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Mass spectrometry analyses

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Abstract

A detailed description for mass spectrometry-based quantitative proteomics, interacted proteins screening, LC–MS metabolomics and GC-MS for isotype-labeled metabolomics.

Quantitative proteomics assay

1 Cells are collected and sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% protease inhibitor cocktail).

2 The remaining debris is removed by centrifugation at 12,000g at 4°C for 10 m.

3 Supernatant is collected, and the protein concentration is determined with BCA kit according to the manufacturer's instructions.

4 The protein solution is reduced with 5 mM dithiothreitol for 30 m at 56°C and alkylated with 11 mM iodoacetamide for 15 m at room temperature in the dark.

5 Protein samples are diluted by adding 100 mM TEAB to a urea concentration less than 2 M.

6 Trypsin is added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h digestion.

7 The peptides are desalted by C18 SPE column and dissolved in solvent A (0.1% formic acid, 2% acetonitrile/in water), directly loaded onto a home-made reversed-phase analytical column (25-cm length, 75/100 µm ID).

8 The peptides are separated with a gradient from 6% to 24% solvent B (0.1% formic acid in acetonitrile) over 70 m, 24% to 35% solvent B over 14 m, and 35% to 80% over 3 m then holding at 80% for 3 m, all at a constant flow rate of 450 nL/m on a nanoElute UHPLC system (Bruker Daltonics).

9 The peptides are subjected to capillary source followed by the timsTOF Pro (Bruker Daltonics) mass spectrometry.

10 The electrospray voltage applied is 1.60 kV.

11 Precursors and fragments are analyzed at the TOF detector, with a MS/MS scan range from 100 to 1700 m/z.

12 The timsTOF Pro is operated in parallel accumulation serial fragmentation (PASEF) mode.

13 Precursors with charge states 0 to 5 are selected for fragmentation, and 10 PASEF-MS/MS scans are acquired per cycle.

14 The dynamic exclusion is set to 30 sec.

15 The resulting MS/MS data are processed using MaxQuant search engine (v.1.6.15.0).

16 Tandem mass spectra are searched against the human SwissProt database (20422 entries)
concatenated with reverse decoy database.

17 Trypsin/P is specified as cleavage enzyme allowing up to 2 missing cleavages.

18 The mass tolerance for precursor ions is set as 20 ppm in first search and 5 ppm in main search, and the mass tolerance for fragment ions is set as 0.02 Da.

19 Carbamidomethyl on Cys is specified as fixed modification, and acetylation on protein N-terminal and oxidation on Met are specified as variable modifications. FDR is adjusted to < 1%.

Interacted proteins screening

20 Cells were lysed with Triton X-100 lysis buffer containing EDTA-free protease inhibitors (Roche).

21 Cell lysates are centrifuged at 12,000 rpm at 4°C for 10 min.

22 The indicated antibody is added to supernatants for immunoprecipitation based on Protein A/G Magnetic Beads.

23 Immunoprecipitates are denatured by boiling in SDS sample buffer and resolved by SDS-PAGE.

24 Protein bands in the whole lane are visualized via Coomassie blue staining (Beyotime).

25 Protein bands are subjected to in-gel digestion in 50 mM ammonium bicarbonate buffer overnight at 37°C with modified sequencing-grade trypsin (0.01 µg/µl).

26 The digested protein samples are separated by liquid phase chromatography and ionized by a nanoESI source, then passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher) for DDA (Data Dependent Acquisition) mode detection.

27 Proteins are identified by searching the fragment spectra against the UniProt protein database (EMBL-EBI) using the Mascot search engine (v.2.3; Matrix Science) with the Proteome Discoverer software program (v.1.4; Thermo Fisher).

LC–MS metabolomics

28 Samples are thawed at 4°C and mixed with cold methanol/acetonitrile (1:1, v/v) to remove protein.

29 The mixture is centrifuged at 14,000g at 4°C for 15 m and dried in a vacuum centrifuge.

30 The samples are re-dissolved in 100 µl acetonitrile/water (1:1, v/v) solvent.

31 To monitor the stability and repeatability of instrument analysis, quality control (QC) samples are prepared by pooling 10 µl of each sample and analyzed together with the other samples.

32 The LC-MS are performed using a UHPLC system (1290 series, Agilent Technologies) coupled to a triple quadruple mass spectrometer (5500 QTRAP, AB SCIEX) in the multiple reaction monitoring (MRM) mode.

33 Energy metabolites are monitored in electrospray negative ionization multiple reaction monitoring (MRM) mode only.

34 Amino column (particle size, l.7 mm,100 mm length × 2.1 mm ID) is used at a temperature of 45°C and a flow rate of 300 µl/m with a 4 µl injected sample volume.

35 The mobile phase A is 15 mM ammonium acetate in 100% water, and solvent B was 100% acetonitrile.

36 The linear gradient for energy metabolites is set as follows: 0-18 min: 0% B to 40% B, 18-18.1 min: 40% B to 90% B, 18.1-23 min: 90% B.

37 ESI-MS/MS conditions are set as follows: sheath gas temperature: 350°C; ion source gas 1: 45 psi; ion source gas 2: 45 psi; curtain gas: 30 psi; ionSapary Voltage Floating: 4500 V; nebulizer pressure: 40 psi.

38 The mass spectrometer is operated with a dwell time of 200 ms.

39 To construct the metabolite MRMlibrary, each metabolite standard (100 mg/ml) is first analyzed in ESI negative mode via flow injection to get the optimal MRM transition parameters.

40 The retention time of each metabolite is determined by measuring the corresponding MRM(Q1/Q3) transition individually on the column.

41 For **absolute quantification** of metabolite, a standard mixture sample containing all metabolites are measured together with biological samples in each experiment, and is used as the reference sample for peak alignment and metabolite identification.

42 LC–MS quantification of energy metabolites are achieved with six-point standard curves using SUCCINIC ACID (D6, 98%, DLM-831-5, CIL) diluted in a relevant matrix matched to the analytical sample.

43 Data acquisition and processing are accomplished using Multiquant software.

GC-MS for isotype-labeled metabolomics (amino acids)

44 Cells cultured with isotype-labeled amino acids are grinded in liquid nitrogen and re-suspended in cold 50% aqueous methanol and inserted in dry ice for 30 m

45 Chloroform is added and vortexed for 30 second before centrifugation for 15 m at 14,000 rpm (4 °C), supernatant is transferred to new tubes to evaporate.

46 O-Isobutylhydroxylamine hydrochloride was added to the dried pellet and incubated for 20 m at 85 °C.

47 After cooling, *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) is added and samples are re-incubated for 60 m at 85 °C before centrifugation for 15 m at 14,000 rpm (4 °C).

48 The supernatant is used for GC/MS analysis. A Shimadzu QP-2020 GC-MS is programmed with an injection temperature of 250°C injection.

49 The GC flow rate with helium carrier gas is 0.92 ml/min.

50 The GC column used is a 30 m x 0.25 mm x 0.25 mm DB-5ms.

51 GC-MS interface temperature is 300°C and (electron impact) ion source temperature is set at 200 °C, with 70 V ionization voltage.

52 The mass spectrometer is set to scan m/z range 50-700, with 1 kV detector.

53 To determine ^{13}C labeling, the mass distribution for known fragments of metabolites was extracted from the appropriate chromatographic peak.

54 For each fragment, the retrieved data comprise mass intensities for the lightest isotopomer (without any heavy isotopes, M0), and isotopomers with increasing unit mass relative to M0.

55 The ratio of universal labeled [^{13}C] amino acids relative to total level of AA is analyzed and Z-score normalized.