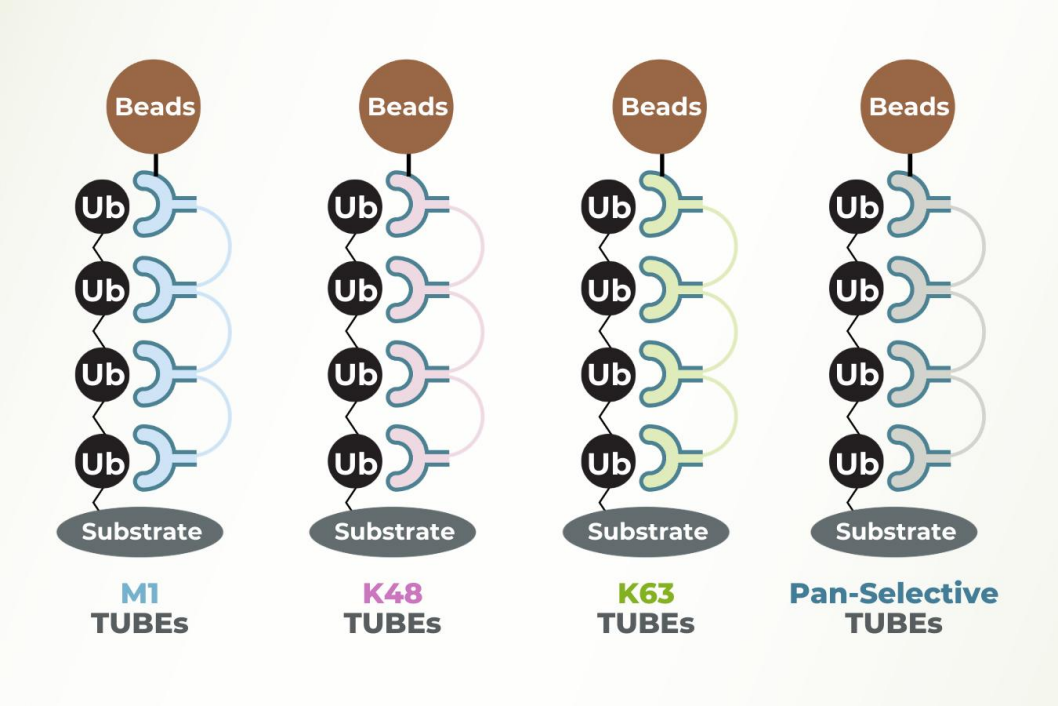
MANUAL

TUBE 2 (His6)

Catalog Number: UM202



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TUBE 2 (His6)

Cat. # UM202

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.

Affinity tagged TUBEs allow for identification and characterization of polyubiquitin proteins by western blotting, as well as isolation of proteins for downstream proteomic studies. **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. In general, the binding profiles are very similar. **Both TUBE 1 and TUBE 2** bind to K6-, K11-, K48- and K63-linked polyubiquitin.

SUGGESTED USES:

1. Pull-down polyubiquitinated protein lysates 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.

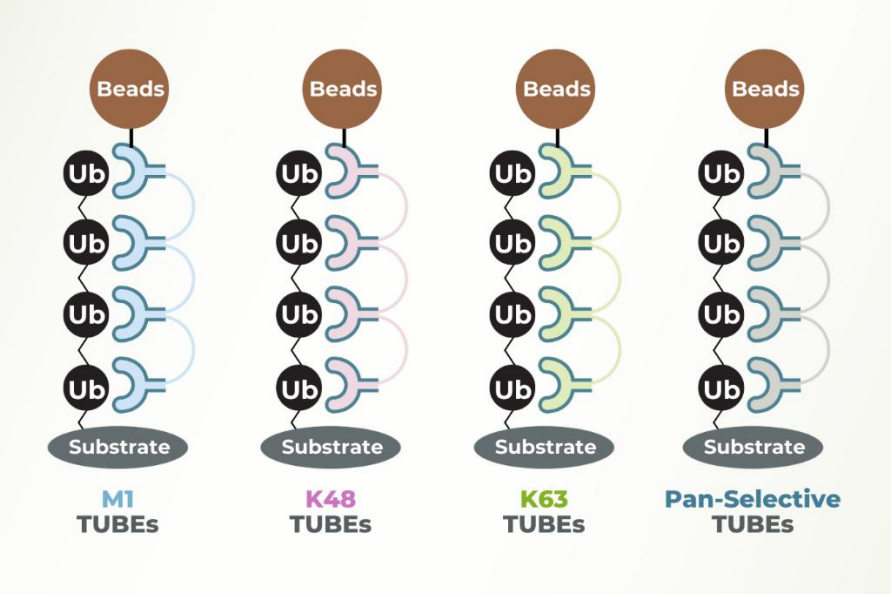

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

TUBE2, His6 (Catalog # UM202)

COMPONENTS

TUBE2 (His6)

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:** 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM 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 the overall yield of polyubiquitinated 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:** 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20 (TBS-T)
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. **Immobilized Affinity Chromatography (IMAC) resin (for HIS_6-TUBEs)**

   Recommended: Qiagen (30210, 25 ml) or GE Healthcare (17-0821-07, 25 ml)

EQUILIBRATION OF AFFINITY RESIN

(Suggested Protocol) Please refer to the manufacturer's recommended protocol before starting.

All steps are for a single pulldown experiment **(scale up 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 the 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 the TUBEs-containing cell lysate (see below).

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 2 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 SDS (reducing sample prep buffer for SDS-PAGE at the following final concentrations of 62.5 mM Tris-HCL (pH6.8), 1.5% SDS, 8.33% Glycerol, 1.5% β-mercaptoethanol, and 0.005% Bromophenol blue).
8. Add the appropriately equilibrated **IMAC** affinity resin to the TUBEs-containing cell lysate. Incubate for at least 2 hours at +4°C
9. 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.
10. Wash beads with **TBS-T**, collect by low-speed centrifugation as above, and aspirate carefully until no liquid remains.
    Optional: if using HIS_6-TUBEs and IMAC resin, perform washes in the presence of 20 mM imidazole.
11. Repeat step 10 two additional times.

12. Polyubiquitinated 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. Normalize to both the INPUT and the UNBOUND FRACTION, if desired. Discard the resin.

14. Elute using 500µl of TBS-T containing 500mM imidazole with a brief vortex. Reducing elution volume may increase sensitivity. Collect supernatant by high-speed centrifugation for 5 minutes for further analysis (immunoprecipitation or MS). Discard the resin.

INHIBITION OF DEUBIQUITINASE ACTIVITY

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

REFERENCES

1. Song, Kyung W., Kyle A. Edgar, Emily J. Hanan, Marc Hafner, Jason Oeh, Mark Merchant, Deepak Sampath et al. (2022) "RTK-dependent inducible degradation of mutant PI3Kα drives GDC-0077 (inavolisib) efficacy." Cancer discovery 12, no. 1: 204-219.

2. Martins-Marques, Tania, Teresa Ribeiro-Rodrigues, Saskia C. de Jager, Monica Zuzarte, Cátia Ferreira, Pedro Cruz, Liliana Reis et al. (2020) "Myocardial infarction affects Cx43 content of extracellular vesicles secreted by cardiomyocytes." Life science alliance 3, no. 12.

3. Li, Yajuan, Qingsong Hu, Chunlai Li, Ke Liang, Yu Xiang, Heidi Hsiao, Tina K. Nguyen et al. (2019) "PTEN-induced partial epithelial-mesenchymal transition drives diabetic kidney disease." The Journal of clinical investigation 129, no. 3: 1129-1151.

4. Weng, Liang, Yi-Peng Han, Atsushi Enomoto, Yasuyuki Kitaura, Shushi Nagamori, Yoshikatsu Kanai, Naoya Asai et al. "Negative regulation of amino acid signaling by MAPK-regulated 4F2hc/Girdin complex." PLoS biology 16, no. 3 (2018): e2005090.

5. Alturki, Norah A., Scott McComb, Ardeshir Ariana, Dikchha Rijal, Robert G. Korneluk, Shao-Cong Sun, Emad Alnemri, and Subash Sad. "Triad3a induces the degradation of early necrosome to limit RipK1-dependent cytokine production and necroptosis." Cell death & disease 9, no. 6 (2018): 1-14.

6. Hjerpe, R., Aillet, F., Lopitz-Otsoa F., Lang, V., England P., and Rodriguez, MS. (**2009**) “Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities.” *EMBO Rep.,* 10(11)1250-8.

7. Hicke, L. (**2005**) “Ubiquitin-Binding Domains.” *Nature Mol Cell Bio* 6: 610-21.

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