Chapter 17

Simple and Reproducible Sample Preparation for Single-Shot Phosphoproteomics with High Sensitivity

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Abstract

The traditional sample preparation workflow for mass spectrometry (MS)-based phosphoproteomics is time consuming and usually requires multiple steps, e.g., lysis, protein precipitation, reduction, alkylation, digestion, fractionation, and phosphopeptide enrichment. Each step can introduce chemical artifacts, in vitro protein and peptide modifications, and contaminations. Those often result in sample loss and affect the sensitivity, dynamic range and accuracy of the mass spectrometric analysis. Here we describe a simple and reproducible phosphoproteomics protocol, where lysis, denaturation, reduction, and alkylation are performed in a single step, thus reducing sample loss and increasing reproducibility. Moreover, unlike standard cell lysis procedures the cell harvesting is performed at high temperatures (99 °C) and without detergents and subsequent need for protein precipitation. Phosphopeptides are enriched using TiO_2 beads and the orbitrap mass spectrometer is operated in a sensitive mode with higher energy collisional dissociation (HCD).

Key words Phosphoproteomics, Phosphopeptide enrichment, Sample preparation, Heated guanidinium chloride lysis, Mass spectrometry

1 Introduction

Mass spectrometry (MS)-based proteomics has emerged as a robust, sensitive, and reliable technology for global characterization of posttranslational modifications (PTMs) of proteins and peptides. Reversible phosphorylation is a ubiquitous PTM and important in defining functional characteristics of a number of proteins involved in virtually all cellular signaling networks, such as signal transduction, cell proliferation, differentiation, apoptosis, and metabolism [1, 2]. Site-specific phosphorylation of proteins is a transient and dynamic event and phosphorylation sites on proteins are often of low stoichiometry and therefore of relatively low abundance [3]. As a result, efficient sample preparation and phosphopeptide enrichment strategies are essential for successful phosphoproteomics studies. In-depth phosphoproteome analysis by

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quantitative mass spectrometry often requires large amounts of starting protein material, extensive fractionation and many hours or days of MS acquisition time, increasing costs and limiting the number of samples that can be measured. The present chapter describes a simplified and robust sample preparation procedure for phosphopeptide enrichment without any fractionation, allowing for comprehensive, in-depth phosphoproteome coverage of up to 10,000 unique phosphopeptides from 1 mg of protein starting material in a single 4 h LC-MS run on a Q-Exactive mass spectrometer. The following protocol has been used with reproducible success down to 10 µg of starting material generating up to 1000 unique phosphopeptides (*see* Fig. 1). For details about ultra-deep phosphoproteome coverage enabled by off-line peptide fractionation the reader is referred to Chapters 10, 11, and 12.

In traditional cell lysis protocols every step of the procedure is performed cold to minimize sample preparation-induced or stress-related alterations of the proteome and protein modification profile. At low temperatures most of the cellular activity is halted preserving the in vivo state of the cells. An alternative way of quenching all cellular activity is by immediate heat denaturation of

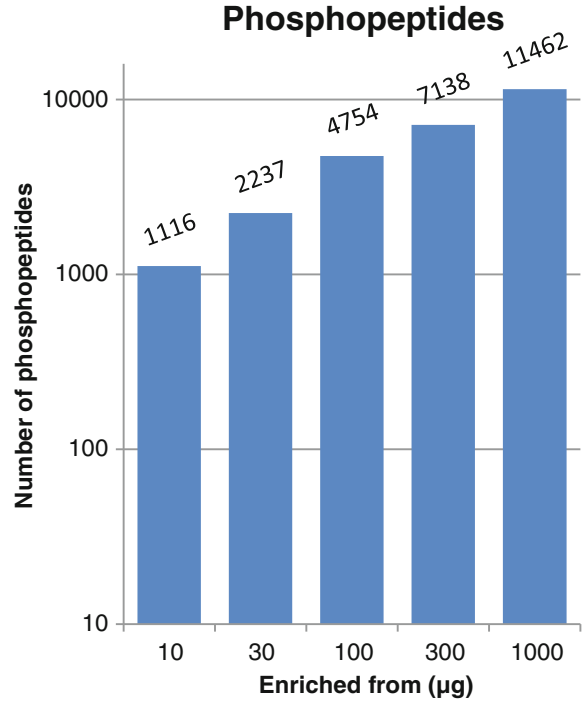

Fig. 1 A dilution series over varying amounts of protein starting material (10 µg–1 mg) was subjected to phosphopeptide enrichments to establish a measure of sensitivity of the protocol

all proteins, which can be achieved by adding hot lysis buffer to the cells. Lysis buffers are typically detergent based. Dialysis or precipitation of the proteins makes it possible to remove the detergents, which are incompatible with peptide separation on reversed-phase C_{18} columns and strongly interfere with peptide ionization in the electrospray source of the mass spectrometer. To minimize sample losses by protein precipitation lysis buffers without detergents, but instead made up of chaotropic agents such as urea and guanidinium hydrochloride (GuHCl), can be used to disrupt the cell membrane [4]. Urea can decompose and form isocyanic acid, which reacts with the primary amines of the protein and peptides. Such carbamylation of peptides will cause increased sample complexity and reduced peptide identification. This process is accelerated at elevated temperatures or slightly acidic conditions [5]; thus the chaotrope of choice for heated lysis buffer is GuHCl [6]. More importantly, when comparing traditional RIPA lysis [7] with boiling GuHCl, we noticed a significant decrease in the number of in vitro artifacts, introduced during sample preparation, with 15 % of the identified peptides modified by in vitro artifacts after RIPA lysis and only 5 % after lysis in warm GuHCl buffer. As expected we also see a higher recovery of protein amounts using the GuHCl lysis procedure.

In the traditional proteomics sample preparation workflow of protein digestion, lysis is often followed by a cysteine disulfide bridge reduction step with dithiothreitol (DTT) and a subsequent alkylation step with either chloroacetamide (CAA) or iodoacetamide (IAA) to prevent reformation of disulfide bonds. These two steps cannot be combined as IAA will react with the thiol groups on DTT and thereby inhibit the reducing effect of the DTT. Using tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent instead of DTT allows for simultaneous reduction and alkylation as TCEP does not contain any thiol groups and will not react with CAA or IAA. In addition, when using GuHCl for lysis, this mix of reducing and alkylating agent can be added directly to the lysis buffer enabling lysis, reduction, and alkylation in one step and thus significantly simplifying the protocol and reducing the time spent on sample preparation (*see* Fig. 2).

Analyzing peptides in complex mixtures by mass spectrometry (MS) is always a compromise between quality and quantity of tandem mass spectra (MS/MS) and MS running time, and for typical proteome samples analyzed on an orbitrap-type instrument the most critical feature and limiting factor is the scan speed of the instrument [8]. However, in phosphoproteomics there are additional challenges in the MS/MS acquisition; apart from identification of phosphopeptides it is also necessary to accurately localize the phosphorylation site(s) with single-amino acid resolution. To achieve this, it is a necessity to generate high-quality MS/MS spectra, which requires longer acquisition scan times. Thus, for

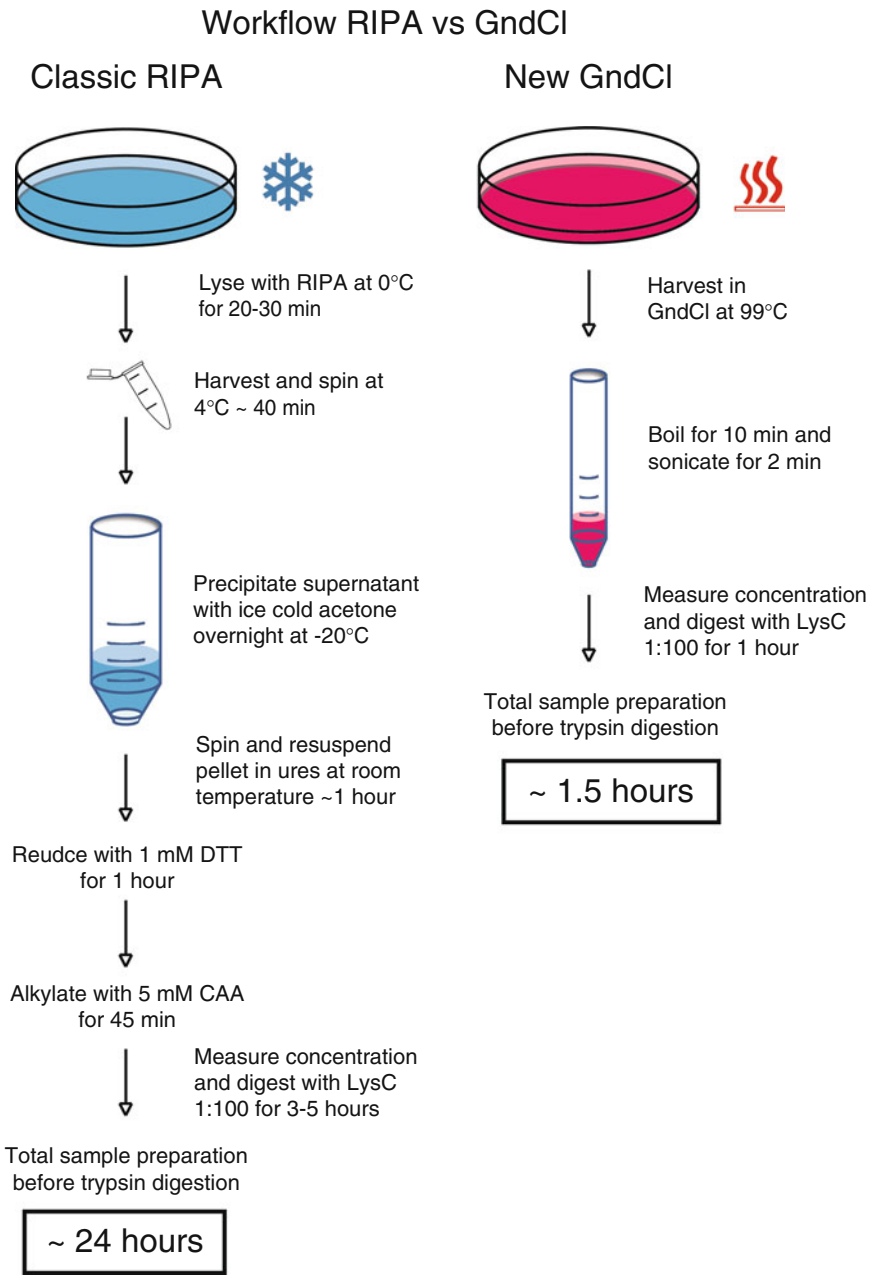

Fig. 2 Comparison of two sample lysis methods for phosphopeptide enrichments. The RIPA lysis protocol comprises multiple steps including lysis, precipitation, reduction, and alkylation, while guanidinium hydrochloride lysis is performed in a single step, where the cells are harvested and lysed in heated GuHCl buffer containing both the reducing and alkylating agents. The extracted proteins are subjected to LysC and tryptic digestions followed by phosphopeptide enrichment

comprehensive analysis of phosphoproteomes longer scan cycles are required than for typical proteome samples, for which faster scanning methods are usually sufficient for peptide identification.

2 Materials

All buffers are made with sequencing grade chemicals and ultrapure water (Milli-Q).

2.1 Cell Lysis

Experiments were performed using the adherent HeLa cervix carcinoma cell line grown in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, supplemented with 10 % fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Use appropriate media for your cell line of choice.

1. Human cervical epithelial cancer cell line HeLa or similar.
2. Sterile petri dishes (150 mm).
3. Lysis buffer: 6 M Guanidinium hydrochloride (GuHCl), 5 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM chloroacetamide (CAA), 100 mM Tris–HCl pH 8.5 (*see* **Note 1**).
4. Phosphate-buffered saline (PBS).
5. Rubber policeman cell scraper.
6. Heat block for centrifuge/microcentrifuge tubes with heating up to 99 °C.
7. Probe sonicator, Sonics Vibra Cell (VCX130).
8. Quick Start™ Bradford 1× Dye Reagent (Bio-Rad) or other Bradford- or BCA-based protein concentration quantification assay.

2.2 Protein Digestion

1. Lysyl endopeptidase (LysC), stock solution 0.5 mg/ml in 50 mM ammonium bicarbonate.
2. Digestion buffer: 25 mM Tris–HCl pH 8.5.
3. Modified trypsin (Sigma-Aldrich), stock solution 0.5 mg/ml in 50 mM acetic acid.
4. Trifluoroacetic acid (TFA).

2.3 Peptide Desalting

1. Reversed-phase C_{18} Sep-Pak cartridge (*see* **Note 2**).
2. Wash buffer I: 100 % acetonitrile (ACN).
3. Wash buffer II: 0.1 % TFA.
4. Elution buffer I: 40 % ACN in 0.1 % TFA.
5. Elution buffer II: 60 % ACN in 0.1 % TFA.
6. NanoDrop 2000 spectrophotometer (Thermo Scientific).
7. SpeedVac concentrator.

2.4 Phosphopeptide Enrichment

1. Reversed-phase C_8 (3 M Empore disk) single-layered StageTips (one per sample per incubation) (*see* **Note 3**).
2. Reversed-phase C_{18} (3 M Empore disk) double-layered StageTips (one per sample per incubation) (*see* **Note 3**).
3. 5 µm TiO_2 beads (Titansphere, GL Sciences) solution in 20 mg/ml 2,5-dihydroxybenzoic acid (DHB), 80 % ACN, 6 % TFA.
4. Enrichment buffer: 80 % ACN, 12 % TFA.
5. Wash buffer A: 10 % ACN, 6 % TFA.
6. Wash buffer B: 40 % ACN, 6 % TFA.
7. Wash buffer C: 60 % ACN, 6 % TFA.
8. Elution buffer A: 5 % ammonium hydroxide solution.
9. Elution buffer B: 10 % ammonium hydroxide solution, 25 % ACN.
10. 100 % Methanol (MeOH).

2.5 Liquid Chromatography and Electrospray Tandem Mass Spectrometry

1. MS buffer A: 0.1 % formic acid (FA).
2. MS buffer B: 0.1 % FA, 80 % ACN.
3. MS buffer C: 1 % TFA, 5 % ACN.
4. 50 cm 75 µm ID fused silica column packed in-house with 1.9 µm reversed-phase C_{18} porous silica beads with 100 Å pore size (Dr. Maisch HPLC GmbH).
5. EASY-nLC 1000 high pressure system (Thermo Fisher Scientific).
6. Q Exactive Plus or Q Exactive HF mass spectrometer (Thermo Fisher Scientific).
7. Data analysis software such as MaxQuant software suite (www.maxquant.org).

3 Methods

3.1 Cell Lysis

Example with adherent HeLa cells grown in 150 mm dishes with a surface area of 17,671 mm^2 (*see* **Note 4**).

1. Heat lysis buffer to 99 °C.
2. Wash cells twice with ice cold PBS and completely aspirate remaining liquid.
3. Add approx. 800 µl of heated lysis buffer and use cell scraper to harvest cells (*see* **Note 5**). Use a pipette to transfer lysate into a centrifuge tube (*see* **Note 6**).
4. Boil in a heat block (99 °C) for 10 min.

5. Sonicate with micro tip probe for 2 min with pulses of 1 s on and 1 s off at an amplitude of 50 %.
6. Measure concentration with Bradford, BCA, or other protein assay to estimate total protein amount (*see* **Note 7**).

   STORAGE OPTION! After this you can store your lysate in the freezer (−20 °C) for a few weeks.

3.2 Protein Digestion

1. Digest lysate with LysC:protein ratio 1:100 (w/w) for 30 min up to 4 h.
2. Dilute to a final concentration of maximum 2 M GuHCl (add minimum two times volume) with 25 mM Tris–HCl pH 8.5 and digest with trypsin 1:100 for 30 min to overnight (*see* **Note 8**).
3. Acidify with TFA to a final concentration of 1 % TFA (pH 1–2).
4. Spin down at max speed for 5 min to pellet insoluble material and keep supernatant (*see* **Note 9**).

3.3 Peptide Desalting

Example with Sep-Pak C_{18} classic cartridge. Adjust the volumes according to the product you are using.

1. Wash Sep-Pak with 3 ml wash buffer I followed by two times 3 ml with wash buffer II using gravity flow.
2. Load sample and after that wash twice with 3 ml wash buffer II.

   STORAGE OPTION! You can keep samples on Sep-Pak in the fridge for 1 week without detectable losses.
3. Elute from Sep-Pak with 3 ml elution buffer I followed by 3 ml elution buffer II.
4. SpeedVac to half volume and measure concentration with Nanodrop A280.

   STORAGE OPTION! Samples can be kept in the freezer for a few months.

3.4 Phosphopeptide Enrichment

1. Prepare in advance C_8 single-layered StageTips (one per sample per incubation) and double-layered C_{18} StageTips (one per sample per incubation) (*see* **Note 3**).
2. Prepare TiO_2 bead slurry to a 1:2 sample:bead ratio (*see* **Notes 10** and **11**).
3. Double your sample volume with enrichment buffer.
4. Add bead slurry and incubate with rotation for 15 min (*see* **Note 12**).
5. Spin down and collect supernatant for second incubation or discard. Leave 20–50 µl of liquid with the beads.

5a. For second and/or third incubation: repeat from **step 4** with the supernatant from **step 5** (*see* **Note 13**).

6. Transfer the bead-peptide suspension to C_8 tips and wash with 50–100 µl wash buffers A, B, and C, respectively, by centrifugation, approximately 500 × *g* for 2 min.
7. Elute phosphopeptides into a 96-well plate or a micro tube with 20 µl of elution buffer A and 20 µl of elution buffer B.
8. SpeedVac until ~5 µl is left.
9. Activate C_{18} StageTips with 50 µl MeOH, followed by 50 µl MS buffer B and 2 × 50 µl MS buffer A.
10. Add 20 µl of MS buffer C to the concentrated sample and load on activated C_{18} StageTips.
11. Wash with 50 µl wash buffer II and elute with 15 µl elution buffer I followed by 15 µl elution buffer II. SpeedVac until <5 µl and add up to 8 µl in total with MS buffer C.

3.5 LC-MS Setup

Example with EASY-nLC 1000 and Q Exactive Plus.

Column was packed in-house using 50 cm long fused silica with 75 µm inner diameter and packed with 1.9 µm C_{18} beads (*see* **Note 14**).

1. Load 5 µl of sample on the column and separate with a MS buffer A and B gradient [% B] going from 5 % to 25 % over 110 min, 25 % to 40 % over 25 min and then a washing step going from 40 % to 80 % over 5 min, staying at 80 % for 5 min and then down to 5 % over 5 min. The flow rate is constant at 250 nl/min.
2. Run the Q Exactive mass spectrometer in the data dependent mode using a top 10 method. Resolution for full scans 70,000, target value 3,000,000, maximum injection time of 20 ms, and scan range from 300 *m/z* to 1750 *m/z*. Resolution for MS-MS scans 35,000, target value 100,000 maximum injection time of 108 ms with normalized collision energy at 28, and a dynamic exclusion of 30 s.

3.6 Data Analysis

1. Analyze the raw files in the free software Max Quant [9] with the Andromeda search algorithm against an appropriate database (e.g., Uniprot or Swissprot human database) for protein identification. Use default settings and add phosphorylation of serine, threonine and tyrosine as variable modification. When running multiple similar phospho raw file use the "match between runs" option in the Global parameter tab for better coverage.
2. Results can be read out from the modificationSpecificPeptides and Phospho (STY) Sites tab-delimited txt output files generated by Max Quant (*see* also Chapters 21 and 22).

4 Notes

1. You can store 6 M GuHCl in the fridge for weeks but TCEP and CAA should be added immediately before use.
2. We use Sep-Pak C_{18} 1 cc cartridge from Waters for up to 1 mg and Sep-Pak C_{18} Classic cartridge for up to 10 mg of protein.
3. Place one or two disks of desired material in a 200 µl pipette tip. A video on how to make StageTips is provided here: http://www.biochem.mpg.de/226863/Tutorials *Note to video*. No special centrifuge is required. Adapters for microcentrifuge tubes can be acquired from Sonation upon special request.
4. If you work with suspension cells skip Subheading 3.1, **steps 2** and **3**, and harvest and wash cell pellet before addition of the heated lysis buffer and proceed from here directly to Subheading 3.1, **step 4**.
5. Adjust volume of lysis buffer to dish or flask size while trying to keep it as low as possible to obtain the most concentrated sample. For suspension cells like HeLa S3 you can use approximately 100 µl per 1,000,000 cells.
6. Be aware that the tube must be compatible with the heat block.
7. Make sure that the method for determining protein concentration is compatible with GuHCl, for example Bradford or BCA.
8. The lower the concentration of GuHCl is the more active trypsin is, so if you are not volume limited dilute up to 10×.
9. Occasionally there is a precipitate appearing after acidification. This will typically not affect the number of identifiable phosphopeptides, but only affect the total protein yield.
10. Example: Use 20 µl bead slurry per 1 mg sample, thus the bead slurry has to be 2 mg/20 µl. This has been tested with up to 20 mg beads per sample. However there is a maximum amount of beads and protein that can be loaded in each StageTip in order to be able to pass liquid through for elution.
11. You can specifically enrich for multiply phosphorylated peptides by adjusting the sample to bead ratio [10]. Usage of scarce amounts of TiO_2 beads typically results in increased number of identified of multiply phosphorylated peptides at the cost of low number of singly phosphorylated peptides. For HeLa lysates, Li et al. (2009) reported an optimal peptide-to-beads ratio of 1:2–1:8 (mass/mass) for obtaining the highest enrichment selectivity and maximum phosphopeptide identifications [10].
12. The beads settle very fast, be sure to have evenly distributed slurry by shaking or tapping the vial before every pipetting event.

13. You can dilute the remaining bead slurry 1:1 with 80 % ACN and 6 % TFA for exceeding incubations.
14. For single shot experiments deeper coverage is obtained with longer columns and gradients. Shorter gradients and 15 cm columns can be used when samples are fractionated.

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