MANUAL

K48 TUBE HF (High Fidelity), FLAG®

Catalog Number:
UM607

This document is the property of LifeSensors Inc. The proprietary ideas mentioned in the document are the subject of patent filings.
This document may not be copied or distributed to others except to the relevant staff of your company.
If you are not the intended recipient, you are hereby notified that any disclosure, copy or distribution of this information,
or taking of any action in reliance on the contents of this transmission is strictly prohibited.
Copyright © 2004 LifeSensors Inc.

All products are for research use only • not intended for human or animal diagnostic or therapeutic uses

LifeSensors, Inc., 271 Great Valley Parkway, Malvern PA 19355 • (p) 610.644.8845 (f) 610.644.8616
techsupport@lifesensors.com • www.lifesensors.com • sales@lifesensors.com

Copyright © 2010 LifeSensors, Inc. All Rights Reserved

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 (TUBE) 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 Kd’s, ~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 (UM607) has a comparable affinity as our existing K63 TUBE (UM604). Both UM607 and UM604 bind to their cognate Ub_4s with a K_d of ~ 10-20 nM. Please note that the old K48 TUBE (UM605) binds K48-Ub_4 with a K_d of ~200 nM.

B. APPLICATIONS

1. Far-Western detection of K48-polyubiquitinated proteins from cell and tissue extracts
2. Isolation and enrichment 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
- Compatible with FLAG® technology, providing flexibility and specificity

D. COMPONENTS

K48 TUBE HF, FLAG

Size: 50µg, 250µg

Buffer: PBS, pH 7.2

Storage: **Stable for up to 12 months at -80C. Aliquot reagent and avoid repeated freeze/thaw cycles.**

E. ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM 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 polyUb chains 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 polyubiquitylated proteins from degradation *(11).*
5. **Wash buffer 1:** 50 mM Tris pH 7.5, 250 mM NaCl, 0.2 % NP-40, 1 mM DTT
6. **Wash Buffer 2:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.05 % NP-40, 1mM DTT

7. **Pull-down buffer:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % NP-40, 1mM DTT
8. **Anti-FLAG® M2 Affinity Gel (Sigma-Aldrich Cat. No. A2220)**
9. **Blocking buffer - 5% (w/v) Bovine Serum Albumin (BSA, Sigma-Aldrich Cat. No. A7030)** in TBS-T (100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA, 0.1% Tween-20)
10. **Anti-FLAG monoclonal Mouse antibody (Sigma-Aldrich Cat. No. F1804)**
11. **HRP labeled anti-Mouse IgG (Jackson ImmunoResearch Cat. No. 715-035-150)**
12. **(Optional) Control Agarose (LifeSensors Cat. No. UM400)**
13. **(Optional) FLAG® peptide (Sigma-Aldrich Cat. No. F3290**) as an alternative to elution with SDS Sample buffer. For additional information regarding the use of these reagents to elute isolated proteins from the affinity gel, please refer to the manufactures' instructions.

A NOTE ON K48 TUBE HF FLAG 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, FLAG 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 to 2.0 µM K48 TUBE HF (with 25 µl of M2 Affinity 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 micro-centrifuge tubes to 4°C. Add PR619 (50-100µM), o-PA (2-5mM), NEM (5mM), protease inhibitor cocktail (see manufacturer's instructions), and K48 TUBE HF FLAG (0.2-2.0 µM, see **A NOTE ON K48 TUBE HF FLAG USE** above) 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 K48 TUBE HF FLAG and inhibitors to cell pellet. As an initial starting point, we recommend using 200 µL 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 1.0-3.0 mg 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. Incubate reaction on ice for 2 hr to overnight to allow binding of K48 TUBE HF FLAG to polyUb chains.
6. Equilibrate FLAG M2 Affinity Resin (25 µl) according to the manufacturer's instructions. Useful tip: use gel loading tips to eliminate loss of beads during the procedure.
7. Remove an aliquot of "INPUT" sample for comparative analysis by Western blotting.

8. Add cell lysate to equilibrated FLAG M2 Affinity Resin and incubate for 1 to 2 hr 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. Useful Tip: After removing the "UNBOUND" fraction adjust the remaining volume to 50-100 µl. For each wash, leave this volume to avoid disturbing the resin bed. After the final wash, use gel loading tips to ensure complete liquid removal and retention of resin for elution.
10. Wash beads with 0.5 to 1 ml of cold Wash Buffer 1, collect by low speed centrifugation and aspirate the supernatant carefully to avoid disturbing the beads. Repeat (Step 10) 2-3 times.
11. Remove excess detergent by washing with Wash Buffer 2. Repeat (Step 11) 2-3 times. Useful Tip: Optimization of components in the wash buffer may required, especially for the isolation of multi-protein complexes.
12. Poly-ubiquitinated proteins can be eluted from M2 Affinity Resin in two ways. For Western blot analysis, proceed to Step 13. For elution prior to proteomic studies, proceed to Step 15.
13. Add 40-50 ul of 2X SDS reducing sample prep buffer to the resin, and heat at ~95ºC for 5 min. The use of reducing agents may result in detection of immunoglobulin light chain components released from the M2 Affinity Resin, depending upon the detection method. Alternative elution procedure is described in Step 15.
14. Centrifuge at 13,000xg for 1 min to collect the resin. Analyze eluted samples by SDS-PAGE/Western blotting in parallel with INPUT and UNBOUND fractions. Discard the resin.
15. (Optional) For further proteomic analysis, elute with FLAG® peptide following manufacturer's instructions.

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 (LifeSensors Cat. No. SI9649), 5 mM NEM, and 20-50 µM PR-619 (LifeSensors Cat. No. 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 PVDF membrane (Western Blot) according to manufacturer's recommendations.
4. Block membrane with 5% BSA in TBS-T buffer for 1 hr at room temperature (RT). Overnight blocking is optional.
5. Incubate with K48 TUBE HF FLAG diluted to a final concentration of 0.5- 3.0 µg/ml in TBS-T containing 5% BSA for 1 hr at RT.
6. Wash blot 3 x 10 min in TBS-T buffer.
7. Incubate with anti-FLAG monoclonal antibody (1 µg/ml) for 1 hr at RT.
8. Wash blot 3 x 10 min in TBS-T buffer.
9. Incubate with HRP-labeled anti-Mouse antibody (0.2 µg/ml) for 1 hr at RT.
10. 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 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. FLAG-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 FLAG-TUBE in TBS-T, 5% BSA.
4. Increase cell lysate amounts, as total levels of K48 polyUb chains will vary.

H. REFERENCES

1. Hershko, A., and Ciechanover, A. (1998) The ubiquitin-proteasome pathway, Ann Rev Biochem 67, 425- 479.
2. Messick, T. E., and Greenberg, R. A. (2009) The ubiquitin landscape at DNA double-strand breaks, J Cell Biol 187, 319-326.
3. Balut, C. M., Loch, C. M., and Devor, D. C. (2011) Role of ubiquitylation and USP8-dependent deubiquitylation in the endocytosis and lysosomal targeting of plasma membrane KCa3.1, FASEB J 25, 3938-3948.
4. Piper, R. C., and Lehner, P. (2011) Endosomal transportation via ubiquitination, Trends Cell Biol 21, 647- 655.
5. Olzman, J. A., and Chin, L. S. (2008) Parkin-mediated K63-linked polyubiquitination. A signal for targeting misfolded proteins to the aggresome-autophagy pathway, Autophagy 4, 85-87.
6. Tan, Y. K., Vu, H. A., Kusuma, C. M., and Wu, A. (2009) Implications of autophagy in anthrax pathogenicity, Autophagy 5, 734-735.
7. Lim, K. L., and Lim, G. G. (2011) k63-linked ubiquitination and neurodegeneration, Neurobiol Dis 43, 9-16.
8. Hjerpe, R., Aillet, F., Lopitz-Otsoa, F., Lang, V., England, P., and Rodriguez, M. S. (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities, EMBO Rep 10, 1250-1258.
9. Hjerpe, R., and Rodriguez, M. S. (2008) Efficient approaches for characterizing ubiquitinated proteins, Biochem Soc Trans 36, 823-827.
10. Sims, J. J., Scavone, F., Cooper, E. M., Kane, L. A., Youle, R. J., Boeke, J. D., and Cohen, R. E. (2012) Polyubiquitin sensor proteins reveal localization and likage-type dependence of cellular ubiquitin signaling, Nat Meth 9, 303-309.
11. Altun, M., Kramer, H. B., Willems, L. I., McDermott, J. L., Leach, C. A., Goldenberg, S., Kumar, K. G., Konietzny, R., Fischer, R., Kogan, E., Mackeen, M. M., McGouran, J., Khoronenkova, S. V., Parsons, J. L., Dianov, G. L., Nicholson, B., and Kessler, B. M. (2011) Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes, Chem Biol 18, 1401-1412.
12. Choo, Y. S., and Zhang, Z. (2009) Detection of protein ubiquitination, J Vis Exp 30, e1293.

LIMITED USE LABEL LICENSE

TUBEs are produced and marketed by LifeSensors, Inc. For information on obtaining a license for commercial purposes, contact: info@lifesensors.com, LifeSensors, Inc., Malvern PA 19355, Phone: 610.644.8845.

The product and/or its derivatives are being furnished to the purchaser under the following conditions: By purchasing the product, the purchaser agrees to comply with the terms of this Limited Use Label License. The product is provided to purchaser for research use only. The purchaser of this product may not transfer or otherwise sell this product or its derivatives to a third party and no rights are given to the purchaser to use the product or its derivatives for commercial purposes as defined below.

Commercial purposes means any activity for which a party receives consideration and may include, but is not limited to,

1. use of the product or its derivatives in manufacturing,
2. use of the product or its derivatives to provide a service, information, or data,
3. use of the product or its derivatives for diagnostic purposes,
4. transfer or sell of protein or chemical component made with the product or derivatives of the product,
5. resell of the product or its derivatives, whether or not such product or its derivatives are resold for use in research.

Product use must follow compliance with all laws and regulations, including but not limited to current EPA, FDA, USDA, and NIH guidelines. THE MATERIALS WILL NOT BE USED IN HUMANS.

Purchaser acknowledges that the product is experimental in nature and provided WITHOUT WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. LIFESENSORS, INC. MAKES NO REPRESENTATION OR WARRANTY THAT THE USE OF THE PRODUCT WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHTS.

In no event shall LifeSensors, Inc. be liable for any use of the product by purchaser. Purchaser agrees to defend, indemnify, and hold harmless LifeSensors, Inc. its officers, employees, and agents from any loss, claim, injury, damage, expense, or liability (including attorney's fees), of whatsoever kind or nature, which may arise from or in connection with this Agreement, including but not limited to purchaser use, handling or storage of the product.