MANUAL

Biotinylated TUBE 2

Catalog Numbers:
UM302

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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. Polyubiqiutination is a reversible process, however, as these chains are degraded and/or removed by proteases known as deubiquitylases (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.

Biotin-TUBEs replace anti-ubiquitin antibodies, as well as the required blot heating, for the detection of polyubiquitylated proteins by ligand blotting ("far western blotting"). The superior nature of TUBEs allows for efficient detection of polyubiquitylated proteins in their native state, while the versatility of TUBEs meets a wide range of experimental needs.

Other TUBEs Product Lines

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.

Affinity tagged TUBEs allow for identification and characterization of polyubiquitin proteins by Western blotting, as well as isolation of proteins for downstream proteomic studies.

APPLICATIONS

1. Pull down polyubiquitylated protein from cells, tissues, and organs
2. Isolate ubiquitinated protein of interest by secondary immunoprecipitation

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

COMPONENTS

1. **TUBE 2**

   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:** 20mM Na_2HPO_4, 20mM NaH_2PO_4 (pH 7.2), 50mM NaF, 5mM tetra-sodium pyrophosphate, 10mM B-glycerophosphate, 2mM EDTA, 1mM DTT, 1% NP-40.

SUGGESTED PROTOCOL

1. Prepare cell extract for Western blot analysis using the extraction buffer of choice in the presence of protease inhibitors. To prevent deubiquitylation of proteins by DUBs during the extraction, we recommend using DUB inhibitor PR-619 (LifeSensors, cat. No.SI9619).
2. Clarify cell lysate by centrifugation for 10 min at 4º.
3. Prepare samples for SDS-PAGE using reducing SDS sample buffer. Load 30-50 µg of total protein per lane. The amount of protein for gel loading should be determined empirically.
4. Transfer to PVDF membrane according to manufacturer recommendations.
5. Block membrane in PBST containing 3%BSA (Cohn fraction V) for 1 h at room temperature (RT).
6. Incubate the membrane with Biotin-TUBE-2 (dilution 0.2-1.0 µg/ml in 3%BSA) for 1h at RT.
7. Wash the membrane with PBST 3 X 10 min.
8. Incubate the membrane with streptavidin conjugated HRP for 1h at RT. Manufacturer and dilutions should be determined empirically.
9. Wash the membrane with PBST at least 4 times, 10 min each prior to the detection of immunopositive protein bands using enhanced chemiluminescence reagent kit (ECL).

PULLDOWN of POLYUBIQUITINATED PROTEINS

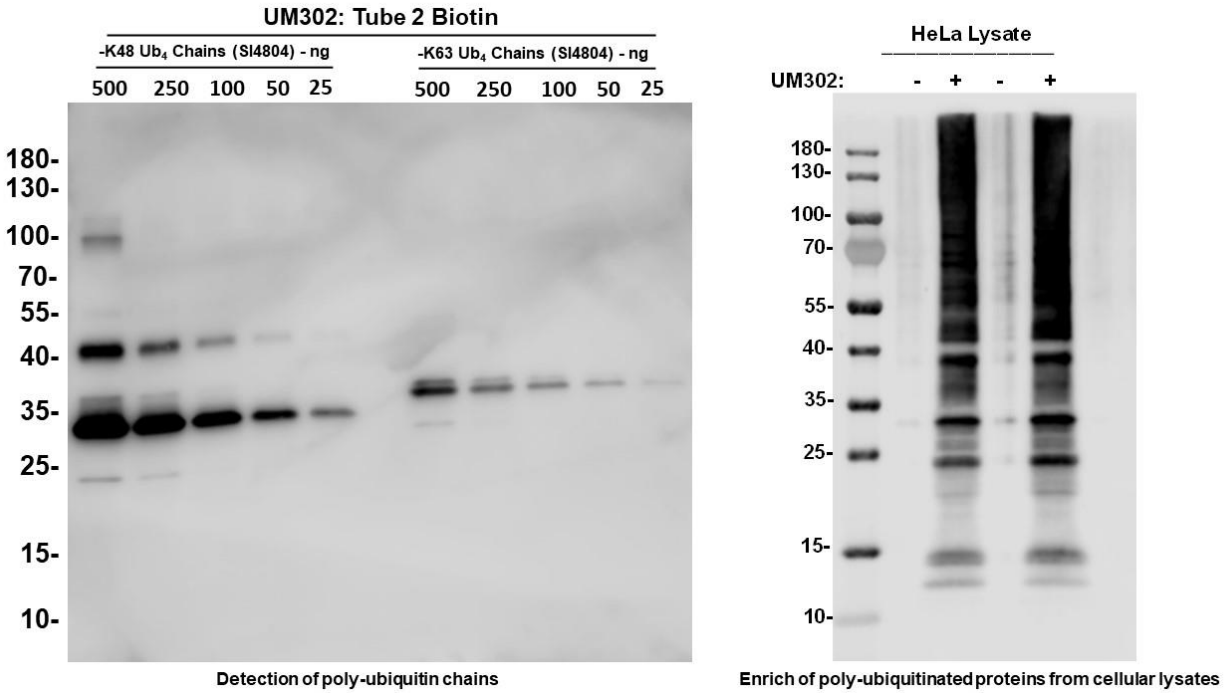
1. Pre-chill cell lysis buffer to 4°C.
2. Add supplied TUBEs to 500µl of lysis buffer to a final concentration of 100-200µg/mL. (1.8-3 µM). Store on ice. Addition of 3µM Biotin TUBE 2 to cell lysis buffer has been shown to effectively protect polyubiquitylated proteins from degradation while maximizing 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 affinity resin to TUBEs containing cell lysate, streptavidin agarose beads (EMD Millipore Sigma, 16-126)
9. Incubate TUBEs containing lysate with affinity resin for at least 2 hours

10. Collect beads by centrifugation (<100xg, 4C) 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. 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 SDSPAGE/western blotting normalizing to both the INPUT and UNBOUND FRACTION, if desired. Discard resin. Please note that when performing a Western Blot with TUBE-Biotin that 5%BSA is to be used, not 5% milk.

SAMPLE DATA



Detection of poly-ubiquitin chains

Enrich of poly-ubiquitinated proteins from cellular lysates

TUBEs conjugated with Biotin allowed detection of poly-ubiquitination chains using far-western and enrichment of poly-ubiquitinated proteins from HeLa lysates. TUBE-Biotin diluted to 1:1000 in 3% BSA (PBS-T) was used to successfully detect -K48 & -K63 poly-ubiquitin chains loaded at different amounts (500, 250, 10, 50, 25 ng loading per well). TUBE2 Biotin was also able to successfully enrich poly-ubiquitinated proteins from 300 µg of HeLa lysates. -/+ indicates whether UM302 was added or not to cell lysates prior to enrichment using streptavidin magnetic beads. Characteristic poly-ubiquitination smears in '+' UM302 condition confirms successful enrichment.

REFERENCES

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