

ZnCl₂ Precipitation-Assisted Sample Preparation for Proteomic Analysis

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Abstract

This manuscript details protocols for the ZnCl₂ precipitation-assisted sample preparation (ZASP) for proteomic analysis. By inducing protein precipitation with ZASP precipitation buffer (ZPB, final concentration of ZnCl₂ at 100 mM and 50% methanol), ZASP depletes harsh detergents and impurities, such as sodium dodecyl sulfate (SDS), Triton X-100, and urea, at high concentrations in solution from protein solutions prior to trypsin digestion. It is a practical, robust, and cost-effective approach for proteomic sample preparation. It has been observed that 90.2% of the proteins can be recovered from lysates by incubating with an equal volume of ZPB at room temperature for 10 min. In 1 h of data-dependent acquisition (DDA) analysis on an Exploris 480, 4,037 proteins and 25,626 peptides were quantified from 1 µg of mouse small intestine proteins, reaching a peak of 4,500 proteins and up to 30,000 peptides with 5 µg of input. Additionally, ZASP outperformed other common sample preparation methods such as sodium deoxycholate (SDC)-based in-solution digestion, acetone precipitation, filter-aided sample preparation (FASP), and single-pot, solid-phase-enhanced sample preparation (SP3). It demonstrated superior performance in protein (4,456 proteins) and peptide identification (29,871 peptides), lower missing cleavage rates (16.3%), and high reproducibility (Pearson correlation coefficients of 0.96 between replicates) with similar protein distributions and cellular localization patterns. Significantly, the cost of ZASP per sample with 100 µg of protein as input is lower than 30 RMB, including the expense of trypsin.

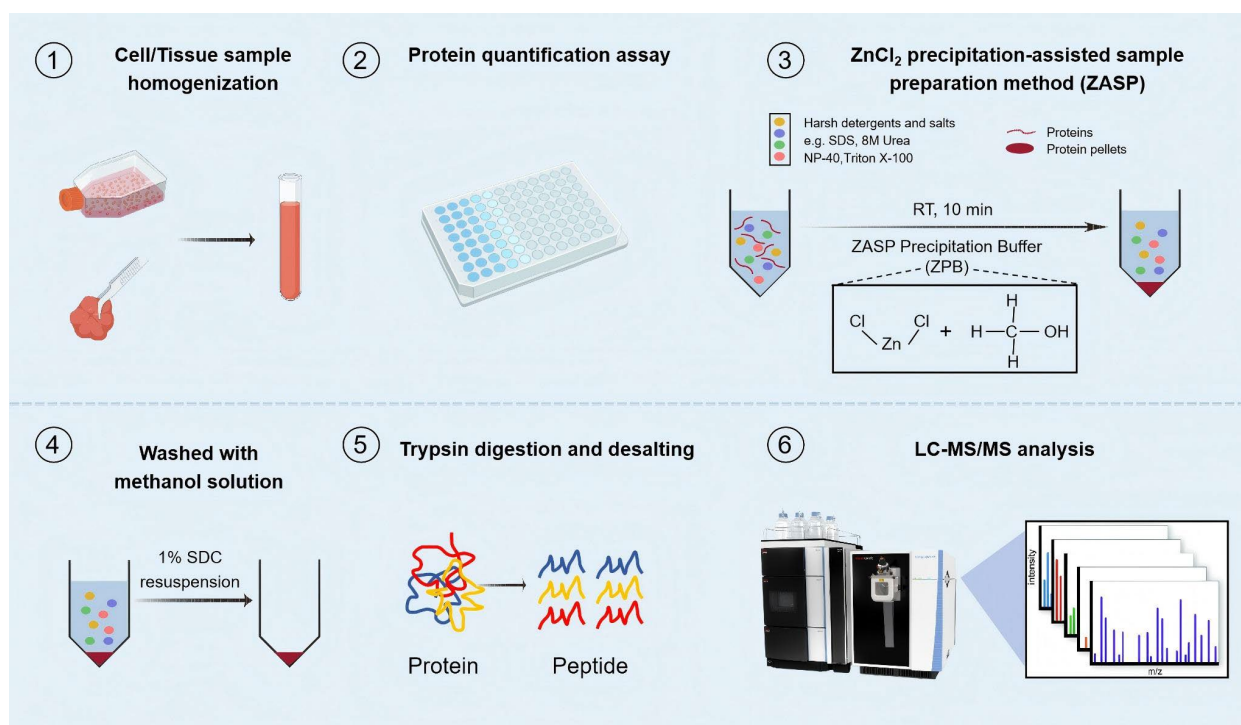
Key features

- ZASP is a user-friendly method that enables efficient, sensitive, and cost-effective proteomic analysis in a common biochemistry lab through simple incubation and centrifugation.
- ZASP enables 90% protein recovery from lysates with various trypsin and LC–MS-incompatible reagents by incubating at room temperature for 10 min.
- ZASP enables unbiased proteomic analysis of diverse biological samples, including cells, optimal cutting temperature (OCT)-embedded tissues, and formalin-fixed paraffin-embedded (FFPE) tissues.

Keywords: Sample preparation, LC–MS-incompatible reagents, ZnCl₂ induced protein precipitation, Protein recovery, Proteomics.

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Graphical overview



Background

Sample preparation is a critical step in proteomics analysis, including protein extraction, denaturation, reduction, alkylation, and digestion, followed by peptide purification. The quality of sample preparation directly impacts the reproducibility, accuracy, and sensitivity of LC-MS analysis [1,2]. Protein extraction is usually enhanced by adding chaotropes and detergents, such as SDS [3,4]. However, most of these additives impair subsequent protein enzymatic digestion and LC-MS analysis, thereby compromising the reliability and sensitivity of the LC-MS results [5,6]. Multiple methods, including affinity-based approaches [7], membrane filtration [8], and protein precipitation [9–11], have been developed to remove them. However, each method has its limitations, such as being labor-intensive or time-consuming, resulting in sample loss, high cost, or low throughput [8,10–14]. We aim to develop a cost-effective, simple, and widely applicable sample preparation technology to resolve these issues and enable high-quality proteomics analysis.

Zn^{2+} -based precipitation methods have been commonly used for the initial fractionation of proteins based on their solubility, such as in the purification of antibodies from cell culture broth [15,16]. ZnSO_4 and acetone aqueous solutions have been reported to rapidly recover peptides generated by trypsin and pepsin digestion prior to MS analysis [17]. Zn^{2+} may bind to histidine and cysteine residues on the surface of proteins via exposed imidazole and thiol groups to form relatively stable complexes [18]. Additionally, as a member of the Hofmeister series, Zn^{2+} can cause protein precipitation by altering the solubility of peptides and proteins in organic solvents [19–21]. Because of the stronger electrostatic interactions, Zn^{2+} is superior to other ions (e.g., Mg^{2+}) in precipitating proteins from solutions [22].

Inspired by these studies, we recently developed a novel sample preparation method based on ZnCl_2 and methanol-induced protein precipitation with in-solution digestion, termed ZnCl_2 precipitation-assisted sample preparation (ZASP) [23]. ZASP achieves efficient recovery of proteins (recovery rate >90%) from lysates containing different trypsin- or LC-MS-incompatible detergents by incubating with an equal volume of ZASP precipitation buffer (ZPB) for 10 min at room temperature. We have demonstrated it as an efficient approach by comparing it with current popular sample preparation methods (e.g., FASP, SP3, SDC-based in-solution digestion) and profiling of different types of samples. The protocol for ZASP is described in this manuscript.

Materials and reagents

Biological materials

1. HEK 293T cells (ATCC, catalog number: CRL-3216)
2. Mouse tissues (C57BL/6) (obtained from The Beijing Proteome Research Center)
3. Yeast (ATCC, catalog number: 204508)
4. *E. coli* (ATCC, catalog number: 25922)

Reagents

1. Tris(hydroxymethyl)aminomethane (Tris) LC-MS grade (Pierce, catalog number: 17926) (keep in a dry, cool, and well-ventilated place)
2. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) $\geq 98\%$ (Sigma-Aldrich, catalog number: 646547) (store at 4 °C)
3. 2-Chloroacetamide (CAA) $\geq 99\%$ (Sigma-Aldrich, catalog number: C0267) (keep in a dry, cool, and well-ventilated place)
4. Trypsin, MS grade (Promega, catalog number: 90057) (store at -20 °C)
5. Formic acid, $\geq 98\%$ (Sigma-Aldrich, catalog number: 33015) (keep container tightly closed in a dry and well-ventilated place)
6. Acetonitrile (ACN) (Thermo Scientific, catalog number: A955-4) (keep container tightly closed in a dry and well-ventilated place)
7. Sodium dodecyl sulfate (SDS) (Solarbio, catalog number: S8010) (keep container tightly closed in a dry and well-ventilated place)
8. Zinc chloride (ZnCl_2) $\geq 98\%$ (Sigma-Aldrich, catalog number: 208086) (keep container tightly closed in a dry and well-ventilated place)
9. Methanol, LC-MS grade) (Sigma-Aldrich, catalog number: 1.06035) (keep container tightly closed in a dry and well-ventilated place)
10. Pierce BCA Protein Assay kit (Thermo Scientific, catalog number: 23227) (keep in a dry, cool, and well-ventilated place)
11. Sodium deoxycholate (SDC) $\geq 97\%$ (Sigma-Aldrich, catalog number: D6750) (keep container tightly closed in a dry and well-ventilated place)
12. Dulbecco's modified Eagle's medium (DMEM) (Gibco, catalog number: C11995500BT) (store at 4 °C)
13. Fetal bovine serum (FBS) (NEWZERUM, catalog number: FBS-CS500) (store at -20 °C)
14. Penicillin-streptomycin (Gibco, catalog number: 15140122) (store at 4 °C)
15. Trypsin-EDTA solution (Gibco, catalog number: 25300054) (store at 4 °C)
16. Phosphate-buffered saline (PBS) (Gibco, catalog number: C10010500BT) (store at 4 °C)
17. Yeast extract peptone dextrose media (YEPD) (Sigma-Aldrich, catalog number: Y1500) (keep container tightly closed in a dry and well-ventilated place)
18. Luria-Bertani medium (LB) (Sigma-Aldrich, catalog number: L2542) (store at 4 °C and keep away from light)

Solutions

1. SDS lysis buffer (see Recipes)
2. ZASP precipitation buffer (see Recipes)
3. SDC lysis buffer (see Recipes)
4. Buffer A (see Recipes)
5. Buffer B (see Recipes)

Recipes

1. SDS lysis buffer

4% SDS (w/v) and 100 mM Tris-HCl in water at pH 8.5: mix 20 mL of 10% (w/v) SDS solution stock with 5 mL of 1 M Tris, followed by the addition of 25 mL of H_2O .

2. ZASP precipitation buffer (ZPB)

Methanol consisting of 0.1% formic acid and 200 mM ZnCl_2 (v/v): dissolve 1,363 mg of ZnCl_2 and 50 μL of formic acid in 50 mL of methanol.

Note: ZnCl₂ is hygroscopic and should be stored in a tightly sealed container under dry conditions. Formic acid is corrosive and should be handled with appropriate protective equipment (gloves, lab coat, and eye protection) in a fume hood.

3. SDC lysis buffer

1% SDC (w/v) and 100 mM Tris-HCl in water at pH 8.5: mix 30 mL of 5% (w/v) SDC solution with 15 mL of 1 M Tris-HCl, then add 90 mL of H₂O.

4. Buffer A

0.1% formic acid aqueous solution: mix 10 µL of formic acid with 10 mL of H₂O.

5. Buffer B

60% acetonitrile solution containing 0.1% formic acid: mix 6 mL of acetonitrile with 4 mL of H₂O, followed by the addition of 10 µL of formic acid.

Laboratory supplies

1. C18 solid phase extraction (SPE) disks (3M Empore, catalog number: 98060402173)
2. C18 powder (Durashell, catalog number: DC930010-L)
3. 0.5 mL centrifuge tubes (Corning, catalog number: 3208)
4. 1.5 mL centrifuge tubes (Corning, catalog number: 3620)
5. 2.0 mL centrifuge tubes (Corning, catalog number: 3213)
6. 10 µL pipette tips (Axygen, catalog number: T-300-L)
7. 200 µL pipette tips (Axygen, catalog number: T-200-C-L)
8. 1,000 µL pipette tips (Axygen, catalog number: T-1,000-C)
9. Cell culture plates (Nunc EasyDish, catalog number: 150468)

Equipment

1. Sonicator Chromatin and DNA Shearing System (Qsonica, catalog number: Q800R3)
2. SpeedVac Integrated Vacuum Concentrator (Thermo Scientific, catalog number: SPD1030A-230)
3. Tissue homogenizer (OMNI, model: Bead Ruptor 24)
4. Centrifuge 5424R (Eppendorf, catalog number: 5404000090)
5. Research[®] Plus pipettes (0.1–2.5 µL) (Eppendorf, catalog number: 3123000217)
6. Research[®] Plus pipettes (2–20 µL) (Eppendorf, catalog number: 3123000292)
7. Research[®] Plus pipettes (20–200 µL) (Eppendorf, catalog number: 3123000250)
8. Research[®] Plus pipettes (100–1,000 µL) (Eppendorf, catalog number: 3123000268)
9. ThermoMixer[®] C (Eppendorf, catalog number: 5382 000015)
10. Freezer (-20 °C) (Haier, model: DW-25L262)
11. Freezer (-80 °C) (Thermo Scientific, catalog number: 907)
12. CO₂ incubator (Thermo Scientific, catalog number: 3111)
13. Shaker (Jingda, model: DHZ-DA)
14. UV/Vis Spectrophotometer (Thermo Scientific)
15. Microplate Reader (BioTek, model: Synergy LX)
16. Orbitrap Exploris 480 Mass Spectrometer (Thermo Scientific, catalog number: BRE725533)

Software and datasets

1. DDA data analysis software (MaxQuant, v.2.0.1.0)
2. Fasta files were downloaded from the Swiss-Prot database, containing entries for human (20387), mouse (17098), *Saccharomyces cerevisiae* (strain ATCC 204508/S288c, Baker's yeast, 6729), and *E. coli* (strain K12, 4529) sequences, released in April 2021.

Procedure

A. Sample collection

1. HEK 293T cells

- a. Culture HEK 293T cells in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in an environment with 5% CO₂ in a 10 cm cell culture dish.
- b. Harvest the cells when they reach 80% confluence.
- c. Discard the medium. Add 1 mL of 0.05% trypsin-EDTA solution and incubate for 1 min to detach cells.
- d. Add 1 mL of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin to terminate the digestion reaction. Centrifuge the cell suspension at 1,000× g for 5 min and remove the supernatant.
- e. Wash the cells three times with PBS: add 5–10 mL of PBS to the cells, gently pipetting to create a suspension. Then, centrifuge the cell suspension at 1,000× g for 5 min and remove the supernatant. Repeat this a total of three times.
- f. Store the cell pellets at -80 °C until further processing.

2. Yeast cells

- a. Cultivate yeasts in YEPD media at 30 °C shaking at 200 rpm in a DHZ-DA shaker.
- b. Collect the cells from a 5–10 mL culture by centrifugation at 4,000× g for 5 min when the optical density at 600 nm (OD₆₀₀) reaches approximately 1.0. Use a UV/Vis spectrophotometer to measure optical density.
- c. Wash the cells three times with PBS buffer: add 5–10 mL of PBS to the cells, gently pipetting to create a suspension. Then, centrifuge the cell suspension at 4,000× g for 5 min and remove the supernatant. Repeat this a total of three times.
- d. Store the cell pellets at -80 °C until further processing.

3. *E. coli*

- a. Grow *E. coli* cells in LB medium at 37 °C overnight with vigorous shaking in a DHZ-DA shaker.
- b. Harvest the cells from a 5–10 mL culture during the stationary phase (OD₆₀₀ between 0.6 and 1) by centrifugation at 4,000× g for 10 min.
- c. Wash the cells twice with PBS buffer: add 5–10 mL of PBS to the cells, gently pipetting to create a suspension. Then, centrifuge the cell suspension at 4,000× g for 5 min and remove the supernatant. Repeat this step twice.
- d. Store the cell pellets at -80 °C until further processing.

B. Protein extraction

1. Add SDS lysis buffer to the cell pellets or tissues and boil at 95 °C for 10 min to denature the proteins. The volume ratio of cell pellets to lysis buffer is 1:10.
2. For tissues, yeast cells, and *E. coli* cells, homogenize samples using an OMNI Bead Ruptor 24 Elite instrument (6.6 m/s, 3 cycles, 30 s on, 15 s off) after being cooled in ice water.
3. Sonicate the lysates or HEK 293T cell suspension for 10 min using a Qsonica Q800R3 sonicator (85% power, 10 s on, 10 s off).
4. Quantify protein concentrations by measuring absorbance at 562 nm using a BioTek Synergy LX Multimode Reader, employing the Pierce BCA Protein Assay kit following the manufacturer's instructions.
5. Aliquot the lysates to 100 µg of protein and store at -80 °C until further use.

C. Protein precipitation

1. Sample volumes are adjusted based on the amount of input protein using SDS lysis buffer: 50 µL for 1–10 µg, 100 µL for 20–100 µg, 200 µL for 500 µg, and 500 µL for 1,000 µg of input protein.
2. Add an equal volume of ZPB to each sample, mixing gently by pipetting. Let the mixture incubate at room temperature for 10 min. For the precipitation step, ensure that the final solution has a concentration of 50% methanol and 100 mM ZnCl₂. **Note:** Cloudiness occurred during incubation for protein inputs exceeding 5 µg.
3. Collect protein pellets by centrifugation at 19,000× g for 5 min at 4 °C. Protein particles are not visible for inputs under 5 µg.

D. Washing protein pellets

1. Wash the protein pellets twice with the same volume of freshly prepared methanol containing 0.1% formic acid as the ZPB, assisted by sonication using a Qsonica Q800R3 sonicator (85% power, 10 s on, 10 s off, 1 min).
2. Evaporate in the air for at least 10 min at room temperature to ensure complete removal of residual formic acid.

E. Resuspension proteins for in-solution digestion

1. Add an appropriate volume of SDC lysis buffer (e.g., 50 μ L for 1–10 μ g, 100 μ L for 20–100 μ g, 200 μ L for 500 μ g, or 500 μ L for 1,000 μ g of input protein) to resuspend the resulting clean protein pellet and sonicate for 1 min (85% power, 10 s on, 10 s off) to aid solubilization.
2. Optional: To calculate the recovery rate of proteins, protein concentrations can be measured using a BCA assay to determine the amount of resuspended protein.
3. Add TCEP stock (pH 7) to a final concentration of 10 mM and CAA stock to a final concentration of 40 mM and incubate for 10 min at 65 °C for reduction and alkylation.

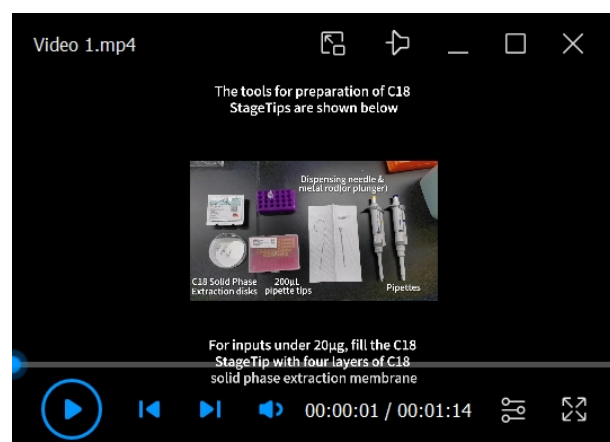
F. In-solution digestion

1. Add trypsin at an enzyme-to-protein ratio of 1:30 and digest at 37 °C for 14 h.
2. After digestion, add formic acid to the sample until the pH reaches 2–3 to terminate the digestion reaction.

G. Preparation of C18 StageTips

1. For inputs under 20 μ g, fill the C18 StageTip with four layers of C18 solid phase extraction membrane with a punch.
2. For inputs between 20 and 100 μ g, fill the C18 StageTip with a C18 solid phase extraction membrane and C18 powder. Weigh the C18 powder (C18 powder is a hydrophobic, reverse-phase silica-based material with a particle size of 3 μ m and a pore size of 150 Å). Add 200 μ L of 100% acetonitrile to reconstitute the C18 suspension. Next, transfer the C18 suspension into 200 μ L tips containing one layer of the C18 disk. The volume of C18 suspension is calculated based on the amount of protein input corresponding to the mass of C18. For reference, 3 mg of C18 powder is suitable for 100 μ g of protein. After centrifuging to remove the acetonitrile, store the tips in a tip box until use. The details of making C18 StageTips are described in Video 1.

Note: The C18 StageTip can be kept in a tip box at room temperature for several months. Although we recommend preparing it one day before use to avoid any issues during storage, this is not strictly necessary. In addition to the homemade C18 StageTip, commercial C18 columns (e.g., Pierce™ C18 Tips for 80 μ g of total peptide, catalog number: 87784) can be used for desalting.



Video 1. Step-by-step guide for the manufacturing of C18 StageTips

3. For higher protein input, Sep-Pak C18 cartridges are a good choice.

H. Desalting

1. For StageTips packed with C18 powder, centrifuge tips at 1,000× g for 45 s at 4 °C before use.
2. Wetting StageTips: Transfer 100% acetonitrile to C18 StageTips and centrifuge at 1,000× g for 2 min to remove acetonitrile. Add buffer B to StageTips and centrifuge at 1,000× g for 2 min at 4 °C.
3. Pre-equilibrium StageTips: Add buffer A to StageTips and centrifuge at 1,500× g for 2 min at 4 °C. Repeat this step 2 times.
4. Load the tryptic peptides: Transfer the digest to StageTips and centrifuge at 1,000× g for around 1.5 min at 4 °C. Collect the flowthrough, transfer it to the StageTips, and then centrifuge at 1,000× g for around 1.5 min at 4 °C.
5. Washing: Transfer buffer A to the StageTips and centrifuge at 1,500× g for 2 min. Repeat this step once.
6. Elution: Transfer buffer B to StageTips. Centrifuge the tips at 1,000× g until they are fully dried (approximately 2 min) at 4 °C.
7. Dry down the elutes and store them at -20 °C before LC–MS analysis. This desalting procedure was performed following established protocols as previously described [24,25]. The detailed volume of buffers added at each step for desalting using different C18 StageTips and cartridges is listed in Table 1.

Notes:

1. Load samples slowly on the StageTip column.
2. Check how long each step of centrifugation will take if it is your first time doing it.
3. Do not let the C18 become fully dried (paper white) before elution.

Table 1. Volume of buffers added during each step of the desalting process

	Wetting with acetonitrile	Wetting with buffer B	Pre-equilibrium with buffer A	Loading samples	Flowthrough	Washing with buffer A	Eluting with buffer B
Homemade C18 StageTip	1×250 µL	1×250 µL	2×250 µL	1×	1×	2×250 µL	1×100 µL
Sep-Pak C18 cartridge		3×1 mL	3×1 mL	1×	1×	5×1 mL	1×0.3 mL

LC–MS/MS analysis

We analyzed the peptides using a 60-min data-dependent acquisition method with an UltiMate™ 3000 RSLC nanoliquid chromatography system (Thermo Fisher Scientific), coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Further details can be found in our original work, published in *Molecular & Cellular Proteomics* [23]. The samples can also be measured using other types of mass spectrometers and methods. Our work primarily focuses on sample preparation.

Data analysis

The raw files were processed via MaxQuant software (version 2.0.1.0). Protein sequences retrieved from the SwissProt database (released in April 2021), encompassing entries for humans (20,387), mouse (17,098), *S. cerevisiae* (strain ATCC 204508/S288c, Baker's yeast. 6729), and *E. coli* (strain K12, 4529) sequences, were utilized. Trypsin/P was specified as the proteolytic enzyme with a maximum of two missed cleavage sites. Cysteine carboxamidomethylation was set as a fixed modification, while methionine oxidation and protein N-terminal acetylation were considered variable modifications.

Protein recovery rate was calculated using the following formula: Protein recovery rate (%) = (precipitated protein amount)/(protein amount in lysate) × 100. The precipitated protein amount = protein concentration × volume of resuspension buffer. Protein concentrations were measured using a Pierce BCA Protein Assay kit (Thermo Scientific). To evaluate digestion efficiency, missed cleavages were assessed. The number of missed enzymatic cleavages is reported in the "Evidence.txt" file generated by MaxQuant. The missed cleavage rate was calculated as follows: Missed cleavage rate (%) = (number of peptides with missed cleavages) / (total number of identified peptides) × 100. The quality of the LC–MS data was assessed based on the shape of the total ion current (TIC) and base peak, the number of MS1 and MS2 scans, and the signals from these scans, along with the results of the database search. During method development, optimization, and evaluation phases, three technical replicates were performed in parallel. The comparison between ZASP and conventional methods focused on the number of identified peptides and proteins, the missed cleavage rate, the correlation between replicates, and the characteristics of identified proteins. Statistical analyses were conducted using two-sided t-tests, with the FDR calculated via the Benjamini-Hochberg method (significance cutoff: 5%).

Validation of protocol

During protocol optimization, the combination of 100 mM ZnCl_2 and 50% methanol (final concentration) was determined to be optimal, achieving the highest protein recovery rate (90.2%) and yielding the most protein and peptide identifications (Figure 1). To assess analytical performance, ZASP was further evaluated using a range of protein input amounts (1–1,000 μg). Remarkably, over 4,000 proteins could be reliably identified from as little as 1 μg of input, with low peptide-level variation (median CV < 20%) and strong reproducibility across replicates and input levels (Figure 2). In addition, the TIC, MS1, and MS2 signal intensities obtained from ZASP were comparable to those from the conventional in-solution digestion, indicating equivalent chromatographic performance and ionization efficiency (Figure 3). Finally, comparative analysis demonstrated that ZASP outperformed conventional sample preparation methods—including acetone precipitation, FASP, and SP3—in both the depth of protein and peptide identifications and lower missed cleavage rates (Figure 4) [23]. The protocol was published and validated in [23].

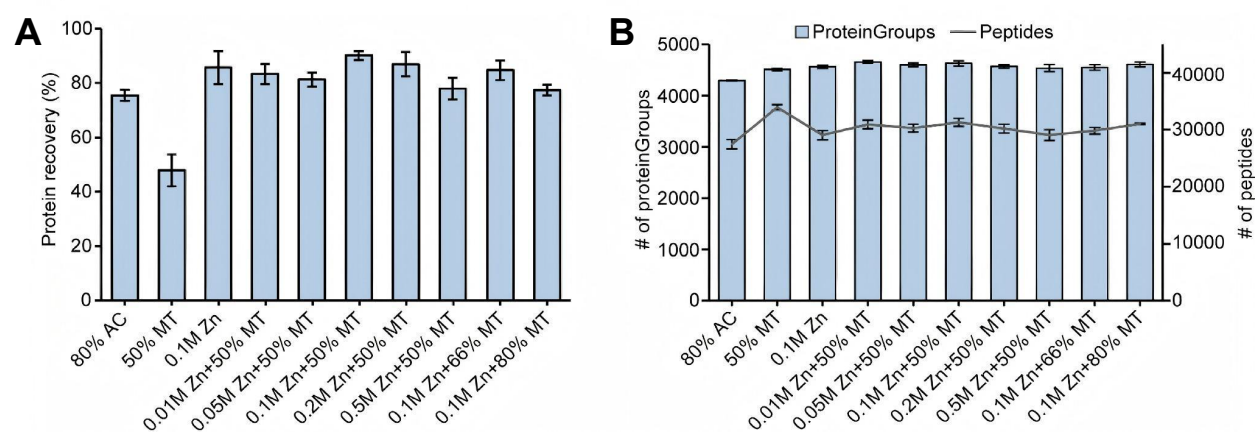


Figure 1. Development and optimization of ZnCl_2 precipitation-assisted sample preparation (ZASP). (A) Protein recovery associated with various ZnCl_2 and methanol mixtures. (B) Enumeration of proteins and peptides identified in HEK 293T cells treated with diverse ZnCl_2 and methanol combinations. For comparative analyses of protein and peptide identifications, only those without missing values were considered. AC and MT denote acetone and methanol, respectively. For each condition, three technical replicates were conducted. The bar plots show the average protein recovery rates and the average number of identified proteins, with error bars indicating the standard deviation.

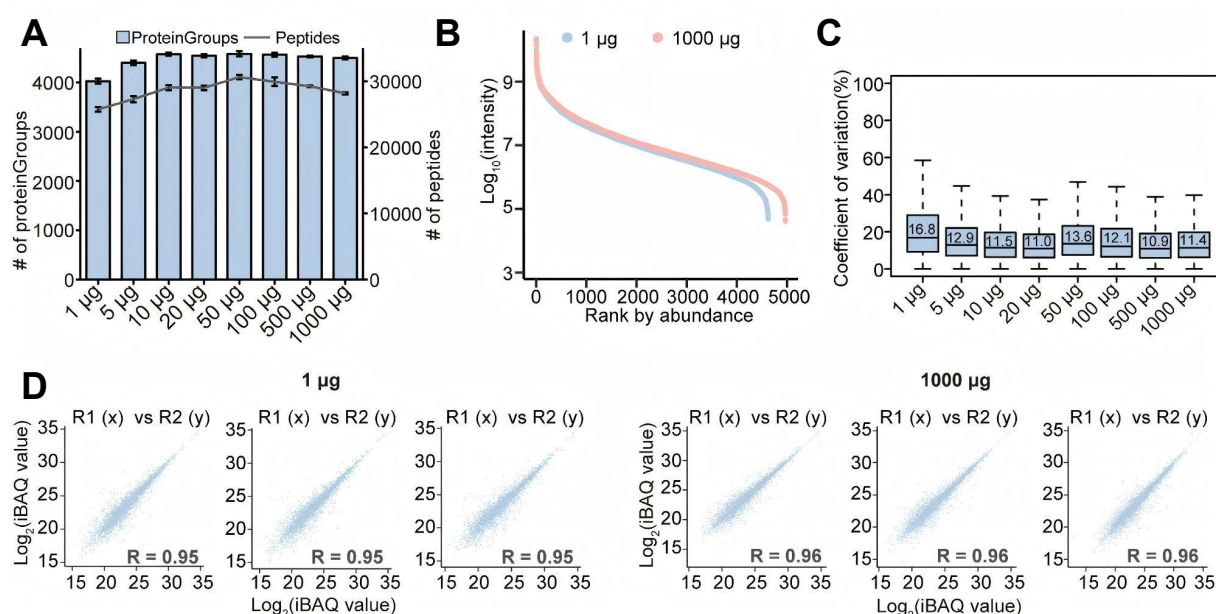


Figure 2. Performance evaluation of ZnCl_2 precipitation-assisted sample preparation (ZASP). (A) The number of proteins identified in mouse small intestinal tissue with protein inputs ranging from 1 μg to 1,000 μg . For each

condition, three technical replicates were performed to determine the average number of identified proteins. The error bars represent the standard deviation of these identifications. (B) Ranked plots illustrating protein abundance in samples with 1 μ g and 1,000 μ g of protein input. (C) Coefficients of variation for peptides across samples with varying protein inputs. (D) Pearson correlation coefficients for protein identification in samples with 1 μ g and 1000 μ g of protein input.

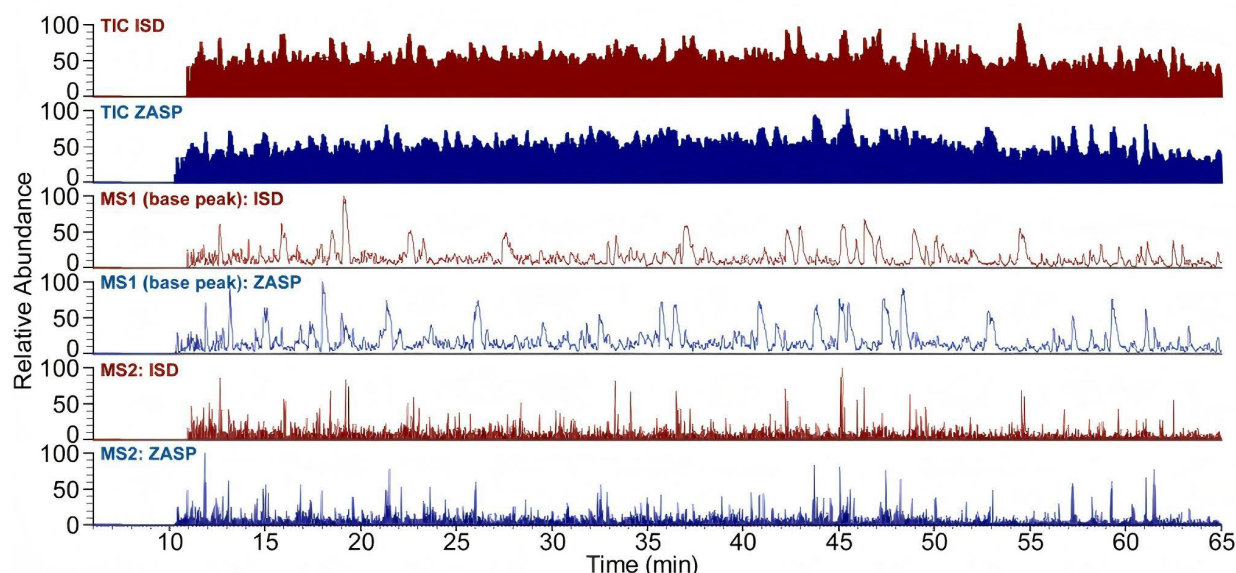


Figure 3. Comparison of total ion current (TIC), base peak (MS1), and MS2 profiles between in-solution digestion (ISD) and ZnCl₂ precipitation-assisted sample preparation (ZASP). All the figures above are reprinted/adapted from Shao et al. [23]. © The Authors. Published by Elsevier Inc. on behalf of the American Society for Biochemistry and Molecular Biology. Distributed under a Creative Commons Attribution License 4.0 (CC BY) <http://creativecommons.org/licenses/by/4.0/>.

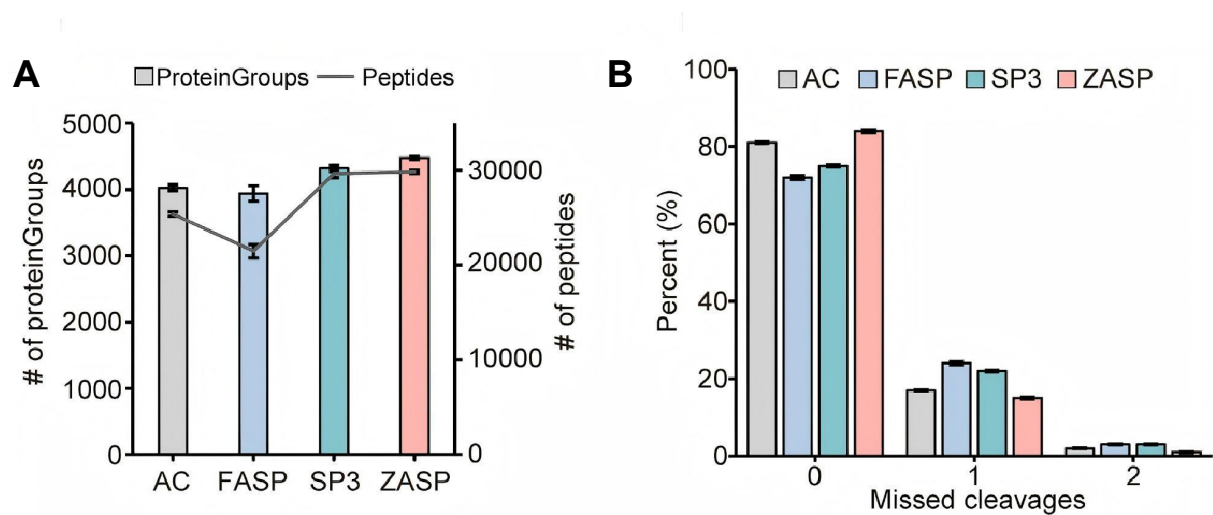


Figure 4. Comparative analysis of ZnCl₂ precipitation-assisted sample preparation (ZASP) and other detergent-depletion sample preparation techniques. (A) Number of proteins and peptides identified using acetone precipitation (AC), filter-aided sample preparation (FASP), solid-phase-enhanced sample preparation (SP3), and ZASP methods. (B) Missed cleavage rates of peptides identified by each of the four methods. For each method, three technical replicates were performed. The bar plots show the average number of identified proteins and the average missed cleavages in three replicates, with error bars indicating the standard deviation.

General notes and troubleshooting

1. Adjust the solvent volumes based on the protein input; use smaller volumes for lower protein amounts and larger volumes for higher input.
2. After precipitation, proteins can be resolubilized in buffers like 1% SDC lysis buffer or other suitable buffers, depending on the analysis requirements.
3. For the reduction and alkylation of proteins, it is recommended to use a TCEP solution with a pH of 7. The TCEP solution, when prepared by dissolving TCEP powder in water, tends to be strongly acidic. This acidity can negatively impact the resolubilization of protein pellets in SDC buffer.
4. Choosing and preparing C18 StageTips depend on the input of proteins. If protein quantity is lower than 20 µg, it is recommended to use C18 StageTips packed with a C18 membrane. For protein amounts between 20 and 100 µg, homemade StageTips filled with C18 powder are a cost-effective option. For higher protein input, Sep-Pak C18 cartridges are a good choice.
5. Commercial tips and columns can also be used instead of homemade C18 StageTips.
6. While standard plasticware was used in this protocol, users working with sub-microgram protein amounts or, particularly, adsorption-sensitive samples, may consider using certified low-binding tubes and tips to further reduce potential sample loss.
7. To ensure reliable performance, all key instruments—particularly high-speed centrifuges and sonicators—should be regularly calibrated and maintained according to the manufacturer's specifications.

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Competing interests

The authors declare no competing interests.

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