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In-Gel Trypsin digestion of Proteins for MS analysis

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Maria da Conceição Almeida: Validated the protocol
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Abstract

This protocol describes a standardized workflow for in-gel 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.
- Use mass-spectrometry-compatible staining methods such as Coomassie Blue (see Material tab). Do not use fixatives such as glutaraldehyde or paraformaldehyde.
- Preferably excise bands under a laminar-flow hood or under water to minimize keratin contamination and on a clean glass plate to avoid plastic-derived contaminants. Clean all tools and the cutting surface with 70% ethanol between each band to prevent cross-contamination.
- Keep gel pieces fully submerged at all times; increase the solution volume as needed to maintain immersion and allow free movement during shaking.
- Use LC-MS grade reagents.
- Use low-protein binding microtubes (avoid autoclaved or PCR-coated microtubes) and low-binding pipette tips.

Materials

- Ice bucket
- Lab coat and hair cover
- Low-binding pipette tips
- Low-protein-binding microtubes (1.5–2 mL)
- Powder-free gloves (preferably nitrile)
- Stainless steel scalpel blade and handle

Kits, Reagents & Solutions

- 70% ethanol
- Acetonitrile (ACN) LC-MS grade
- Ammonium bicarbonate (NH_4HCO_3) – 50 mM in Water and in 50% ACN
- C18 ZipTips (e.g.: OMIX C18 tips ref.: HPAA5700310)
- Dithiothreitol (DTT) – 10 mM in 50 mM NH_4HCO_3
- Formic acid (FA) LC-MS grade
- Hydrochloric acid (HCl) – 1 mM
- Ice
- Iodoacetamide (IAA) – 55 mM in 50 mM NH_4HCO_3
- Mass-spectrometry-compatible stain
- PAGE Gel Preparation Kit
- Trypsin (e.g., Promega V5111) – 0.1 µg/µL in 1 mM HCl
- Water LC-MS grade

Mass-spectrometry-compatible stains

- Coomassie™ Brilliant Blue G-250
- Coomassie™ Brilliant Blue R-250
- Imperial™ Protein Stain (Thermo Scientific)
- PageBlue™ Protein Staining Solution (Thermo Scientific)
- Pierce™ Silver Stain Kit (Thermo Scientific)
- SimplyBlue™ SafeStain (Invitrogen)

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

- Electrophoresis chamber system and power supply
- Fridge and freezer
- Platform rocker

- SpeedVac concentrator
- Spin-down centrifuge
- Thermomixer (capable of 37°C, 56°C, 70°C and 650 rpm agitation)
- Ultrasonic bath
- Variable volume single channel pipettes
- Vortex mixer

Safety warnings

- Be careful not to aspirate the dried gel pieces into the pipette tip as the gel pieces shrink after dehydration and they also can stick to the pipette tip.
- Incubate Iodoacetamide (IAA) step in the dark to avoid side reactions (step 10).
- 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 electrophoresis separation

4h 15m

1 Run the protein samples on an SDS–PAGE gel and stain the gel using a mass-spectrometry-compatible stain such as Coomassie Blue (see Material tab). 2h
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 Destain the gel as thoroughly as possible, as residual dye can interfere with mass spectrometry data acquisition. 2h

3 Excise the protein bands of interest from the gel and cut them into 1–2 mm^3 pieces to improve solvent exchange. Excise only the well-defined, darkest region, prioritizing protein concentration over total band area to increase target purity and improve in-gel digestion efficiency. 15m
Note: *Preferably excise bands under a laminar-flow hood or under water to minimize keratin contamination and on a clean glass plate to avoid plastic-derived contaminants. Clean all tools and the cutting surface with 70% ethanol between each band to prevent cross-contamination.*

4 Transfer the gel pieces into a low-protein binding microtube and spin down to bring them to the bottom of the tube.

Destaining coomassie stained protein band(s)

1h

5 Wash the gel pieces with water for 15 min with gentle agitation. 15m
Remove and discard the supernatant.

6 Add 50 mM ammonium bicarbonate (NH_4HCO_3) prepared in 50% acetonitrile (ACN) and incubate for 15 min with gentle agitation. 15m
Remove and discard the supernatant.

7 Repeat the above steps until the gel pieces are visibly clear (colorless). If needed, heat to 37 °C to help destaining, as residual dye can hinder protease access and peptide recovery. 30m

Protein reduction and alkylation

1h 45m

8 Add ACN, vortex and incubate for 15 min. 15m
Spin down, remove and discard the supernatant.
Note: *After dehydration, the gel pieces shrink and may adhere to the pipette tip. Take care not to aspirate them accidentally or transfer them to another microtube.*

9 Add 10 mM dithiothreitol (DTT) prepared in 50 mM NH_4HCO_3 and incubate for 45 min at 56ºC with agitation (650 rpm) to reduce disulfide bridges.
Spin down, remove and discard the supernatant. 45m

10 Add ACN, vortex and incubate for 15 min.
Spin down, remove and discard the supernatant. 15m

11 Add 55 mM iodoacetamide (IAA) prepared in 50 mM NH_4HCO_3 and incubate for 30 min at room temperature, protected from light, to alkylate the reduced disulfide bridges.
Remove and discard the supernatant. 30m

In-gel protein digestion 18h 10m

12 Add ACN, vortex and incubate for 15 min. Remove and discard the supernatant.
Repeat until the gel pieces are fully dehydrated (typically appearing white, shrunken and rigid). 30m

13 Vortex to separate the gel pieces and spread any residual droplets along the tube walls, then allow them to dry for 10 min at 37 °C with the lid open and without agitation.
Repeat until the gel pieces are fully dry. 30m

14 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

15 Place the required aliquots on ice and dilute each with 90 µL of 50 mM NH_4HCO_3 to obtain a 10 ng/µL working solution.
Note: *Keep the working solution on ice, use immediately and avoid refreezing.*

16 Add the trypsin working solution to the gel pieces and incubate on ice for 60 minutes.
Note: *Check the samples every 15 min. If the initial volume has been fully absorbed by the gel pieces, add additional 50 mM NH_4HCO_3 (without enzyme) to ensure the gel pieces remain completely covered.* 1h

17 Remove any excess buffer and add fresh 50 mM NH_4HCO_3 to fully cover the gel pieces and allow them to move during shaking. 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

18 Spin down and transfer the supernatant (containing the digested peptides) to a new low-protein binding microtube.

19 Stop the digestion by acidifying the digested sample with Formic acid (FA) to a final concentration of 5%.

Peptides extraction with ACN (if necessary) 1h 55m

20 Dehydrate the gel pieces with ACN for 10 min in an ultrasonic bath.
Transfer the ACN extract to the microtube containing the digested sample. 10m

21 Rehydrate the gel pieces with water for 10 min in an ultrasonic bath.
Transfer the remaining water to the same microtube containing the digested sample. 10m

22 Repeat the last two steps. 20m

23 Dry the combined extract completely using a SpeedVac. 1h

24 Resuspend the digested sample in 100 µL of 5% FA by vortexing for 5 min, then sonicate for 10 min to ensure complete peptide solubilization. 15m

Peptides purification with C18 filtered pipet tips 1h 5m

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

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

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

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

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

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

31 Store the purified samples, the flow-throughs and the remaining gel pieces at −20 °C.

Contact Information

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