MANUAL

K63 TUBE, Magnetic

Catalog Number: UM404M

A. BACKGROUND

Ubiquitin and Poly-ubiquitination

The post-translational modification of proteins by ubiquitin (Ub) exerts profound effects on their compartmentalization, degradation, and function (1). While conjugation of a single ubiquitin to a target protein is referred to as mono-ubiquitination, additional Ub moieties can be conjugated to this initial Ub, forming polymer chains. Evidence exists for functional polyUb chains formed through any one of seven lysine residues in Ub, or even at the N-terminus of Ub. Poly-ubiquitination is reversible, with attachment of chains being catalyzed by complex “ligase” machinery, and the degradation or complete removal of polyUb by deubiquitinases (DUBs). The two most well characterized forms of poly-ubiquitination occur through linkage at lysine 48 (K48) or 63 (K63). The most prevalent consequence of K48-linked poly-ubiquitination is proteasome-mediated degradation, while modification by K63-linked poly-ubiquitination has been implicated in, among other cellular processes, the regulation of the DNA damage response (2), endosomal sorting (3, 4), autophagy of misfolded/aggregated proteins (5, 6), and neurodegeneration(7).

Traditional strategies for the characterization of poly-ubiquitinated proteins often require immuno-precipitation of epitope-tagged Ub, usually through over-expression of the protein. Based on their natural affinity for ubiquitin, specific ubiquitin binding associated domains (UBAs) have become useful reagents for the isolation and detection of polyubiquitin chains from a cellular milieu (5). These proteins however, display a relatively low affinity for ubiquitin. For these reasons, determining the ubiquitinated state of many proteins can prove difficult.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

The use of Tandem Ubiquitin Binding Entities (TUBEs) overcomes these problems, and is emerging as an indispensable strategy for ubiquitin research (8, 9). The first generation of these TUBEs bind K48- and K63-linked tetraUb chains with single digit nanomolar Kds, ~100 to 1000-fold more tightly than monomeric UBAs. TUBEs also protect proteins from DUBs and the proteasome, even in the absence of inhibitors normally required to block such activity. This allows efficient isolation of native polyUb chains and attached proteins from cell lines, tissues, and organs under conditions that are less likely to alter cell physiology than those listed above. TUBE1 and TUBE2 have recently been demonstrated to enrich for all polyUb chain linkage types, without discrimination, making these reagents appropriate even if the linkage type is not known for the protein of interest (8).

The next generation of TUBEs: Linkage Specific Isolation of PolyUb Chains

K63 TUBE was developed to show enhanced selectivity for K63-linked polyubiquitin 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 polyubiquitin chain linkage in your substrate protein.

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.

B. APPLICATIONS

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

C. BENEFITS

- Nanomolar affinity for K63 poly-ubiquitin chains
- 100-fold preference for K63 poly-ubiquitin chains over all other linkages
- TUBEs display higher affinity towards polyubiquitins than most ubiquitin antibodies
- Avoids overexpression of epitope-tagged ubiquitin for pull downs

D. COMPONENTS

K63 TUBE, Magnetic

Size: 1ml

Buffer: PBS, pH 7.2, 20% ethanol

Storage: **Stable for up to 6 months at 4ºC. Do not centrifuge at high speeds (>3000g), dry or freeze the beads.**

E. ADDITIONAL ITEMS REQUIRED

1. **Cell lysis buffer**: 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40. The use of other buffer systems should not significantly impact TUBE function; however, the use of alternative detergents e.g. (SDS or deoxycholate) may result in lower recovery efficiency. The inclusion of a protease inhibitor cocktail is strongly recommended to protect from non-specific protein degradation during lysis.
2. **1,10-phenanthroline (*o*-PA), 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.
3. **N-Ethylmaleimide (NEM),** an irreversible inhibitor of all cysteine peptidases.
4. **(Recommended) 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. **Pull-down buffer:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % NP-40, 1mM DTT
6. **Wash buffer 1:** 50 mM Tris pH 7.5, 250 mM NaCl, 0.2 % NP-40, 1 mM DTT
7. **Wash Buffer 2:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.05 % NP-40, 1 mM DTT
8. **Magnetic separation rack for 1.5 ml centrifuge tubes.**

F. 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 500µl of lysis buffer containing 1-2mg of total protein is an appropriate starting point for each experiment.

3. Place the volume of resin necessary for the experiment to the 1.5 mL centrifuge 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 and 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.

G. ENRICHMENT OF K63 POLY-UBIQUITINATED PROTEINS (Suggested Protocol)

1. Pre-chill cell lysis buffer and microcentrifuge tubes to 4°C. Add PR-619 (50-100µM), o-PA (2-5mM), NEM (5mM), protease inhibitor cocktail (see manufacturer's instructions) to the lysis buffer.
2. Wash cells at least 2x with cold PBS. Harvest cells into a centrifuge tube and spin down (~1,000xg, 5min 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 significant loss.
3. Add cold lysis buffer containing inhibitors to cell pellet. As an initial starting point, we recommend using 200µL of lysis buffer for ~$5x10^7$ cells (~1mg of protein.) The optimal number of cells required will depend on cell type and abundance of the protein of interest. As a starting point we recommend 1.0-3.0mg 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 20min at 4°C.
5. Remove an aliquot of "INPUT" sample for comparative analysis by Western blotting (e.g. 5-20µl of cell lysate in 25-50µl 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 hour 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 it stand on the magnetic rack for 2-3 minutes. Save supernatant as an "UNBOUND" fraction and prepare an aliquot for comparative analysis with "INPUT" sample.
8. Wash beads with 0.5 to 1ml of cold Wash Buffer 1, collect beads by letting it stand on the magnetic rack for 2-3 minutes.
9. Repeat step 8, 2-3 times. Useful Tip: Optimization of components in the wash buffer may be required, especially for samples by SDS-PAGE/Western blotting in parallel with INPUT and UNBOUND fractions.
10. Remove excess detergent by washing with Wash Buffer 2

11. For Western blot analysis, add ~25 µl of 2X SDS reducing sample prep buffer to the resin, and heat at ~95ºC for 5 min. Let stand for 3-5 minutes on the magnetic rack. Analyze eluted supernatant carefully to avoid disturbing the beads.

H. REFERENCES

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