K-ε-GG Peptide Enrichment and Analysis by Tandem Mass Tagging-based proteomics

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Materials

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PTMScan Ubiquitin Remnant Motif (K-ε-GG) (D4A7)	Cell Signaling Technology	Custom order
Chemicals, Peptides, and Recombinant Proteins		
Protease Inhibitor Cocktail	Roche	11873580001
PBS (10x)	Santa Cruz	sc-24947
TCEP	Gold Biotechnology	TCEP2
Formic Acid	Sigma-Aldrich	94318
Urea	Sigma-Aldrich	U5378
Acetonitrile	Sigma-Aldrich	34851
Sodium Chloride	Sigma-Aldrich	S9888
MOPS	Sigma-Aldrich	M1254
Trypsin	Promega	Custom order
Lys-C	Wako Chemicals	129-02541
EPPS	Sigma-Aldrich	E9502
2-Chloroacetamide	Sigma-Aldrich	C0267
Protein A Plus Ultralink resin	Thermo-Fisher Scientific	53142
Sodium metaborate	Sigma-Aldrich	S0251
Dimethyl pimelimidate dihydrochloride (DMP)	Siga-Aldrich,	D8388
Critical Commercial Assays		
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868
Tandem Mass Tags	Thermo Fisher Scientific	90406
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
Software and Algorithms		
Other		
Sep-Pak C18 1cc Vac Cartridge, 50 mg	Waters	WAT054955
Empore™ SPE Disks C18	3M Bioanalytical Technologies	2215

BUFFERS:

1. Urea lysis buffer:

Compound	$[Compound]_{final}$
Urea	8 M
NaCl	75 mM
EPPS pH 8.5	50 mM
Protease Inhibitors	1 x

2. EPPS buffer (50 mM EPPS, pH 8.5)
3. IAP buffer (50 mM MOPS pH 7.2, 10 mM Na2HPO4, 50 mM NaCl)
4. Antibody wash buffer: 100 mM sodium borate, pH 9.0.
5. Cross-link buffer is 20 mM DMP in 100 mM sodium borate (pH 9.0).
6. Antibody blocking buffer: Blocking buffer is 200 mM ethanolamine (pH 8.0).
7. Phosphate buffered saline (pH 7.4)

Harvest, precipitation and digestion

1- Cells are lysed in 3mL of lysis buffer and passed through a 21G needle 10 times.
2- Suspensions are centrifuged at 13k rpm (high speed) for 10 min at 4°C and collect supernatant.
3- Quantify protein lysate concentration and 1 mg of lysates are transferred to a clean tube.
4- Lysates are reduced for 20 min at room temperature with 5 mM TCEP, and cysteine residues are then alkylated with 20mM Chloroacetamide (room temperature, 30 min).
5- Extract protein content by methanol-chloroform precipitation and subsequent MetOH washes.

1 Add 4x volumes of MeOH and vortex
2 Add 1x volume of chloroform and vortex
3 Add 3x volume of water and vortex
4 Spin down at RT for 5min at high speed.
5 Remove both the aqueous and organic layers carefully, discard.
6 Add 4x volumes of MeOH and vortex
7 Spin down at RT for 5min at high speed
8 Dry protein pellet down to get rid of MetOH traces.

6- Protein pellets are resuspended in 8 M urea, 50 mM EPPS (pH 8.5) buffer.
7- Samples are then diluted to 4 M urea with 50 mM EPPS (pH 8.5) and digested at 30°C for 2 hr with endoproteinase Lys-C (Wako, Japan) at a 1/200 enzyme/protein ratio.
8- Dilute samples to 1M urea with 50 mM EPPS (pH 8.5). Digest with Trypsin (1:100) o/n at 37ºC.
9- Stop digestion by acidification with formic acid (FA) 5% (v/v) (pH ~ 2).
10- Peptides are subjected to C18 SepPak solid-phase extraction cartridges (SPE Waters) and dried down.
11- Resuspend the desalted peptides in 1.3 ml IAP buffer.

Capture of K-ε-GG containing peptides with α-K-ε-GG Antibody

One IP per sample.
For one IP: 32 μg of α-K-ε-GG Antibody per 40 μl slurry (Pierce™ Protein A Plus UltraLink™ Resin, Cat. No. 53142) - see below for coupling of antibody to resin.

14- Add the resin to a 15 ml Eppendorf tube.
15- Wash 3x with PBS and centrifuge 1' at 1,000g
16- Add the α-K-ε-GG Antibody and add enough PBS to have a total volume of 10 ml in 15 ml tube.
17- Incubate O/N at 4C with gentle rotation.

Chemical cross-linking of K-ε-GG-specific antibody to resin

1. Wash the anti-K-ε-GG Antibody coupled beads 3x with 3 ml 100 mM sodium borate, pH 9.0.
2. Resuspend the beads in 3 ml of 20 mM DMP in 100 mM sodium borate (pH 9.0) and incubate at RT for 30 min with gentle end-over-end rotator.
3. Stop the reaction by washing the beads 2x with 3 ml of antibody blocking buffer (200 mM ethanolamine pH 8.0).
4. Resuspend in 3 ml of Antibody blocking buffer and incubate the Ab for 2 h at 4C with gentle rotation.
5. Wash the cross-linked antibody 3x with 3 ml IAP buffer.

Immunoprecipitation

1. Add each sample to a clean 2 ml Eppendorf tube containing the cross-linked anti-K-ε-GG Antibody (40 μl slurry of resin).
2. Incubate the IPs for 2 h at 4 °C with gentle end-over-end rotation.
4. Centrifuge each IP at 2,000g for 2 min and remove the supernatant. Store supernatant at -80C.
5. Wash the beads 3x with 2 ml of IAP buffer followed with a wash with 2mL PBS.
6. To elute K-ε-GG peptides add 75 µl of elution solution (0.15% TFA), gently tap the bottom of the tube several times and let the tube stand at RT for 5 min.
7. Repeat elution step and combine both eluates.
8. Dry down in speedvac and proceed to stage-tip.

Stage TiP

1. Resuspend samples in 5% FA, 5%ACN.
2. Perform C-18 cleanup:
    a. Wash C-18 with 1 ml 100% ACN.
    b. Equilibrate with 3 ml of 1% FA.
    c. Repeat step b.
    d. Load sample (1 drop per second).
    e. Collect flow through and freeze.
    f. Wash with 3 ml of 1% FA/5% ACN.
    g. Repeat step f.
    h. Elute with 2 x 500 μl 75% ACN/1% FA.
3. Dry down in speedvac.
4. Proceed to labeling.

Labeling

1. Resuspend the peptide pellet in 50 µL of 200 mM EPPS (pH 8.2) containing 20% ACN.
2. Add 3-4 ul of the TMT reagent to each sample.
3. Incubate for 1h at room temperature.
4. Stop the reaction with 4µl of hydroxylamine 5%
5. Combine samples, acidify (5% FA) and speed-vac to dryness (gel like consistency).

Stage TiP

1. Resuspend samples in 5% FA, 5%ACN.
2. Perform C-18 cleanup:
    a. Wash C-18 with 1 ml 100% ACN.
    b. Equilibrate with 3 ml of 1% FA.
    c. Repeat step b.
    d. Load sample (1 drop per second).
    e. Collect flow through and freeze.
    f. Wash with 3 ml of 1% FA/5% ACN.
    g. Repeat step f.
    h. Elute with 2 x 500 μl 75% ACN/1 % FA.
3. Dry down in speedvac.
4. Proceed to B-pH RP
fractionation

Basic-pH RP peptide fractionation kit (follow manufacturer's instructions)

1. Follow manufacturer’s instructions (Thermo Cat# 84868)
2. Elution used: 17.5% ACN, 20% ACN, 22.5% ACN, 25% ACN, 27.5% ACN and 70% ACN
3. Speed vac individual samples to dryness.
4. Proceed to stage-tip

Stage TiP

1. Resuspend samples in 5% FA, 5% ACN.
2. Perform six C18-based stage-tips (one per fraction).
    a. Wash C-18 with 50 μl 100% ACN. Centrifuge at 2000g for 2 min at RT, discard flowthrough.
    b. Equilibrate with 50 μl of 1% FA. Centrifuge at 2000g for 2 min at RT, discard flowthrough.
    c. Repeat step b.
    d. Load sample. Centrifuge at 1500g for 4 min at RT.
    e. Collect flow through and freeze.
    f. Wash with 50 μl of 1% FA/5%ACN. Centrifuge at 2000g for 2 min at RT, discard flowthrough
    g. Repeat step f.
    h. Elute with 1 x 50 μl 75% ACN/ % FA, in mass-spec vial. Centrifuge at 2000g for 2 min at RT.
3. Dry down in speedvac.
4. Resuspend in 10 μl 5%FA, 5% ACN

Mass spectrometry:
The analysis of K-ε-GG peptides by mass spectrometry will depend on the type of instrument/platform used. Typical instrument settings for analysis on a Thermo Fusion Lumos instrument are provided in the following section.

Inject 3 µl for each LC–MS/MS analysis using available mass spectrometer with a 120-minute online LC separation.

Search raw data against UniProt human protein database using any proteomic analysis software with the following parameters:

- Up to 3 missed cleavages allowed for trypsin/LysC digestion
- Carbamidomethyl (C), TMT (N-term peptide and K) set as a fixed modification
- Oxidation (M) and di-glycine (K) set as variable modifications

Extract signal to noise intensity values of each TMT reporter and identified proteins, and further calculate the ratio of each condition to the control sample's intensity.

Instrument settings:
Mass spectrometry data were collected using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC1200 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 µm inner diameter microcapillary column packed in house with ~35 cm of Accucore150 resin (2.6 µm, 150 Å, ThermoFisher Scientific, San Jose, CA) with a gradient consisting of 3%–26% (0-100 min), 26-32% (100-110min) (ACN, 0.1% FA) over a total 120 min run at ~400 nL/min. For analysis, we loaded 1/3 of each fraction onto the column. Each analysis used the Multi-Notch MS^3-based TMT method (McAlister et al., 2014). The scan sequence began with an MS^1 spectrum (Orbitrap analysis; resolution 120,000 at 200 Th; mass range 400−1250 m/z; automatic gain control (AGC) target 1×10^6; maximum injection time 100 ms). Precursors for MS^2 analysis were selected using a Top 4 sec method. MS^2 analysis consisted of collision-induced dissociation (quadrupole Orbitrap analysis; AGC 1×10^5; isolation window 0.7 Th; normalized collision energy (NCE) 35; maximum injection time 300 ms resolution was 7,500 at 200 Th). Monoisotopic peak assignment was used, and previously interrogated precursors were excluded using a dynamic window (120 s ± 7 ppm). As described previously, only precursors with a charge state between 3 and 6 were selected for downstream analysis (Rose et al., 2016). Following acquisition of each MS^2 spectrum, a synchronous-precursor-selection (SPS) MS^3 scan was collected on the top 10 most intense ions in the MS^2 spectrum (McAlister et al., 2014). MS^3 precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap (NCE 65; AGC 2×10^5; maximum injection time 500 ms, resolution was 50,000 at 200 Th).

REFERENCES

- McAlister, G. C. *et al.* MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes. *Analytical chemistry* **86**, 7150 7158 (2014).
- Rose, C. M. *et al.* Highly Multiplexed Quantitative Mass Spectrometry Analysis of Ubiquitylomes. *Cell Syst* **3**, 395-403.e4 (2016).