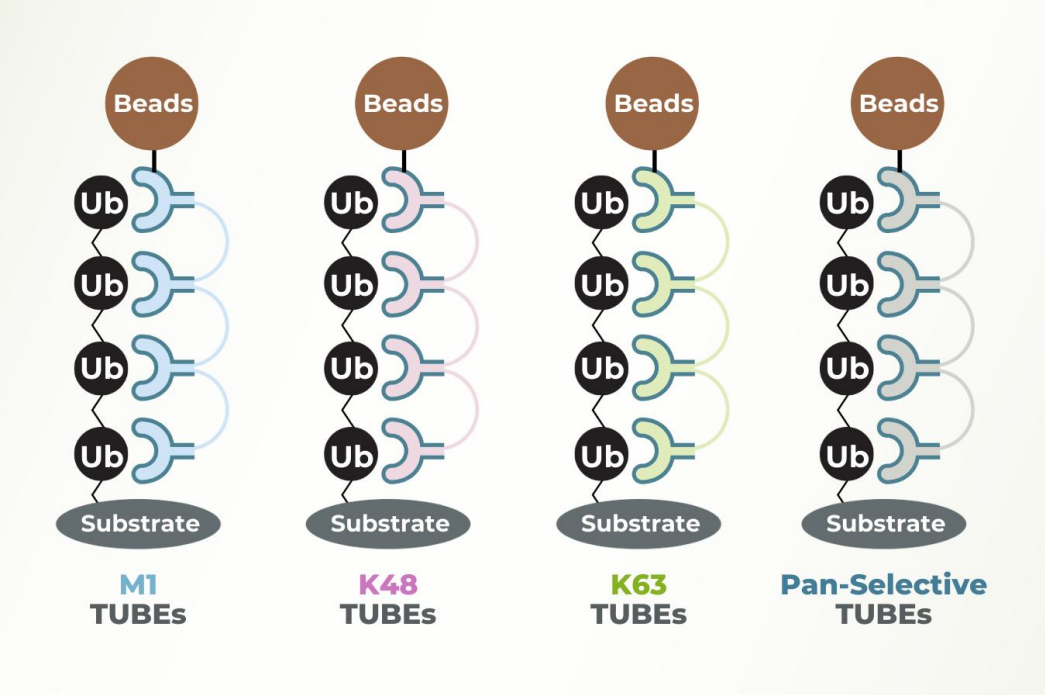
K48 TUBE HF, Biotin (Catalog # UM307)

MANUAL

K48 TUBE HF (High Fidelity), (Biotin)

Catalog Number: UM307



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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.

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

LifeSensors has developed TUBEs that exhibit 100 to 1000-fold higher affinity for polyubiquitin chains compared to monomer ubiquitin-binding domains (UBDs). Our versatile collection of TUBEs includes reagents for the isolation/pulldown of polyubiquitin conjugates, which include both linkage-specific and non-specific reagents, such as M1 Linear TUBE, K63 TUBE, K48 TUBE, and the Pan-Selective TUBE 1 and TUBE 2. K48 TUBE HF was developed to show enhanced selectivity for K48-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 K63 TUBE and M1 (linear) TUBE to investigate polyubiquitin chain linkage in your substrate protein.

Note: The new K48 TUBE HF (UM307) has an affinity comparable to that of the existing K63 TUBE (UM304). Both UM307 and UM304 bind to their respective Ub_4s with a

dissociation constant (Kd) of approximately 10-20 nM. In contrast, the previous K48 TUBE (UM305) binds to K48-Ub4 with a Kd of approximately 200 nM.

Biotin-TUBEs replace anti-ubiquitin antibodies, as well as the required blot heating, for the detection of polyubiquitinated proteins by ligand blotting ("far western blotting"). 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.

SUGGESTED USES:

1. Far-Western detection of K48-polyubiquitinated proteins from cell and tissue extracts.
2. Convenient one step pull-down of K48-polyubiquitinated proteins from cell and tissue extracts
3. Isolation of K48-polyubiquitinated proteins for proteomic studies.
4. Identification of the polyubiquitin linkage-type of your protein of interest.
5. *In situ* labeling for detection of K48-linked polyubiquitinated proteins by histochemistry.

BENEFITS:

1. K48-TUBEs have a nanomolar dissociation constant (Kd) for K48-chains.
2. K48-TUBEs exhibit a 100-fold preference for K48 chains over all other linkage types.
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.

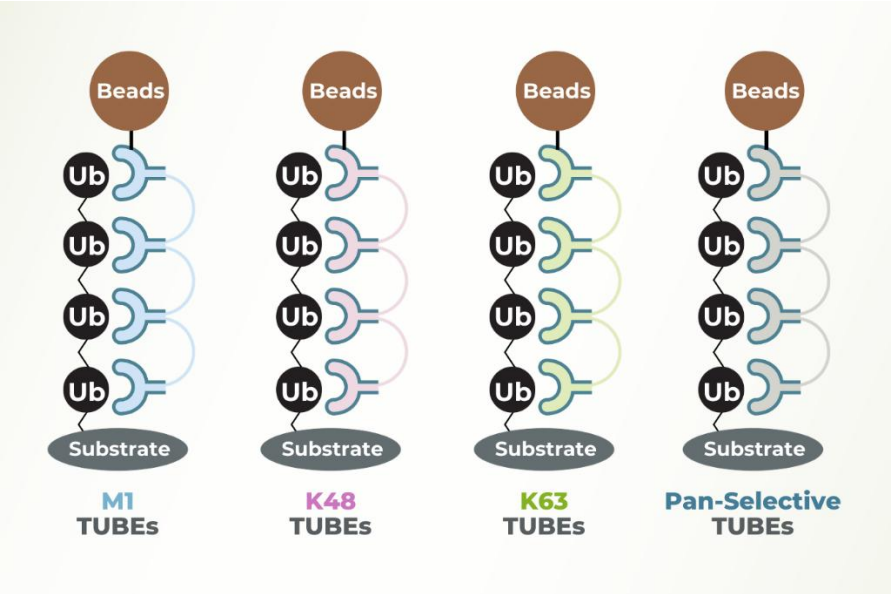

Figure 1. Schematic of the various TUBEs available from Lifesensors Inc.

K48 TUBE HF, Biotin (Catalog # UM307)

COMPONENTS

K48 TUBE HF, (Biotin)

Size	50 µg, 250 µg
Buffer	PBS, pH 7.2
Storage	Stable for up to 6 months at -80°C. Aliquot reagent and avoid repeated freeze/thaw cycles

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:** 100 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% NP-40, 0.5% Triton-X 100.

   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. **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 K48 polyubiquitin chains during cell lysis.

3. **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.

4. **(Optional) N-Ethylmaleimide (NEM), an irreversible inhibitor of all cysteine peptidases.**

   Please note, items 5-8 are required for the enrichment of K48-polyubiquitinated proteins, while Items 9-10 are required for Far Western detection.

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, 1mM DTT.

8. **Streptavidin magnetic beads with a magnetic separation rack, or streptavidin resin.**

9. **Blocking solution (TBS-T plus 5% BSA**): 100 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 0.1% Tween-20 (TBS-T) containing 5% BSA (Sigma-Aldrich).
10. **Avidin-HRP or Streptavidin-HRP conjugate.**

AN IMPORTANT NOTE ON K48 TUBE HF (Biotin) USE

Certain factors must be considered when determining the concentration of this reagent in cell lysates to ensure optimal detection/enrichment of K48 polyubiquitin over other polyubiquitin linkage types. Direct binding studies with K48 TUBE HF for K48- polyubiquitin chains show a dissociation constant (Kd) in the nanomolar range, compared to >2 µM for all other linkages. When following the recommended protocol for immunoprecipitating *in vitro*-synthesized polyubiquitin chains, K48 TUBE HF (Biotin) demonstrates maximal recovery of K48 polyubiquitin and minimal enrichment of other polyubiquitin types between 0.2 – 2.0 µM. Based on these results, we recommend an initial concentration of 0.2 to 2.0 µM K48 TUBE HF (with 25 µl of Affinity Resin) to enrich for K48 polyubiquitinated proteins. Higher concentrations may further enrich K48 polyubiquitinated proteins, though they may also isolate a small fraction of other linkage types. Optimal conditions should be determined by the end user.

PULLDOWN OF K48-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. Wash cells at least 2x with cold PBS. Harvest cells into a 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 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 ~$1.0x10^7$ cells (~ 1 mg protein). The optimal number of cells required will depend on cell type and the abundance of the protein of interest. We recommend starting with 1.0-3.0 mg of total cellular protein. Resuspend cells in lysis buffer by pipetting or vortexing. Keep all reagents cold during lysis.
4. Clarify the lysate by high-speed centrifugation (~14,000xg) for 20 min at 4°C.
5. Add K48 TUBE HF, Biotin (0.2-2 µM, see above **A NOTE ON K48 TUBE HF Biotin USE**) and bring volume of the lysate up with pull-down buffer. Incubate the reaction on a shaker at 4°C for 2 hours to allow binding of K48 TUBE HF, Biotin to polyubiquitin chains.
6. Equilibrate the streptavidin resin in the pull-down buffer according to the manufacturer's instructions. Useful tip: Use magnetic streptavidin beads to minimize bead loss.
7. Save an aliquot of "INPUT" sample for comparative analysis by Western blotting.

8. Add cell lysate to the equilibrated streptavidin resin and incubate for 1 to 2 hours at 4ºC with gentle rocking or rotation. Additional incubation time may be required; optimal time should be determined by the end user.
9. Collect beads by low-speed centrifugation (~5,000xg, 4ºC) for 5 min. For magnetic streptavidin beads, collect the beads by allowing them to stand on the magnetic rack for 2-3 min.
   Save supernatant as an "UNBOUND" fraction and prepare an aliquot for comparative analysis with the "INPUT" sample.
10. Wash the beads with 0.5 to 1 ml of cold Wash Buffer 1 and collect the supernatant as described in step 9.
11. Repeat (Step 10) 2-3 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 UNBOUND fractions.
12. Remove excess detergent by washing with Wash Buffer 2.
13. For Western blot analysis, add approximately 25 µl of 2X Laemmli SDS reducing sample prep buffer to the resin and heat at ~95ºC for 5 minutes. Centrifuge at 13,000 x g for 1 minute to collect the resin. If you use magnetic beads, allow them to stand on the magnetic rack for 3-5 minutes. Carefully transfer the eluate without disturbing the beads.

FAR WESTERN DETECTION (SUGGESTED PROTOCOL)

1. Prepare cell extract for Western blot analysis using the extraction buffer of choice in the presence of protease inhibitors. K48-linked polyubiquitin is particularly sensitive to DUB activity during cell lysis. To ensure maximal protection of K48-polyubiquitin chains, include 1-5 mM 1,10-phenanthroline (LifeSensors Cat. No. SI9649), 5 mM NEM, and 20-50 µM PR-619 (LifeSensors Cat. No. SI9619).
2. Prepare samples for SDS-PAGE using Laemmli reducing SDS sample buffer. Load 30-50 µg of total protein per lane. The optimal amount of protein for gel loading should be determined empirically.
3. Transfer to a membrane (Western Blot) according to the manufacturer's recommendations.
4. Block the membrane with the Blocking Solution for 1 hour at room temperature (RT). Overnight blocking is optional.
5. Incubate with K48 TUBE HF Biotin diluted 1:1,000 in TBS-T containing 5% BSA (Cohn fraction V) for 1 hour at RT.
6. Wash blot 3 x 10 min in TBS-T buffer.
7. Incubate with avidin-HRP conjugate (1:10,000, Rockland Immunochemicals). The manufacturer's instructions and dilutions should be determined empirically
8. Wash the membrane with TBS-T at least 3 times, 10 min each prior to the detection using enhanced chemiluminescence (ECL) reagents as per Manufacturer's instructions.

ADDITIONAL CONSIDERATIONS & TROUBLESHOOTING

Ligand blotting, or 'Far Western,' is a technique that uses a protein or smaller peptide as the primary detection reagent, rather than an immunoglobulin. In this approach, recognition and binding of the primary detection reagent to the immobilized protein of interest often depend on extended interactions, which go beyond the typically narrow epitope requirements of most antibodies. TUBEs have been engineered to recognize polyubiquitin chains in solution under non-denaturing conditions. Biotin-TUBEs have been developed to extend this recognition to polyubiquitin chains immobilized on membranes. However, it is crucial that the membrane NOT be heated, chemically treated, or otherwise subjected to denaturing conditions. Additionally, the following considerations may help enhance signal-to-background:

1. The use of nitrocellulose membranes for electrophoretic transfer.
2. Overnight blocking of the membrane in TBS-T with 5% BSA.
3. Overnight incubation with Biotin-TUBE in TBS-T, 5% BSA.
4. Increased cell lysate amounts, as total levels of K48 polyubiquitin chains may vary.

Avidin/streptavidin-biotin detection systems are sensitive to high background when milk is used as a blocking reagent.

1. The membrane must be blocked with 5% BSA for at least 30min prior to incubation with Biotin-TUBEs. **DO NOT** BLOCK with milk.
2. All dilutions and wash buffers should contain TBS-T to minimize non-specific background inherent in avidin/streptavidin detection systems.

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