr. Manuel Rodriguez, and his team at CIC bioGUNE have developed Tandem Ubiquitin Binding Entities (TUBEs). TUBEs are essentially tandem UBAs with dissociation constants for tetra-ubiquitin in the nanomolar range. TUBEs have also been demonstrated to protect proteins from both deubiquitylation and proteasome-mediated degradation, even in the absence of inhibitors normally required to block such activity. The nanomolar affinity of TUBEs for polyubiquitylated proteins allows for high efficiency in isolation and characterization of these proteins from cell lines, tissues, and organs.

Affinity tagged TUBEs allow for identification and characterization of polyubiquitin proteins by western blotting, as well as isolation of proteins for downstream proteomic studies. **TUBE1 and TUBE 2** are derived from different ubiquitin binding domains and as such may exhibit slight differences in their binding to specific polyubiquitinated target proteins. However, these differences are typically inconsequential. In general, the binding profiles are very similar. **Both TUBE 1 and TUBE 2** bind to K6-, K11-, K48- and K63-linked polyubiquitin.

APPLICATIONS

1. Pull down polyubiquitinated protein from cells, tissues, and organs
2. Isolate ubiquitinated protein of interest by secondary immunoprecipitation
3. Protect polyubiquitylated proteins from degradation during cell lysis

BENEFITS

1. Up to 1000-fold higher affinity for polyubiquitin compared to single UBA form
2. TUBEs have higher specificity and affinity than antibodies
3. Avoid over-expression of epitope tagged ubiquitin for pulldowns
4. Protects polyubiquitylated proteins from degradation in the absence of inhibitors specific to DUB and proteasome activity during cell lysis

COMPONENTS

1. **TUBE 1**

Size:	1 x 200 µg (5mg/ml)
Buffer:	50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol
Storage:	-80°C, avoid cycles of freezing and thawing

Please note that some physical characteristics and protocols are item specific. Please refer to individual product sheets or application notes now available at www.lifesensors.com for further information.

ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 50mM Tris-HCl, pH 7.5, 0.15M NaCl, 1mM EDTA, 1% NP-40, 10% glycerol.

The use of alternative buffer systems should not impact TUBE function; however, the inclusion of detergents e.g. (SDS or deoxycholate) may have a negative impact on overall yield of polyubiquitylated proteins.

The inclusion of a protease inhibitor cocktail is recommended to protect from non-specific protein degradation during lysis and isolation.

2. **Resin Wash buffer:** 20mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.1% Tween-20 (TBS-T)
3. **FLAG Affinity Resin**
4. **Optional:** FLAG® peptide (Sigma-Aldrich Cat. No. F3290) or 0.2M glycine HCl, pH 2.5 as alternatives to elution with SDS-sample buffer. For additional information regarding the use of these reagents to elute isolated proteins from the affinity gel, please refer to the manufacturer's instructions

EQUILIBRATION OF AFFINITY RESIN

(Suggested Protocol) Refer to the manufacture's recommended protocol before starting.

All steps are for a single pulldown experiment **(scale accordingly)**.

1. Add **100µl (slurry)** to 400 µl **TBS-T**.
2. Collect resin by low-speed centrifugation (<1000xg) for 5 min at room temperature.
3. Remove and discard supernatant, being careful not to disturb the resin.
4. Resuspend resin in 500 µl of **TBS-T**; incubate for 5 min on a rocking platform.
5. Repeat collection/wash at least two additional times prior to pulldown.
6. Discard final wash prior to addition to TUBEs containing cell lysate (see below).

PULLDOWN OF POLYUBIQUITYLATED PROTEINS (SUGGESTED PROTOCOL)

1. Pre-chill **cell lysis** buffer to 4°C.
2. Add supplied TUBEs to 500µl of lysis buffer to a final concentration of 100-200µg/mL (1.8-3 µM). Store on ice. **Addition of 3µM TUBE 1 to cell lysis buffer has been shown to effectively protect polyubiquitylated proteins from degradation while maximizing pulldown efficiency.**
3. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500µL of lysis buffer to a 10cm tissue culture dish containing approximately $1.5x10^6$ cells **(The optimal number of cells will depend on the cell line and the abundance of the protein of interest)**.
4. Collect cells by scraping, and transfer lysate to a pre-chilled 1.5mL centrifuge tube.
5. Incubate **TUBEs** containing lysate for 15 minutes on ice.
6. Clarify cell lysate by centrifugation for 10 minutes at ~14,000xg (4°C).
7. Collect supernatant and remove an "INPUT" sample for analysis by Western blotting (e.g., 5µl cell lysate in 50µl 1X SDS reducing sample prep buffer).
8. Add appropriate equilibrated FLAG affinity resin to TUBEs containing cell lysate according to manufacturer's recommendation.
9. Incubate TUBEs containing lysate with affinity resin for at least 2 hours.
10. Collect beads by centrifugation (<1000xg, 4ºC) for 5 minutes. Save supernatant as the "UNBOUND FRACTION," in preparation for analysis in the same manner as INPUT sample.
11. Wash beads with **TBS-T**, collect by low-speed centrifugation as above, and aspirate carefully until no liquid remains. Repeat two additional times
12. Polyubiquitylated proteins can be eluted in one of two ways. For Western blotting analysis, proceed to Step 13. For elution prior to further immunoprecipitation or analysis by mass spectroscopy (MS), proceed to Step 14.
13. Resuspend resin in 1X SDS reducing sample prep buffer (treat by heating at >80ºC for 5 minutes) and centrifuge samples at 13,000xg for 5 minutes. Analyze sample by SDS- PAGE/western blotting normalizing to both the INPUT and UNBOUND FRACTION, if desired. Discard resin.
14. Alternatively, elution with 0.2M glycine HCL, pH 2.5 or 3X FLAG Peptide based on manufacturer recommendation is appropriate for eluting the TUBEs & proteins of interest.

INHIBITION OF DEUBIQUITINASE ACTIVITY

Addition of 3µM TUBEs to cell lysis buffer has been shown to effectively protect polyubiquitylated proteins from degradation. The use of TUBE is more effective than generic cysteine protease inhibitors such as iodoacetamide or N-ethyl maleimide.

REFERENCES

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