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In-Solution Trypsin digestion of Proteins for MS analysis

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Maria da Conceição Almeida: Validated the protocol
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We use this protocol and it's working

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Abstract

This protocol describes a standardized workflow for in-solucion trypsin digestion of protein samples intended for proteomic identification via mass spectrometry (MS). This procedure ensures the production of high-quality peptides while minimizing contamination and ensuring reproducibility and efficiency.

Guidelines

- Always handle samples under clean, keratin-controlled conditions. Wear powder-free gloves (preferably nitrile), a lab coat and a hair cover at all times to minimize the risk of keratin contamination.
- Ideally, the sample should have a protein concentration of 1 mg/mL.
- Precipitate samples to remove contaminants incompatible with trypsin digestion, including protease inhibitors, detergents/surfactants (e.g., SDS, Triton X-100), chaotropes (urea, guanidine), chelators (EDTA/EGTA), organic solvents, salts/buffers (e.g., Tris, phosphate, HEPES), glycerol and unquenched alkylating agents.
- Use LC-MS grade reagents.
- Use low-protein binding microtubes (avoid autoclaved or PCR-coated microtubes) and low-binding pipette tips.

Materials

- Lab coat and hair cover
- Low-binding pipette tips
- Low-protein-binding microtubes (1.5–2 mL)
- Powder-free gloves (preferably nitrile)

Kits, Reagents & Solutions

- Acetone HPLC grade
- Ammonium bicarbonate (NH_4HCO_3) – 50 mM or 1 M in Water
- C18 ZipTips (e.g.: OMIX C18 tips ref.: HPAA5700310)
- Dithiothreitol (DTT) – 250 mM in water
- Formic acid (FA) LC-MS grade
- Hydrochloric acid (HCl) – 1 mM
- Iodoacetamide (IAA) – 500 mM in water
- Protein Quantification Kit
- Trypsin (e.g., Promega V5111), 0.1 µg/µL in 1 mM HCl
- Water LC-MS grade

Alternative Proteases

- Asp-N (e.g., Roche 11420488001)
- Chymotrypsin (e.g., Roche 11418467001)
- Glu-C (e.g., Roche 11047817001)
- Trypsin + Lys-C (e.g., Promega PROMV5072)

Equipment

- Centrifuge with 1.5-2 ml microtubes rotor
- Fridge and freezer
- SpeedVac concentrator
- Spin-down centrifuge
- Thermomixer (capable of 37°C, 56°C and 650 rpm agitation)
- Ultrasonic bath
- Variable volume single channel pipettes
- Vortex mixer

Safety warnings

- Incubate Iodoacetamide (IAA) step in the dark to avoid side reactions (step 3).
- Handle Formic acid (FA) with the appropriate personal protective equipment (PPE) and follow the corresponding waste-management procedures.
- Store Trypsin stock aliquots at -20 °C for up to 6 months and avoid repeated freeze-thaw cycles.

Protein reduction and alkylation

1h 50m

1 Quantify the sample before beginning the protocol. Ideally, the sample should have a protein concentration of 1 mg/mL. 30m
Note: *To minimize keratin contamination, perform all steps of this protocol under clean, keratin-controlled conditions. Wear powder-free gloves (preferably nitrile), a lab coat and a hair cover at all times.*

2 Add 250 mM dithiothreitol (DTT) to the sample to reach a final concentration of 10 mM and incubate for 40 min at 56 °C, with agitation (650 rpm), to reduce disulfide bridges. Allow the sample to cool afterwards. 40m

3 Add 500 mM iodoacetamide (IAA) to the sample to reach a final concentration of 20 mM and incubate for 30 min at room temperature, protected from light, to alkylate the reduced disulfide bridges. 30m

4 Add 250 mM DTT to the sample to raise the final DTT concentration to 21 mM and incubate for 10 min at room temperature, protected from light, to quench any remaining IAA. 10m

Protein precipitation (if necessary)

16h 30m

5 Precipitate the sample by adding six volumes of ice-cold acetone, vortex briefly to mix and incubate overnight at −20°C. 16h

6 Centrifuge at 16,000 × g for 10 minutes using a pre-cooled rotor. Carefully remove the supernatant and allow the pellet to air-dry for 5 min. 15m

7 Resuspend the pellet in 50 mM ammonium bicarbonate (NH_4HCO_3) by vortexing for 5 min, then sonicate for 10 min to ensure complete protein solubilization. 15m

In-sol protein digestion

16h 10m

8 Add 1 M NH_4HCO_3 to non-precipitated samples to reach a final concentration of 50 mM NH_4HCO_3.

9 Reconstitute the contents of one 20 µg vial of trypsin (e.g., Promega V5111) in 200 µL of 1 mM HCl. Aliquot the reconstituted trypsin into 10 µL portions and store at −20°C for up to 6 months. 10m

10 Add trypsin to the sample at a 1:50 (enzyme:protein, w/w) ratio and incubate overnight at 37°C with agitation (650 rpm). 16h

Alternative Proteases and Conditions:

- **Trypsin+Lys-C or Chymotrypsin**

Concentration: 10 ng/µL
Digestion buffer: 50 mM NH_4HCO_3
Temperature: 37ºC

- **Asp-N**

Concentration: 20 ng/µL
Digestion buffer: 50 mM NH_4HCO_3
Temperature: 37ºC

- **Glu-C**

Concentration: 25 ng/µL
Digestion buffer: 25 mM NH_4HCO_3
Temperature: 25ºC

11 Spin down and stop the digestion by acidifying with Formic acid (FA) to a final concentration of 5%.

Peptides purification with C18 filtered pipet tips

1h 5m

12 Aspirate 100 µL of 50% ACN to clean/activate the C18 tip.
Discard the solution and repeat once more. 1m

13 Aspirate 100 µL of 5% FA to equilibrate the C18 tip.
Discard the liquid and repeat once more. 1m

14 Aspirate and dispense the sample five times to ensure maximum peptide binding to the C18 tip. 1m

15 Aspirate 100 µL of 5% FA to clean the peptides.
Discard the supernatant and repeat once more. 1m

16 Aspirate 100 µL of 90% ACN + 0.1% FA and elute the purified peptides into a new low-protein binding microtube. 1m

17 Dry the purified peptides completely using a SpeedVac. 1h

18 Store the purified samples and the flow-throughs at −20 °C.

Contact Information

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