MANUAL

K48 TUBE HF (High Fidelity), His_6

Catalog Number: UM207

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LifeSensors from genomics to proteomics

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 (UM207) binds to K48-Ub_4 with a K_d of ~ 10-20 nM. Please note that the old K48 TUBE (UM205) 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. 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, His_6

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.
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. **Ni-NTA Agarose resin of your choice**

A NOTE ON K48 TUBE HF His_6 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, His_6 displays maximal recovery of K48 polyUb and minimal enrichment of all other poly-Ubs between 0.2-2 µM. Therefore, we recommend an initial concentration of 0.2-2 µM K48 TUBE HF (with 25µl of Ni-NTA 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, His_6 (0.2-2 µM, see **A NOTE ON K48 TUBE HF His_6 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 K48 TUBE HF, His_6 to polyUb chains.
6. Equilibrate Ni-NTA resin in pull-down buffer according to the manufacturer's instructions. Useful tip: Use gel loading tips to minimize loss of resin.
7. Remove an aliquot of "INPUT" sample for comparative analysis by Western blotting.
8. Add cell lysate to equilibrated Ni-NTA 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. 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 the supernatant as 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 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. Analyze eluted supernatant carefully to avoid disturbing the beads.

14. (Optional) For further proteomic analysis, elute with buffer containing imidazole following manufacturer's instructions.

G. REFERENCES

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