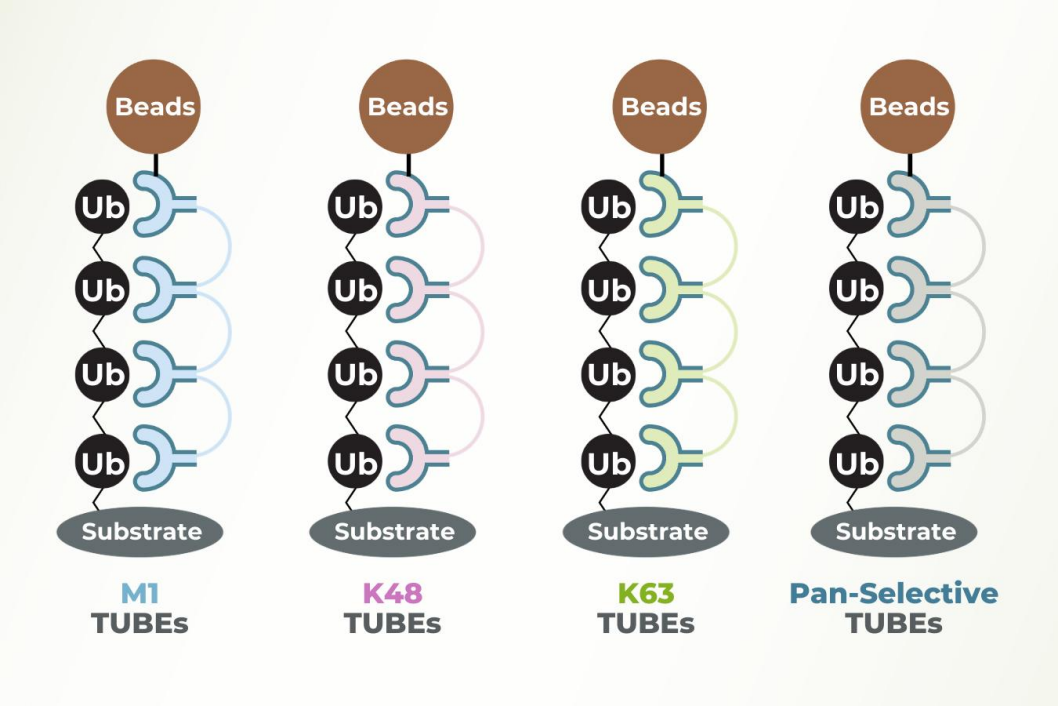
MANUAL

TUBE 1 (Biotin)

Catalog Number: UM301



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BACKGROUND

Ubiquitin and Polyubiquitination

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 monoubiquitination. 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 polyubiquitination. The two most well characterized forms of polyubiquitination occur via linkage at lysine 48 (K48) or lysine 63 (K63). The most prevalent consequence of polyubiquitination is the proteasome-mediated degradation of the target protein. Polyubiquitination is a reversible process, as these chains can be degraded and/or removed by proteases known as deubiquitylases (DUBs). The dynamic nature of this signal represents a major obstacle to the isolation and functional characterization of polyubiquitinated proteins. For this reason, the ubiquitination state of many proteins is unknown or poorly characterized.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

Traditional strategies for characterization of ubiquitinated 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 polyubiquitinated 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 polyubiquitinated proteins. These conditions could alter cell physiology, which in turn may negatively impact the result or introduce experimental artifacts. 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. They have also been shown to protect proteins from both deubiquitination and proteasome-mediated degradation, even in the absence of inhibitors typically required to block such activity. The nanomolar affinity of TUBEs for polyubiquitinated proteins enables highly efficient 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 polyubiquitinated 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

TUBE 1 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, as their binding profiles are generally very similar. **Both TUBE 1 and TUBE 2** bind to K6-, K11-, K48-, and K63-linked polyubiquitin.

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

SUGGESTED USES:

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

BENEFITS:

1. TUBEs exhibit up to 1000-fold higher affinity for polyubiquitin compared to the single UBA form.
2. TUBEs offer higher specificity and affinity for polyubiquitin than ubiquitin antibodies.
3. TUBEs help avoid the overexpression of epitope-tagged ubiquitin in pulldown experiments.
4. TUBEs protect polyubiquitinated proteins from degradation during cell lysis, even in the absence of inhibitors specific to DUB and proteasome activity.

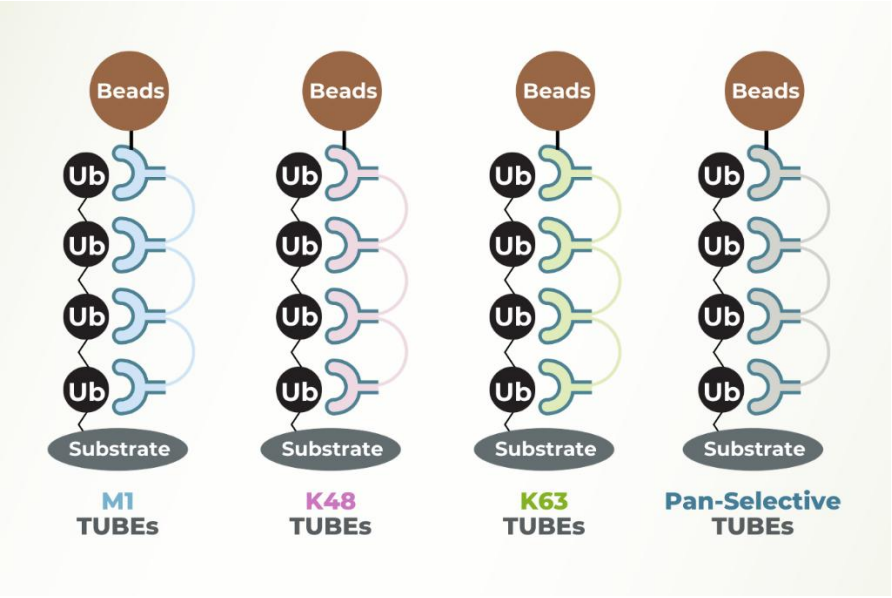

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

TUBE 1, Biotin (Catalog # UM301)

COMPONENTS

TUBE 1 (Biotin)

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 available at www.lifesensors.com for further information.

ADDITIONAL ITEMS REQUIRED BUT NOT INCLUDED IN THE KIT

1. **Cell Lysis buffer:** 20 mM Na2HPO4, 20 mM NaH2PO4 (pH 7.2), 50 mM NaF, 5 mM tetra-sodium pyrophosphate, 10 mM β-glycerophosphate, 2 mM EDTA, 1 mM DTT, 1% NP-40.

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

2. **20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20 (TBST)**
3. **1,10-phenanthroline, 100x (LifeSensors Cat. No. SI9649).** This metal chelator is a potent inhibitor of metalloproteases, including JAMM DUBs, and can help prevent polyUb chain degradation.
4. **PR-619 (LifeSensors Cat. No. SI9619).** This compound is a reversible inhibitor of a wide range of Ub/Ubl proteases and has been shown to protect polyubiquitinated proteins from degradation.
5. **(Optional) N-Ethylmaleimide (NEM), an irreversible inhibitor of all cysteine peptidases.**
6. **Streptavidin agarose beads** (EMD Millipore Sigma, 16-126)

WESTERN BLOT (SUGGESTED PROTOCOL)

1. Prepare cell extracts for Western blot analysis using the extraction buffer of choice, in the presence of protease inhibitors. To prevent deubiquitination of proteins by DUBs during the extraction, we recommend using the DUB inhibitor PR-619 (LifeSensors, cat. No.SI9619).
2. Clarify cell lysate by centrifugation for 10 min at 4°C.
3. Prepare samples for SDS-PAGE using Laemmli reducing SDS sample buffer. Load 30-50 µg of total protein per lane. The optimal amount of protein for gel loading should be determined empirically.
4. Transfer to PVDF membrane according to manufacturer's recommendations.

5. Block membrane in PBST containing 3% BSA (Cohn fraction V) for 1 hour at room temperature.
6. Incubate the membrane with Biotin-TUBE-1 or -2 (dilution 0.2-1.0 µg/ml in 3% BSA) for 1 hour at room temperature.
7. Wash the membrane with PBST three times, 10 min each.
8. Incubate the membrane with streptavidin-conjugated HRP for 1 hour at room temperature. The manufacturer's recommendations and dilutions should be determined empirically.
9. Wash the membrane with PBST at least four times, 10 minutes each, prior to detecting immune-positive protein bands using an enhanced chemiluminescence reagent kit (ECL).

PULLDOWN OF POLYUBIQUITINATED PROTEINS (SUGGESTED PROTOCOL)

1. Pre-chill cell lysis buffer and microcentrifuge tubes to 4°C. Add PR-619 (at a final concentration of 50 µM), o-PA (at a final concentration of 1x), NEM (at a final concentration of 5 mM), and protease inhibitor cocktail (see manufacturer's instructions) to the lysis buffer.
2. Add supplied TUBEs to 500 µl of lysis buffer to a final concentration of 100-200 µg/mL (1.8-3 µM). Store on ice. **Addition of 3 µM TUBE 1 to cell lysis buffer has been shown to effectively protect polyubiquitinated proteins from degradation while maximizing pulldown efficiency.**
3. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500 µL of lysis buffer to a 10 cm tissue culture dish containing approximately $1.0x10^7$ cells (~ 1 mg protein). **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 1.5 mL microcentrifuge 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 save an "INPUT" sample for analysis by Western blotting (e.g., 5 µl cell lysate in 50 µl 1X Laemmli reducing SDS sample buffer).
8. Add the appropriately equilibrated streptavidin agarose beads (EMD Millipore Sigma, 16-126) to the TUBEs containing cell lysate.
9. Incubate the 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 the analysis in the same manner as the INPUT sample.
11. Wash beads with **TBS-T**, collect by low-speed centrifugation as above, and aspirate carefully until no liquid remains.
12. Repeat step 10 two additional times.

13. Resuspend resin in 1X Laemmli reducing SDS sample 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. Normalize to both the INPUT and the UNBOUND FRACTION, if desired. Discard the resin.

Please note that when performing a Western blot with TUBE-Biotin, 5% BSA should be used, not 5% milk.

REPRESENTATIVE DATA

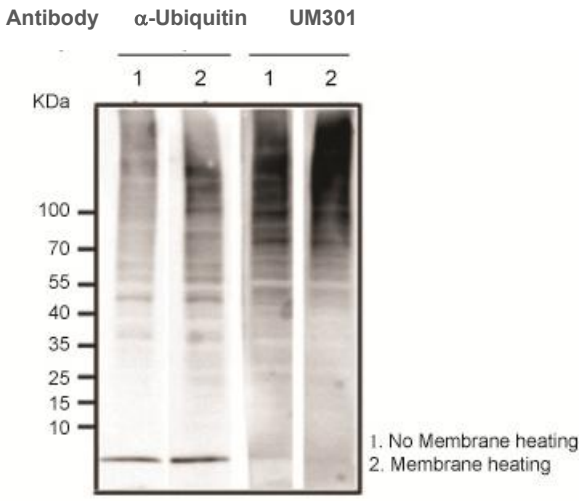

Figure 1: Approximately 40 µg of protein from Neuro 2A cells was subjected to SDS-PAGE, followed by electrophoretic transfer. Probing with α-ubiquitin was performed both with and without pre-treatment of the membrane by heat. Similarly, cellular lysates were probed with biotinylated TUBE1 at a 1:1000 dilution, with and without prior heating of the membrane. As shown in the image above, UM301 outperformed the leading α-ubiquitin antibody in detecting polyubiquitination, both with and without membrane heating.

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