

Chapter 6

In-Depth Analysis of Protein Phosphorylation by Multidimensional Ion Exchange Chromatography and Mass Spectrometry

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Abstract

Protein phosphorylation controls fundamental biological functions that are often deregulated in disease. Therefore, system-level understanding of complex pathophysiological processes requires methods that can be used to profile and quantify protein phosphorylation as comprehensively as possible. Here we present a detailed protocol to enrich phosphopeptides from total cell lysates in a form amenable to downstream analysis by mass spectrometry. Using these techniques, we have detected several thousands of phosphorylation sites in the NIH-3T3 cell line.

Key words: Phosphoproteomics, cell signaling, cancer, quantification.

1. Introduction

Phosphorylation is one of the most common posttranslational modifications known to modulate the biophysical properties of proteins. Protein kinases and phosphatases are the enzymes responsible for catalysing the transfer and the removal of phosphate groups on proteins, respectively. Intense research in this field has shown the relevance of these phosphorylation events for the regulation of many different biochemical pathways in eukaryotic cells. As for their biological function, proliferation, differentiation, apoptosis, transcription, metabolism and migration represent just a short list of all the processes in which protein kinases

and phosphatases play central regulatory roles (1). Indeed, the ubiquitous role of protein phosphorylation in the regulation of cell biology is reflected by the notion that one-third of all proteins in higher eukaryotes are under protein kinase and/or phosphatase control at a given time (2).

Thus, the ability to quantify kinase and phosphatase activities in a comprehensive manner is being instrumental for unravelling many biological processes such as those involved in the development of complex and poorly understood diseases such as cancer. It is therefore well accepted that further advances in our understanding of pathways controlled by phosphorylation at the system level, perhaps the ultimate aim of fields such as signal transduction research, require methods that can be used for comprehensive analysis of phosphorylation. In this regard, recent developments in mass spectrometry-based proteomic approaches have been successfully applied to study protein phosphorylation, allowing identification and quantification of thousands of phosphorylation sites per experiment (3).

These large-scale phosphoproteomic studies are possible because of the recent availability of highly selective phosphopeptide isolation and separation techniques, such as those based on cation exchange chromatography (SCX), immunoprecipitation, immobilised metal ion affinity chromatography (IMAC), titanium dioxide (TiO_2), calcium precipitation and chemical modifications, among others (4). These enrichment techniques are an essential requirement for successful phosphoproteomic experiments because of the low stoichiometry of phosphorylation on phosphoproteins relative to their non-phosphorylated counterparts. In order to reduce the complexity of biological samples and hence increase the fraction of the phosphoproteome analysed, it is also important to perform a separation step prior to the enrichment steps. Thus workflows in which SCX are coupled to IMAC or TiO_2 are common (5–7).

Our laboratory has been implementing and optimising techniques for comprehensive analysis of phosphorylation. The protocol presented here involves the digestion of cellular proteins with trypsin, reducing the peptide mixture complexity by an initial fractionation step using SCX HPLC and phosphopeptide enrichment of the obtained fractions by a combination of orthogonal ion exchange techniques, namely IMAC and TiO_2 chromatography. This combination of enrichment steps was introduced by Thingholm and colleagues (8) as an improved phosphopeptide enrichment strategy that consists of sequentially eluting phosphopeptides from the IMAC material in combination with the different selectivity of TiO_2 , which ultimately allows obtaining a better coverage of the whole phosphoproteome. Here, we present an adaptation of this simple protocol for in-depth analysis of protein phosphorylation.

2. Materials

2.1. Cell Culture and Lysis

1. Murine NIH-3T3 fibroblasts (American Tissue Culture Collection, LGC Standards, Teddington, UK) as a cell model commonly used in cell-signalling studies (*see Note 1*).
2. Phosphate-buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water (*see Note 2*).
3. Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% foetal bovine serum (FBS; HyClone, Ogden, UT), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA).
4. Urea-denaturing lysis buffer supplemented with protease and phosphatase inhibitors: 20 mM HEPES (pH 8.0), 8 M urea, 1 mM sodium vanadate, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate. Prepare the stock lysis buffer (20 mM HEPES, pH 8.0, 8 M urea) and store in aliquots at −20°C. On the day of the experiment, supplement 1 ml of stock lysis buffer with the following: 2 µl of 0.5 N NaF, 10 µl of 100 mM Na₃VO₄, 10 µl of 250 mM sodium pyrophosphate and 1 µl of 1 M β-glycerol phosphate (*see Notes 3 and 4*).
5. Internal standards: α-casein and β-casein. Prepare stock solutions at 4 pmol/µl.
6. Reducing and alkylating solutions are 1 M dithiothreitol (DTT) and 415 mM iodoacetamide, respectively.
7. Bio-Rad protein assay solution (Bio-Rad, Hercules, CA) (*see Note 5*).

2.2. Cell Lysate Protein Digestion

1. Trypsinisation buffer: 20 mM HEPES (pH 8.0).
2. Trypsin digestion is performed using immobilised TLCK-trypsin (Thermo Fisher Scientific, Massachusetts, USA).
3. 10% Trifluoroacetic acid (TFA).

2.3. Solid-Phase Extraction (SPE) Desalting

1. SPE cartridges (Sep-Pack C₁₈ columns; Waters UK Ltd, Manchester, UK).
2. SPE solutions: (A) Conditioning solution: 100% acetonitrile (ACN); (B) loading/washing solution: 0.1% TFA; (C) eluting solution: 50% ACN, 0.1% TFA.

2.4. SCX HPLC

1. SCX mobile phase A: 0.1% formic acid, 25% ACN.
2. SCX mobile phase B: 300 mM ammonium acetate, 25% ACN.
3. Polysulphoethyl A (Poly LC) SCX column 4.6 mm × 100 mm (PolyLC, Columbia, MD, USA).
4. HPLC system with fraction collector (1200 series; Agilent, Wokingham, UK).

2.5. Sequential Elution from Immobilised Metal Ion Affinity Chromatography (SIMAC)

2.5.1. Immobilised Metal Ion Affinity Chromatography

1. Ni(III)-coated Sepharose high-performance beads (GE Healthcare, Little Chalfont, UK).
2. IMAC chelating solution: 200 mM EDTA.
3. IMAC charging solution: 100 mM Fe(III)Cl₃.
4. IMAC loading/washing solution: 50% ACN, 0.1% TFA.
5. IMAC elution solutions: (A) 20% ACN, 1% TFA; (B) NH₄OH (pH 11.3) (prepare by mixing 940 µl of water and 60 µl of 25% NH₄OH; can be stored at −20°C); (C) NH₄OH (pH 11.3) prepared in 50% ACN.
6. 100% Formic acid (FA).

2.5.2. Titanium Dioxide (TiO₂) Chromatography

1. TiO₂ beads (Hichrom Ltd, Theale, UK).
2. 100% ACN.
3. 4 M Urea.
4. 1% Sodium dodecyl sulphate (SDS).
5. TiO₂ loading buffer: 80% ACN, 5% TFA, 1 M glycolic acid.
6. TiO₂ washing solutions: (A) 80% ACN, 5% TFA; (B) 10% ACN.
7. TiO₂ elution buffers: (A) NH₄OH (pH 11.3) (prepare as indicated in **2.5.1 step 5**); (B) 30% ACN.
8. 100% FA.

2.6. MS Identification

Peptide separation is performed by nanoflow reverse-phase chromatography using a C18 column in a high-pressure liquid chromatography (HPLC) system. In our example, we use a nanoflow ultrahigh-pressure liquid chromatograph (UPLC; Acquity, Waters/Micromass) connected on line to a quadrupole

time-of-flight (Q-TOF) mass spectrometer (Waters/Micromass UK Ltd, Manchester, UK). In our example, we used a BEH C₁₈ 100- μ m \times 100-mm column (Waters/Micromass UK Ltd, Manchester, UK).

1. LC-MS/MS mobile phases: solution A (0.1% FA in LC-MS grade water) and solution B (0.1% FA in LC-MS grade ACN).
2. Sample resuspension solution: 0.1% TFA.

3. Methods

Protein phosphorylation is a reversible and dynamic process that regulates essential biological functions in health and disease. The identification of phosphorylation sites can provide mechanistic information at the molecular level and serve as read-outs of pathway activities. In this sense, mass spectrometry combined with phosphopeptide enrichment methodologies has emerged as a powerful method for phosphoproteomic studies (9, 10).

Among the numerous phosphopeptide enrichment strategies available, IMAC and TiO₂ have become the most popular techniques due to their simplicity and great selectivity to isolate phosphopeptides. IMAC is based on electrostatic interactions between the positive charge of the immobilised metal ions (such as Fe(III) or Ga(III)) and the negative charge of phosphopeptides (11). Although non-specific recovery of peptides containing acidic amino acids represents a problem, pH/acid-controlled conditions raise the specificity of IMAC (12). TiO₂ instead has shown higher selectivity for phosphopeptides than IMAC, with lower unspecific binding, and a higher recovery of less acidic (mainly monophosphorylated) peptides (13, 14). These findings suggest that the complementarity of the two approaches may have potential for comprehensive phosphoproteomic profiling. This concept was applied by Thingholm and colleagues to develop a combined approach named 'sequential elution from IMAC' (SIMAC) (8), which is based on differential elution of phosphopeptides according to their acidity from the IMAC material using acidic and basic elution conditions. The less acidic fractions eluted from IMAC (containing a larger proportion of monophosphopeptides) are then further enriched using TiO₂ chromatography.

The SIMAC method produces three fractions per sample. This is often insufficient fractionation when the aim is to perform an in-depth analysis of the phosphoproteome. We propose therefore a combination of SCX sample fractionation followed by phosphopeptide enrichment by SIMAC in order to more com-

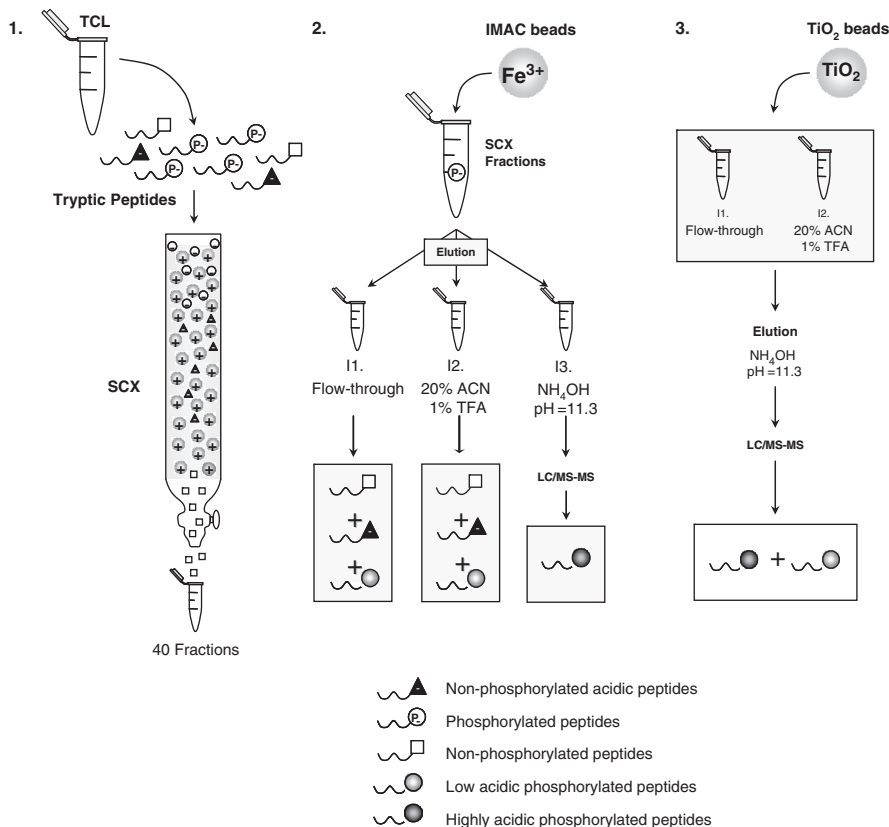


Fig. 6.1. Scheme for the enrichment of phosphopeptides using multidimensional chromatography.

prehensively identify phosphorylated peptides. Here we describe this protocol in detail (the analytical workflow is summarized in Fig. 6.1).

3.1. Sample Preparation for Large-Scale Phosphoproteomics

1. Murine NIH-3T3 fibroblasts are routinely cultured at 37°C in a humidified atmosphere at 5% CO_2 and grown in DMEM medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells are maintained at 10–75% confluency (*see Note 6*).
2. Prior to the experiment, cells are seeded at about 20% confluency and cultured for 48 h when cells reach approx. 75% confluency (*see Note 7*). The use of at least 1×10^7 cells per sample is recommended for samples that are not going to undergo SCX fractionation and $\sim 5 \times 10^8$ cells when the aim is to perform an SCX separation step prior to further phosphopeptide enrichment. The current protocol is given for a sample containing 2×10^8 NIH-3T3.

3. The medium should be changed the day before the experiment.
4. On the day of the experiment, supplement the stock lysis buffer as indicated in **Section 2.1**, Step 4. PBS is also supplemented with 1 mM sodium vanadate and 1 mM sodium fluoride (1 ml of 100 mM sodium vanadate and 200 μ l of 0.5 N sodium fluoride/100 ml of PBS). Both PBS and lysis buffer must be prechilled at 4°C before use and kept on ice (*see Note 8*).
5. Please note that cell harvesting should be performed on ice (*see Note 8*). For harvesting purposes, the DMEM is aspirated from the flask and cells are washed twice with the supplemented PBS. Scrape the cells off the flask at approx. 2×10^7 cells/ml of urea-denaturing lysis buffer. Stand on ice for 15 min to allow lysis to take place. Solubilise cellular proteins further by sonication (three pulses, half intensity) (*see Note 9*).
6. Clear the lysate from debris by centrifugation at $20,000 \times g$ for 10 min at 4°C. The protein concentration of the supernatants is determined using standard protein quantification procedures.
7. Optionally add 20 pmol of internal standards to each sample (*see Note 10*).
8. Reduce and alkylate cell lysate proteins with DTT at a final concentration of 10 mM and iodoacetamide at 120 mM, respectively (*see Note 11*). For 10 ml of cell lysate, add 100 μ l of 1 M DTT and incubate for 45 min at 30°C. Proceed by adding 2,900 μ l of 415 mM iodoacetamide and incubate for 45 min at room temperature in the dark.
9. Dilute protein extracts using 20 mM HEPES (pH 8.0) to a final concentration of 2 M urea (1:4 dilution) to allow protein digestion by trypsinisation. For this, add 30 ml of 20 mM HEPES (pH 8.0) to 10 ml lysate (*see Note 12*).
10. Perform trypsinisation using 300 TAME units of TLCK of trypsin/ 2×10^8 cells for 16 h at 37°C with vigorous shaking (*see Note 13*). Please note that trypsin beads must be washed three times with 20 mM HEPES before adding them to the samples.
11. Stop digestions by acidifying the samples with TFA at a final concentration of 1%.
12. Remove trypsin beads by spinning the samples at $2,000 \times g$ for 5 min.

13. At this stage, the samples can be frozen prior to desalting by solid-phase extraction (SPE).

3.2. Solid-Phase Extraction

1. The peptide solution resulting from the trypsinisation step is desalted using Sep-Pack C₁₈ columns, or any other SPE cartridges (*see* **Note 14**).
2. Condition Sep-Pack C₁₈ 1-ml columns using 10 ml SPE conditioning solution (100% ACN).
3. Equilibrate columns with 10 ml SPE loading/washing solution (0.1% TFA).
4. Load 40 ml sample onto four Sep-Pak C18 columns (*see* **Note 15**).
5. Wash the column with 10 ml SPE loading/washing solution (0.1% TFA).
6. Finally, bound peptides can be eluted with 5 ml SPE eluting solution (50% ACN, 0.1% TFA) (*see* **Note 16**).

3.3. Strong Cation Exchange High-Performance Liquid Chromatography (SCX HPLC)

Desalted peptides can be subjected to phosphopeptide enrichment as explained in **Section 3.4**. Alternatively, peptides can be separated into fractions by SCX HPLC prior to further phosphopeptide enrichment. This optional step reduces the sample complexity (at the expense of increasing LC-MS/MS running time) and hence results in a greater coverage of the phosphoproteome by providing an orthogonal separation step.

1. Connect the SCX column to a HPLC system and set the flow rate to 1 ml/min.
2. Condition the SCX column with SCX solvent B (300 mM ammonium acetate, 25% ACN) for 30 min.
3. Equilibrate SCX column with SCX solvent A (0.1% formic acid, 25% ACN) for at least 10 min and until a stable baseline on the UV trace is achieved.
4. Load the desalted peptides onto the equilibrated SCX column.
5. Apply gradient elution from 0 to 25% B for 20 min followed by 25–100% B for another 20 min. Collect 1-min fractions (40 fractions in total).
6. Combine fractions based on UV absorption to obtain 10 fractions.
7. Dry SCX fractions in speed-vac to remove organic solvents and redissolve in 5 ml SPE loading/washing solution (0.1% TFA).
8. Desalt SCX fractions by SPE as described in **Section 3.2**.

3.4. Sequential Elution from Immobilised Metal Ion Affinity Chromatography (SIMAC)

3.4.1. Immobilised Metal Ion Affinity Chromatography

3.4.1.1. Bead Conditioning

1. Prior to start of IMAC chromatography for phosphopeptide enrichment *per se*, the nickel from the Ni(III)-coated Sepharose beads must be replaced by Fe(III) (**Note 17**).
2. Take 300 μ l of commercial beads (50% slurry) per sample to be enriched. The current protocol is given for one sample, i.e., 300 μ l of beads (*see* **Note 18**). Spin them down at $1,000\times g$ for 2 min, discarding the supernatant that contains the preservative solution. Use these spinning conditions for all the following steps.
3. Wash the beads with 300 μ l of water, spin down and keep the beads.
4. Incubate with 300 μ l IMAC chelating solution (200 mM EDTA) for 5 min to release the nickel from the beads (*see* **Note 19**), spin down and keep the beads. Perform this step twice. The beads must change the colour, from blue to colourless.
5. Wash the beads with 300 μ l IMAC loading/washing buffer (50% ACN, 0.1% TFA), spin down and discard the supernatant. Wash the beads three times.
6. Load the beads with 300 μ l IMAC charging solution (100 mM Fe(III)Cl₃) for 5 min, centrifuge and keep the beads. Repeat the loading once again. The beads should become yellow (*see* **Note 20**).
7. Wash the beads with 300 μ l IMAC loading/washing solution (50% ACN, 0.1% TFA), spin down and discard the supernatant. Wash the beads for six times.
8. The beads will be ready to use after resuspending them in 50% slurry with IMAC loading/washing buffer (50% ACN+ 0.1% TFA). For 300 μ l of starting beads, one obtains 150 μ l of pure beads, which are resuspended with 150 μ l of loading buffer.

3.4.1.2. Phosphopeptide Enrichment

1. Incubate the desalted samples with 300 μ l of conditioned IMAC beads for 30 min at room temperature (*see* **Note 21**).

2. Centrifuge the samples at $1,000\times g$ for 5 min. Take aside the supernatant (unbound fraction) for further enrichment with TiO_2 beads.
3. Beads are now washed with 300 μl of IMAC loading/washing buffer (50% ACN+ 0.1% TFA).
4. Combine the unbound and washed fraction as the I1 fraction for later will TiO_2 enrichment (*see Note 22*).
5. For the elution of non-phosphorylated acidic peptides and less acidic phosphopeptides (mainly monophosphopeptides), treat beads with 300 μl IMAC elution solution A (20% ACN, 1% TFA) for 5 min. Spin down and keep the supernatant as the I2 fraction. This will also be further enriched with TiO_2 beads (*see Note 23*).
6. Highly acidic phosphopeptides, enriched in multiphosphopeptides (I3 fraction), are obtained by eluting with 300 μl IMAC elution solution B (ammonia water, pH 11.3, prepared as indicated in **Section 2.4**) for 5 min. The supernatant obtained after centrifugation constitutes the I3 fraction. Repeat this step twice and combine the two ammonia water elutions.
7. Wash the beads with 50 μl of IMAC elution buffer C (ammonia water, pH 11.3, containing 50% ACN). Combine the resulting supernatant with the previous ammonia elutions rendering the final I3 fraction (*see Note 24*). At this stage the beads can be discarded.
8. Ammonia fraction (I3) should be acidified with 10% FA final concentration. In our example, we will use 65 μl 100% FA for I3 acidification.
9. Lyophilise I2 and I3 fraction.
10. Resuspend the I3 fraction in 10 μl of 0.1% TFA for analysis by LC-MS.

3.4.2. Titanium Dioxide (TiO_2) Chromatography

1. Resuspend the TiO_2 beads with 100% ACN to 50% slurry. Use 10 μl of beads per sample (*see Note 25*).
2. Spin the beads down and remove the ACN supernatant.
3. Wash the beads first with 200 μl of water and then twice with 200 μl TiO_2 loading buffer (1 M glycolic acid in 80% ACN, 5% TFA) (*see Note 26*).
4. Resuspend I2 fraction with 2 μl 4 M urea, 3 μl 1% SDS and 200 μl TiO_2 loading buffer.
5. Incubate fractions I1 and I2 with 10 μl TiO_2 beads for 30 min at room temperature. After this time, centrifuge the samples at $1,000\times g$ for 5 min and discard the supernatant.

6. Wash beads sequentially with 200 μ l TiO₂ loading buffer, then with 200 μ l TiO₂ washing solution A (80% ACN/5% TFA) and finally with 200 μ l TiO₂ washing solution B (10% ACN). Discard the resulting supernatant in each case.
7. Elution of bound phosphopeptides is achieved by incubating the beads with 50 μ l TiO₂ elution solution A (ammonia water, pH 11) (*see Section 2.4*) for 5 min, spinning and collecting the supernatants (*see Note 27*).
8. Beads are subjected to a second elution round with TiO₂ elution solution B (*see Note 28*). Combine this elution with the previous one to obtain the final phosphopeptide-enriched sample.
9. Acidify eluates with 10% FA final concentration, lyophilise and resuspend in 10 μ l 0.1% TFA for analysis by LC-MS/MS (*see Note 29*).

3.5. Identification of Phosphopeptides by LC-MS

1. The identification of phosphopeptides by mass spectrometry can be performed using, for example, a quadrupole time-of-flight (Q-TOF) mass spectrometer connected on line to a nanoflow ultrahigh-pressure liquid chromatograph.
2. HPLC separations can be done using a BEH reverse-phase column. The suggested mobile phases are solution A (0.1% FA in LC-MS grade water) and solution B (0.1% FA in LC-MS grade ACN).
3. The recommended gradient run is from 1% B to 35% B in 100 min followed by a 5-min wash at 85% B and a 7-min equilibration step at 1% B (*see Note 30*).
4. Perform a mass spectrometry data-dependent analysis (DDA) in which the three most abundant multiply charged ions present in the survey spectrum are automatically mass selected and fragmented by collision-induced dissociation in each cycle. MS scans of 500 ms, followed by three MS/MS scans of 1 s each are suggested to be acquired within a mass range of 50–2,000 m/z (*see Note 31*).
5. The phosphopeptide sequences can be identified by loading the MS/MS data files in a protein search engine, such as Mascot, Sequest and Phoenix, among others.

4. Notes

1. This assay can be performed with any cell line to be studied. Here we exemplify the approach by using the murine NIH-3T3 fibroblasts as a cell model commonly used in cell-signalling studies.

2. Routine solutions should be prepared in water that has a resistivity of 18 M Ω cm, unless otherwise stated. The solutions used for both solid-phase extraction and SIMAC protocols, along with all the solvents used in the LC-MS system, must be LC-MS grade. LC-MS grade solvents used here were sourced from LGC Promochem (Middlesex, UK), but any other LC-MS grade solvents should be adequate.
3. The lysis buffer used in this protocol is urea based. Urea acts as a denaturing agent at high concentrations such as that used in the current lysis buffer (8 M). When the lysis buffer is mixed with the cells, the urea denatures the proteins disrupting the cellular structures and acting as a lysing agent. Due to its denaturing nature, urea also protects phosphopeptides from protease degradation and phosphatases activities present in the biological samples. Please note that this compound is toxic; hence one must be extremely careful when handling it.
4. For this type of experiment, it is essential that the lysis buffer contains different protease and phosphatase inhibitors such as Na₃VO₄ and NaF (tyrosine phosphatase inhibitors), sodium pyrophosphate and β -glycerol phosphate (phosphatase inhibitors of broad specificity). This will preserve the phosphosites of the cell lysate proteins.
5. Any other reagent for protein quantification may be suitable.
6. The murine NIH-3T3 fibroblasts used in this example should not be maintained in culture for a long number of passages (maximum 25–30 passages) and should be kept between 10 and 80% confluency. This is due to the high transformation potential of this cell line. When culturing NIH-3T3 at 100% confluency and/or for long passages, they stop behaving like normal fibroblast and become transformed.
7. Cells are harvested at 75% confluency when they are actively dividing and when proliferative signalling networks are activated. This favours the study of kinase activity differences between experimental conditions.
8. Phosphopeptides are very sensitive to degradation by proteases and phosphatases present in the sample. For this reason, when performing phosphoproteomic experiments, it is very important to use prechilled solutions and to keep the sample on ice unless otherwise stated. This will reduce any phosphatase and protease activity that may remain in the sample despite the treatment with urea and inhibitors.

9. In order to obtain a maximum coverage of the phosphoproteome, it is essential to make sure that the proteins located in cellular membranes and nuclei are released and solubilised. This is easily solved by sonicating cell lysates as indicated.
10. Internal standards are phosphoproteins that can be added to the sample at a known concentration. This will allow us to normalise and correct for differences introduced during sample handling.
11. By reducing the cell lysate proteins with DTT, the disulphide bonds are broken. However, in order to prevent disulphide bond reformation, they must also be alkylated with reagents such as iodoacetamide. This step is introduced to facilitate the enzymatic protein digestion, in our example by trypsin.
12. Trypsinisation cannot be performed at 8 M urea due to its denaturing effect. Thus, before trypsinisation, the concentration of urea must be diluted to 2 M or less to preserve trypsin structure and hence function.
13. The use of immobilised TLCK/trypsin has the advantage that it can be removed from the sample after trypsinisation. However, it is very important to ensure vigorous shaking during incubation with trypsin to maintain beads in suspension, otherwise trypsin efficiency is dramatically reduced.
14. Prior to phosphopeptide enrichment, it is essential to remove the added salts since these may interfere with subsequent ion exchange chromatographic steps. For this purpose, we need to desalt the samples by SPE.
15. In this step, the peptides from the sample are retained in the Sep-Pack reverse-phase column, while inorganic salts are not retained and washed away.
16. The Sep-Pack eluting solution is the loading solution for the downstream enrichment step. Therefore, the Sep-Pack eluent can be directly loaded onto the IMAC beads.
17. The principle of the IMAC phosphopeptide enrichment protocol is based on ionic interactions between the negatively charged phosphate groups of such peptides and the positively charged metal ions coating the IMAC beads. One of the most widely used IMAC methods for phosphopeptide enrichment uses Fe^{3+} -coated beads; however other cations such as Ga^{3+} and Al^{3+} have been reported as well (4).
18. In our experience, 300 μl IMAC beads (50% slurry) represent the optimal amount of beads for our specific experimental conditions. However, it is strongly recommended

to optimise the amount of beads needed for efficient binding of phosphopeptides when studying different cell lines.

19. Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that acts by sequestering di- and tricationic metal ions. Thus, after EDTA treatment, the Ni^{3+} is released from the Sepharose beads, which can then be charged with Fe^{3+} ions.
20. Because we have not tested the stability of the Fe^{3+} -conditioned beads, we suggest using them fresh after loading the iron.
21. Fe(III)-IMAC chromatography has been shown to have higher affinity for multiply phosphorylated peptides than for monophosphorylated peptides, which actually represent the vast majority of phosphorylated peptides (14). In contrast, TiO_2 chromatography has proven to be more efficient at yielding monophosphopeptides. The SIMAC approach developed by Thingholm et al. takes advantage of both techniques in order to obtain a broader coverage of the whole phosphoproteome (8).
22. The first fraction obtained from the IMAC beads (I1) mainly contains non-phosphorylated peptides and some less acidic phosphopeptides (mainly monophosphorylated peptides) that did not bind to the IMAC beads during the incubation (8). This fraction will later be further enriched in phosphopeptides using TiO_2 beads.
23. Since acidic conditions have been shown to preferentially elute less acidic phosphorylated peptides from the IMAC beads, by using 20% ACN, 1% TFA, one recovers a fraction (I2) enriched in monophosphorylated peptides along with most of the acidic peptides that also bind to the IMAC beads in an unspecific fashion (8). This fraction will later be further enriched in phosphopeptides using TiO_2 beads.
24. This last elution with an organic solvent allows recovery of any phosphorylated peptides that may remain bound to the IMAC beads due to hydrophobic interactions.
25. TiO_2 chromatography has been shown to have higher selectivity and capacity for phosphopeptides than does IMAC (9). Thus, the volume of beads needed to perform the phosphopeptide enrichment is low in comparison.
26. Besides the high selectivity of TiO_2 chromatography for phosphopeptides, these beads allow the use of higher concentrations of TFA and glycolic acid, both of which further reduce the unspecific binding of non-phosphorylated acidic peptides (1.17 and 1.18). The increased concentration of TFA reduces the pH of the solution, neutralising the charge of non-phosphorylated acidic peptides and

hence avoiding their interaction with the Titanium. Due to the acidic properties of the glycolic acid, this compound competes with the non-phosphorylated acidic peptides for the Titanium binding but it does not compete with the phosphopeptides owing to their higher acidity.

27. The retained phosphorylated peptides are released from the TiO_2 beads by using alkaline buffers such as ammonia water (pH 11.3).
28. This last organic elution allows recovery of any phosphorylated peptides that may remain bound to the TiO_2 beads due to hydrophobic interactions.
29. It is recommended to include in the run one blank sample just containing the digested internal standards (20 pmol α - and β -casein). This will enable computing the percentage of phosphopeptide recovery.
30. Due to the complexity of the sample, we suggest to use a 100-min gradient. This will result in a good separation of the peptides, minimising the problem of MS undersampling at least to some extent.
31. There are different fragmentation techniques that can be used for phosphopeptide identification. In this sense, electron capture/transfer dissociation (ECD/ETD) has proven to be an efficient alternative to collision-induced dissociation (CID). Phosphopeptide fragmentation by CID often results in phosphate neutral loss, which sometimes causes a poor backbone fragmentation and hence low probability of phosphopeptide identification. This problem can be addressed by acquiring MS^3 spectra of the neutral loss product ions in cases where ion trap instruments are available. As an alternative, ECD/ETD fragmentation primarily fragments the peptide backbone chain with no phosphate loss, which in some cases results in an increased probability of detecting phosphopeptides and determining their precise phosphorylation site (10). However, CID may produce better fragmentation spectra than may ECD/ETD for low charge phosphopeptides; therefore, these two fragmentation techniques are complementary.

References

1. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science* **298**, 1912–1934.
2. Bodenmiller, B., Malmstrom, J., Gerrits, B., Campbell, D., Lam, H., Schmidt, A., Rinner, O., Mueller, L. N., Shannon, P. T., Pedrioli, P. G., Panse, C., Lee, H. K., Schlapbach, R., and Aebersold, R. (2007) PhosphoPepa phosphoproteome resource for systems biology research in *Drosophila* Kc167 cells. *Mol. Syst. Biol.* **3**, 139.
3. de la Fuente van Bentem, S., Mentzen, W. I., de la Fuente, A., and Hirt, H. (2008)

- Towards functional phosphoproteomics by mapping differential phosphorylation events in signaling networks. *Proteomics* **8**, 4453–4465.
4. Paradela, A., and Albar, J. P. (2008) Advances in the analysis of protein phosphorylation. *J. Proteome Res.* **7**, 1809–1818.
 5. Olsen, J. V., Blagoev, B., Gnäd, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635–648.
 6. Trinidad, J. C., Specht, C. G., Thalhammer, A., Schoepfer, R., and Burlingame, A. L. (2006) Comprehensive identification of phosphorylation sites in postsynaptic density preparations. *Mol. Cell. Proteomics* **5**, 914–922.
 7. Villen, J., and Gygi, S. P. (2008) The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* **3**, 1630–1638.
 8. Thingholm, T. E., Jensen, O. N., Robinson, P. J., and Larsen, M. R. (2008) SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol. Cell. Proteomics* **7**, 661–671.
 9. Smith, J. C., and Figeys, D. (2008) Recent developments in mass spectrometry-based quantitative phosphoproteomics. *Biochem. Cell Biol.* **86**, 137–148.
 10. Schreiber, T. B., Mausbacher, N., Breitkopf, S. B., Grundner-Culemann, K., and Daub, H. (2008) Quantitative phosphoproteomics: an emerging key technology in signal-transduction research. *Proteomics* **8**, 4416–4432.
 11. Andersson, L., and Porath, J. (1986) Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. *Anal. Biochem.* **154**, 250–254.
 12. Tsai, C. F., Wang, Y. T., Chen, Y. R., Lai, C. Y., Lin, P. Y., Pan, K. T., Chen, J. Y., Khoo, K. H., and Chen, Y. J. (2008) Immobilized metal affinity chromatography revisited: pH/acid control toward high selectivity in phosphoproteomics. *J. Proteome Res.* **7**, 4058–4069.
 13. Pinkse, M. W., Uitto, P. M., Hilhorst, M. J., Ooms, B., and Heck, A. J. (2004) Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem.* **76**, 3935–3943.
 14. Bodenmiller, B., Mueller, L. N., Mueller, M., Domon, B., and Aebersold, R. (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat. Methods* **4**, 231–237.