

# Ub-clipping: an approach for studying ubiquitin chain architecture

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## Method Article

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# Abstract

The post-translational modification of proteins with ubiquitin is a dynamic multifaceted process affecting all aspects of eukaryotic cellular biology. The complexity of ubiquitin modifications arises from their ability to form architecturally distinct polyubiquitin chains<sup>1-3</sup>. Despite our understanding of the importance of these signals, we currently lack tools and methods to study them. Here we describe an approach, termed Ub-clipping, which provides unprecedented insight into ubiquitin chain architecture. This protocol is related to our recent Nature paper titled, "Insights into ubiquitin chain architecture using Ub-clipping". This technology takes advantage of an engineered viral protease, Lb<sup>PRO\*</sup>, which 'clips' ubiquitin such that the information on the site of modification is retained and the remaining ubiquitin and substrate polypeptides are kept intact. The goal of this protocol is to allow researchers to efficiently adapt our new technology to their proteomic workflows. We anticipate this method will continue to shed light on the architecture of ubiquitin signals, and therefore further our understanding of the ubiquitin code across a broad spectrum of biological systems.

## Introduction

In this protocol, we introduce an engineered viral protease, Lb<sup>PRO\*</sup> from foot-and-mouth-disease virus, as a novel tool to study the complexity of ubiquitin modifications. Unlike canonical deubiquitinases which hydrolyze the isopeptide bond following the C-terminal GlyGly motif, Lb<sup>PRO\*</sup> specifically cleaves the peptide bond preceding the GlyGly motif. Consequently, this motif remains attached to the modification site. In our recent paper we show this unique proteolytic activity can be used to dissect the architecture of ubiquitin signals on substrates (i.e. ubiquitinated proteins or polyubiquitin chains) including: 1) *in vitro* assembly reactions, 2) total cell lysates, 3) polyubiquitin chains enriched from cells, and 4) isolated damaged mitochondria. The starting point for this protocol is the expression and purification of Lb<sup>PRO\*</sup>, which routinely yields large quantities of highly pure and active enzyme. After purification, ubiquitinated substrates are digested with Lb<sup>PRO\*</sup>. This results in the collapse of polyubiquitin chains to mono-ubiquitin species. These mono-ubiquitin species consist of ubiquitin moieties with a 'clipped' C-terminus (-114 Da) and ubiquitin molecules with one or more GlyGly-modifications (+114 Da). These mass differences, plus any additional chemical modifications (e.g. phosphorylation or acetylation) are easily distinguishable by mass and can be quantified using standard intact mass spectrometry methods and software. In the last sections of this protocol, we describe procedures for the preparation of ubiquitin samples for Ub-clipping and then discuss the analysis of these samples by mass spectrometry.

## Reagents

### Protein expression

- IPTG (Millipore, cat. no. 420322)

- LB agar plates (i.e. LB agar plates supplemented with 100 µg/ml Ampicillin, 30 µg/ml Chloramphenicol)
- LB medium
- 2x YT medium
- Ampicillin (Serva, cat. no. 13398.01)
- Chloramphenicol (Serva, cat. no. 16785.02)
- pET11d wild-type Lb<sup>pro</sup> or Lb<sup>pro\*</sup> plasmids (Lb<sup>pro</sup> is available from Addgene, plasmid #110759 and Lb<sup>pro\*</sup> is available from the Komander laboratory)
- BL21(DE3) pLysE or pLysS *E. coli* strain (Thermo Fisher Scientific, cat. no. C656503 or C606010)

### **E. coli cell lysis**

- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Hydrochloric acid, 5 M (HCl; Sigma-Aldrich, cat. no. 258148)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. 746398)
- DTT (PanReac AppliChem, cat. no. A1101,0100)
- EDTA (Sigma-Aldrich, cat. no. D2900000)
- Glycerol (Sigma-Aldrich, cat. no. G9012)
- Lysozyme (Sigma-Aldrich, cat. no. L6876)
- DNase I (Sigma-Aldrich, cat. no. DN25-100MG)

### **Lb<sup>pro\*</sup> protein purification**

- Ammonium sulfate (Thermo Fisher Scientific, cat. no. A702-500)
- 3.5K MWCO dialysis tubing (Thermo Fisher Scientific, cat. no. 88244)
- Amicon Ultra-15 centrifugal filters, 10K MWCO (Millipore, cat. no. UFC901096)
- NuPAGE Novex sample buffer (Thermo Fisher Scientific, cat. no. NP0008)
- NuPAGE Novex SDS running buffer (Thermo Fisher Scientific, cat. no. NP0002)
- NuPAGE Novex 4-12% Bis-Tris protein gels (Thermo Fisher Scientific, cat. no. NP0323BOX)

## **Tissue culture**

- Dulbecco's Modified Eagle's Medium + GlutaMax (Gibco, cat. no. 31966-021)
- Heat inactivated fetal bovine serum (user-specific)
- Penicillin-Streptomycin (Sigma-Aldrich, cat. no. P0781)
- Trypsin (Serva, cat. no. 37297.01)
- PBS (Gibco, cat. no. 14190-094 or prepared in-house)
- Oligomycin (Sigma-Aldrich, cat. no. 495455-10MG)
- Antimycin A (Sigma, cat. no. A8674-25MG)

## **Protein extraction**

- For additional reagents see above
- IGEPAL CA-630 (Sigma-Aldrich, cat. no. I3021)
- Chloroacetamide (Sigma-Aldrich, cat. no. C0267)
- Urea (Sigma-Aldrich, cat. no. U5378)
- Bradford reagent (Bio-Rad, cat. no. B6916)
- Transparent 96-well plate (Greiner bio-one, cat. no. 655101)
- Bovine Gamma Globulin Standard (Thermo Fisher Scientific, cat. no. 23212)

## **Total ubiquitin purification**

- Milli-Q water
- Amicon Ultra-4 centrifugal filters, 3.5K MWCO (Millipore, cat. no. UFC800396)

## **Ub chain purification**

- For additional reagents see above
- Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ , Sigma-Aldrich, cat. no. S9390)
- Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ , Sigma-Aldrich, cat. no. RDD007)
- GST 4x ubiquitin 1 TUBE<sup>4</sup> (available from LifeSensors, cat. no. UM401)

- Glutathione Sepharose 4B resin (GE Healthcare, cat. no. 17-0756-01)
- Tween20 (Sigma-Aldrich, cat. no. P1379)
- cOmplete Protease Inhibitor Cocktail (Roche, cat. no. 11873580001)
- PhosSTOP Phosphatase Inhibitor Cocktail (Roche, cat. no. 4906845001)

### **Perchloric acid extraction**

- Perchloric acid (Sigma-Aldrich, cat. no. 311421)
- Slide-A-Lyzer MINI 3.5 K MWCO (Thermo Fisher Scientific, cat. no. 69550)
- Milli-Q water

### **Mitochondrial isolation**

- For additional reagents see above
- HEPES (Sigma-Aldrich, cat. no. H4034)
- Sucrose (Sigma-Aldrich, cat. no. S5016)
- Mannitol (Sigma-Aldrich, cat. no. M4125)
- Sodium carbonate (Sigma-Aldrich, cat. no. S7795)
- BCA Protein Assay Kit (Thermo Fisher Scientific, cat. no. 23225)

### **Mass spectrometry**

- HPLC grade water (Romil, cat. no. H949L)
- HPLC grade acetonitrile (Romil, cat. no. H050)
- Formic acid (Sigma-Aldrich, cat. no. 33015-M)

### **REAGENT SETUP**

- 100% saturated ammonium sulfate
- Buffer A: 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, 5% glycerol (w/v)
- Buffer B: 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 5 mM DTT, 5% glycerol (w/v)

- Extraction buffer: 4 M urea, 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% glycerol, 0.1% IGEPAL CA-630, 5 mM EDTA, 10 mM chloroacetamide, 1x PhosSTOP Phosphatase Inhibitor Cocktail
- Lb<sup>PRO</sup> digestion buffer: 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% glycerol, 0.1% IGEPAL CA-630, 5 mM EDTA
- TUBE lysis buffer: 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% (v/v) IGEPAL CA-630, 2 mM EDTA, 1 mM DTT, 10 mM chloroacetamide, 1x cOmplete Protease Inhibitor Cocktail, 1x PhosSTOP Phosphatase Inhibitor Cocktail
- TUBE digestion buffer: 50 mM Tris pH 8.0, 10 mM DTT
- Mitochondrial isolation buffer: 20 mM HEPES-KOH pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA
- Sodium carbonate solution: 100 mM Na<sub>2</sub>CO<sub>3</sub> pH 11.0
- Mitochondria Lb<sup>PRO</sup> digestion buffer: 50 mM Tris pH 7.4, 50 mM NaCl, 10 mM DTT
- Sucrose storage buffer: 10 mM HEPES-KOH pH 7.6, 0.5 M sucrose
- Reconstitution buffer: 5% acetonitrile (v/v), 95% HPLC water (v/v), 0.1% formic acid (v/v)

## Equipment

- Incubator (user-specific)
- Shaker (user-specific)
- Spectrometer (user-specific)
- Floor centrifuge (user-specific)
- Sonicator (e.g. Misonix, Cole-Parmer, cat. no. EW-04711-81)
- Threaded sonicator probe (e.g. Cole-Parmer, cat. no. GZ-04710-31)
- Magnetic stir bar (user-specific)
- Stir plate (user-specific)
- XCell SureLock Mini-Cell electrophoresis system (Thermo Fisher Scientific, cat. no. EI0001)

- Roller mixer (e.g. Stuart roller mixer SRT6)
- Resource Q column, 6 ml (GE Healthcare, cat. no. 17-1179-01)
- Benchtop centrifuge (e.g. Eppendorf 5424R)
- ÄKTA systems (GE Healthcare, Äkta Pure, Äkta micro)
- HiLoad 26/60 Superdex 75 pg gel filtration column (GE Healthcare, cat. no. 17-1068-01)
- Microtip sonicator probe (user-specific)
- Rotating wheel (user-specific)
- Microplate reader (user-specific)
- Superdex 75 Increase 3.2/300 gel filtration column (GE Healthcare, cat. no. 29-1487-21)
- Dounce homogeniser with drill-fitted pestle (user-specific)
- Table-top centrifuge (user-specific)
- Lyophilizer (user-specific)
- High-performance liquid chromatography (user-specific)
- Mass spectrometer (user-specific)
- Vortex mixer (user-specific)

## Procedure

### Expression and purification of Lb<sup>PRO\*</sup>

**Note:** This is a modified protocol from Kirchweger et al., 1994<sup>5</sup>.

#### (A) Protein expression

1. Transform Lb<sup>PRO</sup> or Lb<sup>PRO\*</sup> plasmid into BL21(DE3) pLysE or pLysS *E. coli* cells using standard methods. Plate the cells onto a LB plate (supplement LB plate with 100 µg/ml Ampicillin and 30 µg/ml Chloramphenicol) and incubate the plate overnight at 37 °C. **Critical Step:** Lb<sup>PRO\*</sup> expression in *E. coli* strains other than pLysE or pLysS may lead to premature lysis and therefore reduced yields.

2. Pick a single colony from the LB plate and inoculate 100 mL of LB medium (supplement LB medium with 100 µg/ml Ampicillin and 30 µg/ml Chloramphenicol).

3. Incubate the culture overnight at 37 °C while shaking at 180 r.p.m.
4. Use the overnight culture to inoculate 900 mL of 2x YT medium (supplement 2x YT medium with 100 µg/ml Ampicillin and 30 µg/ml Chloramphenicol) in a 2 L flask.
5. Incubate the culture at 30 °C while shaking at 150 r.p.m.
6. Once the culture has reached an OD<sub>600</sub> between 0.5 and 0.6 immediately cool the culture for 30 min at 18 °C while shaking at 150 r.p.m.
7. Induce expression of Lb<sup>pro\*</sup> with 0.4 mM IPTG (final concentration).
8. Incubate the culture for 16-20 h at 18 °C while shaking at 110 r.p.m.
9. Harvest cells by centrifugation at 5,000 x g for 15 min at 4 °C.
10. Re-suspend cell pellet in 30 mL Buffer A and store at -80 °C. Alternatively, proceed onto the cell lysis procedure.

## **(B) Cell lysis**

1. Add 1 mg/ml lysozyme and 0.1 mg/ml DNase I to the resuspended cells, mix thoroughly, and incubate the solution on ice for 10 min.
2. Sonicate the resuspended cells on ice (recommended sonication settings: 10 sec burst, 10 sec off, amplitude 60 W for 3 min).
3. Clear the cell lysate by centrifugation at 50,000 x g for 30 min at 4°C.
4. Remove the supernatant and, together with a magnetic stir bar, place into a 100 mL beaker on ice overtop a stir plate at 4 °C.

## **(C) Purification**

### *Ammonium sulfate precipitation*

1. With gentle stirring, slowly add the 100% saturated ammonium sulfate solution to the cleared cell lysate to a final concentration of 30% (v/v).
2. Incubate solution for 2 h at 4 °C with gentle stirring.
3. Centrifuge solution at 50,000 x g for 30 min at 4 °C.
4. Keep the supernatant and discard the pellet.

5. Further precipitate proteins from the supernatant, including Lb<sup>PRO\*</sup>, by adding the 100% saturated ammonium sulfate solution as before to a final concentration of 60%.
6. Incubate solution for 2 h at 4 °C with gentle stirring.
7. Centrifuge solution at 50,000 x g for 30 min at 4 °C.
8. Discard the supernatant and keep the pellet.
9. Re-suspend the pellet in Buffer A (8 mL of Buffer A per 1 L of cell culture) on a roller mixer.
10. Dialyze the resuspended pellet into 1 L of Buffer A at 4 °C overnight to remove excess ammonium sulfate.

### *Anion exchange chromatography*

1. Prior to loading the sample onto the Resource Q anion exchange column, we recommend further diluting the sample 1:10 in Buffer A.
2. Load the diluted sample onto the Resource Q anion exchange column using either a peristaltic pump, sample pump, or superloop.
3. For the Resource Q run, we recommend the following settings (**Fig. 1a**):
  - Column pre-wash            3 CVs, 0% Buffer B
  - Column pre-wash            5 CVs, 100% Buffer B
  - Column pre-wash            3 CVs, 0% Buffer B
  - Sample application        Inject all of the sample
  - Column wash                5 CVs 0% Buffer B
  - Elution                      20 CVs 0-100% Buffer B linear gradient
  - Column wash                5 CVs 100% Buffer B
  - Column wash                5 CVs, 0% Buffer B

Set the flow rate to 6 ml/min for all steps, except for the sample application step which should be set at a flow rate of 3 ml/min.

**Note:** The Resource Q column requires regular maintenance with 2M NaCl/NaOH as per manufacturer's instructions to maintain good resolution.

4. Analyze the ion-exchange run on an SDS-PAGE gel (**Fig. 1b**). **Note:** At this point in the purification it is expected to have contaminating proteins in the Lb<sup>PRO\*</sup>-containing fractions. However, these will be removed in subsequent steps of the purification.

5. Pool the peak Lb<sup>PRO\*</sup>-containing fractions and concentrate to 1.5 mL using an Amicon Ultra-15 centrifugal filter (10K MWCO). **Note:** Multiple anion exchange runs can be pooled for size exclusion chromatography.

### *Size exclusion chromatography*

1. Run the concentrated sample on a size exclusion column (HiLoad 26/60 Superdex 75 pg) equilibrated in Buffer A (**Fig. 1c**).

2. Analyze the size exclusion chromatography run on an SDS-PAGE gel (**Fig. 1d**).

3. Pool the peak Lb<sup>PRO\*</sup>-containing fractions and concentrate the sample using an Amicon Ultra-15 centrifugal filter (10K MWCO). **Note:** Lb<sup>PRO\*</sup> can be concentrated to 20-30 mg/ml.

4. Determine the protein concentration, aliquot and flash-freeze the sample in liquid nitrogen, and store at -80 °C. **Note:** Lb<sup>PRO\*</sup> can be stored for several years at -80 °C, but is unstable at 4 °C and should never be stored at this temperature. Purification routinely yields 5-10 mg per L and is sufficient to perform all steps of the protocol below multiple times.

## **Generation of substrates for Ub-clipping**

Ub-clipping can be performed on any ubiquitinated substrate or sample. Here we describe the preparation and Lb<sup>PRO\*</sup> digestion of three different ubiquitin samples. This includes: **I)** total ubiquitin from whole cell lysate, **II)** purified ubiquitin chains from cells, and **III)** isolated mitochondria. Together, these protocols will not only facilitate the analysis of these substrates, but also serve as a reference point for Ub-clipping analysis of other ubiquitinated proteins.

### **I. Ub-clipping on whole cell lysate**

#### **(A) Culturing and harvesting cells**

1. Culture cells in one 15 cm petri dish containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum and PenStrep (Penicillin and Streptomycin).

2. Grow cells to 90-95% confluence.

3. Place petri dish on ice and remove DMEM medium.

4. Wash cells 2x with 10 mL of ice-cold PBS.

5. Remove excess PBS.
6. Add 500  $\mu$ L PBS to the petri dish.
7. Scrape off cells and transfer to a pre-chilled 1.5 mL tube.
8. Centrifuge at 300 x g for 2 min at 4 °C.
9. Remove and discard the supernatant.
10. Flash-freeze the cell pellet in liquid nitrogen or proceed with the protein extraction procedure described below.

### **(B) Protein extraction**

1. Resuspend cell pellet in 300  $\mu$ L of freshly prepared extraction buffer.
2. Sonicate the sample using a microtip (recommended sonication settings: 10 sec burst, 10 sec off, amplitude 5-10 W for 1 min). **Note:** cells can also be lysed by passing the sample through a 26.5 Gauge needle.
3. Incubate sample on ice for 1 min.
4. Repeat sonication step.
5. Centrifuge cell lysate at 21,000 x g for 10 min at 4 °C.
6. Remove the supernatant and add fresh DTT to a final concentration of 10 mM.
7. Determine the protein concentration with a Bradford assay.
8. Dilute the protein extract 1:4 (vol:vol) with Lb<sup>pro\*</sup> digestion buffer. **Note:** This will reduce the final concentration of urea to 1 M, a concentration in which Lb<sup>pro\*</sup> is active.
9. Proceed with Lb<sup>pro\*</sup> digestion and purification of total ubiquitin.

### **(C) Lb<sup>pro\*</sup> digestion and purification of ubiquitin**

1. Add Lb<sup>pro\*</sup> to the diluted cell lysate and incubate for 16 h at 37 °C. **Note:** Overnight digestion with 10  $\mu$ M Lb<sup>pro\*</sup> is sufficient to collapse the ubiquitin smear (**Fig. 2**). For WT Lb<sup>pro</sup>, we recommend using a concentration higher than 10  $\mu$ M during digestion.
2. Add Lb<sup>pro</sup>-treated sample to a pre-soaked dialysis tubing or cassette (3.5K MWCO).
3. Dialyze sample in cold Milli-Q water overnight at 4 °C with gentle stirring.

4. Remove sample from dialysis and place into a 1.5 mL tube.
5. Centrifuge sample at 21,000 x g for 10 min at 4 °C.
6. Remove the supernatant and discard the pellet.
7. Run the supernatant on a Superdex 75 Increase 3.2/300 gel filtration column using an ÄKTA Micro system. **Note:** We recommend connecting a filter immediately before the column. This will help minimize unwanted damage to the column.
8. Identify ubiquitin-containing fractions with anti-ubiquitin Western blots.
9. Pool the ubiquitin-containing fractions and concentrate using an Amicon Ultra-15 centrifugal filter (3.5K MWCO). **Note:** Minimize contamination of unwanted proteins from the early fractions containing the void elution, as these proteins will interfere with downstream mass spectrometry analysis.
10. Flash-freeze protein in liquid nitrogen or aliquot and lyophilize. Store sample at -80 °C.

## II. Purification of ubiquitin chains and digestion with Lb<sup>PRO\*</sup>

### (A) TUBE pull-down and digestion

**Note:** The TUBE pull-down procedure is a modified protocol from Hjerpe et al., 2009<sup>4</sup>.

1. Culture and harvest one 10 cm petri dish of cells as described above.
2. Resuspend the cell pellets in 300 µL TUBE lysis buffer.
3. Supplement the lysis buffer with 100 µg of GST 4x ubiquilin 1 TUBE<sup>4</sup>.
4. Incubate on ice for 20 min.
5. Clear lysate by centrifugation at 21,000 x g for 10 min at 4 °C.
6. Incubate sample with 25 µL pre-washed Glutathione Sepharose 4B resin on a rotating wheel for 2 h at 4 °C.
7. Centrifuge sample at 300 x g for 2 min at 4 °C and discard supernatant.
8. Wash beads with 500 µL ice-cold PBS + 0.1% Tween20 (v/v).
9. Repeat steps 7-8 two more times.
10. Wash beads with 500 µL PBS.
11. Centrifuge sample at 300 x g for 2 min at 4 °C and discard supernatant.

12. Incubate TUBE-bound ubiquitin chains with 100  $\mu$ L of 10  $\mu$ M Lb<sup>PRO\*</sup> in TUBE digestion buffer for 16 h at 37°C. **Note:** This is an on-bead cleavage assay, where Lb<sup>PRO\*</sup> clips and releases ubiquitin into supernatant.

13. Centrifuge sample at 300 x g for 2 min at 4 °C.

14. Transfer supernatant to a fresh tube and perform perchloric acid extraction.

### **(B) Perchloric acid extraction of ubiquitin**

1. Slowly add perchloric acid to the ubiquitin sample to a final concentration of 0.5% (v/v), mix thoroughly.

2. Incubate sample for 10 min on ice.

3. Centrifuge sample at 21,000 x g for 10 min at 4 °C. **Note:** This step retains the ubiquitin species in the supernatant. Discard the pelleted protein precipitate.

4. Place the ubiquitin-containing supernatant in a pre-soaked Slide-A-Lyzer MINI 3.5 K (MWCO).

5. Dialyze sample in cold 50 mM Tris (pH 7.4) for 4 h at 4 °C with gentle stirring.

6. Dialyze sample in cold Milli-Q water overnight at 4 °C with gentle stirring.

7. Lyophilize sample and store at -80 °C.

### **III. Isolation of damaged mitochondria and digestion with Lb<sup>PRO\*</sup>**

#### **(A) Mitochondrial damage**

1. Culture cells in two 15 cm petri dishes containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum and PenStrep (Penicillin and Streptomycin).

2. Grow cells to 80% confluence.

3. Treat cells with DMEM medium containing OA (10  $\mu$ M oligomycin and 4  $\mu$ M Antimycin A) and incubate for 2 h back in the 37 °C incubator.

4. Place petri dish on ice and remove DMEM medium.

5. Wash cells 2x with 10 mL of ice-cold PBS. **Note:** PBS can be supplemented with 200 mM chloroacetamide to further inhibit deubiquitinase activity.

6. Scrape off cells and transfer to a pre-chilled 1.5 mL tube.

7. Centrifuge at 1,000 x g for 5 min at 4 °C.

8. Remove and discard the supernatant.
9. Wash the cells with PBS and centrifuge again.
10. Remove and discard the supernatant.
11. Store at -80 °C or proceed to the purification of mitochondria.

## B) Purification of mitochondria

**Note:** This protocol was adapted from Lazarou et al., 2007<sup>6</sup>. All steps should be performed on ice or at 4 °C.

1. Resuspend cell pellets in 2.5-5 mL of mitochondrial isolation buffer containing 1x cOmplete Protease Inhibitor Cocktail and 1x PhosSTOP Phosphatase Inhibitor Cocktail.
2. Incubate on ice for 30 min.
3. Transfer resuspended cells to a 5 mL dounce homogeniser and homogenise with 25-30 strokes with a drill-fitted pestle. **Note:** To ensure the cells are properly disrupted without damage to mitochondria, the number of strokes may be varied depending on the torque of the drill.
4. Transfer the sample to a 50 mL tube.
5. Centrifuge at 1,000 x g for 5 min at 4 °C.
6. Carefully split the supernatant across several 1.5 mL tubes.
7. Centrifuge at 10,000 x g for 10 min at 4 °C.
8. Remove the supernatant. **Note:** Steps 1-3 can be repeated with the 1,000 x g pellet to liberate more mitochondria from unbroken cells.
9. Thoroughly resuspend and pool the 10,000 x g pellets in 1 mL of mitochondrial isolation buffer.
10. Centrifuge at 1,000 x g for 5 min at 4 °C. **Note:** This spin will remove any contaminating nuclei. **Critical Step:** Failure to thoroughly resuspend the mitochondria will cause them to be lost at this step.
11. Transfer the supernatant to a fresh 1.5 mL tube.
12. Centrifuge at 10,000 x g for 10 min at 4 °C.
13. Remove the supernatant and resuspend the pellet in 1 mL mitochondrial isolation buffer excluding the inhibitors.
14. Centrifuge at 10,000 x g for 10 min at 4 °C.

15. Remove supernatant.
16. Resuspend the pellet in 1 mL of sucrose storage buffer.
17. Determine the protein concentration using a BCA assay. **Note:** Samples can be stored at -80 °C until needed.

### **(C) Sodium carbonate extraction and Lb<sup>PRO\*</sup> digestion of purified mitochondria**

**Note:** The sodium carbonate extraction procedure was adapted from Fujiki et al., 1982a & 1982b<sup>7,8</sup>

1. If frozen, thaw the isolated mitochondria on ice.
2. Aliquot the desired amount of protein in a 1.5 mL tube. **Note:** The amount of mitochondria can range between 0.2-1 mg per sample and will depend on the downstream application as well as density of ubiquitination.
3. Extract the soluble and peripheral membrane proteins by resuspending the pellet in fresh sodium carbonate solution at a ratio of 1  $\mu$ L per  $\mu$ g mitochondria.
4. Incubate the sample for 30-45 min on ice with occasional vortexing.
5. Centrifuge at 21,000 x g for 30 min at 4 °C.
6. Discard the supernatant and keep the pellet containing integral membrane proteins. **Note:** The degree of extraction depends on the pH of the Na<sub>2</sub>CO<sub>3</sub>, so this should be measured and kept constant across experiments.
7. Resuspend the pellet in mitochondria Lb<sup>PRO</sup> digestion buffer at a ratio of 1  $\mu$ L per 10  $\mu$ g mitochondria.
8. Add an equal volume of mitochondria Lb<sup>PRO</sup> digestion buffer containing 20  $\mu$ M Lb<sup>PRO\*</sup>.
9. Incubate overnight at 37 °C. **Critical Step:** In addition to helping reduce the catalytic cysteine of Lb<sup>PRO\*</sup>, DTT inhibits a mitochondrial carboxypeptidase that cleaves Arg74 of ubiquitin after Lb<sup>PRO\*</sup> cleavage.
10. Centrifuge sample at 21,000 x g for 30 min at 4 °C.
11. Remove the supernatant and transfer to a fresh tube.
12. Protein can be immediately flash-frozen in liquid nitrogen or aliquoted and lyophilized. Store sample at -80 °C.

### **Mass spectrometry & Ub-clipping analysis**

Mass spectrometry analysis will largely depend on the available equipment. As a result, in this section of the protocol we have minimized instrument-specific details and provided general guidelines for analysis and data processing of samples. For specific details on reagents and instrumentation, please see the method section of our paper.

### (A) Mass spectrometry

1. Resuspend sample in reconstitution buffer. **Note:** The resuspension volume will depend on the sample and sensitivity of the mass spectrometer.
2. Desalt your sample using C<sub>4</sub> reverse phase media before mass spectrometry analysis. **Note:** This can be performed before or during HPLC runs.
3. Prior to ionization, separate ubiquitin species with an analytical column packed with C<sub>4</sub> reverse phase media.
4. Standard acetonitrile gradients can be used to elute ubiquitin from the analytical column.
5. We recommend performing mass analysis at the highest mass resolution setting possible.

### (B) Data analysis

1. The analysis of ubiquitin modifications from mass spectrometry data files can be performed by peak integration or spectra deconvolution. **Note:** In many cases both approaches yield comparable results. However, spectra deconvolution is preferred when quantifying both phosphorylation and ubiquitination sites.
2. For peak integration, quantitation should be performed on the ubiquitin species with the highest intensity (e.g. ubiquitin with a charge state of 12) and the nominal mass. After peak integration, export the area under the curve for each species and quantify their relative abundance.
3. For spectra deconvolution, all spectra corresponding to ubiquitin should be averaged and deconvoluted. After spectra deconvolution, export the intensities of each ubiquitin species and quantify their relative abundance.

## Troubleshooting

### Lb<sup>PRO</sup> expression

- If cells lyse during overnight expression or expression levels are unexpectedly low, try expressing Lb<sup>PRO\*</sup> for 5 h at 30 °C while shaking at 150 r.p.m.

## Generation of substrates for Ub-clipping

- The generation of substrates for Ub-clipping analysis is a critical step in this protocol. In our experience, problems often arise due to a lack of initial starting material or inefficient isolation of ubiquitinated substrates. Therefore, as a first resort to troubleshooting this protocol, we often recommend increasing the amount of starting material and optimizing the conditions for substrate isolation.

- After Lb<sup>PRO\*</sup> cleavage, ubiquitin species can be purified in a number of different ways. In this protocol we described two different methods: 1) purification by size-exclusion chromatography and 2) perchloric acid extraction of ubiquitin. These methods are not mutually exclusive. For instance, the perchloric acid extraction method might be used to isolate total ubiquitin from whole cell lysates. Therefore, if your laboratory is not equipped with an Äkta micro, we suggest performing perchloric acid extraction instead.

## Mass spectrometry

- Troubleshooting mass spectrometry data acquisition/analysis parameters will largely be instrument specific, however routine maintenance of instrumentation often helps with reproducibility and sensitivity issues.

## Ub-clipping analysis

- Small chemical post-translational modifications of ubiquitin (e.g. phosphorylation) will alter the charge state of the modified ubiquitin. This will bias the quantitation of ubiquitin species using peak integration, which relies on a uniform charge state. Therefore, in instances where ubiquitin phosphorylation is identified we recommend quantifying by spectra deconvolution.

## Time Taken

5-6 days to express and purify Lb<sup>PRO</sup> (starting point: transformation of plasmid).

3-4 days to complete the isolation of Lb<sup>PRO\*</sup>-treated ubiquitin from whole cell lysate (starting point: confluent petri dish).

3 days to complete the purification of Lb<sup>PRO\*</sup>-treated ubiquitin chains enriched from cells (starting point: confluent petri dish).

2-3 days to purify and digest ubiquitinated substrates on mitochondria (starting point: two confluent 15 cm petri dishes).

30-60 min of mass spectrometry run time per sample (starting point: sample).

## Anticipated Results

This protocol will produce Lb<sup>PRO\*</sup>-generated mono-ubiquitin species from both simple and complex ubiquitin samples (e.g. whole cell lysates, purified ubiquitin chains, purified mitochondria, etc.), while preserving information on linkage-type, additional post-translational modifications, and combinatorial complex modifications, including branched chains. These ubiquitin species can subsequently be analyzed by quantitative mass spectrometry to determine the extent of each ubiquitin modification. Ub-clipping, therefore, provides insight into the complexity of ubiquitin modifications and allows for the modelling of ubiquitin chain architectures.

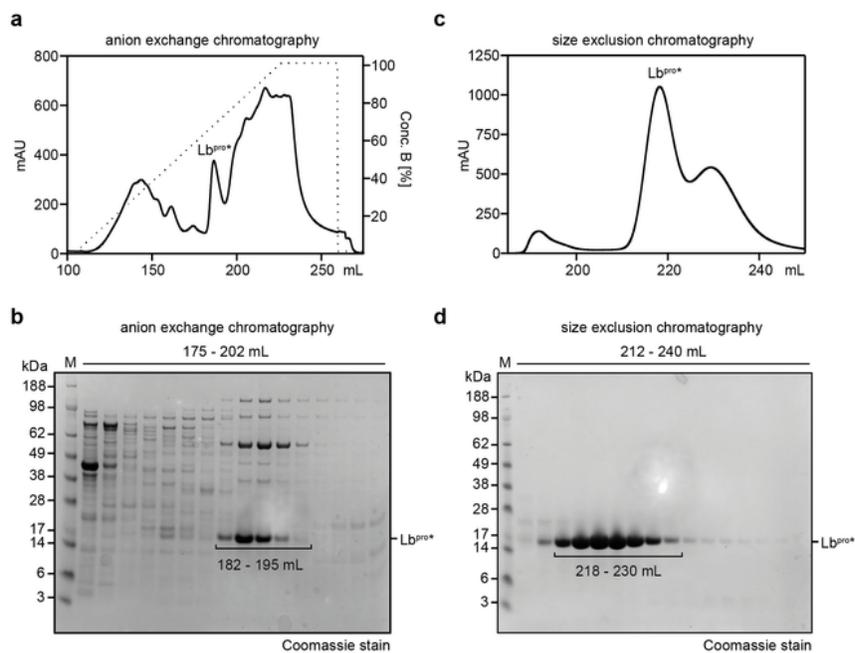
## References

1. Komander, D. & Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203–229 (2012).
2. Swatek, K. N. & Komander, D. Ubiquitin modifications. *Cell Res.* **26**, 399–422 (2016).
3. Yau, R. & Rape, M. The increasing complexity of the ubiquitin code. *Nat. Cell Biol.* **18**, 579–586 (2016).
4. Hjerpe, R. *et al.* Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep.* **10**, 1250–1258 (2009).
5. Kirchweger, R. *et al.* Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4 gamma. *J. Virol.* **68**, 5677–5684 (1994).
6. Lazarou, M., McKenzie, M., Ohtake, A., Thorburn, D. R. & Ryan, M. T. Analysis of the assembly profiles for mitochondrial- and nuclear-DNA-encoded subunits into complex I. *Mol. Cell. Biol.* **27**, 4228–4237 (2007).
7. Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93**, 97–102 (1982).
8. Fujiki, Y., Fowler, S., Shio, H., Hubbard, A. L. & Lazarow, P. B. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* **93**, 103–110 (1982).

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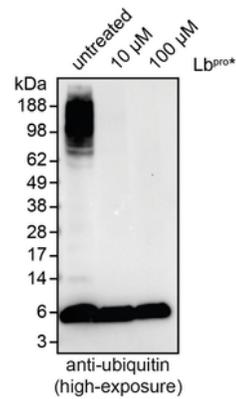
# Figures



**Figure 1**

a, Elution profile of Lbpro\* from Resource Q anion exchange. The absorbance was measured at 280 nm and the peak corresponding to Lbpro\* is labelled. b, Coomassie-stained SDS-PAGE of samples from a. The highlighted Lbpro\*-containing fractions were pooled and concentrated. c, Fractions as in b were

pooled from six separate anion exchange runs and further purified by size exclusion chromatography. The absorbance was measured at 280 nm and the peak corresponding to Lbpro\* is labelled. d, Coomassie-stained SDS-PAGE of samples from c.



## Figure 2

Western blot analysis showing that 10 μM of Lbpro\* is sufficient to collapse a ubiquitin smear from HEK293-F suspension cells. Reactions were performed for 16 h at 37 °C.