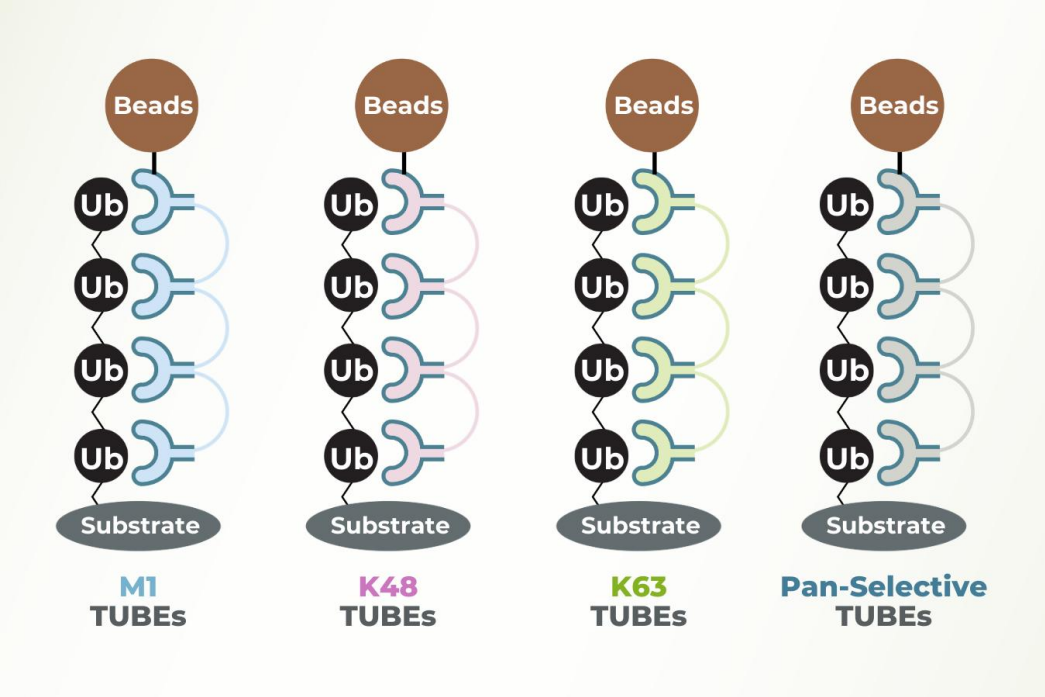
MANUAL

TUBE 1 (Agarose)

Catalog Number: UM401



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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 this are polyubiquitination via lysine at position 48 (K48) or position 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 signaling 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 artifact. Tandem Ubiquitin Binding¬ Entities (TUBEs) have been developed to overcome these problems (1,2) and 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. TUBEs have also been demonstrated to protect proteins from both deubiquitination and proteasome-mediated degradation, even in the absence of inhibitors normally required to block such activity. The nanomolar affinity of TUBEs for polyubiquitinated proteins allows for high efficiency in 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 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. They allow 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 polyubiquitinated 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.

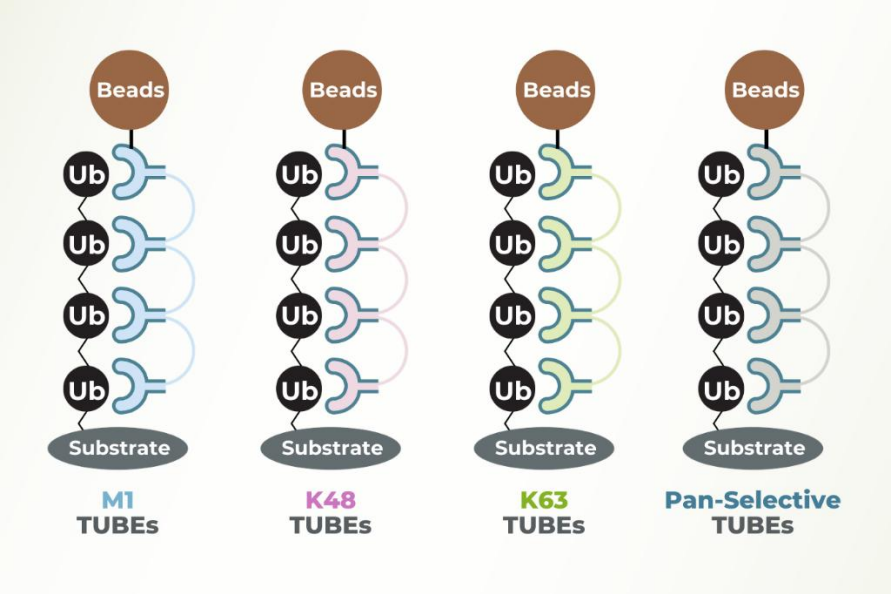

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

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COMPONENTS

TUBE 1 (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 denaturing detergents e.g., SDS or deoxycholate may have a negative impact on 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. **20mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.1% Tween-20 (TBS-T)**
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 1 (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 experiments must be determined empirically by the end user. 10-20 µl of resin in 500 µl of lysis buffer containing 1-2 mg of total protein is an appropriate starting point for each experiment. Larger volumes (100-150 µl) of slurry can be equilibrated and stored for up to one week at 4ºC.

3. Collect the required volume of resin for the experiment by low-speed centrifugation (1000-5000 x g) for 5 minutes at room temperature.
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 losing 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/washing steps at least two times prior to the pull-down.
7. Remove and discard the final wash supernatant, being sure to leave a 50% slurry prior 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 beads (Cat. #UM400) for 30 min at 4°C on the rocker platform. Remove agarose beads 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 Laemmli 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 in step 2 of the previous section and 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.

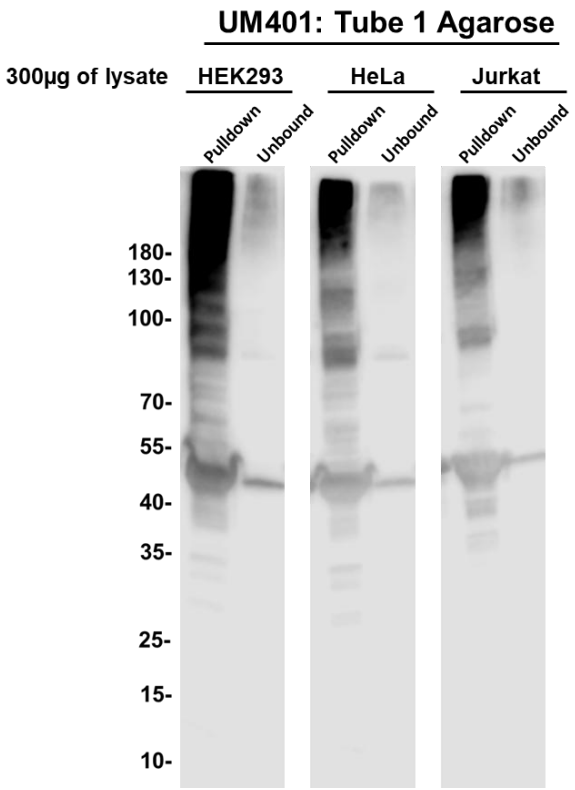
8. Collect beads by low-speed centrifugation (1000-5000xg, 4ºC) for 5 minutes. Save supernatant as the "UNBOUND FRACTION."

9. Wash beads with 1ml TBS-T, collect by low-speed centrifugation and aspirate the supernatant leaving a small volume cushion so as to avoid disturbing the beads.

10. Repeat Step 9 two more times.

11. For Western blot analysis, resuspend resin in 1X Laemmli SDS reducing sample buffer (use of more concentrated SDS reducing sample buffer may allow for flexibility with electrophoresis samples), treat by boiling for 5 minutes, and centrifuge at 13,000xg for 5 minutes. Analyze 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-TUBE1 (UM401). HEK293, HeLa and Jurkat 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 (Control Agarose; LifeSensors cat. # UM400) lysate was determined by Bradford, and 10 µl of Agarose-TUBE 1 resin (LifeSensors Cat. # UM401) was added to 300 µg of cellular lysate. Reactions were rotated for 3.5 hours (4C), washed three times with TBS plus EDTA, and analyzed by SDS-PAGE. The immunoblot was probed for ubiquitin using the VU-1 Ubiquitin Monoclonal Antibody (LifeSensors Cat. # VU101, 1:1000).

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