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Protein Digestion and Mass Spectrometry Analysis

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We use this protocol and it's working

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Abstract

This protocol describes ...

Protein Digestion

1 The beads were resuspended in 80 µL of 2M Urea, 50 mM ammonium bicarbonate (ABC) and treated with DL-dithiothreitol (DTT) (final concentration 1 mM) for 30 minutes at 37°C with shaking at 1100 rpm on a Thermomixer (Thermo Fisher).

2 Free cysteine residues were alkylated with 2-iodoacetamide (IAA) (final concentration 3.67 mM) for 45 minutes at 25°C with shaking at 1100 rpm in the dark.

3 The reaction was quenched using DTT (final concentration 3.67 mM), and LysC (750 ng) was added, followed by incubation for 1h at 37°C at 1150 rpm.

4 Trypsin (750 ng) was added, and the mixture was incubated for 16 hours at 37°C with shaking at 1150 rpm.

5 After incubation, an additional 500 ng of trypsin was added to the sample, followed by a 2-hour incubation at 37°C at 1150 rpm.

6 The digest was then acidified to pH <3 by adding 50% trifluoroacetic acid (TFA), and the peptides were desalted on C18 stage tips (Empore C18 extraction disks).

7 Briefly, the stage tips were conditioned with sequential additions of:

7.1 i) 100 µL methanol,

7.2 ii) 100 µL 70% acetonitrile (ACN)/0.1% TFA,

7.3 iii) 100 µL 0.1% TFA twice.

8 After conditioning, the acidified peptide digest was loaded onto the stage tip, and the stationary phase was washed with 100 µL 0.1% formic acid (FA) twice.

9 Peptides were eluted with 50 µL 70% ACN/0.1% FA twice.

10 Eluted peptides were dried under vacuum in a Speed-Vac centrifuge, reconstituted in 12 µL of 0.1% FA, sonicated and transferred to an autosampler vial.

11 Peptide yield was quantified using a NanoDrop (Thermo Fisher).

Mass Spectrometry Analyses

12 Peptides were separated on a 25 cm column with a 75 µm diameter and 1.7 µm particle size, composed of C18 stationary phase (IonOpticks Aurora 3 1801220) using a gradient from 2% to 35% Buffer B over 90 minutes

12.1 This was followed by an increase to 95% Buffer B for 7 minutes (Buffer A: 0.1% FA in HPLC-grade water; Buffer B: 99.9% ACN, 0.1% FA) with a flow rate of 300 nL/min on a NanoElute2 system (Bruker).

13 MS data were acquired on a TimsTOF HT (Bruker) with a Captive Spray source (Bruker) using a data-independent acquisition PASEF method (dia-PASEF). The mass range was set from 100 to 1700 m/z, and the ion mobility range from 0.60 V.s/cm2 (collision energy 20 eV) to 1.6 V.s/cm2 (collision energy 59 eV), a ramp time of 100 ms, and an accumulation time of 100 ms. The dia-PASEF settings included a mass range of 400.0 to 1201.0 Da, mobility range 0.60-1.60, and an estimated cycle time of 1.80 seconds. The dia-PASEF windows were set with a mass width of 26.00 Da, mass overlap 1.00 Da, and 32 mass steps per cycle.

DIA Data Analysis

14 Raw data files were processed using Spectronaut version 17.4 (Biognosys) and searched with the PULSAR search engine against the Homo sapiens UniProt protein database (226,953 entries, downloaded on 2022/09/23). Cysteine carbamidomethylation was set as fixed modifications, while methionine oxidation, acetylation of the protein N-terminus, and deamidation (NQ) were defined as variable modifications. A maximum of two trypsin missed cleavages was allowed.

15 Searches used a reversed sequence decoy strategy to control the peptide false discovery rate (FDR), with a 1% FDR threshold for identification. An unpaired t-test was used to calculate p-values for differential analysis, and volcano plots were generated based on log2 fold change and q-value (multiple testing corrected p-value). A q-value of ≤0.05 was considered the statistically significant cut-off.