MANUAL

FLAG® K63-TUBEs

Catalog Number:
UM604

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BACKGROUND

Ubiquitin and Polyubiquitination

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 monoubiquitination, 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. Polyubiquitination 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 polyubiquitination occur through linkage at lysine 48 (K48) or 63 (K63). The most prevalent consequence of K48-linked polyubiquitination is proteasome-mediated degradation, while modification by K63-linked polyubiquitination 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 ubiquitinated 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 ubiquitinated 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

Recently, Sims, et al. (*10*) have shown that tandem ubiquitin interacting motifs (UIMs) can be engineered to display remarkable preference in binding specificity for polyUb chains. These constructs bind K63 Ub chains with low nanomolar K_d's, yet bind poorly to both K48 and K11 chains. This 1000 to 10,000-fold preference for K63 polyUb is a function of both the UIM and the length and helical nature of the linker between the tandem UIMs. This linker imparts rigidity and spaces the UIMs precisely for docking with each Ub molecule, enhancing recognition of extended K63 chains. In addition, expression of these tandem UIMs appears to selectively inhibit K63-depedent cellular processes, and protect polyUb chains from degradation *in situ*. These **K63-TUBEs** have been further developed at LifeSensors as tools to study cellular processes that are specifically dependent upon K63 polyubiquitination. **Flag K63-TUBE 1** allows capture and isolation of proteins modified by this linkage type from cell and tissue extracts, or *in vitro* mixtures.

APPLICATIONS

- Isolation and enrichment of K63-polyubiquitinated proteins from cell and tissue extracts
- Isolation of ubiquitinated proteins for proteomic studies

BENEFITS

- Nanomolar dissociation constant (Kd) for K63-chains
- 1000 to 10,000-fold preference for K63 chains over K48- or K11- chains
- Overexpression of epitope-tagged ubiquitin for pull downs is not necessary
- Compatible with FLAG® technology, providing flexibility and specificity

COMPONENTS

FLAG K63-TUBE 1

Size: 50µg

Molecular weight: 9062.7 (calculated)

Buffer: PBS, pH 7.2

Concentration: 1mg/ml (110µM)

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 inhibitors and Flag K63-TUBE1 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 polyubiquitinated proteins from degradation (*11*).
6. **Anti-FLAG® M2 Affinity Gel (Sigma-Aldrich Cat. No. A2220)**
7. **(Optional) Control Agarose (LifeSensors Cat.No. UM400)**
8. **(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 manufactures' instructions.

AN IMPORTANT NOTE ON FLAG K63-TUBE1 USE: Certain factors need to be considered in order to determine the concentration of this reagent that will ensure enrichment of K63 polyUb over other polyUb linkage types. Direct binding studies with K63-selective TUBEs for K63-polyUb chains yields a dissociation constant for this interaction in the nanomolar range, compared to >5µM for either K48- or K11- polyUb. During enrichment of *in vitro*

synthesized polyUb chains, FLAG-K63 TUBE1 displays maximal recovery of K63 polyUb and minimal enrichment of K48 polyUb between 0.1 and 0.3µM. Sims *et al*. have also demonstrated that K63 linkage-specific inhibition of cellular processes *in situ* by K63-selective TUBEs is largely maintained at peptide concentrations at or below 1µM.Based on these observations, we recommend using K63-TUBE 1 at concentrations ranging from 50 to 500nM as a starting point (with 10µl of M2 Affinity Resin) to enrich for K63 polyubiquitinated proteins. **Higher concentrations may further enrich for K63 polyubiquitinated 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 Flag K63-TUBE1 protein, 1-5mM *o*-PA and 5mM of NEM in order to ensure maximal protection of K63-polyUb chains.** Optimal conditions for all components and incubation times must be determined by the end user.

EQUILIBRATION OF CONTROL AGAROSE (Optional Protocol)

1. Allow Control Agarose (UM400) to thaw at room temperature for 10-15min.
2. Gently mix Control Agarose by inverting the vial several times to ensure a homogeneous suspension.
3. Transfer a desired volume of resin (as 50% slurry) with a wide bore ("cut") pipet tip to a 1.5ml microcentrifuge tube. We recommend 10µl of resin for each control experiment (see below). Larger volumes (e.g. 100µl) of resin can be equilibrated and stored for up to one month at 4°C under neutral conditions.
4. Add 10 bed volumes of Tris-buffered saline, pH 7.5 (TBS) to the resin. Mix by inverting or brief vortexing.
5. Collect resin by low speed centrifugation (<5000xg) for 5 minutes (RT).
6. Remove and discard the supernatant.
7. Repeat steps **4** through **6** two-three times. **Useful tip**: With an equal volume of buffer retained on the resin, the equilibrated resin should now be 50% slurry.

ENRICHMENT OF K63-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 manufactures' instructions), and FLAG K63-TUBE 1 (50nM to 500nM, see **A NOTE ON FLAG K63-TUBE1 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 FLAG K63-TUBE1 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 FLAG K63-TUBE1 and all inhibitors accordingly.
6. Incubate reaction on ice for 1 to 2h to allow for binding of FLAG K63-TUBE 1 to polyUb chains.
7. Equilibrate FLAG M2 Affinity Resin according to the manufactures' 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 **FLAG M2 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.** (Optional) Add equilibrated **UM400** to parallel samples reactions to control for the detection of non-

specifically bound proteins.

10. Collect beads by low speed centrifugation (~5,000xg, 4ºC) for 5min. 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. Polyubiquitinated proteins can be eluted from M2 Affinity Resin in three ways. For Western blot analysis, proceed to **Step 14**. For elution prior to proteomic studies, proceed to **Step 15**.

14. 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 M2 Affinity Resin, depending upon the detection method. Alternative elution procedure is described in Step 15.** 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. 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. Alternatively, use **FLAG peptide** for the elution of protein under neutral conditions (follow the manufactures' recommendations). Discard the resin.

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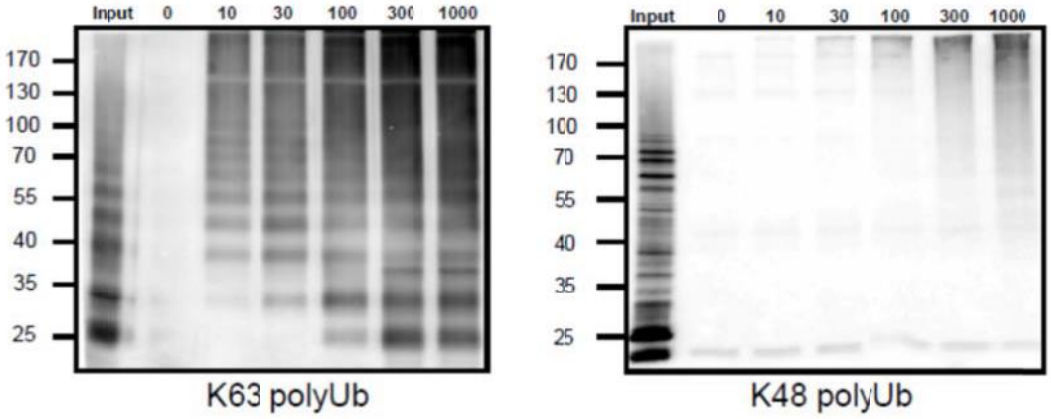
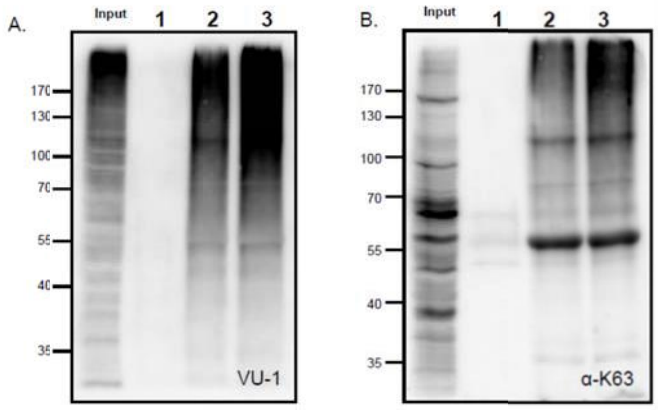
SAMPLE DATA

Enrichment of *in vitro* synthesized polyubiquitin chains with FLAG K63-TUBE 1



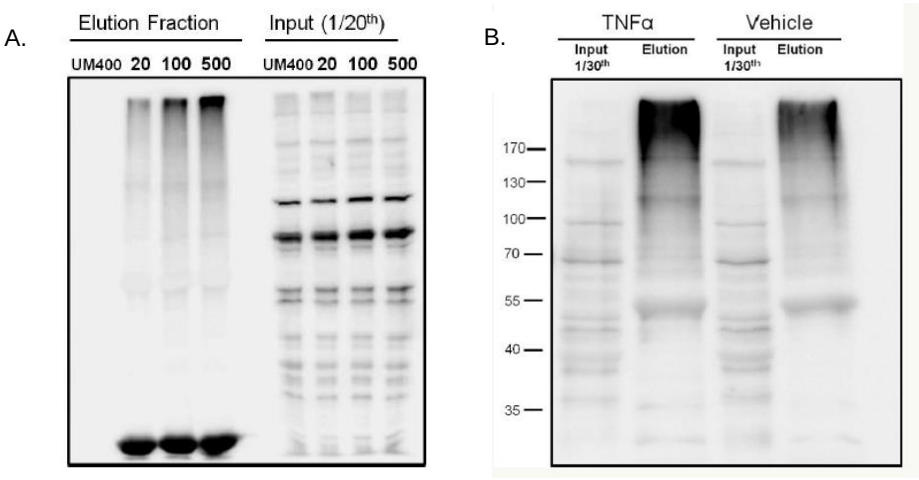
Enrichment of *in vitro* polyUb chains. Approximately 10µg of polyUb chains were diluted into 0.25ml of Isolation Buffer containing indicated nanomolar concentrations of FLAG K63-TUBE1 and incubated for 1.5h (4°C). After addition of FLAG M2 Affinity Resin (10µl, Sigma-Aldrich), reactions were incubated at 4°C with rotation. Captured protein (elution fraction, 20% of total) was resolved on 10% SDS-PAGE followed by Western blot analysis using mouse monoclonal ubiquitin antibody VU-1 (LifeSensors, VU101). Input samples (2% of total) are shown for comparison.

Enrichment of K63-linked Polyubiquitinated Proteins from HEK293T Lysate



Enrichment of K63-polyUb from HEK293T lysate. Pulldown reactions were performed, as described in **Suggested Protocol**, using 0.5µM (lanes 1 & 2) or 1µM (lane 3) FLAG K63-TUBE1 in the presence of FLAG M2 Affinity Resin (10µl, Sigma-Aldrich) or Control Agarose (lane 1) (LifeSensors, UM400). Captured proteins [lanes 2 and 3, 0.5mg (Panel A.); 1.5 mg (Panel B.) were resolved on10% SDS-PAGE followed by Western blot analysis using mouse monoclonal ubiquitin antibody VU-1 (LifeSensors, VU101) (panel A) or a K63-linkage specific ubiquitin antibody. Input samples represent the 5% and 3.3% of total proteins, panel A and B respectively).

Isolation of K63 PolyUb from Jurkat Cells and Macrophages



A. α-CD3 IgG treated cells were washed in PBS, and lysed in TBSE, 10% glycerol, 1% Triton™ X-100, 0.5% CHAPS, 5mM NEM, 50μM PR-619, 5mM 1,10-penanthroline, protease inhibitor cocktail and incubated with the indicated (nM) concentrations of FLAG® K63-TUBE 1. Lysates were clarified, pre-cleared, and rotated with M2 resin (10μl) overnight (4^0 C) and eluted protein was immunoprobed for K63 polyUb.
B. Macrophages were isolated from murine bone marrow of 8 week old pups. Cells were cultured for 8 days in DMEM supplemented with a combination of 10% FCS and conditioned media from the cultured murine L929 fibroblasts. Macrophages were then harvested and treated with either vehicle control or TNFα (20μg/ml) for 15 min to induce up regulation of K63-specific ubiquitylation.

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