# Chapter 5

# Optimized Enrichment of Phosphoproteomes by Fe-IMAC Column Chromatography

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## Abstract

Phosphorylation is among the most important post-translational modifications of proteins and has numerous regulatory functions across all domains of life. However, phosphorylation is often substoichiometric, requiring selective and sensitive methods to enrich phosphorylated peptides from complex cellular digests. Various methods have been devised for this purpose and we have recently described a Fe-IMAC HPLC column chromatography setup which is capable of comprehensive, reproducible, and selective enrichment of phosphopeptides out of complex peptide mixtures. In contrast to other formats such as StageTips or batch incubations using $TiO_2$ or Ti-IMAC beads, Fe-IMAC HPLC columns do not suffer from issues regarding incomplete phosphopeptide binding or elution and enrichment efficiency scales linearly with the amount of starting material. Here, we provide a step-by-step protocol for the entire phosphopeptide enrichment procedure including sample preparation (lysis, digestion, desalting), Fe-IMAC column chromatography (column setup, operation, charging), measurement by LC-MS/MS (nHPLC gradient, MS parameters) and data analysis (MaxQuant). To increase throughput, we have optimized several key steps such as the gradient time of the Fe-IMAC separation (15 min per enrichment), the number of consecutive enrichments possible between two chargings (>20) and the column recharging itself (<1 h). We show that the application of this protocol enables the selective (>90%) identification of more than 10,000 unique phosphopeptides from 1 mg of HeLa digest within 2 h of measurement time (Q Exactive Plus).

**Key words** Phosphorylation, Proteomics, Phosphocapture, LC-MS

## Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| AGC | Acquisition gain control |
| CAA | Chloroacetamide |
| DTT | Dithiothreitol |
| FA | Formic acid |
| FCS | Fetal calf serum |
| HCD | Higher energy collision induced dissociation |
| HCl | Hydrochloride |
| HPLC | High-performance liquid chromatography |

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| | |
|---|---|
| I.D. | Inner diameter |
| IMAC | Immobilized metal ion affinity chromatography |
| MeOH | Methanol |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| PBS | Phosphate buffered saline |
| Ppm | parts per million |
| PSM | Peptide spectrum match |
| pY/pS/Pt | Phosphotyrosine, -serine, -threonine |
| TFA | Trifluoro acetic acid |
| $TiO_2$ | Titanium dioxide |
| Tris | Tris(hydroxymethyl)aminomethane |
| v/v | Volume/volume |
| w/w | Weight/weight |
| $ZrO_2$ | Zirconium dioxide |

## 1 Introduction

Reversible protein phosphorylation is a posttranslational modification that plays a key role in signal transduction and aberrant regulation has been implicated in a number of diseases [1]. As a consequence, mass spectrometry-based large-scale identification of phosphorylation events has received considerable attention over the last years. Due to the low abundance and sub stoichiometric levels of the phosphorylation events, enrichment of phosphopeptides or phosphoproteins is required prior to mass spectrometric detection [2, 3]. In the field of phosphoproteomics, various large-scale enrichment strategies have been developed over the last years, all with their own strengths and weaknesses. The most widely used enrichment strategies utilize the affinity of phosphate groups to metal ions (immobilized on a solid support). Examples of such strategies are metal oxide affinity chromatography ($TiO_2$, $ZrO_2$) [4, 5], immobilized metal ion affinity chromatography (IMAC) with different metal ions ($Fe^{3+}$, $Ga^{3+}$, or $Zr^{4+}$) [6–8] and Ti-IMAC [9]. It is thought that there is a high degree of complementarity between these different enrichment materials [9–12]; however, we have recently shown that such complementarity can be attributed to the format of the enrichment, the inefficient elution from the material, as well as the insufficient acquisition speed of the mass spectrometer, rather than the material [13]. To date, most phosphopeptide enrichments are still performed in either batch mode or in micro-column format with the material packed in gel-loader tips. These formats suffer from a high degree of variability due to the multitude of manual handling steps in these protocols. In addition, variability is further increased by the use of, for example, different loading solvents, different washing procedures and incubation times [14, 15]. More importantly, the enrichment efficiency and selectivity is largely dependent on the so called

bead-to-sample ratio [16, 17]. Consequently, batch- and tip-based enrichment strategies require optimization for each sample. Here, we describe the workflow for the reproducible and comprehensive enrichment of phosphopeptides using Fe-IMAC HPLC columns that overcomes most of these issues. As reported previously, the Fe-IMAC column does not suffer from bead-to-sample ratio issues and allows for the comprehensive depletion of phosphopeptides from digests without showing any bias in the type of phosphopeptides that are enriched [13]. The protocol describes the entire workflow starting from sample preparation to data analysis. Moreover, it includes several improvements over the published method such as shortened gradient length and improved column recharging, ultimately resulting in considerably increased throughput. We provide a detailed description of the column setup and operation (including charging of the columns and gradients) and describe guidelines for monitoring column performance. Finally, we apply the protocol to the enrichment phosphopeptides from 1 mg cell line digest which led to the identification of >10,000 unique phosphopeptides in 2 h of measurement time.

## 2 Material

Unless stated otherwise, all solvents and solutions are prepared fresh, using ultrapure water and analytical grade reagents. Devices such as centrifuges, vacuum centrifuges/lyophilizer, thermoshaker, or refrigerators (−20 °C/−80 °C) are not explicitly listed.

### *2.1 Preparation of Proteome Digests for Phosphopeptide Analysis*

1. Cell culture: RPMI 1640 medium supplemented with 10% fetal calve serum (FCS). Add 55 ml FCS to 500 ml RPMI 1640 medium. Sterile phosphate buffered saline (PBS) without calcium and magnesium. 150 × 20 mm cell culture dishes. Cell scraper. HeLa S3 cervix carcinoma cells (DMSZ, Braunschweig, Germany).
2. Lysis buffer: Prepare a 40 mM Tris(hydroxymethyl)aminomethane (Tris)–HCl, pH = 7.6 solution, containing 8 M urea, protease and phosphatase inhibitors. Prepare a stock solution of 2 M Tris–HCl by dissolving 2.42 g Tris in 5 ml water, adjust the pH to 7.6 using a 5 M HCl solution and fill up to 10 ml with water. A 100 fold stock solution of phosphatase inhibitor cocktail 1, 2, and 3 is commercially available (Sigma Aldrich, Munich, Germany). Add 4.8 g of urea to a 15 ml falcon tube, add 200 µl Tris–HCl stock solution, add one protease inhibitor tablet complete mini EDTA-free (Roche, Mannheim, Germany) and 100 µl of each phosphatase inhibitor stock solution and fill up to 10 ml with water. Store the lysis buffer on ice.
3. Reducing agent: 1 M stock solution of dithiothreitol (DTT) in water. Dissolve 1.54 g DTT in a falcon tube and fill up to 10 ml

with water. Prepare 200 µl aliquots and store the reducing agent at −20 °C.

4. Alkylating agent: 550 mM stock solution of CAA (CAA) in water. Dissolve 514 mg CAA in a falcon tube and fill up to 10 ml with water. Prepare 200 µl aliquots and store the alkylating agent at −20 °C.
5. 40 mM Tris–HCl solution, pH 7.6: Prepare a stock solution of 2 M Tris–HCl by dissolving 2.42 g Tris in 5 ml water. Adjust the pH to 7.6 using a 5 M HCl solution and fill up to 10 ml with water. Take 200 µl of the 2 M stock solution and fill up to 10 ml with water.
6. Trypsin stock solution: Prepare a stock solution of 1 µg/µl trypsin (sequencing grade modified trypsin, Promega) in 50 mM acetic acid, store at −80 °C.
7. Sep-Pak C-18 peptide purification: 50 mg Sep-Pak cartridges (Waters Corp., Eschborn, Germany). Solvent A: 0.07% (v/v) TFA in water. Dilute 70 µl of 100% TFA in 99.93 ml water. Solvent B: 50% (v/v) ACN, 0.07% (v/v) TFA in water. Prepare 50 ml ACN and 70 µl of TFA and dilute in 49.93 ml water. Store at 4 °C.
8. Vacuum manifold for Sep-Pak desalting.

### 2.2 Phosphopeptide Enrichment by Fe-IMAC Column Chromatography

To avoid column clogging, solvents should be degassed and vacuum-filtered prior to use.

1. Formic acid solvent: 100 ml of 0.1% FA (v/v) in water.
2. IMAC charging solvent: 250 ml of 25 mM $FeCl_3$ (reagent grade, Sigma-Aldrich, Product No. 157740) in 100 mM acetic acid. Put 200 ml water into a cylinder, add 1.43 ml acetic acid and fill up to 250 ml with water. Add 1.014 g $FeCl_3$ and use a magnetic stirrer to dissolve $FeCl_3$. Leave the solution stirring for 30 min and vacuum filter it afterwards to remove insoluble $FeCl_3$ remnants. Store the filtered solution at 4 °C.
3. IMAC stripping solvent: 250 ml of 50 mM EDTA in water, pH 8. Use a magnetic stirrer to dissolve 3.653 g EDTA in 200 ml water. Add 5 M NaOH solution until the EDTA is dissolved, adjust the pH to 8 and fill up to 250 ml with water. Vacuum filter the solvent to remove insoluble EDTA remnants and store at 4 °C.
4. IMAC loading solvent: 1 l of 0.07% (v/v) TFA in 30% (v/v) ACN. Always prepare fresh.
5. IMAC elution solvent: 100 ml of 0.3% (v/v) $NH_4OH$ in water (caution, *see* **Note 1**).
6. ProPack IMAC-10 column: 10 µm, nonporous, polymeric beads; 4 mm inner diameter × 50 mm length (Thermo Scientific, Product No. 063276).

7. HPLC system with the following requirements: flow rates ranging from 0.1 ml/min to 4 ml/min; 0.5–1 ml sample loop; UV detector set to read fixed wavelengths of 214 nm and 280 nm; stable at pH 2–12.

### 2.3 Desalting of the Fe-IMAC Eluate

1. Preparation of the C-18 StageTips: A detailed instruction on how to construct micro-column tips is provided in Chapter 8. Prepare a StageTip containing five C-18 disks (Empore Octadecyl C-18 47 mm Solid Phase Extraction Disks #2215, 3 M Purification, Eagan, MN, USA).
2. Desalting solvents: Solvent A: prepare 5 ml of 0.07% (v/v) TFA in water. Solvent B: prepare 5 ml of 0.07% (v/v) TFA and 60% (v/v) ACN in water.

### 2.4 LC-MS/MS and Data Analysis

1. 50 mM citric acid and 1% (v/v) FA in water. Dissolve 2.1 g of citric acid in 9.9 ml of water and add 100 µl of FA.
2. LC-MS/MS: nano-HPLC setup coupled to a high resolution mass spectrometer. Here, we use an Eksigent NanoLC-Ultra 1D+ (Eksigent, Dublin, CA) coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen). LC-trap column: 75 µm × 2 cm, packed with 5 µm Reprosil-Pur ODS-3 C-18 material (Dr. Maisch, Ammerbuch, Germany). Analytical column: 75 µm × 42 cm, packed with 3 µm Reprosil-Gold C-18 material (Dr. Maisch, Ammerbuch, Germany).
3. Nano-HPLC solvents: Loading solvent: 0.1% (v/v) FA in water. Solvent A: 0.1% (v/v) FA and 5% (v/v) DMSO [18] in water. Solvent B: 0.1% (v/v) FA and 5% (v/v) DMSO in ACN.
4. Data analysis: Freely available MaxQuant [19] software package (e.g., version 1.5.2.8) with the integrated search engine Andromeda [20]. Protein sequence database in FASTA format (e.g., UniprotKB).
5. Spreadsheet editor or the freely available Perseus software package.

## 3 Methods

### 3.1 Preparation of Proteome Digests for Phosphopeptide Analysis

A schematic overview of the experimental steps covered in this protocol is provided in Fig. 1.

1. Seed HeLa cells under sterile conditions in RPMI 1640 medium supplemented with 10% FCS. Use 30 ml medium for 150 mm cell culture dishes. Grow the cells to 80% confluency under humidified atmosphere, 5% $CO_2$ at 37 °C. For lysis, place the cell culture dishes on ice or work at 4 °C. Wash cells two times with cold PBS. Use a pipette to aspirate residual PBS from cell culture plates after the final washing step (*see* **Note 2**).

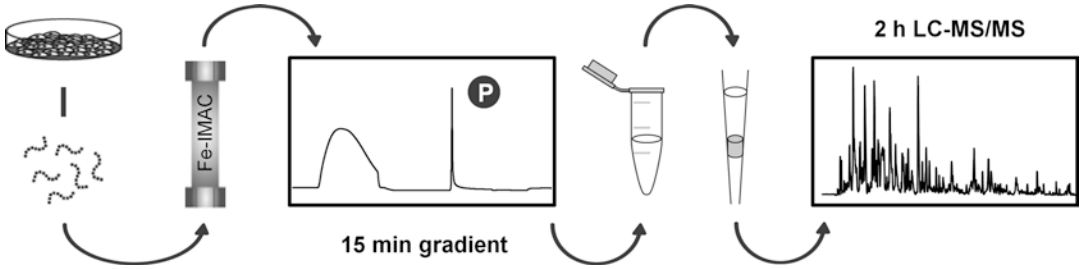


**Fig. 1** Experimental workflow for comprehensive phosphopeptide enrichment depicting cell culture and lysis, phosphopeptide enrichment using the Fe-IMAC column, desalting of enriched phosphopeptides, and LC-MS/MS analysis

2. Add 550 µl of precooled lysis buffer to cell culture dishes. Carefully pan the dish to distribute the lysis buffer evenly over all cells and incubate the dishes for 10 min on ice. Use a cell scraper to mix cell lysate in the cell culture plates. Transfer cell lysates to 1.5 ml reaction vessels and spin down insoluble debris for 20 min at 21,000 × *g* and 4 °C. Transfer supernatant to a new tube. Use a Bradford assay (or similar photometric assay) to determine the protein concentration. Store lysates at −80 °C or continue directly.
3. Use 1 mg of protein lysate. To reduce disulfide bonds, 1 M DTT stock solution is added to a final concentration of 10 mM (1:100 dilution). Incubate in a thermoshaker for 40 min at 37 °C and 700 rpm.
4. For alkylation of cysteine residues, add 550 mM CAA to a final concentration of 50 mM (1 : 10 dilution). Carefully invert the sample once and incubate for 30 min at room temperature in the dark.
5. Dilute sample with four volumes of 40 mM Tris–HCl (pH 7.6) to decrease urea concentration to 1.2 M (*see* **Note 3**). Add trypsin in a protease-to-protein ratio of 1:100 (w/w) and predigest 4 h in a thermo-shaker at 37 °C and 700 rpm. Add another 1:100 (w/w) trypsin and incubate the digestion mixture over night in a thermo-shaker at 37 °C and 700 rpm.
6. Cool samples down to room temperature and acidify the sample by addition of 0.5 % (v/v) TFA. Centrifuge acidified peptides at 5000 × *g* to precipitate insoluble matter. Use 50 mg Sep-Pak columns and place them into a vacuum manifold (*see* **Note 4**). Prime Sep-Pak columns by adding 1 ml of solvent B. Equilibrate column by adding 2 × 1 ml of solvent A. Transfer the supernatant of the acidified sample to the column and load slowly (*see* **Note 5**). Reapply flow-through a second time and discard it afterwards. Wash the column with 3 × 1 ml solvent A. Elute peptides with 2 × 150 µl solvent B into a 1.5 ml reaction vessel. Adjust the volume to 0.5 ml by

addition of solvent A (*see* **Note 6**). The Sep-Pak eluate has a final concentration of 30% ACN and can thus be directly applied to phosphopeptide enrichment (*see* **Note 7**) or alternatively stored at −80 °C.

### 3.2 Phosphopeptide Enrichment by Fe-IMAC Column Chromatography

For first time use, the column can be directly charged with $FeCl_3$ solvent (it does not have to be stripped) (*see* **Note 8**). The column is usually operated below 1000 psi. Column stripping and charging should be repeated after 20 enrichments or in case the column has not been used for more than one week (*see* **Note 9**).

1. Column stripping: Connect the IMAC column to your HPLC system and rinse it with ultrapure water (1 ml/min, 10 ml). Inject 1 ml of IMAC stripping solvent into the sample loop and let it run through the column (1 ml/min). After 1 min, inject another 1 ml IMAC stripping solvent. Repeat this step eight more times. Make sure that the sample loop is flushed with water afterwards. Rinse the column with ultrapure water (2 ml/min, 5 ml).
2. Column charging: Inject 1 ml of IMAC charging solvent into the sample loop and let it run over the column (0.2 ml/min). After 5 min, inject another 1 ml of IMAC charging solvent. Repeat this step four more times. Wash the sample loop with 3 × 1 ml EDTA to scavenge remaining $Fe^{3+}$ ions (from both syringe and sample loop) and 10 × 1 ml water to get rid of residual EDTA. Rinse the column with 50 ml FA solvent to wash away unbound $Fe^{3+}$ ions (2 ml/min).
3. Fe-IMAC enrichment: Connect the IMAC loading solvent and IMAC elution solvent to the HPLC system. Flush the column with 5 ml of 50% IMAC elution solvent (3 ml/min). Re-equilibrate the column with 20 ml IMAC loading solvent (3 ml/min) (*see* **Note 10**). Perform a standard enrichment to ensure proper charging (15 min gradient, *see* Table 1, *see* **Note 10**).

**Table 1**
**Settings for a 15 min Fe-IMAC column enrichment, displaying the programmed time, the flow (in ml/min), and percentages IMAC elution solvent used**

| Time [min] | Flow [ml/min] | IMAC elution solvent [%] |
|---|---|---|
| 0–0.1 | 1 | 0 |
| 0.1–5.1 | 0.2 | 0 |
| 5.1–6.72 | 3 | 0 - 16 |
| 6.72–11.68 | 0.55 | 16 - 26.25 |
| 11.68–12.35 | 3 | 26.25 - 50 |
| 12.35 | 0 | 50 - 0 |
| 12.35–15.02 | 3 | 0 |

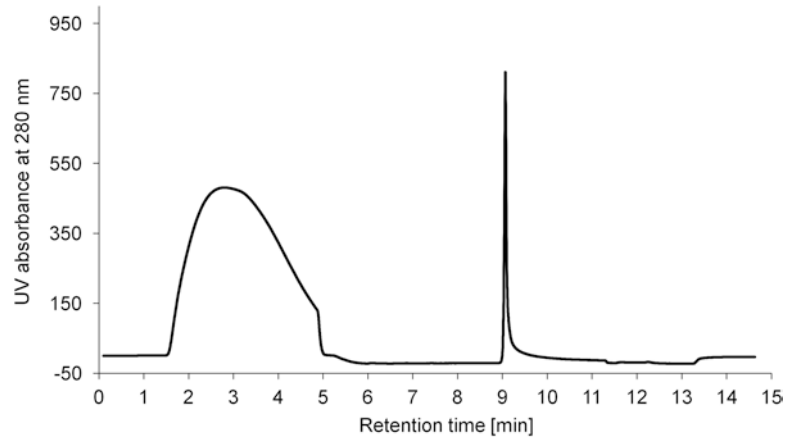


**Fig. 2** Typical chromatogram of a 15 min Fe-IMAC column enrichment using 1 mg of HeLa digest. The first peak (retention time between 2 and 5 min.) contains the non-phosphorylated peptides. The second peak (retention time around 9 min) contains the phosphorylated peptides

4. Inject the desalted sample (*see* **Note 11**) dissolved in 0.5 ml IMAC loading solvent. Prepare two 1.5 ml reaction vessels for collecting flow-through and phosphopeptide eluate. Start the 15 min gradient (the gradient setup is shown in Table 1). Monitor the absorption at 280 nm (*see* Fig. 2) and collect 1.3 ml of the flow-through in a 1.5 ml eppendorf tube. The IMAC eluate peak containing the phosphopeptides is collected in another 1.5 ml eppendorf tube (total volume of 1 ml, *see* Fig. 2 and Table 1). Freeze both eppendorf tubes at −80 °C and subsequently dry the samples down using a vacuum centrifuge or a lyophilizer. A typical 15 min Fe-IMAC enrichment chromatogram is depicted in Fig. 2.
5. There is no need to run blanks in between consecutive enrichments. The carryover is minimal. You can reinject the Fe-IMAC column flow-through in order to monitor (absorption at 214 and 280 nm) if all the phosphopeptides were properly depleted.

### *3.3 Desalting of the Fe-IMAC Eluate*

Although most of the ammonia will evaporate during the vacuum centrifugation/lyophilization step, residual ammonia salts might remain. Hence, it is recommended to desalt Fe-IMAC eluates using C-18 StageTips [21]. Pass all liquids through the tips by centrifugation (~800 × *g*, room temperature; *see* **Note 12**).

1. Dissolve the dried sample in 250 µl of solvent A and keep the sample on ice while the StageTips are prepared. Check the pH of the dissolved peptide solution and, if required, adjust it to pH 2 using FA.
2. Sequentially activate the tips using 250 µl of MeOH, 250 µl of solvent B and equilibrate with 250 µl of solvent A. Empty the eppendorf tube in between.

3. Load the dissolved sample and reapply the flow-through. Discard the flow-through afterwards and wash the column with 250 µl solvent A.
4. Use 40 µl of solvent B to elute the peptides of the C-18 material. Transfer the eluate into a 96-well plate and dry the sample down using a vacuum centrifuge/lyophilizer. At this point, the plate can be stored at −20 °C.

### 3.4 LC-MS/MS and Data Analysis

1. Reconstitute desalted IMAC eluate in 20 µl of 1% FA in 50 mM citrate (*see* **Note 13**).
2. Perform LC-MS/MS measurements by coupling an Eksigent NanoLC-Ultra 1D+ to a Q Exactive Plus instrument. 5 µl of IMAC enriched phosphopeptides corresponding to the enrichment from 250 µg peptide digest are delivered to the trap column at a flow rate of 5 µl/min in loading solvent (0.1% FA in water). During 10 min of sample loading chelated iron is washed out while phosphopeptides are retained.
3. Transfer peptides to the analytical column and separate at a flow rate of 300 nl/min using a 110 min gradient from 0 to 27% solvent B (0–2 min: 0% B; 2–100 min: 0–27% C; 100–101 min 27–80% B, 101–105 min: 80% B, 105–106 min: 80–0% B, 106–110 min: 0% B) (*see* **Note 14**).
4. Operate the Q Exactive Plus in data-dependent mode, automatically switching between MS1 and MS2. Acquire full-scan MS spectra at 360–1300 m/z, 70,000 resolution with acquisition gain control (AGC) target value of $3 \times 10^6$ charges and maximum injection time of 100 ms for MS1. Allow up to 20 precursor ions for HCD fragmentation in tandem mass spectra. Acquire MS2 spectra at 17,500 resolution, AGC target value of $1 \times 10^5$ charges and max injection time of 50 ms (*see* **Note 15**). Set precursor ion isolation width to 1.7 Th and dynamic exclusion to 20 s. Figure 3 shows an expected MS1 base peak intensity chromatogram of the LC-MS/MS measurement.

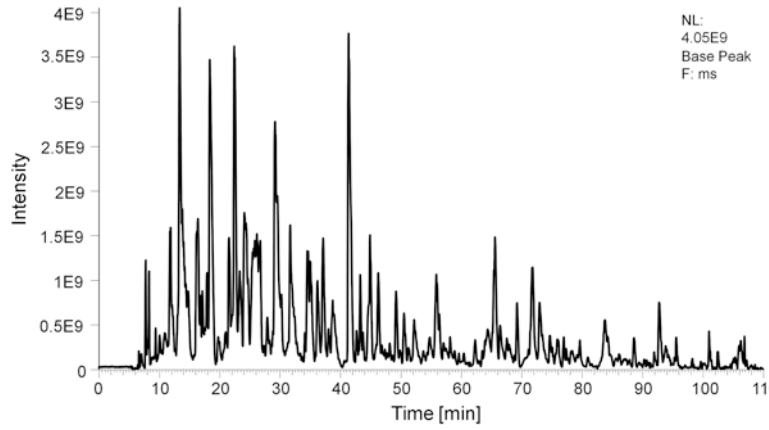


**Fig. 3** Typical base peak intensity chromatogram of a desalted Fe-IMAC phosphopeptide eluate (1/4 of a 1 mg HeLa enrichment) measured on a Q Exactive Plus

5. Analyze data using a proteomics software capable of label-free quantification. All results shown in this chapter are based on peptide identifications by search of raw data against the UniProtKB human database, version July 2013 (88,354 sequences) using the freely available MaxQuant version 1.5.2.8 and its built-in Andromeda search engine. Parameters used are specified in Table 2.
6. To facilitate any kind of data analysis, filter the MaxQuant evidence.txt or the phospho(STY)sites.txt output file to remove reverse sequences and potential contaminants. To determine the selectivity of the phosphopeptide enrichment, the reported number of peptides annotated with a phosphorylation sites is divided by the total number of identified sequences. The intensity-based selectivity is acquired similarly by dividing the summed intensity of phosphorylated peptides by the total intensity. Filter the "Modified Sequence" column for duplicates to remove redundancies and obtain the number of

**Table 2**
**Group-specific and global parameters for data analysis using MaxQuant version 1.5.2.8**

| | |
|---|---|
| Group-specific parameters | |
| Type | Standard |
| Label | No |
| Variable modifications | Acetyl (Protein N-term), Oxidation (M), Phospho (STY) |
| Digestion mode | Specific (Trypsin/P) |
| Max. missed cleavages | 2 |
| Main search peptide tolerance | 5 parts per million (ppm) |
| Max. number of modifications per peptide | 5 |
| Global parameters | |
| Database | UniProtKB |
| Fixed modifications | Carbamidomethyl |
| PSM FDR | 0.01 |
| Protein FDR | 0.01 |
| Site decoy fraction | 0.01 |
| Min. peptide length | 7 |
| Min. score for (un)modified peptides | 0 |
| Min. delta score for (un)modified peptides | 0 |
| MS/MS match tolerance | 20 ppm |
| Second peptide search | Enabled |

**Table 3**
**Overview of results typically expected from a single Fe-IMAC enrichment, measured on a 2 h LC-MS/MS gradient on a Q Exactive Plus. The Fe-IMAC column eluate was reconstituted in 20 µl of 50 mM Citrate, 1 % FA and 5 µl were injected**

| | |
|---|---|
| Phosphopeptides (MaxQuant—evidence.txt) | |
| Identified unique phosphopeptides | 10089 |
| Quantified unique phosphopeptides | 9392 |
| Mono phosphorylated | 8392 (83 %) |
| Multiply phosphorylated | 1697 (17 %) |
| Identification-based phosphopeptide selectivity | 81 % |
| Intensity-based phosphopeptide selectivity | 94 % |
| Phosphorylation sites (MaxQuant—Phospho(STY)sites.txt) | |
| Identified phosphorylation sites | 8973 |
| Quantified phosphorylation sites | 7451 |
| Class I sites (Loc. prob. > 0.75) | 6566 |
| pS sites (class I) | 5674 (86 %) |
| pT sites (class I) | 727 (11 %) |
| pY sites (class I) | 165 (3 %) |

unique phosphopeptides. Similarly, the phospho (STY)sites.txt is used to determine the number of unique and quantifiable sites. Filter for "Localization probability" ≥ 0.75 to obtain the number of class I sites [22].

7. Table 3 shows expected results in terms of unique phosphopeptides and phosphorylation sites obtained from processing 1 mg of HeLa digest (1/4 of the enrichment was subjected to MS measurement) according to the procedures described in this protocol.

## 4 Notes

1. We noticed that ammonia is evaporating if the $NH_4OH$ bottle is not tightly sealed or if it has been opened and closed repeatedly. Thus, the 0.3 % (v/v) in the IMAC elution solvent refers to a freshly opened bottle of $NH_4OH$ and might have to be adjusted upon prolonged use. This can be delayed by ensuring proper sealing of the bottle or alternatively by working at 4 °C.

2. Aspiration of remaining PBS is important to avoid dilution of the lysis buffer. Low concentrations of the chaotropic reagent urea might result in insufficient protein denaturation.
3. Considering the additional volume of the lysed cells and the remaining PBS, the concentration of urea is reduced from 8 M to roughly 6 M. Hence, a 1:4 dilution is sufficient to reduce the urea concentration down to 1.2 M.
4. Sep-Pak sorbent weight has to be chosen according to the amount of digest you intend to load. As a rule of thumb, the capacity of Sep-Pack cartridges equals 5% of the sorbent weight (e.g., 2.5 mg for the 50 mg sorbent weight cartridges and 10 mg for the 200 mg sorbent weight cartridges).
5. Load the sample slowly onto the Sep-Pak column. Lower the flow rate by adjusting the vacuum at the vacuum manifold. Loading should take at least 10 min to ensure proper binding of the phosphopeptides. Reapplying the flow-through increases recovery.
6. Avoid letting the columns run dry.
7. Adjusting the volume to 0.5 ml using solvent A enables direct sample loading onto the IMAC column without the need to intermediately dry the sample down.
8. Upon first time use, the column should be thoroughly flushed with water followed by 0.1% (v/v) FA solvent. It is advisable to note down the column backpressure for different types of solvents. This facilitates to monitor column performance over time. If the pressure increase is too severe, the column should be exchanged.
9. From our experience, 20 enrichments can be performed without any performance decrease. However, if the column is not used for a longer period of time (conservatively more than one week), column performance seems to be decreasing. If you want to verify or monitor column performance, run a standard before and after your enrichment set.
10. Make sure to properly equilibrate the column after charging. The absorption at 280 nm has to have reached a stable baseline. We recommend to always run a standard or a blank run before you enrich your first sample.
11. The 4 mm I.D. column can be applied to sample amounts ranging between 0.5 and 3 mg. Please be aware that the enrichment efficiency is also cell line dependent as the degree of cellular phosphorylation is highly dynamic and may therefore vary considerably.
12. Using volumes of 250 µl prevents columns from running dry even upon prolonged centrifugation. This is especially beneficial when parallelized fractionation is intended as not all col-

umns run at the same speed. If only one sample is intended to be desalted, the procedure can be accelerated by manually pushing the liquids through the tips using a 5 ml Eppendorf CombiTip. The volumes can be scaled down accordingly (~40 µl for each step).

13. Citric acid acts as a chelating agent for residual $Fe^{3+}$ ions that might co-elute from the Fe-IMAC column. Remaining $Fe^{3+}$ ions can stick to the trap/analytical nano-HPLC columns and deplete phosphopeptides. Ever since we use citrate we have not detected any iron contamination [23]. If you are in doubt, specify iron as a variable modification during data processing and check if any iron-bound peptides are identified.
14. Phosphopeptides are generally more hydrophilic than non-phosphopeptides. Compared to full proteome separations, we use a shallow gradient which leads to a more efficient use of gradient and MS time.
15. Phosphopeptides often show a pronounced loss of the phosphate group upon fragmentation. Because this neutral loss peak may constitute a big part of the fragment ion intensity, backbone fragment ions might get lost. Therefore you may want to evaluate if increased MS2 injection time and or increased MS2 target values (by a factor of 2) leads to an increase in phosphopeptide identifications. The substantially higher identification rate compensates for decreased scan numbers. Moreover, we found that this AGC/injection time increase is beneficial for phosphosite localization.

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