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 We use this protocol and it's working

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

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ABSTRACT

This protocol details K-ε-GG peptide enrichment and analysis by tandem mass tagging-based proteomics.

ATTACHMENTS

[dhzabiez.pdf](#)

MATERIALS

REAGENT or RESOURCE:

Antibodies:

PTMScan Ubiquitin Remnant Motif (K-ε-GG) (D4A7)

Chemicals, Peptides, and Recombinant Proteins:

- ⊗ cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #11873580001**
- ⊗ Phosphate Buffered Saline: powder for 5 L of 10X **Santa Cruz Biotechnology Catalog #sc-24947**
- ⊗ TCEP-HCl **Gold Biotechnology Catalog #TCEP2**
- ⊗ Urea **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U5378**
- ⊗ Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich) Catalog #34851**
- ⊗ Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9888**
- ⊗ 3-(N-Morpholino)propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1254**
- ⊗ Lysyl EndopeptidaseR (Lys-C) **Wako Catalog #129-02541**
- ⊗ EPPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E9502**
- ⊗ 2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C0267**

Pierce™ Protein A Plus UltraLink™ Resin Thermo Fisher Scientific Catalog #53142
Sodium metaborate tetrahydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #S0251
Dimethyl pimelimidate dihydrochloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8388

Critical Commercial Assays:

Pierce™ High pH Reversed-Phase Peptide Fractionation Kit Thermo Fisher Catalog #84868
TMT10plex™ Isobaric Label Reagent Set Thermo Fisher Scientific Catalog #90406
Bio-Rad Protein Assay Dye Reagent Concentrate Bio-Rad Laboratories Catalog #5000006

Other:

Sep-Pak C18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge 55-105 µm 100/pk Waters Catalog #WAT054955
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A	B	C
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

A	B	C
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-FisherScientific	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 FisherScientific	84868
Tandem Mass Tags	Thermo FisherScientific	90406
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
Other		
Sep-Pak C18 1cc Vac Cartridge, 50 mg	Waters	WAT054955
Empore™ SPE Disks C18	3M BioanalyticalTechnologies	2215

BUFFERS:

1. Urea lysis buffer:

A	B
Compound	[Compound]final
Urea	8M
NaCl	75mM
EPPS pH 8.5	50mM
Protease Inhibitors	1x

2. EPPS buffer ([mM] 50 millimolar (mM) EPPS, pH 8.5)

3. IAP buffer:

A	B
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A	B
MOPS pH 7.2	50mM
Na ₂ HPO ₄	10mM
NaCl	50mM

4. Antibody wash buffer: 100 millimolar (mM) sodium borate, pH 9.
5. Cross-link buffer is 20 millimolar (mM) DMP in 100 millimolar (mM) sodium borate (pH 9).
6. Antibody blocking buffer: Blocking buffer is 200 millimolar (mM) ethanolamine (pH 8).
7. Phosphate buffered saline (pH 7.4).

Harvest, precipitation and digestion

3h 10m

- 1 Lyse cells in 3 mL of lysis buffer and pass through a 21G needle 10 times.
- 2 Centrifuge suspensions at 13000 rpm (high speed) for 00:10:00 at 4 °C and collect supernatant. 10m
- 3 Quantify protein lysate concentration and transfer 1 mg of lysates to a clean tube.
- 4 Reduce lysates for 00:20:00 at Room temperature with 5 millimolar (mM) TCEP. 20m
- 5 Alkylate cysteine residues with 20 millimolar (mM) Chloroacetamide (Room temperature, 00:30:00). 30m

6 Extract protein content by methanol-chloroform precipitation and subsequent MetOH washes.

6.1 Add 4x volumes of MeOH and vortex.

6.2 Add 1x volume of chloroform and vortex.

6.3 Add 3x volume of water and vortex.

6.4 Spin down at Room temperature for 00:05:00 at high speed.

5m

6.5 Remove both the aqueous and organic layers carefully, discard.

6.6 Add 4x volumes of MeOH and vortex.

6.7 Spin down at Room temperature for 00:05:00 at high speed.

5m

6.8 Dry protein pellet down to get rid of MetOH traces.

7 Resuspend protein pellets in 8 Molarity (M) urea, 50 millimolar (mM) EPPS (8.5) buffer.

8 Dilute samples to 4 Molarity (M) urea with 50 millimolar (mM) EPPS (8.5) and digest at 30 °C for 02:00:00 with endoproteinase Lys-C (Wako, Japan) at a 1/200 enzyme/protein ratio.

2h

9 Dilute samples to 1 Molarity (M) urea with 50 millimolar (mM) EPPS (8.5).

10 Digest with Trypsin (1:100) o/n at 37 °C .

11 Stop digestion by acidification with formic acid (FA) 5% (v/v) (pH ~ 2).

12 Subject peptides to C18 SepPak solid-phase extraction cartridges (SPE Waters) and dry down.

13 Resuspend the desalted peptides in 1.3 mL IAP buffer.

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

14

Note

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.

Add the resin to a 15 mL Eppendorf tube.

15

Wash 3x with PBS and centrifuge 1' at 1000 x g .

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 4 °C with gentle rotation.

Chemical cross-linking of K-ε-GG-specific antibody to resin 2h 30m

18

Wash the anti-K-ε-GG Antibody coupled beads 3x with 3 mL 100 millimolar (mM) sodium borate, pH 9.0.

19 Resuspend the beads in 3 mL of 20 millimolar (mM) DMP in 100 millimolar (mM) sodium borate (pH 9.0) and incubate at Room temperature for 00:30:00 with gentle end-over-end rotator. **30m**

20 Stop the reaction by washing the beads 2x with 3 mL of antibody blocking buffer (200 millimolar (mM) ethanolamine pH 8.0).

21 Resuspend in 3 mL of Antibody blocking buffer and incubate the Ab for 02:00:00 at 4 °C with gentle rotation. **2h**

22 Wash the cross-linked antibody 3x with 3 mL IAP buffer.

Immunoprecipitation

2h 7m

23 Add each sample to a clean 2 mL Eppendorf tube containing the cross-linked anti-K-ε-GG Antibody (40 µL slurry of resin).

24 Incubate the IPs for 02:00:00 at 4 °C with gentle end-over-end rotation. **2h**

25 Centrifuge each IP at 2000 x g for 00:02:00 and remove the supernatant. Store supernatant at -80 °C. **2m**

26 Wash the beads 3x with 2 mL of IAP buffer followed with a wash with 2 mL PBS.

27 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 Room temperature for 00:05:00 .

5m

28 Repeat elution step and combine both eluates.

29 Dry down in speedvac and proceed to stage-tip.

Stage TiP

30 Resuspend samples in 5% FA, 5% ACN.

31 Perform C-18 cleanup:

31.1 a. Wash C-18 with 1 mL 100% ACN.

31.2 b. Equilibrate with 3 mL of 1% FA.

31.3 c. Repeat step b.

- 31.4** d. Load sample (1 drop per second).
- 31.5** e. Collect flow through and freeze.
- 31.6** f. Wash with 3 mL of 1% FA/5% ACN.
-
- 31.7** g. Repeat step f.
- 31.8** h. Elute with 2 x 500 μ L 75% ACN/1% FA.

32 Dry down in speedvac.

33 Proceed to labeling.

Labeling

1h

- 34** Resuspend the peptide pellet in 50 μ L of 200 millimolar (mM) EPPS (pH 8.2) containing 20% ACN.

35 Add 3 μL 4 μL of the TMT reagent to each sample.

36 Incubate for 01:00:00 at Room temperature .

1h

37 Stop the reaction with 4 μL of hydroxylamine 5%.

38 Combine samples, acidify (5% FA) and speed-vac to dryness (gel like consistency).

Stage TiP

39 Resuspend samples in 5% FA, 5% ACN.

40 Perform C-18 cleanup:

40.1 a. Wash C-18 with 1 mL 100% ACN.

40.2 b. Equilibrate with 3 mL of 1% FA.

- 40.3** c. Repeat step b.
- 40.4** d. Load sample (1 drop per second).
- 40.5** e. Collect flow through and freeze.
- 40.6** f. Wash with 3 mL of 1% FA/5% ACN.
-
- 40.7** g. Repeat step f.
- 40.8** h. Elute with 2 x 500 μ L 75% ACN/1 % FA.
- 41** Dry down in speedvac.
- 42** Proceed to B-pH RP fractionation.

Basic-pH RP peptide fractionation kit (follow manufacturer'...

43 Follow manufacturer's instructions (Thermo Cat# 84868).

Note

Elution used: 17.5% ACN, 20% ACN, 22.5% ACN, 25% ACN, 27.5% ACN and 70% ACN.

44 Speed vac individual samples to dryness.

45 Proceed to stage-tip.

Stage TiP

12m

46 Resuspend samples in 5% FA, 5% ACN.

47 Perform six C18-based stage-tips (one per fraction).

47.1 a. Wash C-18 with 50 µL 100% ACN. Centrifuge at 2000 x g for 00:02:00 at Room temperature, discard flowthrough.

2m

47.2 b. Equilibrate with 50 µL of 1% FA. Centrifuge at 2000 x g for 00:02:00 at Room temperature, discard flowthrough.

2m

47.3 c. Repeat step b.

47.4 d. Load sample. Centrifuge at 1500 x g for 00:04:00 at Room temperature .

4m

47.5 e. Collect flow through and freeze.

47.6 f. Wash with 50 μ L of 1% FA/5% ACN. Centrifuge at 2000 x g for 00:02:00 at Room temperature , discard flowthrough.

2m

47.7 g. Repeat step f.

47.8 h. Elute with 1 x 50 μ L 75% ACN/% FA, in mass-spec vial. Centrifuge at 2000 x g for 00:02:00 at Room temperature .

2m

48 Dry down in speedvac.

49 Resuspend in 10 μ L 5% FA, 5% ACN.

Mass spectrometry

50

Note

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.

51 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.

52 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

2h

53 Collect mass spectrometry data 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).

54 Separate peptides on a 100 micromolar (µM) inner diameter microcapillary column packed in house with ~ 35 cm of Accucore150 resin (2.6 micromolar (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 02:00:00 run at ~ 400 nL/min .

2h

55 For analysis, load 1/3 of each fraction onto the column.

Note

Each analysis used the Multi-Notch MS3-based TMT method (McAlister et al., 2014). The scan sequence began with an MS1 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).

- 56** Select precursors for MS2 analysis using a Top 4 sec method.

Note

MS2 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)

- 57** Use monoisotopic peak assignment, and exclude previously interrogated precursors using a dynamic window ($120 \text{ s} \pm 7 \text{ ppm}$).

- 58** As described previously, select only precursors with a charge state between 3 and 6 for downstream analysis (Rose et al., 2016).

- 59** Following acquisition of each MS2 spectrum, collect a synchronous-precursor-selection (SPS) MS3 scan on the top 10 most intense ions in the MS2 spectrum (McAlister et al., 2014).

- 60** Fragment MS3 precursors by high energy collision-induced dissociation (HCD) and analyze using the Orbitrap (NCE 65; AGC 2×10^5 ; maximum injection time 500 ms, resolution was 50,000 at 200 Th).