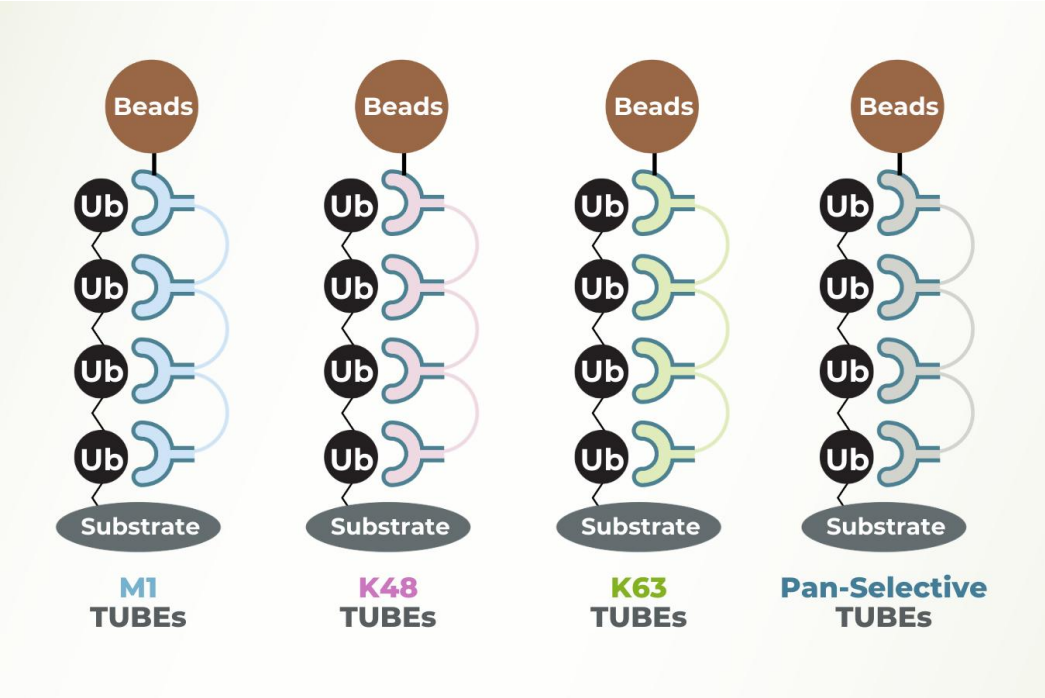
MANUAL

TUBE 2 (Magnetic Beads)

Catalog Number: UM402M



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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 the 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. Tandem Ubiquitin Binding Entities (TUBEs) have been developed to overcome these problems (1,2) and they are licensed by LifeSensors, Inc. from Dr. Manuel Rodriquez at CIC bioGUNE. 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 allows for a highly efficient isolation and characterization of these proteins from cell lines and tissues. TUBE1 and TUBE2 have been demonstrated to bind to all 7 linkage types (3). However, being derived from different ubiquitin-binding domains it is expected that TUBE1 and TUBE2 may have different specificity profiles for the various linkage types. The superior nature of TUBEs allows for an efficient detection of polyubiquitinated proteins in their native state, while the versatility of TUBEs meets a wide range of experimental needs.

Magnetic-TUBEs are TUBE moieties directly coupled to magnetic beads, for the identification and characterization of polyubiquitinated proteins by western blotting and/or downstream proteomic studies. Magnetic-TUBEs facilitate convenient "one-step" pull-down of polyubiquitinated proteins.

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SUGGESTED USES:

1. Convenient one step pull-down of polyubiquitinated proteins from cell and tissue extracts
2. Isolation of ubiquitinated proteins for proteomic studies.

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 than antibodies.
3. TUBEs help avoid the overexpression of epitope-tagged ubiquitin in pulldown experiments.
4. TUBEs protect polyubiquitinated proteins from degradation during cell lysis and storage.
5. Magnetic beads increase the pull-down efficiency and reduce background.

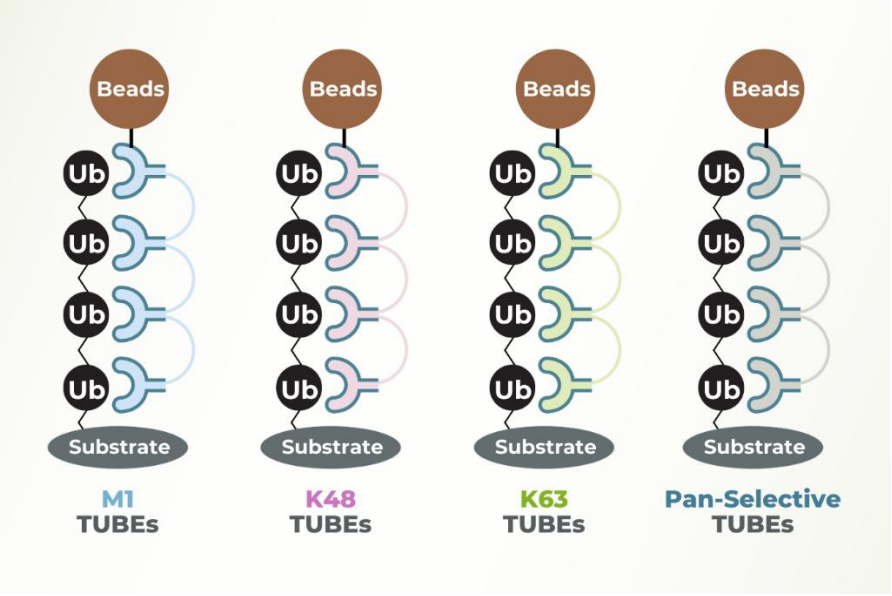

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

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COMPONENTS

TUBE 2 (Magnetic Beads)

Size 1 ml of slurry.
100 ul slurry is recommended for 1-2 mg of total cell lysate pull down.

Buffer PBS, pH 7.2, 0.05% sodium azide.

Storage 4°C.

Do not centrifuge above 3000 rpm, dry or freeze the beads.

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. **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. **Magnetic rack for 1.5 mL microcentrifuge tubes.**

EQUILIBRATION OF MAGNETIC-TUBEs

1. Gently mix Magnetic-TUBE by inverting the vial several times to ensure a homogeneous suspension.
2. Determine the amount of resin required for the experiment. The amount of polyubiquitin in samples can vary with cell or tissue type, experimental conditions, and the presence or absence of deubiquitinating enzyme or proteasomal inhibitors. Therefore, the optimal amount of Magnetic-TUBE for pull down needs to be determined empirically by the end-user. 100 µl of resin (Slurry) in 500 µl of lysis buffer containing 1-2 mg of total protein is an appropriate starting point for each experiment.
3. Place the volume of resin necessary for the experiment in the 1.5 mL

microcentrifuge tube, then place the tube into a magnetic stand, collect the beads and discard the supernatant.

4. Suspend the resin in 5-10 volumes of TBST, place it back into a magnetic stand, collect the beads, and discard the supernatant.
5. Repeat washing the resin at least two times prior to pull-down.
6. Resuspend in 160uL of pulldown buffer. (*Note: Pull down buffer contains 3% BSA which helps to reduce non-specific protein binding to the beads. If BSA is expected to interfere with your downstream applications such as proteomics, exclude BSA from the pull-down buffer and/or try pre-clearing the lysates using control magnetic beads that are not coated with proteins. Control magnetic beads are not included)*

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. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500 µL of lysis buffer to a 10 cm2 tissue culture dish containing ~5-10x10^6 cells (80% confluence). The optimal number of cells will depend on the cell line and the abundance of the protein of interest.
3. Collect cells by scraping and transfer the lysate to 1.5 mL tube.
4. Clarify lysate by high-speed centrifugation (~14,000xg) for 10 min at 4ºC.
5. Save an "INPUT" sample for analysis by western blotting (e.g., 5-20 µl of cell lysate in 25-50 µl 1X SDS reducing sample buffer.)
6. Add the amount of cell lysate to the amount of equilibrated Magnetic-TUBE determined from the previous section and incubate for 2 hours at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.
7. Place the tube into a magnetic stand, collect the beads and save the supernatant as the "UNBOUND FRACTION."
8. Wash the beads by re-suspending with 1ml TBST, Place the tube into a magnetic stand, collect the beads and discard the supernatant.
9. Repeat step 8 three times.
10. For Western blot analysis, resuspend resin in SDS reducing sample buffer (use of a more concentrated SDS reducing sample buffer may allow for greater flexibility with electrophoresis samples), treat by boiling for 5 minutes, and centrifuge at 13,000xg for 5 minutes. Analyze the eluted samples by SDS-PAGE/western blotting, alongside the INPUT and UNBOUND FRACTION. Discard the resin.

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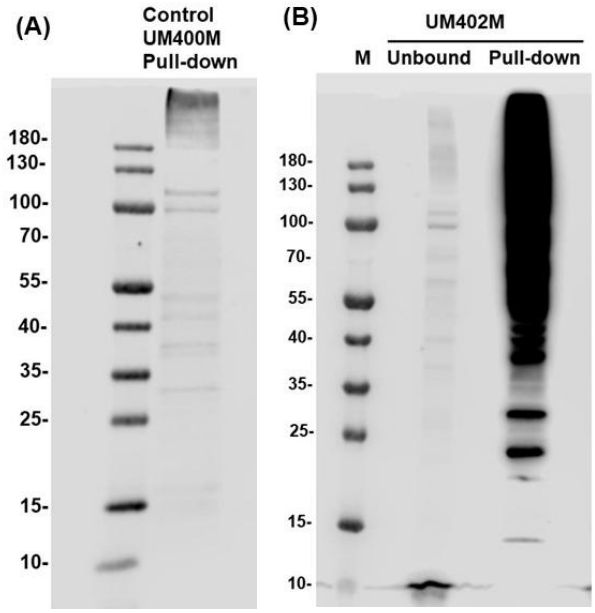
REPRESENTATIVE DATA



Enrichment of polyubiquitinated proteins from lysate with Magnetic-TUBEs: Cells were lysed in RIPA buffer containing Protease Inhibitor Cocktail (Calbiochem). Cell lysates (0.3 mg) were applied to magnetic beads coupled or uncoupled to TUBEs. Image shows enriched polyubiquitinated proteins from HEK293T cells with magnetic TUBE 2 (UM402M) and the enrichment with control magnetic beads with no TUBE (UM400M) The amount of unbound protein shows the efficiency of binding to TUBEs. Samples were analyzed by SDS-PAGE and immunoblotting for ubiquitin using the VU-1 Ubiquitin Monoclonal Antibody (LifeSensors Cat. # VU101, 1:1000).

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