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Protocol nanoLC-MALDI MS and MS/MS analysis of peptides

1. Solutions

1. Loading solvent: 2% (v/v) ACN containing 0.05% (v/v) trifluoroacetic acid (TFA).
2. Mobile phase A: 0.05% (v/v) TFA.
3. Mobile phase B: 80% (v/v) ACN containing 0.05% (v/v) TFA.
4. Matrix stock solution: saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 90% (v/v) ACN containing 0.1% (v/v) TFA.
5. Matrix working solution: 748 µL of 95% (v/v) ACN containing 0.1% (v/v) TFA is mixed with 36 µL of the saturated CHCA solution, 8 µL of 10% (v/v) TFA and 8 µL of 100 mM $(NH_4)H_2PO_4$.
6. Peptide standard stock solution: the protocol has been optimized with the Peptide Calibration Standard II (Bruker Daltonik, Bremen, Germany); the whole content of the tube is dissolved in 125 µL of 0.1% (v/v) TFA according to manufacturer's instructions; 10-µL aliquots are stored frozen at -80 °C. Any other peptide calibration standard applicable for MS of peptides is applicable.
7. Solvent for peptide standard: 748 µL of 85% (v/v) ACN containing 0.1% (v/v) TFA is mixed with 36 µL of the saturated CHCA solution, 8 µL of 10% (v/v) TFA and 8 µL of 100 mM $(NH_4)H_2PO_4$.
8. Peptide standard working solution: 2 µL of the Peptide Calibration Standard II aliquot are added to 300 µL of the above solvent.
9. Solvent for dissolving peptides from digests (LC-MALDI): 0.1% (v/v) TFA.
10. Washing solvent for external pump at the spotter: isopropanol.
11. Washing solvents for MALDI target: (a) isopropanol and (b) 30% (v/v) ACN containing 0.1% (v/v) TFA.

2. Consumables, tools and instruments

1. Non-powdered protective gloves.
2. Pipettes and appropriate tips.
3. Plastic tubes (0.5, 1.5 and 2 mL) with appropriate racks.
4. Analytical scales.
5. Centrifuge for sample tubes.
6. Vortex.
7. Laminar flow box.
8. Vacuum centrifuge.
9. Ultrasonic bath.
10. 1-mL glass vials for HPLC ("total recovery").
11. Dionex UltiMate3000 RSLCnano liquid chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Proteineer fc II fraction collector (Bruker Daltonik, Bremen, Germany) or any other system with the same or similar parameters.
12. Syringe: Precision glass syringe for solvents, capacity of 1 mL.
13. Pre-column for nanoLC-MALDI-MS/MS: the protocol has been optimized using a reversed-phase Nano Trap column (100 µm × 20 mm; nanoViper inlet/outlet) packed with Acclaim PepMap® C18 silica particles (5 µm particle size, 100 Å pore size) by Thermo Fisher Scientific (Waltham, MA, USA); any other nano column of the same or similar parameters is applicable.
14. Analytical column for nanoLC-MALDI-MS/MS: The protocol has been optimized using a reversed-phase Acclaim PepMap® RSLC column (75 µm × 150 mm; nanoViper inlet/outlet) with C18 silica particles (2 µm particle size, 100 Å pore size)

by Thermo Fisher Scientific (Waltham, MA, USA); any other nano column of the same or similar parameters is applicable.

15. MALDI target: The protocol has been optimized with an MTP AnchorChip™ 800-384 target (Bruker Daltonik, Bremen, Germany) but any other comparable target can be used depending on the available mass spectrometer.
16. ultrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with a smartbeam-II laser (Bruker Daltonik, Bremen, Germany) or any other instrument with similar parameters
17. Computers for database searches: 1st with the database search engine Mascot Server (Matrix Science, London, UK) and in-house versions of protein databases such as Swiss-Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) or NCBInr (National Center for Biotechnology Information, Bethesda, MD, USA); 2nd with a proteomic software platform such as ProteinScape 3.1 (Bruker Daltonik, Bremen, Germany)

3. **Methods**

NanoLC-MALDI MS and MS/MS analysis of peptides (**Note 1**)

1. Add 15 µL of 0.1% (v/v) TFA to each peptide sample in a glass vial and mix the content thoroughly by a repeated pipetting and aspirating to dissolve the desalted peptides. Close the vials by screw neck caps with PTFE/silicone septa. Transfer the vials into an ultrasonic bath and sonicate the content for 10 min. Then spin down the liquid on a centrifuge.
2. Similarly, when stored in 0.5-ml test tubes, dissolve peptides as above; sonicate the content for 5 min. Then spin down the liquid on a centrifuge. Transfer the liquid into the glass vials for chromatography.
3. Place the vials with samples to be analyzed into the cooled autosampler of the nanoflow liquid chromatograph (5-8 °C).
4. Filter the loading solvent and mobile phases using Millicup filters and perform a degassing step by placing the bottles into the ultrasonic bath for 15 min (see **Note 2**).
5. Place the loading pump tubing into the loading solvent and the tubing leading to both nano pumps into the respective mobile phases. Purge the pumps (via the control software or manually, if applicable) and adjust the loading solvent and mobile phase A flow rates at 10 µl/min and 300 nl/min, respectively. Keep the pumps running for at least 1 h in order to equilibrate the trap column as well as the analytical column. When new columns are attached, it is recommended to wash them consecutively by both mobile phases with a final equilibration in the mobile phase A (**see Note 3**).
6. Prepare the MALDI target according to manufacturer's instructions. The washing steps include wiping off impurities with a tissue paper wetted by isopropanol, sonication of the target submerged in isopropanol for 15 min repeated by a sonication in 30% (v/v)

ACN containing 0.1% (v/v) TFA. Finally, the target is dried on air or using a flow of nitrogen gas.

7. Transfer the working solution of peptide calibration standard by pipette to the respective calibration spots (aliquots of 0.5 µl) on the target and wait for drying out the stuck drops: matrix-peptide cocrystals are formed.
8. Place the target into the spotting device. Launch the control software (e.g. Hystar by Bruker Daltonik). Using the software, adjust a correct horizontal as well as vertical positioning of the spotting needle at sample spots. Mount the syringe filled with the working matrix solution to the internal pump and attach it to the tubing (see **Note 4**). Perform priming of the internal pump system. Check the level of the washing solvent at the external pump.
9. Open the sample table as a new window in the software and input information about samples in the autosampler. Select a method to be used, which contains important parameters for separation runs such as the injection mode, flow rate values, gradient programming, and eluate collection time window (including how many fractions are collected in total and what is the time interval for each spotting). The procedure has been optimized for injecting 5-µl sample aliquots, the flow rates are as above and gradient has the following composition: 0 min, 4% mobile phase B; 7 min, 4% B; 45 min, 60% B; 48 min, 90% B; 57 min, 90% B; 59 min, 4% B; 70 min, 4% B. The eluate is usually collected in 17-second fractions (120 fractions in total) starting from 20 min and spotted after mixing with the working matrix solution. The total drop volume is 420 nL. The total run time is 70 min.
10. Run automatic separations and collect eluate fractions. This results in obtaining matrix-peptide cocrystals for each fraction at the surface of the target (sample spots).

11. Launch the acquisition software for MALDI mass spectrometry (MALDI MS), e.g. flexControl by Bruker Daltonik. Place MALDI target into the instrument and wait for re-establishing vacuum in the ion source. Adjust target geometry by a manual or automatic position teaching, select a method for acquisition peptide mass spectra and check the quality of selected calibration spots by switching on the laser and acquiring a few hundred calibration spectra. Optionally you may do this briefly for selected sample spots.
12. Set up automatic spectra acquisition runs. This is done by opening the corresponding LC-MALDI control software (such as the WarpLC by Bruker Daltonik) and customizing parameters. Select sample spots to be analyzed, indicate the positions of the calibration spots. Select a folder into which data will be stored on the computer for each sample (referring to a single digest) and a folder in the proteomic platform (e.g. ProteinScape by Bruker Daltonik), where database search results will appear. Select proper acquisition methods for calibration, MS and MS/MS measurements, spectra processing and database searches.
13. Switch on automatic spectra acquisition runs (see **Note 2**). During the procedure, mass spectra are acquired and calibration repeatedly checked. MS peaklists are built, which is followed by selecting precursors for fragmentation. Then tandem mass spectra are acquired and MS/MS peaklists are built.
14. Database searches utilize the MS/MS peaklists and result in lists of proteins assigned for the collected eluate of each sample (spread e.g. over 120 spots) in the database.

1. Notes

1. Always wear non-powdered protective gloves and work in a bio-safety hood while working with electrophoresis chemicals. Do not touch the equipment and polyacrylamide gels by bare hands in order to prevent a keratin contamination of the samples.
2. Be sure that the mobile phases and loading solvent for liquid chromatography have been filtered to prevent from a column clogging.
3. Always use fresh working solutions for all nanoLC-MS/MS analyses!
4. NanoLC-MALDI: be sure that the syringe with working matrix solution is properly mounted at the internal pump in the eluate fraction collector (spotter) and the attached tubing is not clogged.

Abbreviations

ACN	Acetonitrile
CHCA	α-Cyano-4-hydroxycinnamic acid
HPLC	High-performance liquid chromatography
LC-MALDI-MS	Liquid chromatography coupled to matrix-assisted laser desorption/ionization mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
TFA	Trifluoroacetic acid