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Comparative Sample Preparation Methods for a Label-Free Proteomic Analysis

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Mass spectrometry (MS) is the cutting-edge platform that propels biological and clinical research. MS-based bottom-up proteomics has expedited the characterization of signal transductions and aberrant proteomes of disease specimens through protein identification, protein-protein interaction discovery, and post-translational dynamics profiling. The typical pipeline of the bottom-up strategy for MS involves protein digestion prerequisite to peptide separation by liquid chromatography and data acquisition on a mass spectrometer.[1] The refinement of the sample processing is critical to fulfilling the quest for augmenting proteome coverage, robustness, and throughput, alongside the quantum leap of the instrument and computational analysis.

Enzymatic digestion of proteomes into peptides is a hallmark of bottom-up proteomics, influencing the quality of protein identification and quantitation. To deduce the optimal condition for protease activity, many studies have been conducted using different lysis reagents and buffers.[2-5] The conventional in-solution digestion method using urea is the most feasible and cost-effective. Urea is a chaotropic denaturant that can disrupt intraprotein interactions, resulting in protein unfolding to facilitate trypsin accessibility. As a hydrophilic compound, urea can be removed by reversed-phase liquid chromatography (RP-HPLC), hence its compatibility with mass spectrometry. However, carbamoylation modification on proteins and peptides' amines caused by urea under heat hinders digestion sites.[6,7] Sodium dodecyl sulfate (SDS) is an alternative to urea with more efficient solubilization of membrane proteins, but it can damage the RP-HPLC. Evidence and concerns were also raised regarding the ion suppression and poor spectra in electrospray ionization mass spectrometry, which resulted from residual SDS exceeding 0.01%.[8,9] To resolve this, filter-aided sample preparation (FASP) was established to remove the ionic detergent by centrifugation and retain proteins above the molecular weight cut-off.[3,5] Another imperative method is the in-gel digestion approach that utilizes size-based separation assisted by negatively charged protein-SDS complexes. Not only does it dissipate SDS during electrophoresis, but it also enhances the depth of proteome characterization by reducing sample complexity.[10,11]

DataOn is a web-based resource established by Korea Institute of Science and Technology Information to improve re-analysis of registered data. In this protocol, we exemplified in-gel, in-solution and FASP digestion strategies. The demonstration videos entitled "Comparative sample preparation methods for label-free proteomic analysis (videos)" can be accessed at: https://doi.org/10.22711/idr/966, and through Supplementary Materials.

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Procedure

Cell lysis

Reagents

- Sodium dodecyl sulfate (SDS, Sigma)
- UltraPure^{TM} Tris (Invitrogen)
- Hydrochloric acid (Sigma-Aldrich)
- Urea (ThermoScientific)
- HPLC water (J.T.Baker, ThermoScientific)

Reagent setup

Stock solution:

√ 20% SDS (w/v) in HPLC water. Can be stored at room temperature for up to 1 year.
*SDS is flammable, irritative to skin and eyes, and harmful if swallowed or inhaled. Handle with care and avoid inhalation.

√ 1 M Tris in HPLC water and bring to pH 8.0 by adding HCl. Can be stored at room temperature for up to 1 year.
*Hydrochloric acid is volatile and corrosive. It causes skin burns, eye damage, and respiratory irritation. Open the bottle in a fume hood or a well-ventilated area.

Working solution:

√ 4% SDS (v/v) in 50 mM Tris-HCl (pH 8.0) and HPLC water.

√ 8 M Urea in 50 mM Tris-HCl (pH 8.0) and HPLC water.

Apparatus

- Sonic dismembrator (Fisher Scientific)
- Micro centrifuge (Smart R17 Plus, Hanil)
- Eppendorf ThermoMixer C (Eppendorf)
- HyperVAC speed vacuum (Gyrozen)

1. Wash adherent cells with 5 mL cold PBS before harvesting.
2. Centrifuge cells at 1500 × g for 5 minutes at 4°C. Remove the supernatant.
3. Lyse approximately 1×10^5 to 10^6 cells in the presence of 200 μL 8 M urea, 50 mM Tris-HCl (pH 8.0) for in-solution digestion or 200 μL of 4% SDS, 50 mM Tris-HCl (pH 8.0) for in-gel and FASP digestion methods.
4. Apply probe sonication with 10 seconds interval and 10% power to reduce the viscosity.
5. Cool the samples on ice for 5 seconds.
6. Repeat step 4 & 5 for three times.
7. Heat samples at 95°C for 5 minutes.
*Note: Digestion with urea should not be boiled to prevent carbamylation of proteins.
8. Centrifuge samples at 14 000 x g for 15 minutes to collect proteins in the supernatant.
*Note: Samples can be stored at -80°C for the future process but freeze-thaw cycles should be avoided.

In-solution digestion

Reagents

- Dithiothreitol (DTT, Sigma)
- Iodoacetamide (IAA, Sigma)
- HPLC water (J.T.Baker, Thermo Scientific)
- Trifluoroacetic acid (TFA, Sigma-Aldrich)
- Ammonium bicarbonate (ABC, Sigma-Aldrich)
- Pierce™ Trypsin protease, MS-grade (Thermo Scientific)

Reagent setup

Stock solution:

√ 1 M DTT in HPLC water. Can be stored at -20°C for 1 year

√ Prepare 10% TFA (v/v) by diluting with HPLC water in a Duran bottle. Can be stored at room temperature for up to 1 month.
*TFA is volatile and corrosive. It causes skin burns, eye damage, and respiratory irritation. Open the bottle in a fume hood or a well-ventilated area.

Working solution:

√ Prepare 100 mM DTT by diluting stock DTT in HPLC water.
*DTT is susceptible to oxidation. Prepare freshly before use.

√ Dissolve IAA in HPLC water to a final concentration of 200 mM.
*IAA is unstable and light-sensitive. Prepare freshly before use and perform experiments in the dark.

√ Prepare 50 mM ABC buffer (pH 7.8) by dissolving ABC in HPLC water.

√ Reconstitute 20 μg of trypsin with 100uL of 50 mM ABC (pH 7.8) to obtain a final concentration of 0.2 μg/μL.

Apparatus

- Eppendorf ThermoMixer C (Eppendorf)

1. Reduce the disulfide bonds of proteins with 22 μL of 100 mM DTT to a final concentration of 10 mM at 56°C for 30 minutes.
2. Alkylate the sulfhydryl functional groups of proteins with 25 μL of 200 mM IAA to a final concentration of 20 mM. Cover samples in foil and incubate at room temperature for 30 minutes.
3. Dilute samples with 2 mL of 50 mM ABC to decrease the urea concentration below 1 M.
4. Incubate samples overnight at 37°C with sequencing-grade trypsin with 1:50 of an enzyme-to-protein ratio.
5. Acidify samples with 20 μL of 10% TFA to quench trypsin activity.

FASP

Reagents

- Urea (Thermo Scientific)
- Triethylammonium bicarbonate buffer (TEAB, Merck)
- Dithiothreitol (DTT, Sigma)
- Iodoacetamide (IAA, Sigma)
- Trifluoroacetic acid (TFA, Sigma-Aldrich)
- HPLC water (J.T.Baker, Thermo Scientific)
- Pierce™ Trypsin protease, MS-grade (Thermo Scientific)

Reagent setup

Stock solution:

√ 1 M DTT in HPLC water. Can be stored at -20°C for 1 year.

√ Prepare 10% TFA (v/v) by diluting with HPLC water in a Duran bottle. Can be stored at room temperature for up to 1 month.
*TFA is volatile and corrosive. It causes skin burns, eye damage, and respiratory irritation. Open the bottle in fume hood or in a well-ventilated area.

Working solution:

√ Make 50 mM TEAB (v/v) (pH 8.0) in HPLC water.

√ Prepare 8 M urea in 50 mM TEAB (pH 8.0) with HPLC water.
*Urea reaction with water is endothermic. However, increasing temperature decomposes urea to ammonia and isocyanic acid, so the reaction should be kept at room temperature. Urea crystal might be hard to be solubilized, mix vigorously by a magnetic stirrer if needed.

√ Prepare 100 mM DTT by diluting stock DTT in HPLC water.
*DTT is susceptible to oxidation. Prepare freshly before use.

√ Dissolve IAA in HPLC water to a final concentration of 200 mM (w/v).
*IAA is unstable and light-sensitive. Prepare freshly before use and perform the experiment in the dark.

√ Reconstitute 20 μg of trypsin with 100uL of 50 mM TEAB (pH 8.0) to obtain a final concentration of 0.2 μg/μL.

Apparatus

· 3kDa Amicon Ultra-0.5 centrifugal filter unit (Millipore, Merck)
· Micro centrifuge (Smart R17 Plus, Hanil)
· Eppendorf ThermoMixer C (Eppendorf)

1. Follow step 1 and 2 as in the in-solution digestion method.
2. Dilute samples with 8 mL of 8 M urea to decrease the concentration of SDS from 4% to less than 0.1%.
3. Transfer 300 μL of samples to a 3k-Da filter and centrifuge for 15 minutes at 14,000 × g. Discard the flow-through.
*Note: 15 mL Amicon filter can be used to accelerate the process.
4. Repeat step 3 until the remaining volume is finished.
5. Centrifuge the sample-containing filter with 200 μL of 8 M urea in 50 mM TEAB (pH 8.0) at 14,000 × g for 15 minutes.
6. Repeat step 5 for three times.
7. Exchange 8 M urea in 50 mM TEAB (pH 8.0) with 200 μL of 50 mM TEAB at 14 000 xg centrifugation for 15 minutes.
8. Repeat step 7 for three times.
*Note: Approximately 100 μl of the sample will remain in the filter after centrifugation.
9. Recover the samples by placing the filter upside down in the collection tube and centrifuge at 1000 xg for 2 minutes.
10. Incubate samples overnight at 37°C with sequencing-grade trypsin (1:50 enzyme-to-protein ratio).
11. Acidify samples with 20 μL of 10% TFA to quench trypsin activity.

In-gel digestion

Reagents

· Ammonium bicarbonate (Sigma-Aldrich)
· Acetonitrile (ACN, J.T.Baker, Thermo Scientific)
· Dithiothreitol (Sigma)
· Iodoacetamide (IAA, Sigma)
· Pierce™ Trypsin protease, MS-grade (Thermo Scientific)
· SimplyBlue™ SafeStain (Invitrogen)

Reagent setup

Stock solution:

√ 1 M DTT in HPLC water. Can be stored at -20°C for 1 year.

√ 1 M ABC (pH 7.8) (w/v) in HPLC water. Can be stored at room temperature for up to 1 year.

Working solution:

√ Prepare 50 mM ABC buffer (pH 7.8) by diluting stock 1 M ABC in HPLC water.

√ Prepare the destaining buffer with the final concentration of 10 mM DTT and 50 mM ABC in 40% acetonitrile.
*DTT is susceptible to oxidation. Prepare freshly before use.

√ Dissolve IAA in HPLC water to a final concentration of 200 mM. Further, dilute it 10-fold to obtain a final concentration of 20 mM.
*IAA is unstable and light-sensitive. Prepare freshly before use and perform the experiment in the dark.

√ Reconstitute 20 μg of trypsin with 2mL of 50 mM ABC (pH 7.8) to obtain a final concentration of 0.01 μg/μL.

√ Make harvest buffer containing 40% acetonitrile (v/v) and 0.1% formic acid (v/v) in HPLC water.

Apparatus

· Gel cutter (SPL Life Science)
· Micro centrifuge (Smart R17 Plus, Hanil)
· Eppendorf ThermoMixer C (Eppendorf)

1. After electrophoresis, stain the gel with SimplyBlue™ SafeStain solution according to manufacturer's protocol.

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2. Excise the band of interest by an extraction tool and transfer it into a 1.5mL Eppendorf tube.
3. Dice the gel bands into smaller cubes with a pipette tip (approximately 1 mm^3)
4. Cover the gel with 300 µL of DTT-containing destaining buffer.
5. Shake the gel-filled tube at 900 rpm at room temperature for 10 minutes.
6. Remove the destaining solution and repeat steps 4 & 5 until there is no visible blue stain.
7. Fill the tube with 300 µL of 20 mM IAA solution at room temperature for 30 minutes, then remove the excess solution.
8. Add 200 µL of 100% acetonitrile to dehydrate the gel and change the buffer every 5 minutes until the gel pieces are rigid.
9. Remove acetonitrile and evaporate the remaining solution in the samples by speed vac.
10. Incubate sample with 100 µL of 50 mM ABC buffer containing 1 µg trypsin on ice until the white gel pieces become transparent.
11. Remove the excess trypsin, add the ABC buffer just cover gel pieces and incubate at 37°C overnight.
12. Transfer the ABC inside the gel-filled tube to a new 1.5 mL Eppendorf tube.
13. Add 200 µL of harvest buffer into the gel tube and shake at 900rpm for 20 minutes.
14. Collecting the solution into the 1.5 mL tube of the previous step 12.
15. Repeat steps 13 and 14 for two more times.
16. Dry the collected peptide solution in the speed vac at 4°C overnight.

Discussion

Taken together, our protocol amalgamates the popular approaches for MS-based bottom-up proteomics, enabling their further application and evaluation to elucidate biological and pathophysiological questions. There is no universal protein preparation method for bottom-up proteomic research, albeit it is arguably the most critical process. Previously, we employed the complementary protein fractionation by SDS-PAGE and peptide fractionation by reversed-phase liquid chromatography to delineate a draft map of the human proteome, substantiating it as a powerful routine to reduce proteome's complexity.[10] Of note, the inevitable pitfall of this method entails massive keratin contamination, incomplete peptide recovery, and incompatibility for high-throughput investigation.[11,12] The alternative strategy, FASP, is touted to be conceptually promising for removing interfering chemicals by utilizing a spin filter and centrifugation. However, its inherent advantages are weighed down by sample loss and incomplete SDS elimination.[2,5] We successfully applied the in-solution digestion method for identifying albumin modifications in human serum and FASP for profiling the proteome of the autism spectrum disorder mice.[13,14] In addition, buffers in this protocol (ABC, TEAB, and Tris-HCl) can be used interchangeably depending on the experimental goals. Thus far, the decision for the optimal approach is subject to the samples' nature and the ultimate purposes of the investigation.

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Supporting information

Supplementary Information is available at https://drive.google.com/file/d/1ELX6Rcg9UvBPzcvBNZiZtCNS2ppl-iCL/view?usp=share_link

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