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Sample Preparation (Urea/trypsin digestion) for Proteomics - Mammalian Organisms

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We use this protocol and it's working

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Disclaimer

For research purpose only

Abstract

This procedure is to be used for preparation of peptide samples from proteins from mammalian samples. The peptide samples will be used for mass spectrometry based proteomic analysis.

Materials

Chemical supplies

1. NH_4HCO_3 (e.g., Thermo Scientific Catalog number 393212500), prepared in solution : 50 mM pH8 buffer
2. Urea (e.g., in solid form from Thermo Scientific, Catalog number 036428.A3)
3. Dithiothreitol (DTT) - (e.g., Pierce, No-Weigh Format,Catalog number A39255)
4. Iodoacetamide (e.g., Pierce, No-Weigh Format, Catalog number A39271)
5. 1 M $CaCl_2$ in MilliQ water (e.g., Thermo Scientific, Catalog number L13191.0I)
6. Sequencing grade modified Trypsin (e.g., Promega, Catalog number V5111)
7. BCA protein assay reagents (e.g., Pierce, Catalog number 23225)
8. HPLC-grade methanol (e.g., Thermo Scientific, Catalog number 325740025)
9. HPLC-grade trifluoroacetic acid (TFA)(e.g., Thermo Scientific, Catalog number 393212500) at 0.1% v/v in ultrapure water
10. A solution of HPLC-grade 95:5 H_2O:Acetonitrile (ACN), 0.1% TFA (example of ACN source, Thermo Scientific Catalog number 044630.AY)
11. A solution of 80:20 ACN:H_2O, 0.1% TFA
12. Ultrapure water (e.g. Milli-Q, Nanopure)

Non-Chemical Supplies

1. Vortexer mixer
2. ThermoMixer with Thermoblock for the size of tubes you are using
3. Low-retention microcentrifuge tubes (0.6, 1.5, and/or 2.0 mL, the tube have to be low retention and tested to not leach plastic mono/polymers that would be detrimental for mass spectrometry)
4. 15- and/or 50-mL centrifuge tubes
5. Incubator at 37 °C
6. Centrifugal vacuum concentrator (e.g. ThermoSavant SpeedVac or Labconco Centrivap)
7. Vials compatible with the LC-MS system
8. 1 mL capacity 50- or 100-mg C18 Solid Phase Extraction (SPE) column/tube
9. Vacuum manifold with controlled vacuum for SPE tubes
10. Glass test tubes
11. Pipets
12. Pipet tips

Safety warnings

If the samples to be prepared are from human origin or may contain infectious organisms, this procedure must be performed in conditions meeting the bio-safety laboratory (BSL) requirements at your facility and adapted to the type of samples. This usually requires the use of Biosafety Cabinets (BSC's) for the procedure.

Protein digestion

1 Use the method of your choice to extract the proteins from the samples (e.g. MPLEx procedure, sonication, tissue homogenization, etc.).
Ensure that a BCA has been performed and that you have calculated the sample volume.

2 Add powdered form of Urea to sample to a target concentration of 8 M (0.745 * Sample Volume = mg of Urea).
Note that the sample volume will increase to approximately: Sample Volume + (0.550*Sample Volume)

3 Vortex sample for 5-10 sec on highest setting or gently rotate end-over end until Urea is completely dissolved.

4 Prepare a 500 mM solution of Dithiothreitol (add 100 µL to 7.7 mg DTT No-Weigh tube). Add an appropriate volume of the DTT solution to obtain a 5 mM concentration in the sample.

5 Incubate in a thermomixer (or equivalent dry bath) at 37ºC for 1 hour, at 600-800 rpm.

6 Weigh out sufficient amount of iodoacetamide to have a final concentration of 400 mM (74 mg per 1 mL Nanopure water). Add an appropriate volume of iodoacetamide to obtain a 40 mM concentration in the sample.

7 Incubate in a thermomixer (or equivalent dry bath) at 37ºC for 1 hour, in the dark, at 600-800 rpm.

8 Pre-activate trypsin by incubating for 10 minutes at 37ºC.

9 Dilute the sample 8-fold with 50 mM NH_4HCO_3 to reduce the salt concentration.

10 Add sufficient amount of a 1M solution of $CaCl_2$ to obtain a sample concentration of 1 mM $CaCl_2$.

11 Digest sample for 3 hours with Trypsin @ 37ºC at a concentration of 1 µg trypsin/50 µg protein.
(Note: at this stage after a quick freeze of the resulting peptides in liquid nitrogen, it can be stored at –80ºC until needed for analysis.)

Sample SPE cleanup

12 Choose an appropriate solid phase extraction (SPE) C18 column. The capacity of each column is usually 5% of the bed weight. Therefore, a 25 mg column can hold up to 1.25 mg of digested peptides. C18 SPE cleanups are good for samples that do not contain any detergents.

13 Condition column with 3 mL of MeOH.
(note : Columns can withstand going to dryness between steps as long as the dry period is extended for no longer than 30 seconds. However, the columns cannot be allowed to go dry between the Methanol and 0.1% TFA steps.)

14 Rinse column with 2 mL acidified water (0.1% TFA)

15 Slowly put sample solution through the column (no faster than 1 mL/minute).

16 Wash column containing sample with 4 mL of 95:5 H_2O:ACN, 0.1% TFA.

17 Allow column to go to dryness and blot “needles” below columns dry.

18 Put collection tubes under column, and while SPE tubes are closed off from vacuum add 1 mL of 80:20 ACN:H_2O, 0.1% TFA.

19 Allow elution buffer to flow slowly through column until column is dry.

20 Remove sample from SPE vacuum chamber.

21 Concentrate sample in speedvac (or equivalent) to a volume of ~50-100 µL.

22 Perform a BCA assay on sample, following the supplier's instructions.
(Note: at this stage after a quick freeze of the sample in liquid nitrogen, it can be stored at −80ºC until needed for analysis.)

Peptide vialing

23 Dilute the samples in ultrapure water to the concentration of 0.1 µg of peptide per µL (or to the concentration of your choice).
(note : we typically run 5 µL at 0.1 µg/µL in the Thermo Orbitrap instruments)

24 Vial sample with an excess volume to ensure that the volume to be injected by the autosampler will be smaller than the volume contained in the vial
(note : we typically vial between 12 and 50 µL 0.1 µg/µL of peptides to ensure one or multiple injections of 5 µL at 0.1 µg/µL in the Thermo Orbitrap instruments)

25 Freeze the samples at -20 ºC until the samples are placed in the LC-autosampler.
(note: to reduce peptide degradation, the samples should be run in the 2 months following preparation)