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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

dhzabiezx.pdf

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KEYWORDS

K-ε-GG Peptide Enrichment, Tandem Mass Tagging-based proteomics, ASAPCRN

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May 13, 2021

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OWNERSHIP HISTORY

May 13, 2021 Urmilas

May 25, 2021 Felix Kraus

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49908

MATERIALS TEXT

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 **Sigma Aldrich Catalog #U5378**

Acetonitrile **Sigma Aldrich Catalog #34851**

Sodium Chloride **Sigma Catalog #S9888**

3-(N-Morpholino)propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) **Millipore Sigma Catalog #M1254**

Lysyl EndopeptidaseR (Lys-C) **Wako Catalog #129-02541**

EPPS **Sigma Aldrich Catalog #E9502**

2-Chloroacetamide **Sigma – Aldrich Catalog #C0267**

Pierce™ Protein A Plus UltraLink™ Resin **Thermo Fisher Scientific Catalog #53142**

Sodium metaborate tetrahydrate **Sigma Aldrich Catalog #S0251**

Dimethyl pimelimidate dihydrochloride **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 SignalingTechnology	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-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 ([M] **50 Milimolar (mM)** EPPS, pH 8.5)

3. **IAP buffer:**

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A	B
MOPS pH 7.2	50mM
Na2HPO4	10mM
NaCl	50mM

4. Antibody wash buffer: [M]**100 Milimolar (mM)** sodium borate, pH**9**.

5. Cross-link buffer is [M]**20 Milimolar (mM)** DMP in [M]**100 Milimolar (mM)** sodium borate (pH**9**).

6. Antibody blocking buffer: Blocking buffer is [M]**200 Milimolar (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 10m

Centrifuge suspensions at **13000 rpm** (high speed) for **00:10:00** at **4 °C** and collect supernatant.

3 Quantify protein lysate concentration and transfer **1 mg** of lysates to a clean tube.

4 20m

Reduce lysates for **00:20:00** at **Room temperature** with [M]**5 Milimolar (mM)** TCEP.

5 30m

Alkylate cysteine residues with [M]**20 Milimolar (mM)** Chloroacetamide (**Room temperature** , **00:30:00**).

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.

5m

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6.4 

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

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

6.6 

Add 4x volumes of MeOH and vortex.

6.7 

5m

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

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

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

8 

2h

Dilute samples to **4 Molarity (M)** urea with **50 Milimolar (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.

9 Dilute samples to **1 Molarity (M)** urea with **50 Milimolar (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.

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13 Resuspend the desalted peptides in **1.3 mL** IAP buffer.

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

14 

One IP per sample.
For one IP: **32 µg** of a-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 a-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 Milimolar (mM)** sodium borate, pH 9.0.

19 30m

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

20 

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

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21 2h

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

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 2h

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

25 2m

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

26

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

27 5m

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** .

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.

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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 µl** 75% ACN/1% FA.

32 Dry down in speedvac.

33 Proceed to labeling.

Labeling 1h

34 Resuspend the peptide pellet in **50 µl** of [M] **200 Milimolar (mM)** EPPS (pH 8.2) containing 20% ACN.

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35 

Add **3 µl** - **4 µl** of the TMT reagent to each sample.

36 

1h

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

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.

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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 µl** 75% ACN/1 % FA.

41 Dry down in speedvac.

42 Proceed to B-pH RP fractionation.

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

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

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 2m

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

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47.2 2m

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

47.3 c. Repeat step b.

47.4 4m

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

47.5 e. Collect flow through and freeze.

47.6 2m

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

47.7 g. Repeat step f.

47.8 2m

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

48 Dry down in speedvac.

49 Resuspend in **10 µl** 5% FA, 5% ACN.

Mass spectrometry

50

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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 Seperate peptides on a [M]**100 Micromolar (µM)** inner diameter microcapillary column packed in house with ~ **35 cm** of Accucore150 resin ([M]**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.

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 1×106; maximum injection time 100 ms).

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

MS2 analysis consisted of collision-induced dissociation (quadrupole Orbitrap analysis; AGC 1×105; 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 s ± 7 ppm).

58 As described previously, select only precursors with a charge state between 3 and 6 for downstream analysis (Rose et

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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).

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