MANUAL

K48 TUBE HF (High Fidelity), Biotin

Catalog Number: UM307

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

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 a comparable affinity as our existing K63 TUBE (UM304). Both UM307 and UM304 bind to their cognate Ub_4s with a K_d of ~ 10-20 nM. Please note that the old K48 TUBE (UM305) binds K48-Ub_4 with a K_d of ~200 nM.

B. APPLICATIONS

1. Isolation and enrichment of K48-polyubiquitinated proteins from cell and tissue extracts
2. Far-Western detection of K48-polyubiquitinated proteins from cell and tissue extracts
3. Isolate K48-polyubiquitinated proteins for proteomic studies

C. BENEFITS

- Nanomolar affinity for K48 poly-ubiquitin chains
- 100-fold preference for K48 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

K48 TUBE HF, Biotin

Size: 50µg, 250µg

Buffer: PBS, pH 7.2

Storage: **Stable for up to 6 months at -80C. Aliquot reagent and avoid repeated freeze/thaw cycles.**

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 K48 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.

   Items 5-8 required for enrichment of K48-polyubiquitinated proteins and Items 9-10 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, 1 mM DTT
8. **Streptavidin magnetic beads and magnetic separation rack or streptavidin resin**
9. **Blocking solution (TBS-T plus 5% BSA):** 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA, 0.1% Tween-20 (TBS-T) containing 5% BSA (Sigma-Aldrich).
10. **Avidin-HRP or Streptavidin-HRP conjugate.**

A NOTE ON K48 TUBE HF Biotin USE: Certain factors need to be considered in order to determine the concentration of this reagent in cell lysates that will ensure detection/enrichment of K48 polyUb over other polyUb linkage types. Direct binding studies with K48 TUBE HF for K48-polyUb chains yields a dissociation constant for this interaction in the nanomolar range, compared to >2 µM for all other linkages. Using the recommended protocol below to immuno-precipitate *in vitro* synthesized polyUb chains, K48 TUBE HF, Biotin displays maximal recovery of K48 polyUb and minimal enrichment of all other poly-Ubs between 0.2-2.0 µM. Therefore, we recommend an initial concentration of 0.2-2.0 µM K48 TUBE HF (with 25µl of streptavidin resin) to enrich for K48 poly-ubiquitinated proteins. **Higher concentrations may further enrich for K48 poly-ubiquitinated proteins, while also potentially isolating a small fraction of other linkage types**. Optimal conditions must be determined by the end user.

F. ENRICHMENT OF K48 POLY-UBIQUITINATED PROTEINS (Suggested Protocol)

1. Pre-chill cell lysis buffer and microcentrifuge tubes to 4°C. Add PR619 (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. Add K48 TUBE HF, Biotin (0.2-2.0 µM, see **A NOTE ON K48 TUBE HF Biotin USE** above) and bring up volume of the lysate with pull-down buffer. Incubate reaction on a shaker at 4°C for 2hr to allow binding of Biotin-K48 TUBE HF to polyUb chains.
6. Equilibrate streptavidin resin in pull-down buffer according to the manufacturer's instructions. Useful tip: Use magnetic streptavidin beads to minimize loss of beads.
7. Remove an aliquot of "INPUT" sample for comparative analysis by Western blotting.
8. Add cell lysate to equilibrated streptavidin resin and incubate for 1-2 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. In case of magnetic streptavidin beads, 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.

10. Wash beads with 0.5 to 1ml of cold Wash Buffer 1, collect by method of choice from step 9.
11. Repeat step 10, 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.
12. Remove excess detergent by washing with Wash Buffer 2
13. For Western blot analysis, add ~25 µl of 2X SDS reducing sample prep buffer to the resin, and heat at ~95ºC for 5 min. Centrifuge at 13,000xg for 1 min to collect the resin. If using magnetic beads, let stand for 3-5 minutes on the magnetic rack. Analyze eluted supernatant carefully to avoid disturbing the beads.

G. 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. The inclusion of 1-5 mM 1,10-phenanthroline (Cat# SI9649), 5 mM NEM, and 20-50 µM PR-619 (Cat# SI9619) ensures maximal protection of K48-polyUb chains.
2. Prepare samples for SDS-PAGE using reducing SDS sample buffer. Load 30-50 µg of total protein per lane. The amount of protein for gel loading should be determined empirically.
3. Transfer to membrane according to manufacturer recommendations.
4. Block membrane with Blocking Solution for 1h 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 1hr at RT.
6. Wash 3 x 10 min in TBS-T buffer.
7. Incubate with avidin-HRP conjugate (1:10,000, Rockland Immunochemicals). Manufacturer 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).

ADDITIONAL CONSIDERATIONS & TROUBLESHOOTING

Ligand blotting, or “Far Western,” is a technique that employs a protein or smaller peptide as a primary detection reagent, as opposed to an immunoglobulin. As such, recognition and binding of the primary detection regent to the immobilized protein-of-interest is often dependent upon extended interactions 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 polyUb chains immobilized on membranes. However, it is important that the membrane NOT be heated, chemically treated, or otherwise subjected to denaturing conditions. In addition, the following considerations may also 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-TUBEs in TBS-T, 1% BSA.
4. Increased cell lysate amounts, as total levels of K48 polyUb chains will 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 in 5% BSA for at least 30min prior to incubation with Biotin-TUBEs. DO NOT BLOCK in milk.
2. All dilutions and wash buffers should contain TBS-T, in order to reduce non-specific background inherent in Avidin/streptavidin detection systems.

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