MANUAL

Anti-M1 TUBE, His_6, Biotin, or Flag®

Catalog Numbers:
UM206, UM306,
UM606

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

Ubiquitin and Polyubiquitylation

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 monoubiquitylation, 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. Polyubiquitylation 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 polyubiquitylation occur through linkage at lysine 48 (K48) or 63 (K63). The most prevalent consequence of K48-linked polyubiquitylation is proteasome-mediated degradation, while modification by K63-linked Polyubiquitylation 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 characterization of ubiquitylated proteins often require immunoprecipitation 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, the determining the ubiquitylated state of many proteins can prove difficult.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

The use of Tandem Ubiquitin Binding Entities (TUBEs), developed by Dr. Manuel Rodriquez at CIC bioGUNE, 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 K_d'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

LifeSensors has developed TUBE (Tandem Ubiquitin Binding Entity) technology for the detection, characterization and isolation of polyubiquitylated proteins from cells and tissue extracts. TUBEs capitalize on the linkage of multiple ubiquitin interacting motifs (UIMs) to generate reagents with high affinity for polyubiquitin. TUBEs have 100 to 1000-fold higher affinity for polyubiquitin chains compared to monomer ubiquitin binding domains (UBDs). TUBEs both stabilize and bind to ubiquitylated proteins, serving as an indispensable tool for ubiquitologists. Our versatile collection of TUBEs includes reagents for the isolation/pulldown of polyubiquitin conjugates include both linkage-specific and non-specific reagents including Anti-M1 (Linear) TUBE, Anti-K63 TUBE, Anti-K48 TUBE, and the non-specific reagents Anti-Ubiquitin TUBE1 and TUBE2.

We have di- (cat # NC0102, tri- (cat # NC0103), tetra (cat # NC0104), and penta (cat # NC0105)- M1 (linear) ubiquitin substrates that are resistant to cleavage by Otulin, which can be used as controls or in competition based experiments. Additionally, we sell native linear chains. Our LUB9 antibody can be used to detect linear ubiquitylated substrates after pull down with TUBES.

APPLICATIONS

- Isolation and enrichment of M1-polyubiquitinated proteins from cell and tissue extracts
- Isolation of ubiquitylated proteins for proteomic studies

BENEFITS

- Nanomolar dissociation constant (Kd) for M1-chains
- 1000 to 10,000-fold preference for M1 chains over K48- or K63- chains
- Overexpression of epitope-tagged ubiquitin for pull downs is not necessary
- Compatible with Ni^{+2}IMAC, streptavidin, or FLAG® -based isolation technologies, providing flexibility and specificity

COMPONENTS

Anti-M1 TUBE

Size: 50µg

Molecular weight:

UM206 – 33.4kDa

UM306 – 33.4kDa

UM606 – 23.1kDa

Buffer: PBS, pH 7.2; 5% glycerol

Concentration: Lot dependent

Storage: **Stable for up to 1 year at -80ºC. Aliquot reagent and avoid repeated freeze/thaw cycles.**

ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA, 1% NP-40, 0.5% Triton-X 100. 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 general protease inhibitor cocktail is strongly recommended to protect from non-specific protein degradation during lysis and isolation. See also recommendations 4 and 5 below.**
2. **Reaction buffer:** 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA, **0.1% NP-40,** 0.05% Triton-X 100. Prior to addition of FLAG reagents, NP-40 must be diluted to 0.1% or below. **Important: Maintain the concentration of protease inhibitors and Anti-M1 TUBE during this dilution step.**
3. **Wash buffer:** 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA, **0.05% NP-40.**
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.

   N-Ethylmaleimide (NEM), an irreversible inhibitor of all cysteine peptidases.
5. **(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*).
6. **Ni^{+2}IMAC-Magnetic Beads (Millipore Cat. No. LSKMAGH02), MagnaLink Strepavidin Magnetic Beads (Solulink Cat. No. M-1003-010), or Anti-FLAG® M2 Affinity Gel (Sigma-Aldrich Cat. No. A2220)**
7. **(Optional) FLAG® peptide (Sigma-Aldrich Cat. No. F3290**) or **0.2M glycine HCl, pH 2.5** as alternatives 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 manufacturer's instructions.

AN IMPORTANT NOTE ON Anti-M1 TUBE USE: Certain factors need to be considered in order to determine the concentration of this reagent that will ensure enrichment of M1-linked polyUb over other polyUb linkage types. Direct binding studies with M1-selective TUBEs for M1-polyUb chains yields a dissociation constant for this interaction in the 10-20 nanomolar range, compared to >5µM for either K48- or K63- polyUb. During enrichment of *in vitro* synthesized polyUb chains, Anti-M1 TUBE displays maximal recovery of M1 polyUb and minimal enrichment of K48 polyUb between 0.1 and 0.5µM. Based on this observation, we recommend using Anti-M1 TUBE at concentrations ranging from 50 to 500nM as a starting point (with 10µl of affinity resin) to enrich for M1 polyubiquitylated proteins. **Higher concentrations may further enrich for M1 polyubiquitylated proteins, but may also result in isolation of other linkage types**. Increasing the amount of total protein in lysate or homogenate in order to increase yield should not significantly impact linkage type specificity. **Cell lysis should be carried out in the presence of Anti-M1 TUBE protein, 1-5mM *o*-PA and 5mM NEM in order to ensure maximal protection of polyUb chains.** Optimal conditions for all components and incubation times must be determined by the end user.

ENRICHMENT OF M1-POLYUBIQUITINATED PROTEINS (Suggested Protocol)

n.b. An educational video demonstrating cell lysis and IP can be found in Ref. (*12*).

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), and Anti-M1 TUBE (50nM to 500nM, see **A NOTE ON Anti-M1 TUBE 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 Anti-M1 TUBE and inhibitors to cell pellet. As an initial starting point, we recommend using 100-200µL of lysis buffer for ~$1.5x10^6$ cells. **The optimal number of cells required will depend on cell type and abundance of the protein of interest**. 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. Dilute clarified lysate 5-10 fold in 100mM Tris-HCl, pH 8.0, 0.15M NaCl, 5mM EDTA to reduce concentration of NP-40 to 0.1-0.2% and Triton X-100 to 0.05% (**Reaction buffer).** Adjust the concentration of Anti-M1 TUBE and all inhibitors accordingly.
6. Incubate reaction on ice for 1 to 2h to allow for binding of Anti-M1 TUBE to polyUb chains.
7. Equilibrate appropriate affinity resin according to the manufacturer's instructions. **Useful tip**: use gel loading tips to eliminate loss of beads during the procedure.
8. Remove an aliquot of "INPUT" sample for comparative analysis by Western blotting.
9. Add cell lysate to equilibrated **affinity 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.**
10. Collect beads by low speed centrifugation (~5,000xg, 4ºC) for 5min or with a magnetic bead stand. Save supernatant as an "UNBOUND" fraction and prepare an aliquot for comparative analysis with "INPUT" sample.
11. Wash beads with 0.5 to 1ml of **cold Wash Buffer**, collect by low speed centrifugation and aspirate the supernatant carefully to avoid disturbing the beads. **Useful Tip:** After removing the "UNBOUND" fraction adjust the remaining volume to 50-100ul. 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.
12. Repeat (**Step 11**) 2-3 times. **Useful Tip:** Optimization of components in the wash buffer may be required, especially for the isolation of multi-protein complexes.
13. Polyubiquitylated proteins can be eluted from affinity resin in three ways. For Western blot analysis or proteomic studies, proceed to **Step 14**. Alternative methods are outlined in **Step 16 and 17**.
14. Centrifuge at 13,000xg for 10min to collect the resin. Analyze eluted samples by SDS-PAGE/Western blotting in parallel with INPUT and UNBOUND fractions. Discard the resin.
15. Add 10-20ul of 6X SDS reducing sample prep buffer to the resin, and heat at ~96ºC for 5 min. **The use of reducing agents may result in detection of immunoglobulin light chain components released from the affinity resin depending upon the detection method.**

16. For further proteomic analysis, elute material with 50 to 100µl of **0.2M glycine HCl, pH 2.5** for at least 1hr (4°C) through gentle mixing/rotation. Pellet resin by high speed centrifugation (13,000xg) for 5min and collect supernatant without disturbing resin. Neutralize the reaction with 1M Tris, pH 8.0 or 1M HEPES, pH 7.5. For Discard the resin.

17. **His_6 Anti-M1 TUBE** can be eluted from Ni^{+2}IMAC resins with 0.5M imidazole**,** while **Flag Anti-M1 TUBE** can be eluted from anti-Flag M2 supports with the **FLAG peptide** (follow the manufacturer's recommendations).

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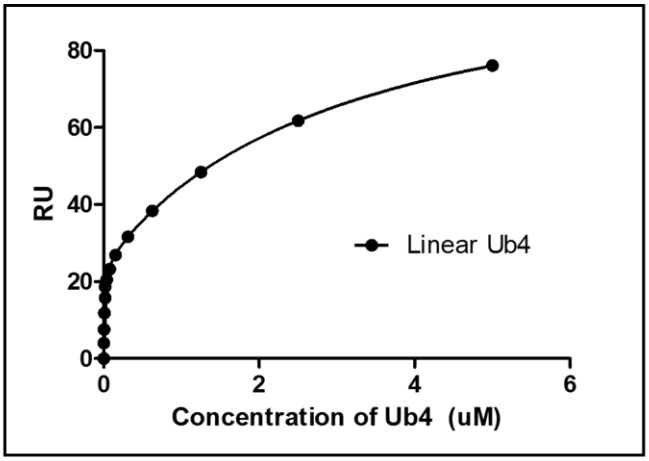
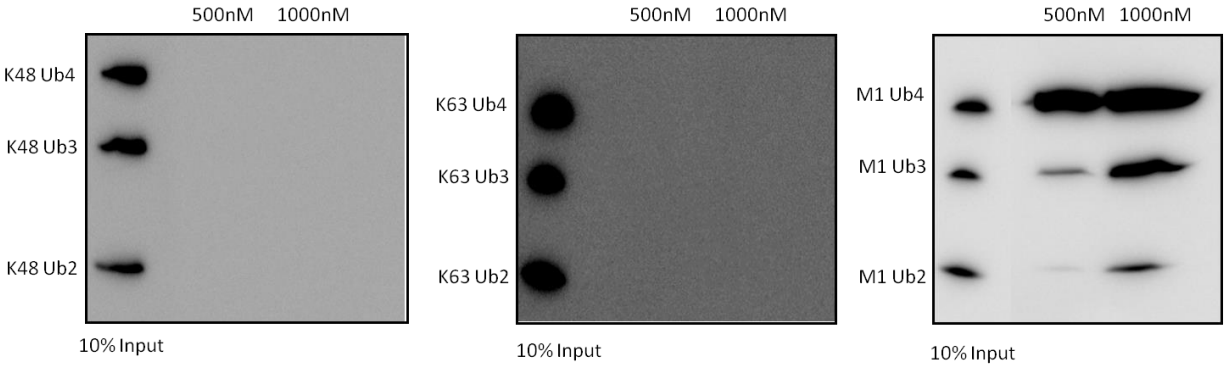
SAMPLE DATA



Anti-M1 (Linear) TUBEs show a strong affinity for M1 ubiquitin (KD ~15nM) as measured by SPR.



Anti-M1 (Linear) TUBE shows strong specificity. No K48 or K63 chains were pulled down with 500 or 1000nM anti-M1 TUBE. Pull down was done with anti-M1 TUBE and then probed with VU1.

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