Protein Digestion and Mass Spectrometry Analysis Protocol

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ABSTRACT

This protocol details a protocol for the trypsin digestion of PI3KC3-C1 and subsequent analysis of digested peptides using LC-MS/MS.

ATTACHMENTS

852-2203.pdf

MATERIALS

Materials

- PI3KC3-C1 (mCherry-ATG14|VPS15-TSF) protein sample
- Urea
- tris(hydroxymethyl)aminomethane (TRIS) pH 8.0
- tris(2-carboxyethyl)phosphine (TCEP)
- Iodoacetamide (IAA)
- Trypsin (Gold Trypsin, 0.5 mg/mL Promega V5280)
- $CaCl_2$
- $NH_4CH_3CO_2$
- Acetonitrile
- Formic acid (Optima LC-MS grade, 99.9% minimum)
- Water (purified using the Milli-Q Gradient system)
- Zorbax 300SB-C8 Micro Bore Rapid Resolution column (150 mm length, 1.0 mm inner diameter, 3.5 µm particle size, Agilent)
- LC-MS system (e.g., Agilent 1200 series LC and Thermo Fisher Scientific LTQ-Orbitrap-XL mass spectrometer)
- Proteome Discoverer software (version 1.3, Thermo Fisher Scientific)

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Protocol status: Working
We use this protocol and it's working

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Protein Denaturation and Reduction

20m

1 Mix PI3KC3-C1 (mCherry-ATG14|VPS15-TSF) protein with [M] 8 Molarity (M) urea, [M] 50 millimolar (mM) TRIS pH 8.0, and [M] 10 millimolar (mM) TCEP.

2 Add Iodoacetamide (IAA) to achieve a final concentration of [M] 15 millimolar (mM).

3 Incubate the mixture at Room temperature for 00:20:00 to alkylate cysteine residues. 20m

Trypsin Digestion Setup

4 Prepare a trypsin digestion solution containing Gold Trypsin (0.5 mg/mL), [M] 50 millimolar (mM) TRIS pH 8, and [M] 100 millimolar (mM) $CaCl_2$.

Trypsin digestion solution

	A	B
	Gold Trypsin (0.5 mg/mL)	8 uL
	50 mM TRIS pH 8	160 uL
	100 mM $CaCl_2$	2 uL

5 Mix the trypsin digestion solution with the alkylated protein sample.

Digestion Incubation

20m

6 Incubate the digestion mixture at 37 °C Overnight with shaking at 200 rpm. 20m

Confirmation by SDS-PAGE

7 Analyze the digested proteins by SDS-PAGE to confirm successful digestion. The absence of bands corresponding to the PI3KC3-C1 components indicates successful digestion.

Liquid Chromatography (LC) Setup

8 Prepare LC mobile phase solvents: Solvent A and Solvent B.

Solvent A

	A
	99.9% water
	0.1% formic acid

Solvent B

	A
	99.9% acetonitrile
	0.1% formic acid

9 Set up the LC system with a Zorbax 300SB-C8 Micro Bore Rapid Resolution column (150 mm length, 1.0 mm inner diameter, 3.5 µm particle size).

10 Maintain the column compartment at 50 °C.

LC-MS Analysis

2h

11 Load a 10 µL sample onto the column.

12 Implement the following gradient elution program: 2h

- Isocratic flow at 1% (volume/volume) B for 00:02:00.
- Linear gradient to 35% B over 01:30:00.
- Linear gradient to 95% B over 00:01:00.
- Isocratic flow at 95% B for 00:06:00.
- Linear gradient to 1% B over 00:01:00.
- Isocratic flow at 1% B for 00:20:00.

13 Set the flow rate to 120 µL/min.

Mass Spectrometry Analysis

14 Acquire full-scan, high-resolution mass spectra in positive ion mode over the m/z range of 340 to 1800 using the Orbitrap mass analyzer with a mass resolution of 60,000 (at m/z = 400, FWHM).

Data-Dependent MS/MS Analysis

15 Select the ten most intense ions exceeding an intensity threshold of 10,000 raw ion counts from each full-scan mass spectrum for tandem mass spectrometry (MS/MS) analysis using collision-induced dissociation (CID).

16 Acquire MS/MS spectra using the linear ion trap.

Data Analysis

17 Use Proteome Discoverer software (version 1.3, SEQUEST algorithm) to search raw data files against the amino acid sequences of mCherry-labeled Class III phosphatidylinositol 3-kinase complex I (mCherry-PI3KC3-C1) proteins.

18 Consider tryptic peptides with up to three missed cleavages and specify post-translational modifications.

19 Validate peptide assignments by manually inspecting MS/MS spectra.