Chapter 6

Quantitative Analysis of Tissue Samples by Combining iTRAQ Isobaric Labeling with Selected/Multiple Reaction Monitoring (SRM/MRM)

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Abstract

Mass spectrometry-based phosphoproteomics is an indispensible technique used in the discovery and quantification of phosphorylation events on proteins in biological samples. The application of this technique to tissue samples is especially useful for the discovery of biomarkers as well as biological studies. We herein describe the application of a large-scale phosphoproteome analysis and SRM/MRM-based quantitation to develop a strategy for the systematic discovery and validation of biomarkers using tissue samples.

Key words Phosphoproteome, iTRAQ, SRM, MRM, IMAC

1 Introduction

Advances have recently occurred in mass spectrometry-based phosphoproteomics due to improvements in both phosphopeptide enrichment [1] and isotope labeling [2, 3] technologies. Therefore, it is now possible not only to identify several thousand phosphopeptides within one large-scale analysis [4–8], but also to accurately quantify these phosphopeptides [9–12].

A common technique for phosphopeptide enrichment is immobilized metal ion affinity chromatography (IMAC), in which metal ions are chelated to nitrilotriacetic acid- or iminodiacetic acid-coated beads, thereby forming a stationary phase to which negatively charged phosphopeptides in a mobile phase can bind [1]. Phosphopeptides in a peptide mixture prepared from biological samples by enzymatic digestion show increased affinity for the IMAC resin.

Isotope labeling techniques have been classified into two groups: metabolic labeling such as SILAC (stable isotope labeling by amino acids in a cell culture) [2] and chemical labeling such as iTRAQ (isobaric tags for relative and absolute quantification) [3].

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Chemical labeling techniques are particularly useful for quantitatively comparing proteomes between tissue samples (for example obtained from human patients). Thereagainst, metabolic labeling cannot be applied to the tissue samples, in which protein synthesis does not occur preventing the replacement of the amino acids in the proteins with isotope-labeled ones [13, 14]. Moreover, large-scale phosphoproteome analyses can be performed by combining chemical labeling with the phosphopeptide enrichment techniques, and have recently been applied to the discovery of biomarkers using tissue samples [15].

On the other hand, extensive validation for tens or hundreds of biomarker candidates identified by a large-scale phosphoproteome analysis is needed prior to their application as biomarkers. A targeted proteomic approach using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) [16] is more appropriate for the validation of these candidates than an antibody-based approach. Antibodies with sufficient specificity and sensitivity for this validation are commonly not available, especially for phosphoproteins, and the high cost and long development time required to generate high-quality reagents are limiting factors. SRM can quantify target proteins without antibodies by monitoring the ions matching the precursor-product ion pair of m/z (SRM transition) of the target peptides. SRM using stable isotope peptides was recently used to validate candidate protein biomarkers in human tissue samples [13–15].

We herein describe the application of a large-scale phosphoproteome analysis and SRM-based quantitation to develop a strategy for the systematic discovery and validation of biomarkers using tissue samples. We first identify differentially expressed phosphopeptides using IMAC coupled with the iTRAQ technique. The phosphopeptides identified are then validated by the SRM analysis. This systematic approach has enormous potential for the discovery of *bona fide* disease biomarkers (*see* Fig. 1).

2 Materials

2.1 Homogenizing Tissue Samples and Enzymatic Digestion (See Note 1)

1. Phase-transfer surfactant A (PTS-A) buffer: 50 mM ammonium bicarbonate. Dissolve 1.0 g ammonium bicarbonate in 250 mL water.
2. Phase-transfer surfactant B (PTS-B) buffer: PTS-A buffer, 12 mM sodium deoxycholate, 12 mM sodium *N*-lauroyl sarcosinate.
3. Lysis buffer: PTS-B buffer, 1× PhosSTOP phosphatase inhibitor cocktail. Prepare the lysis buffer just before the experiment, and use it for the day.

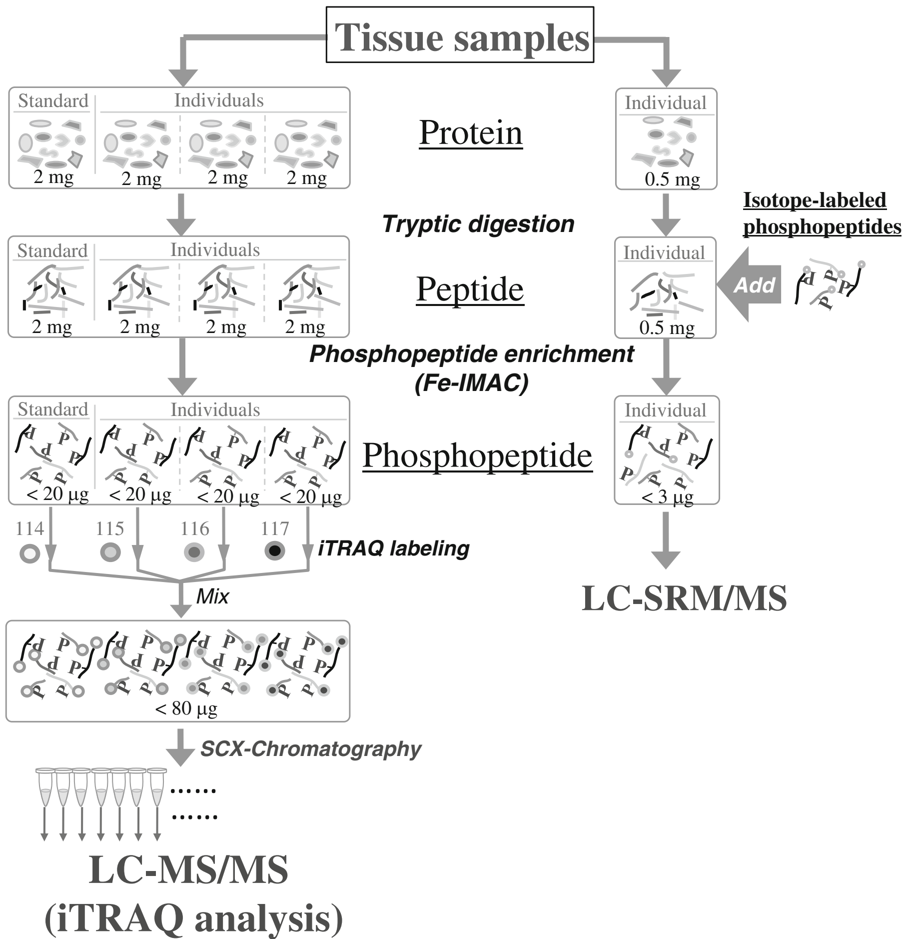


Fig. 1 Two workflows of iTRAQ analysis (*left*) and SRM analysis (*right*) are shown. In the iTRAQ analysis, Standard, which is a mixture of all analytical samples, and three individual samples are each processed into peptides and applied to Fe-IMAC to enrich the phosphopeptides. The resulting samples are labeled by iTRAQ reagents and followed by mixing the four samples. The mixture is fractionated by SCX chromatography and each fraction is analyzed by LC-MS/MS. In the SRM analysis, the individual sample is processed into peptides and followed by adding of a mixture of the isotope labeled peptides of targeted phosphorylation sites. The resulting sample is applied to Fe-IMAC to enrich the phosphopeptides and analyzed by SRM analysis

4. LysC stock solution: 1 μg/μL Lysyl endopeptidase (LysC). Dissolve lyophilized LysC in water (*see* **Note 2**). Store at −80°C.
5. Trypsin stock solution: 1 μg/μL Trypsin. Dissolve lyophilized trypsin in 10 mM HCl (*see* **Note 3**). Store at −80°C.
6. Dithiothreitol (DTT) solution (×10): 100 mM DTT. Weigh DTT powder and transfer it to a centrifuge tube. Store at 4 °C until it is used. Add 65 μL of PTS-A buffer to 1 mg of DTT and dissolve immediately prior to use.

7. Iodoacetamide (IAA) solution (×10): 500 mM IAA. Weigh IAA powder and transfer it to a centrifuge tube. Store at 4 °C in the dark until it is used. Add 10.8 μL of PTS-A buffer to 1 mg of IAA and dissolve immediately prior to use.
8. DC Protein Assay Kit.
9. Tissue grinder.
10. Liquid nitrogen.
11. Phosphate-buffered saline (PBS) buffer.
12. Bovine serum albumin (BSA), e.g., Pierce BSA Protein Assay Standards.
13. Benchtop centrifuge.
14. Sonicator, e.g., Bioruptor-UCD-250 (Cosmo Bio Japan).
15. Speed Vac.
16. 100 % Ethylacetate (sequence grade).
17. 100 % Trifluoroacetic acid (TFA) (HPLC grade).
18. 1.5 mL microtubes.

2.2 Desalting Peptide Mixtures by C18 Stage Tip (See Note 4)

1. 47 mm Empore™ C18 disk (3 M).
2. 200 μL pipet tips.
3. Methanol (LC-MS grade).
4. 80 % acetonitrile, 0.1 % TFA: Mix acetonitrile (LC-MS grade), distilled water (LC-MS grade), and TFA (HPLC grade).
5. 2 % acetonitrile, 0.1 % TFA.
6. 60 % acetonitrile, 0.1 % TFA.

2.3 IMAC

1. ProBond™ Nickel-Chelating Resin (Life Technology).
2. 50 mM EDTA-2Na in water.
3. 0.1 % acetate: Dilute acetate 1000 times in water.
4. 100 mM $FeCl_3$ in 0.1 % acetate.
5. 2 % Acetonitrile, 0.1 % TFA.
6. 60 % Acetonitrile, 0.1 % TFA.
7. 1 % Phosphate: Dilute phosphoric acid (HPLC grade, 85 %) 85 times with water.

2.4 iTRAQ Labeling

1. iTRAQ reagents: Isobaric tags for relative and absolute quantification (iTRAQ) reagents (4 plex) (Applied Biosystems).
2. 1.0 M Triethylammonium bicarbonate.
3. Ethanol.
4. pH strips.
5. Benchtop centrifuge.
6. 1.5 mL microtubes.
7. Speed Vac.

2.5 Strong Cation-Exchange Chromatography

1. Strong cation-exchange (SCX) buffer A: 25 % acetonitrile, 10 mM H_3PO_4 (pH 3). Mix 250 mL of acetonitrile (HPLC grade), approximately 650 mL of water, and 685 μL of phosphoric acid (HPLC grade, 85 %). Adjust to pH 3.0 by adding KOH solution (approximately 1.0 mL of 50 % KOH) and to 1 L with water.
2. SCX buffer B: 25 % acetonitrile, 10 mM H_3PO_4 (pH 3), 1 M KCl. Mix 250 mL of acetonitrile (HPLC grade), approximately 650 mL of water, 685 μL of phosphoric acid (HPLC grade, 85 %), and 74.55 g of KCl. Adjust to pH 3.0 by adding KOH solution (approximately 1.0 mL of 50 % KOH) and to 1 L with water.
3. HPLC system, e.g., Prominence UFLC (Shimadzu, Japan).
4. SCX column, e.g., 50 mm × 2.1 mm, 5 μm, 300 Å, ZORBAX 300SCX (Agilent Technology).
5. Sample vial/sample plate for HPLC system.
6. 1.5 mL microtubes.

2.6 iTRAQ-Based or SRM Mass Spectrometry Analysis

1. Buffer-A: 0.1 % formic acid, 2 % acetonitrile.
2. Buffer-B: 0.1 % formic acid, 90 % acetonitrile.
3. Stable isotope-labeled peptides (SI peptides) (crude grade) (Thermo Fisher Scientific) (*see* **Note 5**). Dissolve 1 μg/μL of the peptide in water. Store at −80 °C.
4. Sample vial/sample plate for MS analysis.
5. Mass spectrometer for iTRAQ analysis, e.g., LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific).
6. Mass spectrometer for SRM analysis, e.g., TSQ Vantage triple-quadruple mass spectrometer (Thermo Scientific).
7. Nano-LC system, e.g., nano-Advance UHPLC system (Bruker Daltonics).
8. Analytical column, e.g., a self-made ESI column (*see* **Note 6**).
9. Trap column, e.g., L-column2 ODS (Chemicals Evaluation and Research Institute, Japan).
10. Software for iTRAQ-based MS analysis, e.g., Mascot (Matrix Science), which is used to identify protein and phosphorylation site and Proteome Discoverer 1.3 (Thermo Scientific), which is a platform to analyze qualitative and quantitative data of the identified proteins and phosphorylated peptides (*see* **Note 7**).
11. Software for SRM/MS analysis, e.g., Pinpoint (Thermo Scientific), software to obtain the peak areas (quantitative data of targeted peptides) from the raw data of SRM analysis as well as to develop the SRM methods.

12. 2 % acetonitrile, 0.1 % TFA.
13. 2 % acetonitrile, 0.1 % TFA, 25 μg/mL EDTA.
14. 1 pmol/μL BSA digest solution.

3 Methods

3.1 Grinding Frozen Tissues

1. Chill the stainless tissue pulverizer in liquid nitrogen. Place a piece of frozen tissue in the chilled device and pulverize the tissue by striking the device with a mallet several times.
2. Check how small the particles are that the tissue has been crushed into. Rearrange the particles with a chilled spoon and keep striking the device again until there are no large pieces left in the particles.
3. Transfer the grinded tissue into the chilled tube. Store at −80 °C.

3.2 Homogenizing Tissue

1. Place part of the grinded tissue into a microcentrifuge tube and weigh the amount required for analysis. In our case, at least 40 mg of tissue was used to obtain at least 3.17 mg protein (*see* **Note 8**). If the degree of contamination by blood is predicted to be high, wash the sample by adding an appropriate volume of PBS, spin down the pellet, and discard the supernatant.
2. Add cold lysis buffer, approximately 15 μL per 1 mg of tissue (e.g., for 40 mg of tissue, add 600 μL) (*see* **Note 9**). Suspend the grinded tissue lightly by pipetting.
3. Immediately homogenize the tissue by sonication, which is performed by placing the tubes containing the sample into an ice-water bath in the Bioruptor-UCD-250 and sonicating for 10 min (30 s on/30 s off) several times in the device with the amplitude set to 250 W (*see* **Note 10**).
4. Centrifuge the sample at 100,000 × *g* for 30 min at 4 °C. Collect the supernatant into a new tube. Place a small amount of the sample into another tube to determine the protein concentration. Store the remainder at −80 °C.
5. Dilute the sample for determining the protein concentration several times with TBS. The protein concentration is determined by a DC protein assay kit using BSA as the standard.

3.3 Protein Digestion

1. Add the protein extract to a new tube: 2 mg protein from individual tissue sample and 2 mg protein from standard mixture for iTRAQ analysis and 500 μg protein from individual tissue sample for SRM analysis. Dilute the protein with the lysis buffer to a concentration that is constant between all samples (*see* **Note 11**).

2. Reduce cysteine residues in the proteins in the homogenate with 10 mM DTT for 30 min at 37 °C.
3. Alkylate the residues with 50 mM IAA for 30 min at 37 °C in the dark.
4. Dilute the sample five times with PTS-A buffer.
5. Digest the proteins by 1:100 (w/w) LysC for 8 h at 37 °C.
6. Sequentially digest the sample by 1:100 (w/w) trypsin for 12 h at 37 °C.
7. Add an equal volume of ethyl acetate to the resulting peptide mixture. Acidify the sample by adding 1/200 volume of TFA (i.e., 0.5 % TFA) in order to transfer the detergents from the lysis buffer into the ethyl acetate layer while the peptides exist in the water layer. Mix the ethyl acetate layer and water layers well by vortexing the tube. Centrifuge the tube at 10,000 × *g* for 10 min at room temperature to separate both layers. Discard the upper ethyl acetate layer.
8. Dry the resulting peptide mixture using Speed Vac.
9. Store at −80 °C until starting the following enrichment of phosphopeptides.

3.4 Preparing Fe-IMAC Resin

1. Suspend Probond™ nickel-chelating resin in 20 % ethanol. Transfer the resin to empty spin columns (*see* **Note 12**). Centrifuge the resin at 150 × *g* for 2 min. Discard the flow-through.
2. Add 50 mM EDTA-Na solution (3 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min to release the nickel ions from the resin. Repeat this step twice more (*see* **Note 13**).
3. Add water (3 mL of water per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min. Discard the flow-through.
4. Add 1 % acetate solution (3 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min. Discard the flow-through. Repeat this step once more.
5. Add 100 mM $FeCl_3$ in 0.1 % acetic acid (2 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min to chelate iron ions to the resin. Repeat this step once more.
6. Add 1 % acetate solution (3 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min to wash the resin. Repeat this step twice more.
7. Add 60 % acetonitrile and 0.1 % TFA (3 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min. Discard the flow-through. Repeat this step once more.

8. Add 60 % acetonitrile and 0.1 % TFA to the resin with the bottom end of the column plugged and then suspend and transfer the resulting Fe-IMAC resins to a tube (*see* **Note 14**).

3.5 Enrichment of Phosphopeptides for the Large-Scale Analysis (iTRAQ Analysis)

1. Dissolve the peptide mixture prepared from the tissue sample in 60 % acetonitrile and 0.1 % TFA.
2. Add Fe-IMAC resin (1 mg of the resin for 2 mg of protein) into an empty spin column. Centrifuge at 150 × *g* for 2 min to discard the flow-through.
3. Add 60 % acetonitrile and 0.1 % TFA to the resin in the column. Centrifuge at 150 × *g* for 2 min. Discard the flow-through.
4. Load the peptide mixture in 60 % acetonitrile and 0.1 % TFA to the resin in the column. Centrifuge at 150 × *g* for 2 min. Discard the flow-through.
5. Add 60 % acetonitrile and 0.1 % TFA (3 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min to wash off the non-phosphopeptides. Repeat this step twice more.
6. Add 2 % acetonitrile and 0.1 % TFA (3 mL of the solution per 1 mL of the resin) to the resin in the column. Centrifuge at 150 × *g* for 2 min. Discard the flow-through.
7. Add 1 % phosphate solution (1 mL of the solution per 1 mL of the resin) to the resin in the column and centrifuge at 150 × *g* for 2 min. Collect the eluate into a tube. Repeat this step once more and then collect the second eluate into the same tube.
8. Desalt the eluate with a disposable solid-phase extraction (SPE) device such as Sep-Pak C18 or C18 Stage Tip.
9. Dry the sample using Speed Vac.
10. Store at −80 °C until iTRAQ labeling.

3.6 Labeling of Phosphopeptides by iTRAQ Reagents

1. Dissolve the sample enriched by IMAC in 30 μL of 1.0 M triethylammonium bicarbonate solution.
2. Return the iTRAQ reagent to room temperature and then add 70 μL of ethanol to each iTRAQ reagent vial.
3. Vortex each vial for 1 min to dissolve the iTRAQ reagent and then spin down.
4. Transfer one iTRAQ reagent to one sample tube (*see* **Note 15**). Vortex each tube to mix and then spin down.
5. Incubate the tubes at room temperature for 1 h.
6. Terminate the reaction by adding an equal volume of water. Vortex each tube to mix and then spin down.

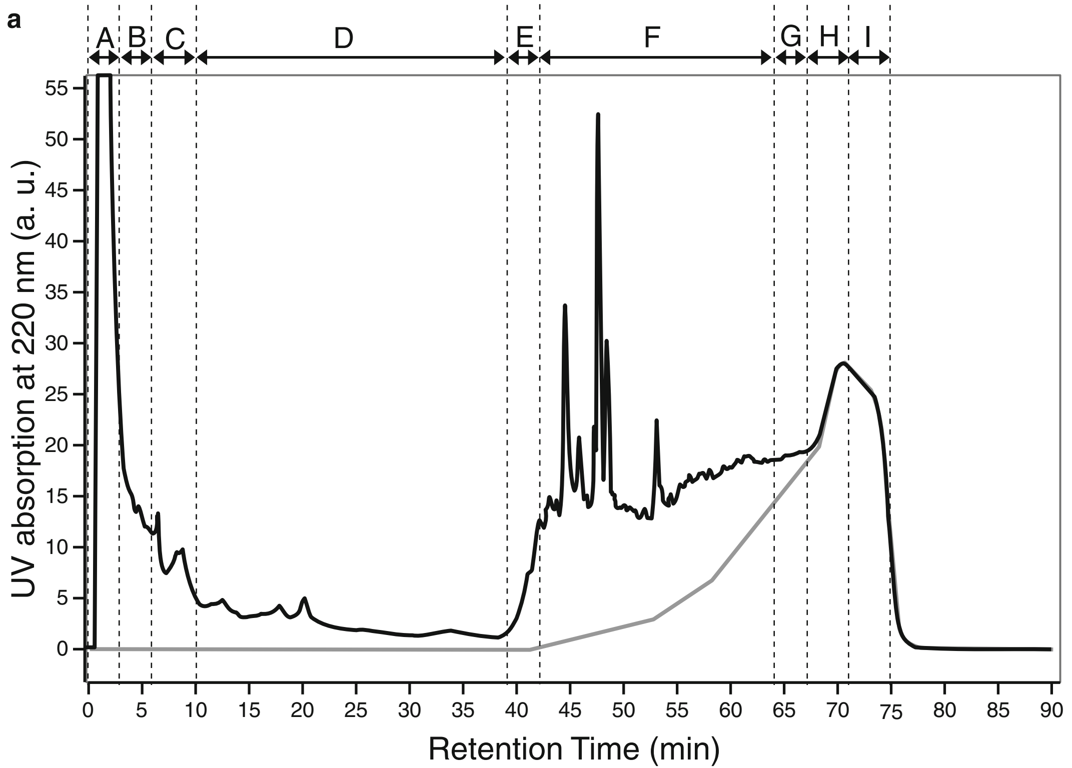
7. Combine all of the iTRAQ-labeled samples in a new tube. Vortex the tube to mix and then spin down.
8. Dry the resulting mixture using Speed Vac.
9. Dissolve the sample in 2 % acetonitrile and 0.1 % TFA, and then check the pH. If the sample is not acidic, acidify by adding TFA. After acidifying, desalt the sample with a disposable SPE device such as C18 Stage Tip.
10. Dry the sample using Speed Vac.
11. Store at −80 °C until SCX fractionation.

3.7 Strong Cation-Exchange Chromatography

1. Dissolve the iTRAQ-labeled sample in SCX Buffer A (*see* **Note 16**).
2. Fractionate the sample using an HPLC system fit with an SCX column. Separate the sample using a gradient of SCX Buffers A and B and sequentially collect the eluted sample in microfuge tubes every 1 min (*see* **Note 17**).
3. Dry the fractions collected every 1 min using Speed Vac.
4. Decrease the number of the fractions for the subsequent MS analysis by combining the fractions collected every 1 min based on the peak intensity on the HPLC chromatogram (*see* **Note 18** and Fig. 2).
5. Desalt the combined fractions with a disposable SPE device such as C18 Stage Tip.
6. Elute the sample into a sample vial for MS analysis and then dry it using Speed Vac.
7. Store at −80 °C until MS analysis.

3.8 Comprehensive Analysis by LC-MS/MS

1. Add 10 μL of 2 % acetonitrile and 0.1 % TFA to each sample vial.
2. Vortex each vial for 1 min to dissolve the fractionated peptides and then spin down.
3. Set the operating parameters of the mass spectrometer for iTRAQ analysis (*see* **Note 19**).
4. Analyze each sample by LC-MS/MS (*see* **Note 20**).
5. Apply the acquired raw file to the search software such as MASCOT to identify and quantify the phosphopeptides (*see* **Note 21**).
6. Select the phosphopeptide that has to be validated in the subsequent SRM analysis by quantitatively comparing the identified phosphopeptides.



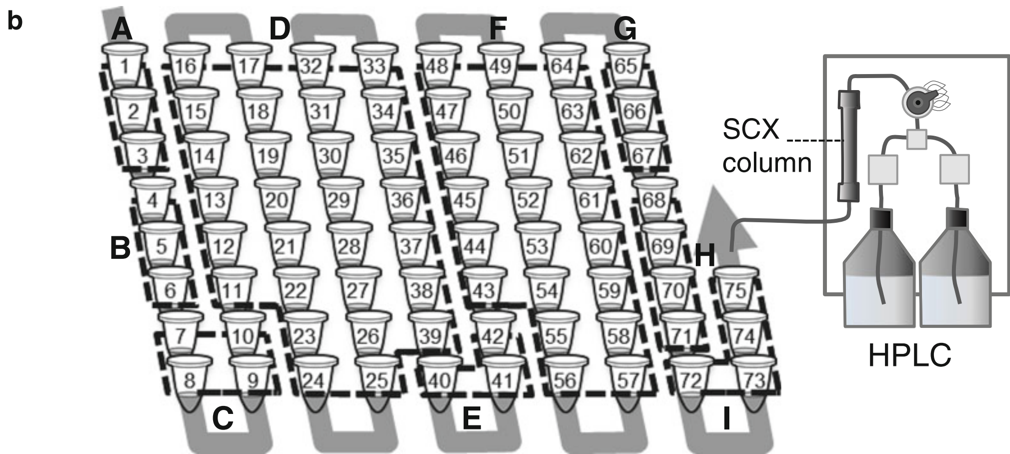


Fig. 2 A SCX chromatography from our study is shown in (**a**). The black trace is the result of iTRAQ-labeled phosphopeptides, which was prepared as indicated in Fig. 1. The gray trace is the baseline. The eluate of the chromatography was fractionated into 75 fractions every 1 min (**b**). The fractions were separated into nine groups (Group-A–I) in a manner dependent on the intensity in the chromatogram as shown in (**a**) and (**b**). The eight groups other than the group-F have low intensities and so all fractions of their groups are each combined into a single fraction

3.9 Sample Preparation for SRM Analysis (for up to 100 μg of Proteins (See Note 22)

1. Prepare a homogenate by processing tissue samples according to the "Homogenizing Tissue" section.
2. Digest the homogenate according to "Protein Digestion" section, **steps 1** and **6**, and prepare the peptide mixture from which the detergent has not yet been removed.
3. Add all SI peptides to each sample (*see* **Notes 23** and **24**).
4. Extract the peptides from the sample according to **steps 7–9** in "Protein Digestion" section.
5. Desalt the resulting sample with a disposable SPE device such as C18 Stage Tip.
6. Prepare the IMAC-C18 Stage Tip by packing 2 layers of C18 resin at the end of a 200 μL pipet tip and then loading 50 μL of Fe-IMAC resin on the C18 resin. Stick the IMAC-C18 Stage Tip to the microcentrifuge tube with a hole made in the center of the lid and remove the solution by centrifugation at 800 × *g* for 2 min.
7. Load the desalted sample in 60 % acetonitrile and 0.1 % TFA to the IMAC-C18 Stage Tip and then centrifuge at 600 × *g* for 5 min.
8. Add 200 μL of 60 % acetonitrile and 0.1 % TFA to the IMAC-C18 Stage Tip and then centrifuge at 800 × *g* for 4 min to wash non-phosphopeptides off the IMAC resin. Repeat this step twice more.
9. Add 200 μL of 0.1 % TFA to the IMAC-C18 Stage Tip and then centrifuge at 800 × *g* for 4 min to equilibrate the C18 resin under the IMAC resin.
10. Add 100 μL of 1 % phosphate to the IMAC-C18 Stage Tip and then centrifuge at 800 × *g* for 2 min to release the phosphopeptides from the IMAC resin and then bind them to the C18 resin. Repeat this step once more.
11. Add 200 μL of 0.1 % TFA to the IMAC-C18 Stage Tip and then centrifuge at 2,300 × *g* for 2 min to wash the C18 resin.
12. Elute the phosphopeptides bound to the C18 resin to a sample tube using 60 μL of 60 % acetonitrile and 0.1 % TFA.
13. Dry the sample using Speed Vac.
14. Store it at −80 °C until MS analysis.

3.10 Targeted Analysis by LC-SRM/MS

1. Analyze a mixture of the stable isotope-labeled peptide (SI peptides), which has the same sequence as the phosphopeptide selected in the former comprehensive analysis, by LC-MS/MS.
2. Create a primary method for the subsequent SRM analysis by analyzing the acquired MS data (*see* **Note 25**) and selecting the precursor ions of each target observed with a strong signal intensity (doubly, triply, or higher charged ions) and the

product ions generated from the precursor ion with a strong signal intensity (*see* **Note 26**).

3. Optimize the parameters (*m/z* of product ions and CE) of the SRM method by LC-SRM/MS (*see* **Note 27**).
4. Add 10 μL of 2 % acetonitrile, 0.1 % TFA, and 25 μg/mL EDTA to each sample.
5. Vortex each vial for 1 min to dissolve the fractionated peptides and then spin down.
6. Set the optimized SRM method and other operating parameters for the SRM analysis (*see* **Note 28**).
7. Analyze each sample by LC-SRM/MS (*see* **Note 29**).
8. Apply the acquired raw data to the software for quantification (*see* **Note 30**). Quantitatively compare the target peptides between the samples by calculating the peak area in a chromatogram of each SRM transition and then normalizing the values of the endogenous targeted peptides to those of the corresponding SI peptides.

4 Notes

1. The procedures used for homogenizing tissue samples and enzymatic digestion are based on phase transfer surfactant (PTS)-aided trypsin digestion as described in a previous study [17].
2. Add 1 mL of water to a bottle containing 1 g of lyophilized LysC.
3. Add 100 μL of 10 mM HCl to a bottle containing 100 μg of lyophilized trypsin.
4. Peptide mixtures are desalted using C18 Stage Tip or another solid-phase extraction (SPE) device such as Oasis HLB. Desalting by C18 Stage Tip is performed as described in a previous study [18]. Briefly, a small 47 mm Empore™ C18 disk is stamped out using a blunt-ended syringe needle (16 G), and then the layers are placed in a 200 μL pipet tip by pushing them from the top of the tip using a plunger. Methanol (for swelling), 80 % acetonitrile, 0.1 % TFA (for washing), and 2 % acetonitrile and 0.1 % TFA (for equilibrating) are passed through by centrifugation the C18 resin in this order. After the sample is passed through to absorb the peptides to the C18 resin, 2 % acetonitrile, and 0.1 % TFA (for washing) is passed through. Elution of the peptide mixture is performed by 60 % acetonitrile and 0.1 % TFA. Loading capacity is 20 μg per layer of C18 resin. The volume of all solutions is 20 μL per layer of C18 resin. When another SPE device is used, desalting is performed according to the instructions of each manufacturer.

5. We mostly replace lysine or arginine at the C-terminal of target peptides with isotope-labeled lysine (13C6, 15 N2) or arginine (13C6, 15 N4) in order to make y-ions heavier. When the amino acid at the C-terminal is not lysine or arginine (e.g., the C-terminal of a protein), we replace the other amino acids (e.g., alanine) at or near the C-terminal with the other isotope-labeled one (e.g., Alanine-13C3–15 N1).
6. We make an ESI column by packing C18 particles by a capillary column packer into a glass capillary needle (200 mm length × 100 μm for the inner diameter) which is made by laser puller.
7. By using Proteome Discoverer 1.3, we obtain the list in which there are the identified phosphopeptides associated with quantitative data (quantitative values obtained from iTRAQ-reporter ions) and qualitative data (Mascot ion score and probability of phosphorylation sites). We export the list into an excel file and follow by editing it (filtering the phosphopeptides by score and merging the results of multiple analysis).
8. In our study, we need 2 mg protein +0.67 mg protein (to make standard mixture) for iTRAQ analysis and 0.5 mg protein for SRM analysis prepared from each sample. To obtain enough amount of protein, we use more than 40 mg of tissue if possible.
9. By adding the buffer to samples at this ratio, we can generally obtain a solution containing 5–15 mg of proteins per mL.
10. After several rounds of sonication, we examine the contents in the tubes in order to check the residual pieces of the tissue. If the tissues are completely dissolved, we stop the sonication. If not, a few rounds of sonication are additionally performed until the size of residual tissues remains the same. At this stage, we consider the proteins to be sufficiently extracted from the tissue and stop the sonication.
11. We use 2 mg of protein from individual tissue sample and 2 mg of protein from standard mixture for iTRAQ analysis and 500 μg protein from individual tissue sample for SRM analysis. The reasons for the amounts of the protein used are as follows. Maximum amount of peptides to load on our LC-MS system is 2–3 μg considering the separation ability of our analytical column with an inner diameter of 100 μm and the robustness of the LC-MS systems. As shown in Figs. 1 and 2, less than 80 μg phosphopeptide is estimated to be obtained for iTRAQ analysis after phosphopeptide enrichment and less than 3 μg phosphopeptide in each combined fraction after SCX chromatography because the amount of phosphoprotein is estimated to be 1 % of the total protein (also accounting for sample loss during our procedures). Similarly, in case of SRM analysis, we estimate less than 3 μg phosphopeptide to be obtained after phosphopeptide enrichment.

12. The ProBond resin is initially provided as 50 % slurry in 20 % ethanol. We use 1 mL of the resin (or 2 mL of the suspension) for up to 2 mg of proteins.
13. When nickel ions are released from the resin by EDTA and washed away, the color of the resins turns from blue to white. If the nickel ions are not released sufficiently based on the color of the resins, repeat this step once more.
14. We store the Fe-IMAC resin as 50 % slurry at 4 °C and use it within 1 week.
15. When we quantitatively compare more than 4 samples by iTRAQ analysis, we use iTRAQ 115, 116, and 117 to label individual samples and iTRAQ 114 as the reference sample. This reference sample is the mixture of an aliquot of all samples and is used as the standard in all iTRAQ experiments.
16. The volume of SCX buffer A needed to dissolve the sample depends on the HPLC systems used. We dissolve the sample in 110 μL of SCX buffer A according to the maximum volume (100 μL) of the autosampler in our HPLC system. 100 μL of the sample is loaded onto the HPLC equipment. The remainder (10 μL) is used to assess iTRAQ labeling by MS analysis.
17. We use a flow rate of 200 μL/min and four-step linear gradient for the separation, as follows: 0 % B for 30 min, 0–10 % B in 15 min, 10–25 % B in 10 min, 25–40 % B in 5 min, 40–100 % B in 5 min, and 100 % B for 10 min.
18. We combine the 75 fractions collected every 1 min into 30 fractions. The flow-through fraction is not combined in case polymer-like contaminants are found in it. If the fractions collected every 1 min are collected at the time when the peak intensities are lower in the HPLC chromatogram, a larger number of samples is combined. If the fractions are collected when the peak intensities are higher, we combine a few fractions or use it for MS analysis as a single fraction (*see* Fig. 2).
19. When we perform iTRAQ analysis using the LTQ-Orbitrap XL or Velos mass spectrometer, the operating parameters are set as follows: full MS scans are performed in the orbitrap mass analyzer (scan range 350–1500 *m/z*, with 30 K FWHM resolution at 400 *m/z*). The three (LTQ XL) or five (LTQ Velos) most intense precursor ions are selected for the MS/MS scans. MS/MS scans are performed using collision-induced dissociation (CID) and higher energy collision-induced dissociation (HCD, 7500 FWHM resolution at 400 *m/z*) for each precursor ion. Collision energy is set to 35 % for CID and 50 % for HCD. A dynamic exclusion option is implemented with a repeat count of 1 and exclusion duration of 60 s. The values of automated gain control (AGC) are set to 5.00e+05 for full MS, 1.00e+04 for CID MS/MS, and 5.00e+04 for HCD MS/MS.

20. We analyze the fractionated peptides using an LTQ-Orbitrap XL or Velos mass spectrometer equipped with a nano HPLC system and HTC-PAL autosampler. The analytical column is self-made by packing C18 particles (L-column2 ODS, 3 μm) into a self-pulled needle (200 mm length × 100 μm for the inner diameter). The mobile phases consist of buffers A (0.1 % formic acid, 2 % acetonitrile) and B (0.1 % formic acid, 90 % acetonitrile). Samples are loaded onto the trap column. The nano LC gradient is delivered at 500 nL/min and consists of a linear gradient of Buffer B developed from 5 to 30 % B in 135 min. A spray voltage of 2000 V is applied.
21. To identify the phosphopeptides, the CID and HCD raw spectra are extracted and searched separately against the forward and reverse-decoy human IPI database (version 3.68) using Proteome Discoverer 1.3 and Mascot v2.2. The precursor mass tolerance is set to 3 ppm and a fragment ion mass tolerance is set to 0.6 Da for CID and 0.01 Da for HCD. The search parameters allow for one missed cleavage for trypsin, fixed modifications (carbamidomethylation at cysteine and iTRAQ labeling at lysine and the N-terminal residue), and variable modifications (oxidation at methionine, iTRAQ labeling at tyrosine, and phosphorylation at serine, threonine, and tyrosine). The score threshold for peptide identification is set at 1 % false-discovery rates (FDR). Peptides identified at a threshold with 5 % FDR are also accepted in cases in which the peptide with the same sequence is identified at a threshold with 1 % FDR in any three other iTRAQ experiments. To quantify the phosphopeptides, we obtain the iTRAQ quantitation values automatically calculated from the intensity of the iTRAQ reporter ions in the HCD scans using the Proteome Discoverer 1.3. Quantitation of peptides identified from CID scans is performed using the reporter ion information extracted from the HCD spectra of the same precursor peptide. In cases in which peptides with the same sequence are identified repeatedly from different precursor peptides in the same iTRAQ experiment, the median of their quantitation values is calculated. The iTRAQ quantitation values of individual samples (iTRAQ 115, 116, and 117) are normalized with the values of the reference sample (iTRAQ 114) in each iTRAQ experiment for comparisons among all of iTRAQ experiments.
22. We use tissue samples containing 500 μg of proteins for SRM-based validation of the results of our large-scale phosphoproteome analysis. Each homogenate containing 500 μg of proteins is equally divided into five tubes, and processing is then performed according to the protocol.
23. In the case of crude SI peptides, the purities are very different between the products especially for phosphopeptides. In addition, the ionization efficiency depends on peptide

sequences. As a result, the signal intensities of SI peptides can be very different. To maintain the robustness of the experimental system, the signal intensity of each SI peptide should be checked by LC-MS/MS before mixing and then the amount of each SI peptide added should be modified based on the signal intensity.

24. When we perform serial dilutions of the SI peptides for the addition of small amounts of peptides, we perform the dilution with a 1 pmol/μL BSA digest solution to prevent adsorption.

25. The SRM method consists of SRM transitions, which mean pairs of *m/z* of the precursor/product ions, the collision energies (CEs), and retention time of the nano HPLC.

26. In our case, the SI peptide mixture is analyzed by LC-MS/MS using LTQ-Orbitrap XL (CID mode) and the msf file is generated using Proteome Discoverer and Mascot. The msf file is opened with Pinpoint software (version 2.3.0, Thermo Scientific) and the list of MS/MS fragment ions derived from SI-peptides is generated. A total of multiple product ions (4–10 product ions) are selected for the SRM transitions of each target peptide based on the following criteria: y-ion series, strong ion intensity, at least 2 amino acids in length, and no neutral loss fragment.

27. At first, we optimize collision energy (CE) for every SRM transition around the theoretical value calculated according to the formulas; $CE = 0.044 \times m/z + 5.5$ for doubly charged precursor ions, and $CE = 0.051 \times m/z + 0.55$ for triply charged precursor ions. In cases in which the theoretical value is over 35 eV, the value is set to 35 eV. After this optimization, the 4 most intense transitions are selected for each target peptide.

28. In addition to the SRM method (SRM transitions, CE, and the retention time for the each target peptide), the parameters of the instrument are set as follows; a scan width of 0.002 *m/z*, Q1 resolution of 0.7 FWHM, cycle time of 1 s, and gas pressure of 1.8 mTorr. Data are acquired in the time-scheduled SRM mode (retention time window: 8 min).

29. We use the TSQ-Vantage triple quadruple mass spectrometer equipped with the LC system described above. The nanoLC gradient is delivered at 300 nL/min and consists of a linear gradient of mobile phase B developed from 5 to 23 % B in 45 min. A spray voltage of 1800 V is applied.

30. We use Pinpoint, software for the analysis of SRM data. SRM transitions with more than 1×10^3 ion intensity at the peak are used for quantitation. We check that the ratios among the peak areas of individual SRM transitions for each targeted phosphopeptide are comparable to those of the corresponding SI peptide.

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