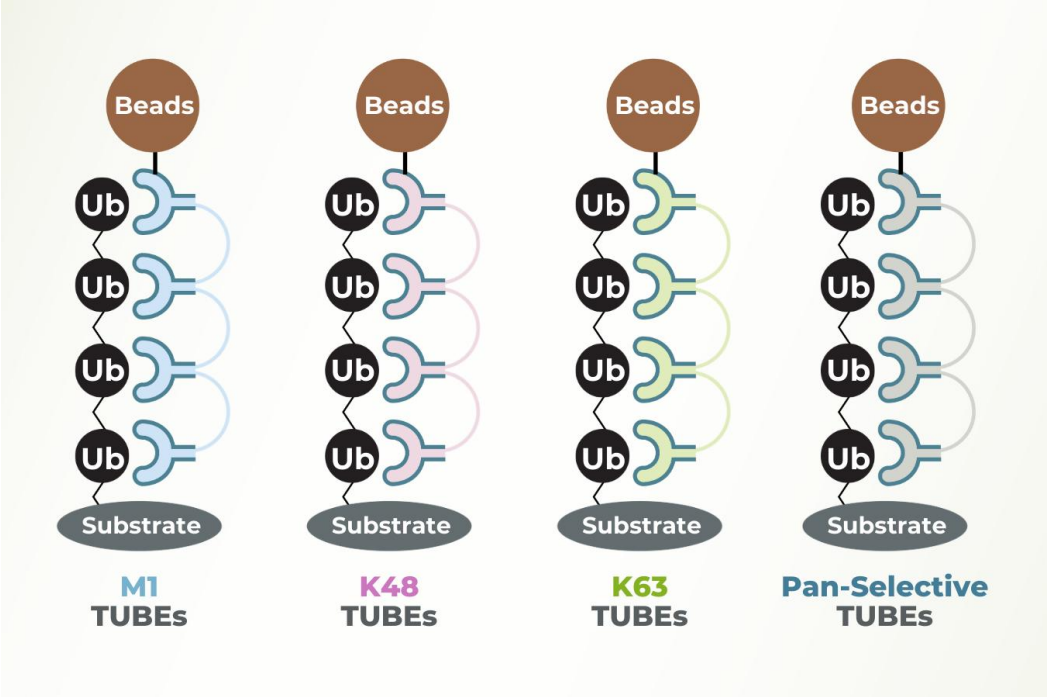
MANUAL

K63 TUBE Magnetic

Catalog Number: UM404M



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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, however, as these chains are 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.

The Next Generation of TUBEs: Linkage Specific Isolation of Poly Ubiquitin Chains

K63 TUBE was developed to show an enhanced selectivity for K63 linked poly-ubiquitin chains (~20 nM) over all other linkages (>2 µM). It can be used alone or in conjunction with our other TUBE products, especially K48 TUBE HF and M1 (linear) TUBE to investigate poly-ubiquitin chain linkage in your substrate protein.

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Magnetic-TUBEs are TUBE moieties directly coupled to magnetic beads. They allow 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.

SUGGESTED USES:

1. Isolation and enrichment of K63 poly-ubiquitinated proteins from cell and tissue extracts.
2. Isolate K63 poly-ubiquitinated proteins for proteomic studies.

BENEFITS:

1. K63-TUBEs have a nanomolar affinity for K63 poly-ubiquitin chains.
2. K63-TUBEs exhibit 100-fold preference for K63 chains over K48- or K11-chains.
3. TUBEs offer higher specificity and affinity for polyubiquitin than ubiquitin antibodies.
4. TUBEs help avoid the overexpression of epitope-tagged ubiquitin in pulldown experiments.
5. TUBEs protect polyubiquitinated proteins from degradation during cell lysis and storage.
6. Magnetic beads increase the pull-down efficiency and reduce background.

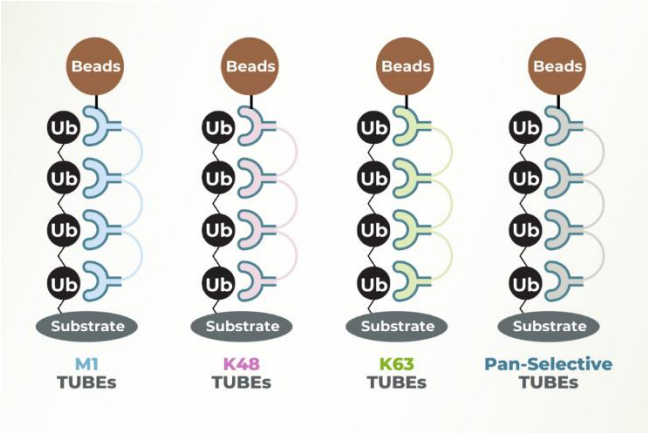

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

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COMPONENTS

K63 TUBE Magnetic

Size: 1 mL

Buffer: PBS. pH 7.2, 20% ethanol

Storage: Stable for 6 months at 4°C.

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

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. **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 K63 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. **Pull-down buffer:** 20 mM HEPES pH 7.5, 300 mM NaCl, 3% BSA.
7. **Wash buffer 1:** 50 mM Tris pH 7.5, 250 mM NaCl, 0.2 % NP-40, 1 mM DTT
8. **Wash Buffer 2:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.05 % NP-40, 1 mM DTT
9. **Magnetic separation rack for 1.5 ml centrifuge 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 depending on 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 experiments must be determined empirically by the end-user. An appropriate starting point for each experiment is to use 100 µl of resin slurry in 500 µl of lysate containing ~1–2 mg of total protein.

3. Transfer the required volume (160uL for 1mg of total cell lysate) of resin into a 1.5 mL microcentrifuge tube. To wash the resin (K63-TUBE–conjugated magnetic beads), place the tube on a magnetic stand to separate the beads, then carefully remove and discard the supernatant.
4. Suspend the resin in 5-10 volumes of TBST and place it back into the magnetic stand. Collect the beads and discard the supernatant.
5. Repeat washing the resin at least two times prior to the 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 POLYUBIQUINATED 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. Wash cells at least 2x times with cold PBS. Harvest cells into a 1.5 ml microcentrifuge tube and spin down (~1,000xg, 5 min at 4°C).

   For best results, proceed immediately to cell lysis (Step 3). Cell pellets may be flash-frozen and stored at -80°C for later use without a significant loss.
3. Add cold lysis buffer containing the inhibitors to the cell pellet. As an initial starting point, we recommend using 200 µL of lysis buffer for ~1x10^7 cells (~1mg protein). The optimal number of cells required will depend on cell type and the abundance of protein of interest. As a starting point we recommend 1-2 mg of total cellular protein. Resuspend cells in lysis buffer by pipetting or vortexing. Keep all reagents cold during lysis.
4. Clarify lysate by high-speed centrifugation (~14,000xg) for 30 min at 4ºC.
5. Save an "INPUT" sample for analysis by western blotting (e.g., 6-18µl of cell lysate in 1-3 µl 6X Laemmli SDS reducing sample buffer).

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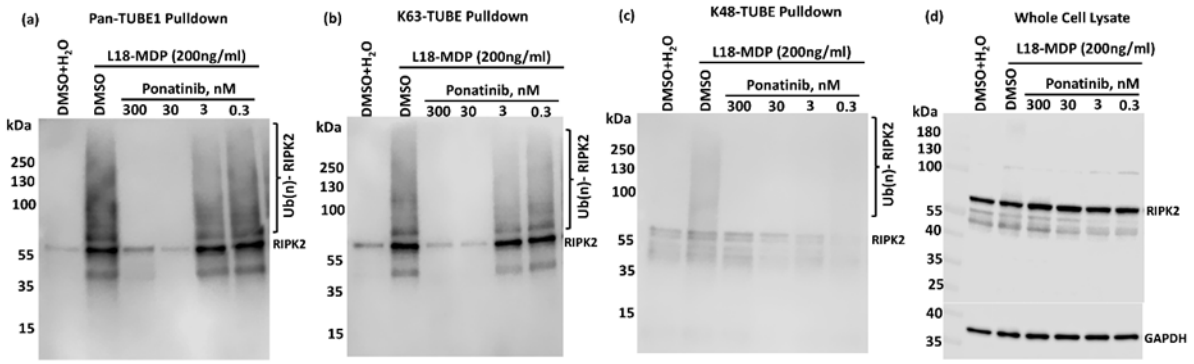
6. Add the amount of cell lysate to the amount of equilibrated Magnetic-TUBE determined in the previous section and incubate for 2 hours at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.
7. Collect beads by letting them stand on the magnetic rack for 2-3 minutes. Save supernatant as an "UNBOUND" fraction and prepare an aliquot for comparative analysis with the "INPUT" sample.
8. 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.
9. Repeat Step 8 two to three times.
   Useful Tip: Optimization of components in the wash buffer may be required, especially for samples analyzed by SDS-PAGE/Western blotting in parallel with the "INPUT" and the "UNBOUND fractions".
10. Remove excess detergent by washing with Wash Buffer 2
11. For Western blot analysis, add ~25 µl of 2X Laemmli SDS reducing sample prep buffer to the resin, and heat at ~95ºC for 10 min. Let the resin stand for 3-5 minutes on the magnetic rack. Analyze the eluted supernatant carefully to avoid disturbing the beads.
12. Alternatively, for Mass spectrometry analysis, elute with ~30 µL of elution buffer followed by the neutralization buffer from UM411B LifeSensors buffer elution kit.

REPRESENTATIVE DATA



Analysis of K63 polyubiquitination of endogenous RIPK2. THP1 cells were pre-treated with indicated doses Ponatinib for 30 minutes followed by treatment with water (control) or 200 ng/ml L18-MDP for 30 minutes. Cell lysates were subjected to pull downs using pan-selective TUBE (a), K63-selective TUBE (b) or K48-TUBE (c). 30 µg whole cell lysate (d) was used detect RIPK2 expression. Pull down samples and whole cell lysates were immunoblotted with anti-RIPK2 antibody. GAPDH was used as loading control for whole cell lysate.

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