MANUAL

Biotinylated K63-TUBE 1

Catalog Numbers:
UM304

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BACKGROUND

Ubiquitin and Polyubiquitylation

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to Ɛ-amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. 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 polyubiquitylation. The two most well characterized forms of polyubiquitylation occur via linkage at lysine 48 (K48) or lysine 63 (K63). The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein. Polyubiqiutination is a reversible process as these chains are degraded and/or removed by proteases known as deubiquitylases (DUBs). The dynamic nature of this signaling represents a major obstacle to the isolation and functional characterization of polyubiquitylated proteins. For this reason, the ubiquitylation state of many proteins is unknown or poorly characterized.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

Tandem Ubiquitin Binding Entities (TUBEs)[1] have proven utility for isolation (Agarose TUBEs [UM401/402], GST-[UM101/102] and His6-TUBEs [UM 201/202]) and identification (Fluorescent TUBEs [UM502F/502T] and Biotin TUBEs [UM301/302]) of polyubiquitylated proteins irrespective of linkage type. The nanomolar affinity of TUBEs for polyubiquitylated proteins allows for high efficiency in isolation and characterization of these proteins from cell lines, tissues and organs.

Biotinylated-K63 TUBE 1

Sims et al.[2] have engineered high-affinity Lys63 polyubiquitin-binding peptides with selectivity for K63- over K48- and K11-linkages. These engineered proteins are the basis for Biotin-K63 TUBE 1, a new tool for the sensitive and selective detection of K63-linked polyubiquitylated proteins. Biotin-K63 TUBE 1 displays 1000-10,000-fold higher affinity for K63 ubiquitin chains over K48- or K11- chains. These K63-specific TUBEs are not based on the identity of the UBA itself, but rather on the structural context of the UBA within the TUBE as a consequence of the helical nature of the linkers that separate each domain. Biotin-K63 TUBE 1 is a sensitive and effective tool for detection of K63-linked polyubiquitylated proteins in cell and tissue lysates by “far Western”.

SUGGESTED APPLICATIONS

1. Detection of K63-linked polyubiquitinylated proteins by ligand (far Western) blotting
2. Identification of the polyubiquitin linkage-type of your protein of interest
3. Inhibition of K63-dependent processes in lysates
4. Purification of K63-linked polyubiquitinylated proteins from cell and tissue lysates using avidin supports
5. *In situ* labeling for detection of K63-linked polyubiquitinylated proteins by histochemistry

BENEFITS

1. Selective for K63-linked polyubiquitin chains
2. Superior to a standard antibody-based detection
3. Convenient detection via standardized ligand blotting (far western) techniques
4. High sensitivity
5. Low cost per blot

COMPONENTS

Biotin K63-TUBE 1: 50μg in PBS, pH 7.2 at 0.5mg/ml.

Store at -80°C. Avoid repeated thaw/freeze cycles

ADDITIONAL ITEMS REQUIRED

1. Cell lysis buffer
2. Blocking solution (TBHS-T plus 5% BSA): 20mM Tris, pH 7.6, 0.3M NaCl, and 0.1% Tween-20 (TBHS-T) containing 5% BSA (Sigma-Aldrich).
3. Avidin-HRP or Streptavidin-HRP conjugate.
4. 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.
5. N-Ethylmaleimide (NEM), an irreversible inhibitor of all cysteine peptidases.
6. (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.

SUGGESTED PROTOCOL

1. Prepare cell extract for Western blot analysis using the extraction buffer of choice in the presence of protease inhibitors.

   K63-linked polyubiquitin is particularly sensitive to DUB activity during cell lysis. The inclusion of 1-5mM 1,10-phenanthroline (Cat#SI9649), 5mM NEM, and 20-50μM PR-619 (Cat# SI9619) ensures maximal protection of K63-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 Biotin-K63 TUBE 1 diluted 1:1,000 in TBHS-T containing 1% BSA (Cohn fraction V) for 1hr at RT.
6. Wash 3 x 10 min in TBHS-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 TBHS-T at least 4 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

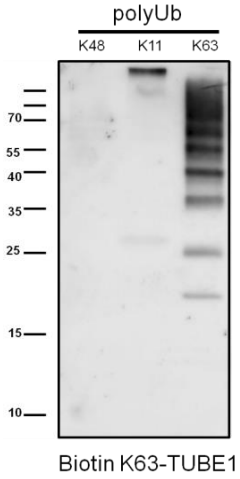
be heated, chemically treated, or otherwise subjected to denaturing conditions. In addition, the following considerations may also enhance signal to background:

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 50mM Tris, pH 7.5, 0.3M NaCl, 0.1% Tween-20, 1% BSA.
4. Increased cell lysate amounts, as total levels of K63 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 0.3M NaCl and 0.1% Tween-20, in order to reduce non-specific background inherent in Avidin/streptavidin detection systems.

SAMPLE DATA



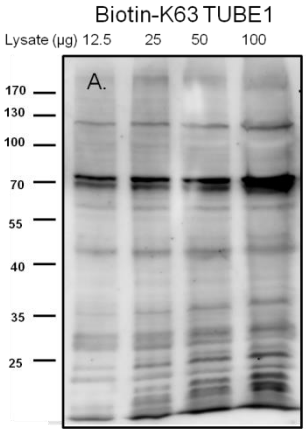


Figure 1. Biotin-K63 TUBE 1 selectively recognizes K63-linked over K48- and K11-linked polyubiquitin chains. *In vitro* synthesized K48-, K11- and K63-linked ubiquitin chains were separated by SDS-PAGE, transferred to PVDF, and the membrane was probed with Biotin-K63 TUBE 1 according to the protocol above.

Figure 2. Biotin-K63 TUBE 1 recognizes K63-linked polyubiquitylated proteins in HEK293 lysates. The indicated amount of HEK293 protein lysate was separated by SDS-PAGE, transferred to PVDF, and the membrane was probed with Biotin-K63 TUBE 1 according to the protocol above.

REFERENCES

1. Hjerpe, R., Aillet, F., Lopitz-Otsoa F., Lang, V., England P., and Rodriguez, MS. (**2009**) "Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities." *EMBO Rep.,* 10(11)1250-8.

2. Sims, JJ, Cooper, EM, Scavone, F, Kane, LA, Youle, RJ, Roeke, JD, and Cohen, RE. (**2012**) "Ubiquitin sensor peptides reveal localization and linkage-type dependence of cellular polyubiquitin signaling." *Nature Methods* 9(3):303-9.

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