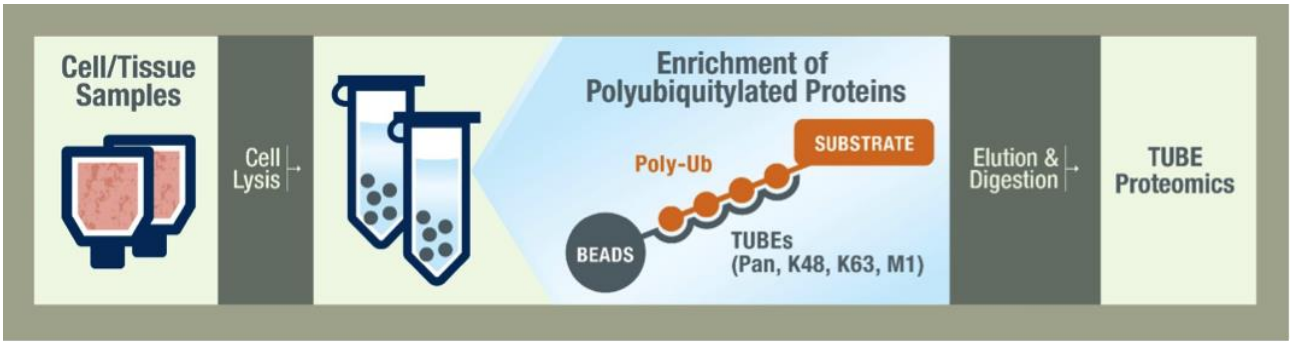
Ubiquitin Mass Spectrometry Kit

(Label free ubiquitin proteome analysis)

Instruction Manual

Cat. No. UM420



Background

Ubiquitin, Polyubiquitination, and Ubiquitin Proteasome System

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitination. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The two most well characterized forms of this are polyubiquitination via lysine at position 48 (K48) or position 63 (K63). The most prevalent consequence of polyubiquitination is the proteasome-mediated degradation of the target protein *via* the ubiquitin proteasome system (UPS). The dynamic nature of this signaling represents a major obstacle to the isolation and functional characterization of polyubiquitinated proteins. For this reason, the ubiquitination state of many proteins is unknown or poorly characterized.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

Traditional strategies for characterization of ubiquitinated proteins often require immunoprecipitation. of overexpressed ubiquitin with an epitope tag or the use of ubiquitin antibodies (expensive for large scale studies). Alternatively, isolation of polyubiquitinated proteins can be achieved with certain antibodies and UBDs, but these proteins display a low affinity for ubiquitin. All the ubiquitin antibodies that are commercially available are poor in IP and lack chain selectivity. Tandem Ubiquitin Binding Entities (TUBEs) have been developed to overcome these problems. TUBEs are essentially tandem UBDs with dissociation constants for tetra-ubiquitin in the nanomolar (or sub-nM) range. TUBEs have also been demonstrated to protect proteins from both deubiquitination and proteasome-mediated degradation, even in the absence of inhibitors normally required to block such activity. The nanomolar affinity of TUBEs for polyubiquitinated proteins allows for high efficiency in isolation and characterization of these proteins from cell lines and tissues. LifeSensors has developed a number of *selective* TUBE products that bind to polyubiquitin molecules of specific linkage topologies. These include:

TUBE1 and TUBE2, pan-selective (bind to all linkages)
K48 TUBE HF, K48 linkage selective
K63 TUBE, K63 linkage selective
M1 TUBE, M1 (linear) linkage selective

Chain-selective binding in TUBE technologies remains a grand challenge in the UPS field. The superior nature of TUBEs allows for efficient detection of polyubiquitinated proteins in their native state, while the versatility of TUBEs meets a wide range of experimental needs.

Need for studying polyubiquitination on target proteins – Mass spectrometry.

Mass spectrometry (MS) proteomics, especially when combined with TUBE technologies for protein enrichment, can be used in several innovative and impactful applications. Quantitatively profiling the ubiquitome can lead to the identification of ubiquitination sites on target proteins, and by extension unveiling the mechanisms of undiscovered E3 ligases with their substrates. MS can also inform small molecule drug discovery for these targets (e.g. PROTACs, molecular glues) by quantitatively reporting on how much target protein is degraded in the presence or absence of a drug candidate. This approach can also greatly aid the discovery and development of novel diagnostic biomarkers for challenging disease targets and development of clinically monitoring disease progression. All these applications require a simple, label-free, rapid ubiquitome approach. This kit is designed to address this for rapid yet reproducible proteomics.

A Mass Spec Approach to Ubiquitin Bioinformatics

This kit is designed to aid the user in identifying ubiquitylated targets from cell lysates. The first step involves lysing the cells and treating the lysates with beads coated with TUBEs to enrich the ubiquitylated targets. Next, the beads are isolated, washed, and eluted to enrich total ubiquitinated proteome. A simple digestion of eluted proteome with trypsin followed by analysis using LC-MS/MS allows for extremely sensitive and reproducible monitoring of changes in ubiquitinated proteins. The quantitative changes in overall ubiquitome and site-specific lysine modification with Di-glycine (GG), which is a signature for ubiquitination, can be evaluated by performing database search using advanced bioinformatics tools.

Applications

1. Convenient, one-step pull down of polyubiquitinated protein from cell and tissue extracts.
2. Isolate label-free ubiquitinated proteins for proteomic studies.
3. Identifying new ligases and/or their substrates.
4. Mechanistic studies of E3 ligases and substrates.
5. Efficient screening of small-molecule drug candidates (e.g. PROTACs, molecular glues).
6. Diagnostic/clinical biomarker discovery.

Benefits

- ✓ Up to 1000-fold higher affinity for polyubiquitin compared to single UBA
- ✓ TUBEs have higher specificity and affinity than antibodies for IP applications
- ✓ Avoid overexpression of epitope-tagged ubiquitin for ubiquitin proteome enrichment
- ✓ TUBEs protect polyubiquitinated proteins from degradation and DUBs.
- ✓ Specially designed polymeric magnetic beads offer efficient pulldown for MS analysis with lower non-specific binding
- ✓ Label free and extremely user-friendly.
- ✓ Highly reproducible with higher proteome coverage compared traditional ubiquitin GG-remnant antibody approaches
- ✓ Enriches only ubiquitinated proteome compared to GG-remnant antibodies enrich peptides resulted from other post-translational modifications derived from trypsin digestion. (note: Trypsin digestion results in GG-remnant from ubiquitin like protein besides ubiquitin)

Components

Mammalian Cell Lysis Buffer:	5 mL Lysis Buffer, (contains protease inhibitors) (***Store at -80°C***)
UPS Inhibitor Cocktail:	50 µL, 100X (***Store at -80°C***)
Magnetic TUBE Reagent:	10 reactions/vials (***Store at 4°C***)
Decomplexing Reagent:	50 µL, 10X (***Store at -80°C***)
TUBE Wash Reagent:	1000 µL, 10 reactions (***Store at 4°C***)
TUBE Elution Reagent:	300 µL, 10 reactions (***Store at 4°C***)
TUBE Neutralization Reagent:	100 µL, 10X, 10 reactions (***Store at 4°C***)
Digestion Buffer:	5 mL, 10 reactions (***Store at 4°C***)
Reducing solution:	1000 µL, 10 reactions (***Store at -80°C***)
Alkylating solution:	1000 µL, 10 reactions (***Store at -80°C***)
Trypsin:	1 vial, 20 ug (***Store at -80°C***)
Gel Wash Buffer:	20 mL, 10 reactions (***Store at -80°C***)

Components required but not supplied

- 1x concentration phosphate-buffered saline and phosphate-buffered saline with 0.1% Tween-20 (PBS / PBS-T)
- 100% Acetonitrile (HPLC grade)
- 50 mM acetic acid
- Alternate Lysis Buffer: The use of alternative buffer systems should not impact TUBE function; however, the inclusion of denaturing detergents e.g. SDS or deoxycholate may have a negative impact on overall yield of polyubiquitinated proteins. The inclusion of a protease inhibitor cocktail is recommended to protect from non-specific protein degradation during lysis and isolation.
- Pipettes, tips, and proper disposables (gloves, Eppendorf tubes, etc.)
- Magnetic rack for 1.5 mL centrifuge tubes.
- BCA Protein Quantification Assay
- SDS-Page Loading Dye, Gels, and Apparatus
- LC-MS/MS instrument

Optional

LifeSensors offer fee-for-service MS analysis where you can ship the eluted samples for MS analysis.

Additional Notes

1. Each kit comes with enough supplied material to run 10 reactions total.
2. Magnetic TUBEs should be stored at 4°C for long term storage. **DO NOT STORE MAGNETIC TUBEs**

AT -80°C. This will compromise their efficacy during pulldown.

3. All other components can be stored long-term at -80°C.

4. Use high-purity, nanopure water and solvents to avoid contamination.

5. Handle gel pieces carefully to avoid transfer loss. We recommend using commercial grade, pre-cast gels.

6. To avoid multiple freeze-thaw cycles, we recommend the user aliquot the Trypsin Solution provided into 5 µL aliquots to thaw when needed. These aliquots can be stored at -20°C for up to two months with minimal activity loss.

7. Appropriate controls should be determined by the end-user for best results and interpretation.

Detailed Protocol

Note: This protocol described below is for a single reaction/sample preparation. The kit comes with enough material for 10 total reactions.

EQUILIBRATION OF MAGNETIC-TUBEs

1. Gently mix Magnetic-TUBE by inverting the vial several times to ensure a homogeneous suspension/slurry.

2. Place the 100 µL of magnetic slurry into 1.5 mL centrifuge tube, then collect the beads by placing the tube into a magnetic stand. Discard the supernatant.

3. Suspend the resin 500 µL of PBS and place it back into a magnetic stand, collect the beads, and discard the supernatant. Repeat this step two more times. Finally, resuspend the magnetic beads in 100 µL of PBS-T wash buffer, which is ready for pulldown.
   Note: This step ensures removal of storage solution that contains ethanol which is detrimental to protein lysates.

CELL LYSIS AND PULLDOWN OF POLYUBIQUITINATED PROTEINS

1. Pre-chill Mammalian Cell Lysis Buffer AND Eppendorf tubes to 4°C.

2. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 495 µL of lysis buffer to a 10 cm^2 tissue culture dish containing ~5-10x10^6 cells (80% confluence).
   Note: The optimal number of cells will depend on the cell line and the abundance of the protein of interest.

3. Collect cells by scraping and transfer the lysate to chilled 1.5 mL Eppendorf tube. Add 5 µL of UPS Inhibitor Cocktail and gently invert 5 times to ensure even mixing. Allow to lyse for 15 minutes over ice.

4. Clarify lysate by high-speed centrifugation (~14,000xg) for 10 minutes at 4ºC.

5. Gently transfer the supernatant from the insoluble pellet and place in chilled microcentrifuge tubes, being careful not to disrupt the pellet. Save a 10 µL aliquot and label it “INPUT”— This will be a reference to the proteins that you start the assay with in the SDS-PAGE analysis.

6. Add 50 µL Decomplexing Buffer (10x) to every mL of clarified lysate. Aspirate a few times to ensure even mixing. Incubate for 15 minutes over ice.

7. Quantify the amount of protein in the supernatant by performing a BCA Protein Quantification Assay (not provided in kit).

8. Add 100 µL of Magnetic TUBE slurry with 1-3 mg of available total protein with pulldown volume being 1000 µL. The final volume of 1mL can be adjusted using PBS. LifeSensors has noticed superior proteome coverage with at least 2 mgs of protein lysate with the lowest recommended amount being 1 mg. Incubate these mixtures overnight at 4°C either using an end-to-end rotator or a standard nutator.
   Note: With suggested conditions LifeSensors has demonstrated that you can identify at least 2000-3000 proteins in the enriched fraction with at least 3-5% ubiquitination lysine sites mapped. You may need to adjust the lysate amount required for the experiment based on the experiment or the cell line. The amount of polyubiquitin in samples can vary with cell or tissue type, experimental conditions, and the presence or absence of deubiquitinating enzyme or proteasomal inhibitors.

9. Place the tube into a magnetic stand, collect the beads and save the supernatant as the "UNBOUND FRACTION."

10. Wash the remaining beads (now bound with ubiquitylated proteins) by removing the tube from the magnetic rack and resuspending the beads with 1 mL of PBS-T. Place back in the magnetic rack, decant the solution, and discard the solution. Repeat these steps 3 more times.

11. Wash the beads with 80 µL of TUBE Wash Buffer and discard the supernatant. This step is to equilibrate the beads to allow efficient elution in the next steps. Immediately proceed to protocol for Elution, SDS-PAGE, and Trypsin Digest.

ELUTION, SDS-PAGE, AND TRYPSIN DIGEST

1. Resuspend and incubate the beads in 30 µL TUBE Elution Buffer at room temperature for 15 minutes with mixing. Place the tube in the magnetic rack, remove the supernatant, and place it into a new Eppendorf tube. This solution contains eluted, ubiquitylated proteins.

2. Neutralize the elution product by adding 3.3 µL Neutralization Buffer
   Note: At this point, samples can be stored at -80°C for long-term storage

3. Spin the sample down by centrifugation for 5 minutes. Transfer a 20 µL aliquot of the sample to a new Eppendorf tube containing 4 µL of 6x Laemmli Buffer.
   Note: All 33.3 µL can be loaded incase the enrichment is inadequate or if protein coverage is inadequate.

4. Mix the SDS-PAGE sample and boil at 90°C for 5 minutes. Spin down the sample briefly.

5. Run the SDS-PAGE on an acrylamide gel of your choosing. Let the gel run until the Laemmli buffer dye front is ca. 0.5 cm from the top of the gel (see sample data to get an idea of where to stop the gel run).

6. Stain the gel with Coomassie Brilliant Blue for 15 minutes. Destain the gel with destaining solution until the dye is removed.

7. Carefully excise the gel lane of interest with a clean scalpel or gel cutter and place into a clean Eppendorf. Try to minimize the gel volume as much as possible – cut at the stain margin to remove essentially all the stained gel, but minimize the amount of unstained gel in the excised fragment.

8. Wash excised gel fragment with 400 µL Gel Wash Buffer. Incubate for 30 min at 37°C with shaking. Aspirate and discard the solution. Repeat this step two more times. Allow the gel to air dry for 15 minutes.

9. To alkylate the gel, add 100 µL Reducing Solution and incubate for 15 min at 37°C. Aspirate and discard the solution. Add 100 µL Alkylating Solution for 1 hour at 37°C. Aspirate the solution with a pipette and discard. **THIS STEP MUST BE DONE IN THE DARK.**

10. Wash the gel fragment with 400 µL Gel Wash Buffer, incubate for 15 min at 37°C. Aspirate and discard the solution. Wash the gel fragment with 400 µL Digestion Buffer, incubate for 15 min at 37°C. Aspirate and discard the solution. Wash the gel fragment with 400 µL Gel Wash Buffer, incubate for 15 min at 37°C. Aspirate and discard the solution. Allow the gel to air dry for 5-10 minutes.

11. Prepare the Trypsin Stock: The kit comes with a vial of 20 µg lyophilized trypsin. Resuspend the trypsin powder in 10 µL of 50 mM acetic acid (final concentration of 2 µg/µL). This stock can be stored at -20°C for > 1 year without any significant loss of activity. Alternatively, prepare 1 µL aliquots for one-time use and store at -80°C and to avoid multiple freeze-thaws.

12. Prepare the Trypsin Working Solution: Add 199 µL Digestion Buffer to 1µL of 2 µg/µL aliquot. This will generate 100 µL of Trypsin Working Solution (0.02 µg/µL Trypsin in Digestion Buffer).

13. Add 50-100 µL of the Trypsin Working Solution to the gel fragment and allow digest to proceed overnight at room temperature with mixing. Add just enough Trypsin Working Solution until the gel is completely submerged.

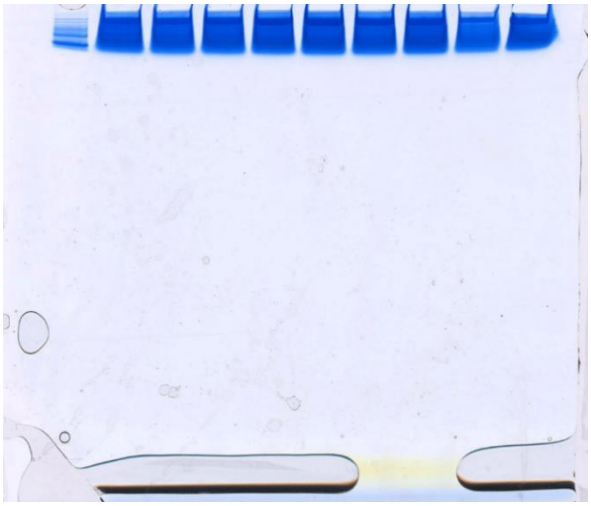
LC-MS/MS

1. Prepare digest protein samples in desired LC-MS sample solution (e.g. 0.1% Trifluoroacetic acid in water). The samples should be acidified in TFA for efficient peptide binding.

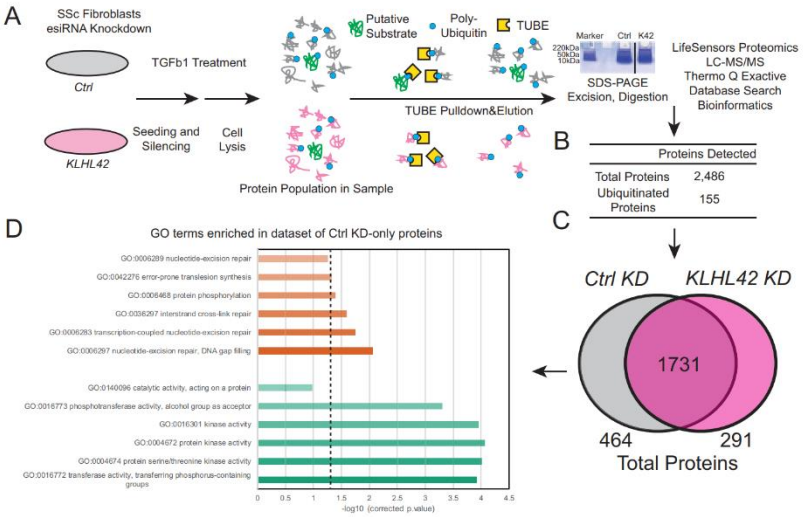
2. Prepare an LC-MS method for analysis. As a starting point, we recommend a 150-minute LC run. This is on a Thermo Q Exactive HF mass spectrometer. A 30-minute blank should be injected in between samples. Ultimately the LC-MS method may need to be optimized to suit the end-user's needs.

3. MS data can be analyzed against a proteome database at the user's preference.

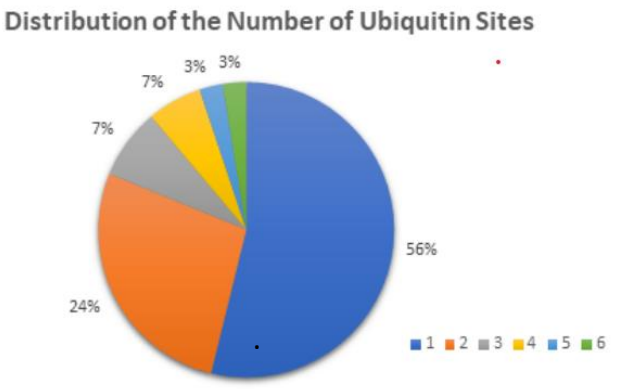
SAMPLE DATA



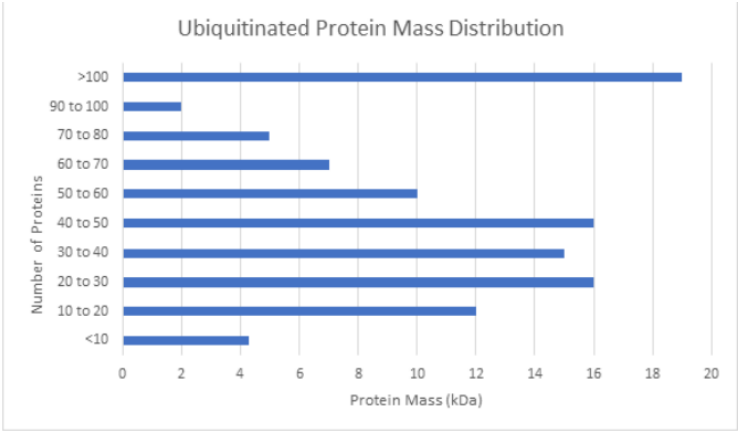
Enrichment for polyubiquitin with Magnetic-TUBE. SDS-PAGE gel with Coomassie stain/destain prior to excision and trypsin digestion.



Experimental workflow from Lear *et al*. Ubiquitin proteomics were performed on SSc fibroblasts and KLHL knockdown mutants TUBE enrichment was performed on cellular lysates and led to the discovery of the KLHL42 as an E3 ligase that controls TGF-B-mediated profibrotic signaling.



Distribution of number of ubiquitin sites. Analysis of the ubiquitome allows for sensitive detection of the number of ubiquitin sites on a given protein. In this case over half of the protein detected were monoubiquitinated.



Mass Distribution of Ubiquitinated Proteins. Global analysis of proteome and ubiquitome. The number of ubiquitylated proteins detected was assigned to different intervals of protein mass. Here there is a interesting cluster of proteins around 30 kDa, and over 100 kDa. The ubiquitin pathway is clearly playing a role in clearing and otherwise influencing larger proteins in this experiment.