Chapter 15

High-Throughput Characterization of Histidine Phosphorylation Sites Using UPAX and Tandem Mass Spectrometry

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Abstract

Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) is key for the characterization of phosphorylation sites in a high-throughput manner, and its application has proven essential to elucidate the phosphoproteome of many biological systems. Following proteolytic digestion of proteins extracted from tissues or cells, phosphopeptides are typically enriched by affinity chromatography using TiO_2 or metal-ions (*e.g.*, Fe^{3+}) coupled to solid-phase materials, prior to LC-MS/MS analysis. Separation of relatively low abundance phosphopeptides from nonphosphorylated peptides in these types of extremely complex mixtures is essential to maximize coverage of the phosphoproteome. Maintaining acidic conditions during these IMAC or TiO_2-based enrichment minimizes the concurrent unwanted binding of highly acidic peptides. However, while peptides containing phosphomonoesters, namely, phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr), are stable under these acidic binding conditions, phosphopeptides containing acid-labile phosphate group such as phosphohistidine (pHis), are not. Consequently, hydrolysis of these types of phosphopeptides occurs during standard phosphopeptide enrichment, and subsequent phosphosite identification by LC-MS/MS is severely compromised. Here we describe UPAX, unbiased phosphopeptide enrichment using strong anion exchange, for the separation of both acid-stable (pSer, pThr, pTyr) and acid-labile phosphopeptides (including those containing pHis) from nonphosphorylated peptides. We outline how implementation of UPAX prior to a minimally modified standard proteomics workflow can be used to identify sites of pHis as well as other acid-labile, as well as acid-stable phosphosites.

Key words Phosphohistidine, pHis, Phosphoproteomics, Mass spectrometry, Strong anion exchange, Enrichment

1 Introduction

Over the last two decades, mass spectrometry (MS)-based phosphoproteomics has proved invaluable for defining sites of phosphorylation on serine, threonine and tyrosine residues, in both low- and high-throughput studies. Although histidine phosphorylation is known to be important in relaying the extracellular signals that drive intracellular responses in a variety of organisms,

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characterization of sites of phosphohistidine (pHis) by MS (or other analytical strategies) has remained a significant challenge, and interrogation of pHis lags far behind its canonical counterparts. The phosphoramidate bond of pHis is both heat- and acid-labile, with a -ΔG value of −12 to −14 kcal/mol compared to approximately −6.5 to −9.5 kcal/mol for the phosphoester bonds found in phosphoserine, or phosphothreonine [1]. Consequently the phosphate group of pHis is unstable under the acidic conditions typically used in standard phosphoproteomics workflows and the rapid hydrolysis hampers phosphosite mapping [2, 3].

A key stage in any phosphoproteomics workflow is the enrichment of phosphopeptides following proteolysis of a complex protein extract. Separation of phosphopeptides from the background of nonphosphorylated peptides which are present in vast excess, is essential for sensitive phosphopeptide identification and phosphorylation site identification by tandem mass spectrometry (MS/MS) due to both the relatively low abundance of phosphopeptides, where modification of an individual residues is typically sub-stoichiometric, and the compromising effect that addition of the negatively charged phosphate group can have on peptide ionization efficiency. Most of the currently available phosphopeptide enrichment approaches rely on acidic conditions to minimize unwanted binding of acidic peptides, which is not suitable for pHis due to the significant hydrolysis observed for both 1- and 3-pHis.

Strong anion exchange (SAX) chromatography can facilitate peptide separation based on electrostatic interactions of negatively charged groups with a positively charged stationary phase. Elution from the SAX column can be elicited by either a decreasing pH gradient [4–6], or increasing salt concentration at a constant pH [7]. At low pH, the negative charge of a single phosphate group is often not sufficient to overcome the electrostatic repulsion conferred by the N-terminus and the side chain of the C-terminal amino acid, resulting in poor retention of singly phosphorylated peptides. However, above ~pH 6 a second negative charge is acquired, making SAX chromatography feasible for separation of negatively charged phosphopeptides from the majority of peptides which do not contain a net negative charge [6]. Crucially, as the phosphate group of pHis peptides is stable above pH 6, SAX fractionation under these conditions is an attractive strategy for separation of acid-labile phosphopeptides, such as those containing pHis, from the majority of nonphosphorylated peptides, facilitating MS-based site identification. Here we describe a procedure termed UPAX (*u*nbiased *p*hosphopeptide enrichment by strong *a*nion e*x*change) for the separation and enrichment of phosphopeptides, including acid-labile pHis-containing peptides, permitting pHis phosphosite characterization by LC-MS/MS [8, 9].

2 Materials

Use HPLC-grade solvents and acids to prepare all solutions, and analytical grade buffers throughout. Low-bind Eppendorf tubes (or similar) should be used to minimize sample loss through adsorption to the vessel.

2.1 Sample Preparation

1. Dithiothreitol (DTT): 100 mM DTT in 50 mM ammonium bicarbonate (AmBic).
2. Iodoacetamide (IOA): 250 mM IOA in 50 mM AmBic.
3. Sequencing grade modified trypsin: reconstitute in 50 mM acetic acid at 0.5 mg/mL (*see* **Note 1**).
4. Heating block.
5. Benchtop centrifuge.

2.2 Strong Anion Exchange (SAX) Chromatography

1. Sonicating water bath.
2. SAX buffer A: 20 mM ammonium acetate, pH 6.8, 10% (v/v) acetonitrile (MeCN).
3. SAX buffer B: 300 mM triethylammonium phosphate, pH 6.8, 10% (v/v) MeCN (*see* **Note 2**).
4. SAX column: PolySAX LP column (PolyLC; 4.6 mm × 200 mm, 5 μm particle size, 300 Å).
5. HPLC system such as the Dionex U3000 HPLC instrument, equipped with a fraction collector and a UV detector capable of measurement at 280 nm (*see* **Note 3**).
6. Vacuum centrifuge.

2.3 Peptide Desalting

1. StageTips: 200 μL pipette tip containing 3 discs of C18 material (Empore™ Octadecyl C18, 47 mm) (*see* **Note 4**).
2. C18 StageTip elution buffer: 50% (v/v) MeCN in H_2O.
3. Methanol.

2.4 Liquid Chromatography (LC)-Tandem Mass Spectrometry (MS/MS) Analysis

1. MS dilution buffer: 3% (v/v) MeCN in H_2O.
2. C18 trap wash buffer: 2% (v/v) MeCN, 0.1% (v/v) TFA in H_2O.
3. LC-MS buffer A: 0.1% (v/v) formic acid in H_2O.
4. LC-MS buffer B: 0.1% (v/v) formic acid, 80% (v/v) MeCN in H_2O.
5. High resolution mass spectrometry system with online nanoUPLC system capable of high energy (beam-type) CID. For these studies we use a Thermo Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific) attached to an UltiMate 3000 nano system (Dionex).

6. C18 trapping column: PepMap100, C18, 300 μm × 5 mm (Thermo Scientific).
7. C18 analytical column: Easy-Spray C18, 75 μm × 500 mm, 2 μm bead diameter.

2.5 LC-MS/MS Data Processing

1. Proteome Discoverer (PD) with *ptm*RS node (for these studies we used PD version 1.4).
2. Mascot (Matrix Science).

3 Methods

3.1 Sample Preparation

Proteins should be extracted from the required cell line or tissue according to sample specific protocols, based on the individual requirements of the biological material (*see* **Note 5**). To avoid heating of the sample or treatment under acidic conditions, proteins are typically extracted in a urea-based buffer (8 M urea) (*see* **Note 6**), and ~2 mg of protein per sample is digested (*see* **Note 7**).

1. Add 100 mM DTT to the protein lysate solution to achieve a final DTT concentration of 3 mM. Incubate at 30 °C for 20 min (*see* **Note 8**). Cool the sample to room temperature.
2. Add 250 mM IOA to achieve a final IOA concentration of 14 mM. Incubate at room temperature in the dark for 45 min.
3. Quench excess IOA by addition of sufficient DTT to achieve a final concentration in the protein sample of 7 mM. Dilute the sample by addition of 50 mM AmBic such that the urea concentration is at or below 2 M.
4. Add 2% (w/w) trypsin and incubate at 30 °C with shaking at 650 rpm overnight (~16 h).
5. Following overnight digestion, dry the samples to completion by vacuum centrifugation. Dried samples can be stored at −20 °C if necessary, or ideally subjected immediately to SAX fractionation.

3.2 SAX Fractionation

1. Wash the SAX column with 20 column volumes (CV) of SAX buffer B, then equilibrate with at least 20 CV of SAX buffer A (*see* **Note 9**).
2. Resolubilize the dried digested peptide samples in 180 μL SAX buffer A. Leave in a sonicating water bath for 5 min to aid recovery of peptides from the Eppendorf tubes (*see* **Note 10**).
3. Load the resolubilized peptide sample onto the column in 100% SAX buffer A using the same flow rate as for **step 1** (*see* **Notes 9** and **11**). Wash the column for 5 min with 100% SAX buffer A. Collect unbound peptides and store on ice.

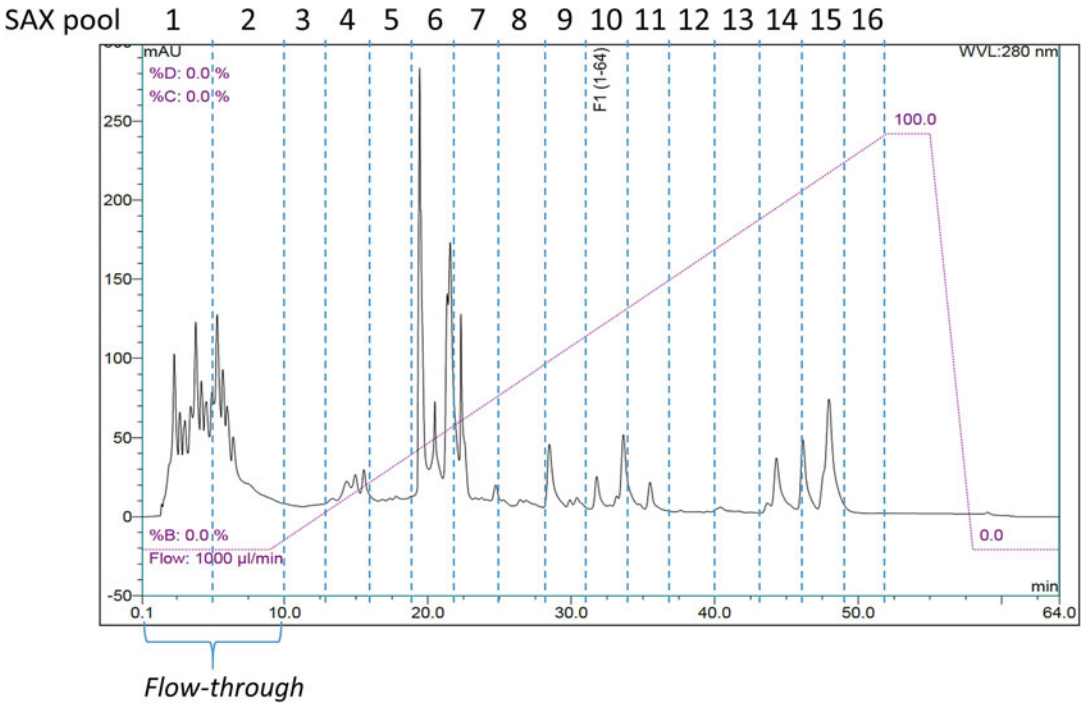

Fig. 1 Example SAX chromatogram (280 nm absorbance) following separation of 2 mg HeLa cell tryptic peptides on a PolySAX LP column. SAX buffer A: 20 mM ammonium acetate (pH 6.8), 10% MeCN; SAX buffer B: 300 mM triethylammonium phosphate (pH 6.8), 10% MeCN. Fractions are collected every minute (1 mL/min flow rate) and pooled as indicated

4. Elute bound peptides with a gradient of increasing concentration of SAX buffer B to 100% over 43 min. Maintain at 100% SAX buffer B for 5 min before equilibration to start conditions (100% SAX buffer A). Collect fractions every minute until the end of the gradient (48 min) and store them on ice. An example SAX chromatogram, with pooled fractions is shown in Fig. 1.
5. Pool the fractions to reduce the overall number of fractions for LC-MS/MS analysis to 16 (*see* **Note 12**). Reduce the volume of the pooled samples by vacuum centrifugation so that the volume of each pooled sample is ~500 µL (*see* **Notes 13** and **14**).

3.3 Peptide Desalting

1. Pack three discs of C18 material into a 200 µl pipette tip (*see* **Note 4**), repeat to create 16 StageTips, one for each set of pooled SAX fractions. Place the tips into Eppendorf tubes, held in place with a plastic stopper (*see* **Note 15**).
2. Condition each of the StageTips tips by sequential addition of 100 µL methanol, 100 µL 50% MeCN in H_2O and 100 µL H_2O, centrifuging for 2 min at 2000 × *g* between each step to pass the liquid through the tip (*see* **Note 16**).

3. Load 150 μL of sample onto each tip, centrifuge as in **step 2**, load the flow through back into the tip and centrifuge again (*see* **Note 17**). Ensure all the liquid has passed through the tip at each stage.
4. Wash each of the tips with 100 μL H_2O, then elute bound peptides with 50 μL 50% MeCN in H_2O into a fresh low-bind Eppendorf tube (*see* **Note 18**).
5. Dry eluents to completion by vacuum centrifugation. Samples are now ready for LC-MS/MS analysis and can be stored at −20 °C until needed.

3.4 LC-MS/MS Analysis

1. Resolubilize the dried desalted SAX pools in an appropriate volume of MS dilution buffer. Sonicate the fractions for 5 min in the sonicating water bath. Centrifuge in a bench-top at 15,000 × *g* for 15 min, then transfer to glass LC-MS vials.
2. Using a nanoUPLC system arranged in-line with the mass spectrometer, load the peptides onto a C18 trapping column using partial loop injection, for 7 min at a flow rate of 9 μL/min with C18 trap wash buffer. Resolve bound peptides at 300 nL/min using the C18 analytical column using an LC gradient from 3.8% LC-MS buffer B (96.2% LC-MS buffer A) to 50% buffer B (50% buffer A) over 90 min.
3. Acquire a full mass spectrum over *m/z* 350–2000 in the Orbitrap (120K resolution at *m/z* 200). Perform data-dependent MS/MS analysis using a top speed approach (cycle time of 3 s), with HCD (collision energy 32%, max injection time 35 ms) and neutral loss-triggered (Δ80 and Δ98 amu) EThcD (ETD reaction time 50 ms, max ETD reagent injection time 200 ms, supplemental activation energy 25%, max injection time 50 ms) for fragmentation. Detect all product ions in the ion trap (rapid mode) (*see* **Note 19**).

3.5 Data Processing

1. Convert .raw files to .mzml using ProteoWizard's msconvert tool in order to perform MS2-level deisotoping.
2. Using Proteome Discoverer (PD), split scans for each raw file into those arising from HCD and EThcD events using a collision energy (CE) filter (HCD: min CE 0, max CE 34; EThcD: min CE 35, max CE 1000) to generate two separate .mgf files. An example Proteome Discoverer workflow is shown in Fig. 2.
3. Search the .mgf files in PD using the MASCOT search algorithm against the appropriate database. For human derived cell extracts, we use the UniProt Human database. Set search parameters as follows: MS1 tolerance of 10 ppm; MS2 mass tolerance of 0.6 Da; enzyme specificity as trypsin, with two missed cleavages allowed; fixed modification: carbamidomethylation of Cys; variable modifications: phosphorylation of Ser, Thr,

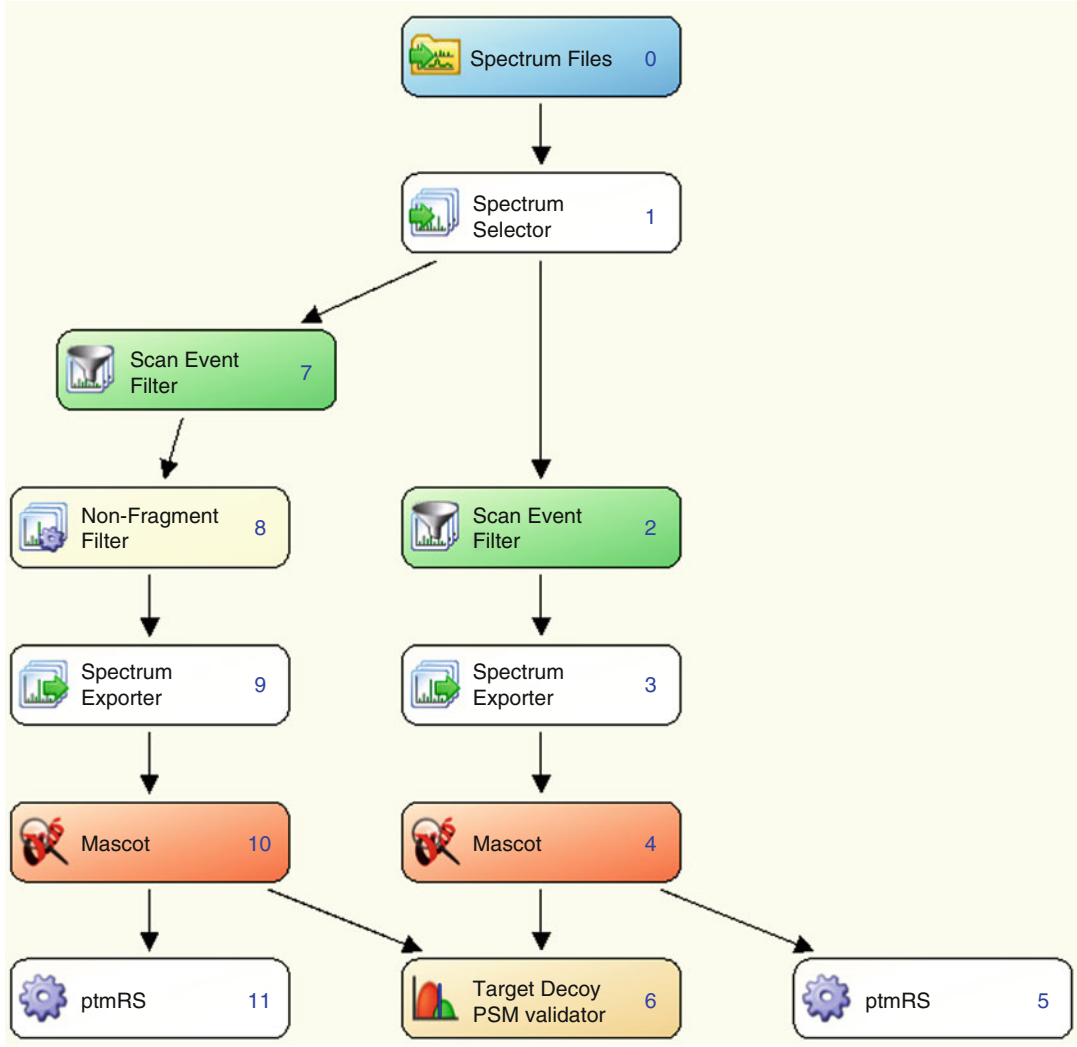

Fig. 2 Proteome discoverer data processing pipeline. Tandem mass spectra are separated according to fragmentation type prior to searching with MASCOT and application of ptmRS for phosphosite localization

Tyr, and His, oxidation of Met; instrument type set as ESI-Quad-TOF for HCD files and CID + ETD for EThcD files (*see* **Note 20**).

4. Analyze phosphopeptide spectra using the ptmRS node, with the "treat all spectra as EThcD" option selected for EThcD data (*see* **Note 21**).
5. Apply a peptide false discovery rate (FDR) filter (typically 1% or 5%) and export files to .csv for further data processing.
6. Process the exported .csv to identify phosphosites above desired ptmRS score cut-off. Scores above 0.75 are generally considered to be "localized," although we typically apply a

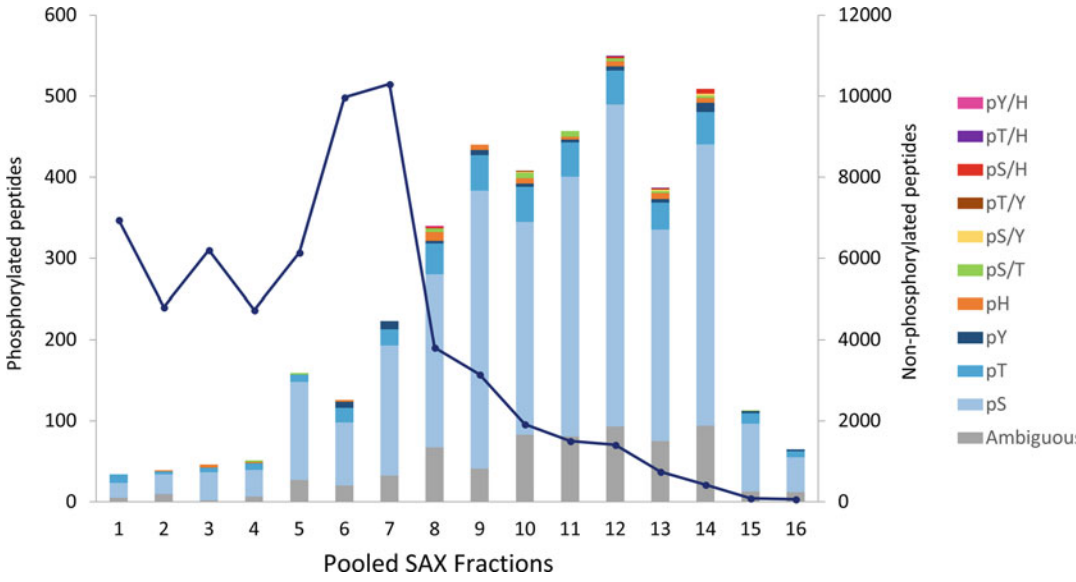

Fig. 3 Phosphopeptides identified in each pooled SAX fraction following separation of peptides from 2 mg HeLa cell lysate. Phosphosites with ptmRS score >0.75 are considered localized, whilst any peptide with an unassigned site is shown as "Ambiguous." The number of nonphosphorylated peptides in each fraction is also plotted, showing a proportional decrease in the number of non-phosphorylated peptides identified in the later SAX fractions, coinciding with an increase in the number of phosphopeptide identifications

more stringent 1% false localization rate (FLR) cut-off defined according to the mode of MS2 fragmentation and analysis [9, 10] (Fig. 3). More detailed strategies for analysis of this type of MS data are presented in Chapter 16.

4 Notes

1. Acidic stock solutions of trypsin can be aliquoted and frozen at 20 °C for up to 6 months.
2. Make up SAX buffer B by adding 30% volume of 1 M phosphoric acid to a graduated cylinder. Adjust the pH to 6.8 with trimethylamine – this is typically at least 10 mL per 250 mL total buffer volume. Add 20% volume of MeCN and make up to volume with water.
3. Any offline HPLC system can be used, ideally one that also has a fraction collector. If a fraction collector is not available, fractions can be collected manually, although this is more time consuming. Retain all fractions on ice.
4. Using a Plunger Assembly for a Hamilton Syringe, punch a disk of 3M-C18 material and carefully dispense into a 200 µl tip. Repeat twice so that for each sample, you have constructed a 200 µl C18-StageTip with three layers of C18 material [11].

5. pHis-containing samples should be kept on ice as much as possible. Temperatures above 30 °C should be avoided to minimize heat-induced loss of the labile pHis group. All sample processing procedures should be carried out as quickly as possible within minimal time between stages. Be sure to include phosphatase and protease inhibitors in cell/tissue lysis buffers. Care must be taken to avoid sample processing procedures that require acid-based treatment.
6. Mammalian cells grown in culture can be lysed in 50 mM AmBic, 8 M Urea with protease/phosphatase inhibitors. We have not tested any other lysis buffer compositions for compatibility with the SAX fractionation.
7. For large-scale phosphoproteome analysis we typically prepare 2 mg of protein and fractionate all of this by SAX. The amount of protein in the sample should be measured, for example, by Bradford assay or using a NanoDrop system, and used to calculate the amount of trypsin required (2% w/w).
8. The temperature of these samples must be carefully controlled — all incubation/digestion steps are performed at a maximum of 30 °C to minimize loss of pHis.
9. For the PolySAX LP column (4.6 mm × 200 mm) described here, we used a flow rate of 1 mL/min.
10. To minimize sample adsorption, we use low-bind tubes at all steps.
11. Sample injection onto the HPLC column will be dependent on the size of the injection loop. If the sample loop is at least 200 μL, all 180 μL can be injected in a single step. For smaller injection loops, two or three injections of smaller volumes can be made, that is, load 60 μL into the loop, switch the valve to wash sample onto the column for 90 seconds, switch the valve back to load a further 60 μL into the loop, switch again to load this portion of the sample onto the column then repeat again to load the final 60 μL. The column can then be washed with 100% SAX buffer A for 5 min as described in **step 3**. If the sample is delivered in multiple steps, then the fractions corresponding to this loading portion should be combined.
12. Fractions can be combined every 3, so that SAX fractions 1–3 become pool 1, fractions 4–6 pool 2 etc. Alternatively, as the latter fractions are more enriched in phosphopeptides, SAX fractions can be differentially combined so that more fractions in the earlier part of the gradient (which are less phosphopeptide rich) are combined (e.g., SAX fraction 1–6 combined to generate pool 1), while latter fractions are kept as separate pools. This can increase the phosphopeptide coverage.

13. Fractions are only dried to 500 μL (rather than to completion) as the high concentration of nonvolatile salt in the later fractions prevents total drying. Volumes smaller than ~500 μL have such a high salt concentration that C18 StageTip desalting becomes problematic; liquid does not easily pass through the tip.
14. Pooled SAX fractions can be stored frozen (−20 °C) after drying to 500 μL, although we recommend proceeding to the desalting step as soon as possible (i.e., within a day) to minimize sample degradation.
15. High concentrations of triethylammonium phosphate following SAX fractionation must be removed prior to LC-MS/MS analysis. C18 StageTips prepared in-house are a suitable choice for desalting [11], although commercial prepacked tip or column options are also available for peptide desalting.
16. When using StageTips be sure that the liquid in the Eppendorf tube does not reach the bottom of the tip. We typically use 2 mL low bind Eppendorf tubes to reduce the risk of this happening, although the 1.5 mL tubes also work.
17. For the workflow described here, desalting 150 μL of sample (out of the 500 μL volume remaining after drying) is sufficient based on a 2 mg starting amount of protein. However, if the amount of material in a given pool is low (based on A_{280}) or a different concatenation strategy is employed where fewer SAX fractions are pooled, it may be necessary to desalt more (or all) of each sample.
18. Following desalting (and prior to drying) peptide concentration can be estimated by NanoDrop. Peptide concentration estimates can be used to assist in determining the amount of each sample to load for LC-MS/MS analysis.
19. Suggested starting parameters for a Thermo Orbitrap Fusion mass spectrometer are described. Both HCD and ETD are implemented, with ETD fragmentation triggered by neutral loss of 80 or 98 amu from the precursor ion. It is recommended that sample loading and mass spectrometer parameters are optimised for a specific instrument set-up. While we describe low-resolution ion trap-based MS2 analysis, fragment ion analysis can also be performed in the orbitrap.
20. Search parameters should be adjusted according to the instrument set up: acquisition of MS2 data in the orbitrap will require a reduction to MS2 tolerance to 10 ppm. Alternative modifications may also be considered (e.g., Asn deamidation, N-terminal acetylation, phosphorylation of residues other than Ser, Thr, Tyr, and His).
21. For phosphopeptide identification it is important to have confidence in both the sequence and the site of modification.

Determining with confidence the position of modification when there is more than one possibility can be challenging. It is therefore beneficial to apply a localization score to indicate confidence in a given site of modification. A suggested pipeline for processing LC-MS/MS data utilises Proteome Discoverer with the MASCOT search engine [12] and ptmRS for phosphosite localization [13].

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