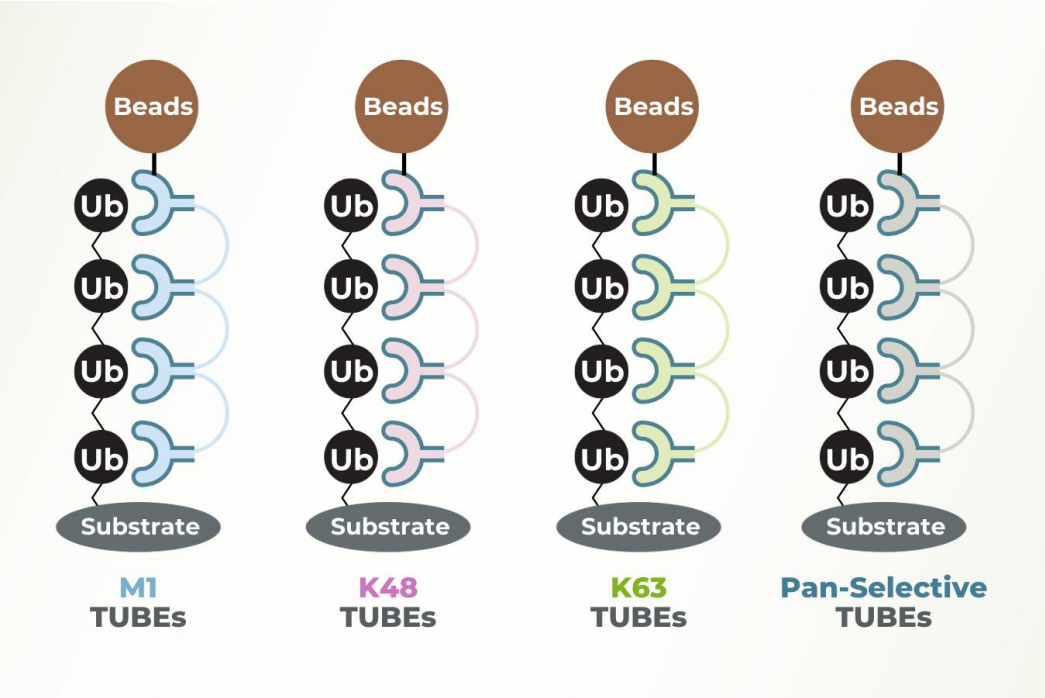
MANUAL

TUBE 2 (Agarose)

Catalog Number: UM402



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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.

Agarose-TUBEs are TUBE moieties directly coupled to agarose beads, designed for the identification and characterization of polyubiquitinated proteins by Western blotting and/or downstream proteomic studies. Agarose-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 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 and storage.

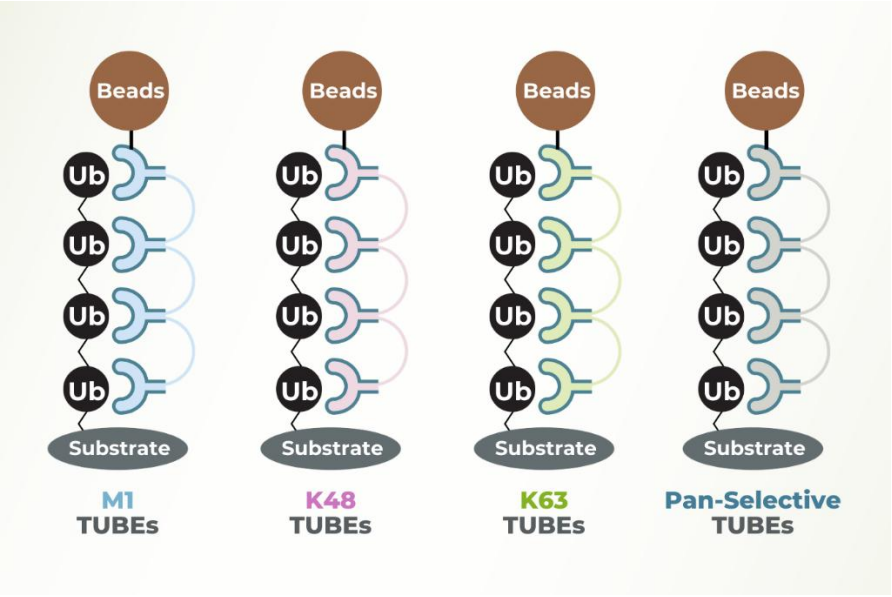

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

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COMPONENTS

TUBE 2 (Agarose)

Size: 1 ml of a 50% slurry.

Buffer: PBS, pH 7.2, 20% ethanol.

Storage: -20°C. Avoid storage at lower temperature. Small batches of equilibrated resin can be stored at 4ºC for up to 1 week.

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. **Control agarose (LifeSensors Cat. No. UM400)**
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. The inclusion of PR-619 in the lysis buffer can increase the yield of polyubiquitinated proteins during the preparation of cell and tissue extracts.
5. **1,10-phenanthroline (*o*-PA), 100X (LifeSensors Cat. No. SI9649).** This metal chelator is a potent inhibitor of metalloproteases, including JAMM DUBs, and helps prevent the degradation of polyubiquitin chains during cell lysis.
6. **(Optional) N-Ethylmaleimide (NEM), an irreversible inhibitor of all cysteine peptidases.**

EQUILIBRATION OF TUBE 2 (AGAROSE)

1. Allow the slurry to equilibrate to 4˚C by incubating at room temperature for 20-30 min or at 4ºC for up to 2 hours. Gently mix Agarose-TUBEs 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 depending on cell or tissue type, experimental

conditions, and the presence or absence of deubiquitinating enzymes or proteasomal inhibitors. Therefore, the optimal amount of Agarose-TUBEs for pull-down needs to be determined empirically by the end user. A starting point of 10-20 µl of resin in 500 µl of lysis buffer containing 1-2 mg of total protein is appropriate for each experiment. Larger volumes (100-150 µl) of slurry can be equilibrated and stored for up to one week at 4°C

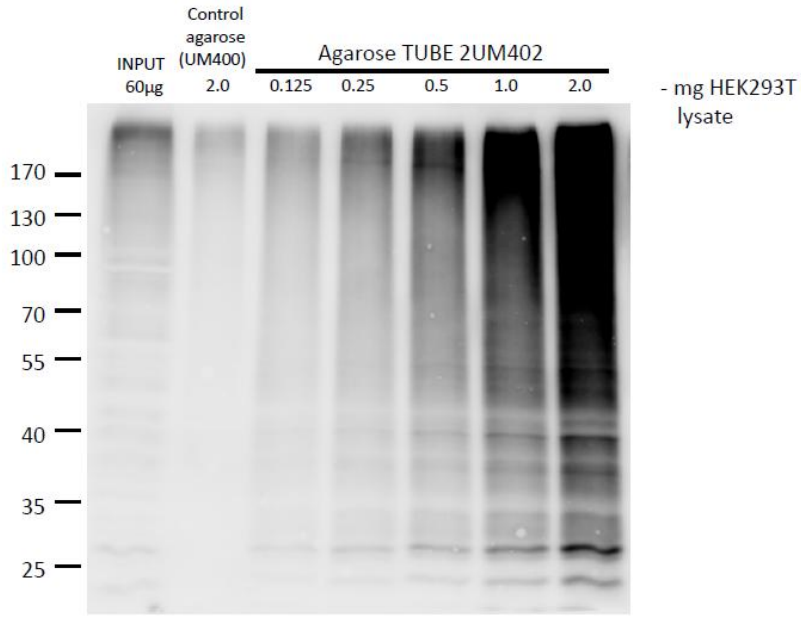
3. Collect the volume of resin necessary for the experiment by low-speed centrifugation (1000-5000xg) for 5 minutes (RT).
4. Remove and discard the supernatant, being careful not to disturb the resin. **Useful tip:** when working with agarose matrices, use gel loading tips to collect supernatants to avoid loss of beads.
5. Suspend the resin in 5-10 volumes of TBS-T and incubate for 5 minutes on a rocker platform at room temperature.
6. Repeat the collection/wash steps (steps 3-5) at least two times before proceeding with the pull-down.
7. Remove and discard the final wash supernatant. Make sure to leave a 50% slurry before proceeding to Step 7 of the protocol 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. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500 µL of lysis buffer to a 10 cm^2 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 microcentrifuge tube.
4. Clarify lysate by high-speed centrifugation (~14,000xg) for 10 min at 4ºC.
5. **Optional control for non-specific binding**: Incubate the clarified cell lysate with uncoupled agarose (Cat. #UM400) for 30 min at 4°C on a rocker platform. Remove agarose by centrifugation and transfer the clarified supernatant to a new 1.5 ml microcentrifuge tube.
6. 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 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).

7. Add the cell lysate to the appropriate amount of equilibrated Agarose-TUBEs determined from step 2 of the previous section. Incubate for 1 hour at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.
8. Collect beads by low-speed centrifugation (1000-5000xg, 4ºC) for 5 minutes. Save the supernatant as the "UNBOUND FRACTION."
9. Wash beads with 1 ml TBS-T. Collect by low-speed centrifugation and aspirate the supernatant, leaving a small volume cushion to avoid disturbing the beads.
10. Repeat Step 9 two more times.
11. 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 the UNBOUND FRACTION. Discard the resin.

REPRESENTATIVE DATA



Enrichment of polyubiquitinated proteins with Agarose-TUBE2 (UM402). HEK293T cells were lysed in TBS containing 5 mM EDTA, 1% NP-40, Protease Inhibitor Cocktail (Calbiochem), 50 µM PR619 (LifeSensors Cat. # SI9619), and 5 mM o-phenanthroline (LifeSensors Cat. #SI9649). Total protein content of pre-cleared (using Control Agarose; LifeSensors cat. # UM400) lysate was determined by Bradford, and 10 µl of Agarose-TUBE 2 resin (LifeSensors Cat. # UM402) was added to each sample. Samples were incubated by rotation for 3.5 hours at 4°C, washed three times with TBS plus EDTA, and analyzed by SDS-PAGE followed by immunoblotting for ubiquitin using the VU-1 Ubiquitin Monoclonal Antibody (LifeSensors Cat. # VU101, 1:1000).

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