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Determining *in vivo* Phosphorylation Sites using Mass Spectrometry

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Abstract

Phosphorylation is the most studied protein post-translational modification (PTM) in biological systems since it controls cell growth, proliferation, survival, etc. High resolution/high mass accuracy mass spectrometers are used to identify protein phosphorylation sites due to their speed, sensitivity, selectivity and throughput. The protocol described here focuses on two common strategies: 1) Identifying phosphorylation sites from individual proteins and small protein complexes, and 2) Identifying global phosphorylation sites from whole cell and tissue extracts. For the first, endogenous or epitope tagged proteins are typically immunopurified (IP) from cell lysates, purified via gel electrophoresis or precipitation and enzymatically digested into peptides. Samples can be optionally enriched for phosphopeptides using immobilized metal affinity chromatography (IMAC) or titanium dioxide (TiO₂) and then analyzed by microcapillary liquid chromatography/tandem mass spectrometry (LC-MS/MS). Global phosphorylation site analyses that capture pSer/pThr/pTyr sites from biological sources sites are more resource and time-consuming and involve digesting the whole cell lysate, followed by peptide fractionation by strong cation exchange chromatography (SCX), phosphopeptide enrichment by IMAC or TiO₂ and LC-MS/MS. Alternatively, one can fractionate the protein lysate by SDS-PAGE, followed by digestion, phosphopeptide enrichment and LC-MS/MS. One can also IP only phospho-tyrosine peptides using a pTyr antibody followed by LC-MS/MS.

Keywords

phosphorylation; mass spectrometry; LC-MS/MS; SCX; IMAC; immunoprecipitation; SDS-PAGE; nano-LC; phosphoproteomics

Introduction

Phosphorylation of proteins plays an important role in cellular signaling events and metabolic processes and is therefore the most studied post translational modification (Choudhary and Mann; Zarei *et al.*; White, 2008). The determination of phosphorylated peptides in a biological sample is most easily achieved using mass spectrometry due to sensitivity, selectivity and throughput (Yates *et al.*, 2009). The phospho-proteome of mammalian cells and tissues is complex and displays a wide dynamic range of varying concentration. In order to overcome the human proteome complexity and determine the phospho-proteome content, it is necessary to use either enrichment, purification or sample fractionation at the protein or peptide level (Eyrich *et al.*). These methods utilize gel electrophoresis, ion exchange chromatography, and microcapillary HPLC.

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Proteomic approaches to analyzing phosphorylation usually involve selective isolation of phosphopeptides and subsequent fragmentation in a mass spectrometer to identify both the peptide sequence and phosphorylation site. Suitable mass spectrometers are capable of high resolution and high mass accuracy and include hybrid instruments containing orbitrap analyzers and time-of-flight (TOF) mass analyzers (Ahmed, 2008). In hybrid linear ion trap-orbitrap style mass spectrometers (LTQ-Orbitrap XL, Velos Pro Orbitrap or Velos Elite Orbitrap series, ThermoFisherScientific) (Makarov and Scigelova), peptide precursor ions are analyzed in the orbitrap at high resolution/high mass accuracy at $\leq 1\text{--}2$ ppm mass accuracy and up to 100,000 resolution and can then either be fragmented in the ion trap or in a collision cell for sequence/phosphorylation analysis. Alternatively, a hybrid quadrupole-TOF instrument (QqTOF) can be used (such as Xevo from Waters; 5600 from AB/SCIEX; 6500 series from Agilent, and microTOF from Bruker). The QqTOF is made up of two quadrupole mass spectrometers followed by a high resolution TOF, peptide precursors are analyzed in the TOF, fragmented in the collision cell and analyzed in the TOF analyzer for both sequence and modification determination. The mass accuracy is similar to orbitrap instruments at sub 2 ppm; however, resolution is lower on the order of $\sim 25\text{K}$, though sufficient for most phosphopeptide applications.

It is also important to use an online HPLC instrument capable of nanoliter flow rates coupled directly to the mass spectrometer in order to achieve optimal sensitivity in the low femtomole to high attomole range since many phosphorylation events are present in very low abundance (Washburn, 2008). One can use either splitless or manual split HPLC system with microcapillary columns that are capable of flow rates down to ~ 200 nL/min (e.g. EASY-nLC from ThermoFisherScientific; NanoAcquity from Waters; NanoLC Ultra from Eksigent, etc.). Tandem mass spectrometry (capable of fragmentation) is used in combination with nano-HPLC is referred to as microcapillary liquid chromatography/tandem mass spectrometry (LC-MS/MS).

In addition to the equipment required, informatics software is necessary to identify peptide sequences and their phosphorylated counterparts. For this purpose, database search engines such as commercially available Mascot (Perkins *et al.*, 1999) and Sequest (Yates *et al.*, 1995) are commonly used to interrogate protein databases for peptide/protein/PTM identifications as well as freely available MaxQuant (Andromeda) (Cox *et al.*) and XTandem! (Falkner and Andrews, 2005).

This protocol focuses on determining phosphorylation sites using LC-MS/MS. It is important to note that matrix-assisted laser desorption/ionization (MALDI) based systems are also capable of identifying phosphorylation sites (Asara and Allison, 1999; Bennett *et al.*, 2002); however, far more work has been performed using LC-MS based systems.

Basic Protocol 1: Single protein (protein complex) phosphorylation site mapping

In order to comprehensively study the functional role of phosphorylation from a specific protein of interest from a cell or tissue source, it is important to identify all phosphorylation sites of the protein from one or more biological conditions. The first step is purifying the protein in sufficient amounts (micrograms) for successful LC-MS/MS analysis. Since the stoichiometric level of phosphorylated peptides can be extremely low compared to the unmodified peptides, the goal is to purify as much protein as possible for optimal success. This is typically done through an epitope tag such as FLAG, HA or Myc or through immunoprecipitation (IP) with a suitable antibody. The next step involves protein purification using either SDS-polyacrylamide gel electrophoresis (SDS-PAGE) especially for the case of antibody IPs followed by gel band excision proteolytic digestion followed by

tandem mass spectrometry (LC-MS/MS). For the case of epitope tagged proteins, solution based digestions are possible if detergent-free and low salt-containing buffers are used for protein elution (Alternative Protocol 1). In addition, one can choose additional steps prior to LC-MS/MS to enrich phosphorylated peptides using either metal ion affinity chromatography (IMAC) (Basic Protocol 2) or titanium dioxide beads (TiO₂) (Support Protocol 1). Figure 1 shows a flowchart describing the sequential steps used in the following protocol for identifying/mapping phosphorylation sites on single proteins and simple protein mixtures.

Materials for Basic Protocol 1

Lysis and IP-buffer: (0.5% NP-40, 1% TritonX-100, NaCl 150 mM, Tris-HCl pH=7.4, EDTA pH=8.0 1 mM, EGTA 1 mM)

Protease and phosphatase inhibitors (1 mM of Na₃VO₄, 1 mM Aprotinin, 1 mM Leupeptin, 1 mM Pepstatin, 10 mM NaF, 1 mM PMSF, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate)

Liquid nitrogen (−196°C)

Stainless steel mortar with ceramic pestle

Bradford assay (Biorad)

HPLC grade solvents (methanol (MeOH), water (H₂O), acetonitrile (ACN))

LC/MS grade water and acetonitrile

Cold acetone (−20°C freezer)

A/G-protein agarose beads

Mini-gel SDS-PAGE apparatus (Bio-Rad or other vendor) with power supply

1X SDS Sample buffer (31.25 mM Tris, pH=6.8; 5% Glycerol; 1% SDS; 0.36 M beta-Mercaptoethanol; 0.0025% Bromophenol blue)

SDS-PAGE Tris-Glycine polyacrylamide gels 10-well, 1-mm (10% fixed or 4–20% gradient, Lonza #58511)

Tris-Glycine SDS Running Buffer (1X formulation: 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH=8.3.)

Coomassie blue stain (15% methanol, 10% acetic acid, 2 g Coomassie Brilliant Blue)

Coomassie destain (15% methanol, 10% glacial acetic acid)

0.5 mL microcentrifuge tubes

1.5 mL microcentrifuge tubes

2.0 mL microcentrifuge tubes

15 mL polypropylene conical centrifuge tubes

50 mL polypropylene conical centrifuge tubes

10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (NH₄HCO₃)

55 mM iodoacetamide (IAA) in 100 mM NH₄HCO₃

100 mM, 50 mM and 20 mM NH₄HCO₃

Trypsin, TPCK modified sequencing grade (Promega Corp. # 9PIV5113, 20 µg/vial)

Acetic acid (HA): 50 mM

Shaking incubator (37°C)

Refrigerated (4 °C) centrifuge 14,000g speed for microcentrifuge tubes (Eppendorf)

Refrigerated (4°C) centrifuge for 15 mL and 50 mL conical tubes (Beckman)

2% Formic acid (FA)/40% acetonitrile (ACN)

SpeedVac concentrator (ThermoFisherScientific)

TiO₂ SpinTips Sample Prep Kit (Protea Biosystems #SP-154-24, Containing TiO₂ SpinTips for 20–1000 µL volumes, 25 mL TiO₂ Reconstitution and Wash 1 solution, 25 mL of Wash 2 solution, 25 mL of TiO₂ Elution solution)

TiO₂ Phos-Trap kit (Perkin-Elmer #PRT301001KT, Containing 20x Phos-trapTM Magnetic Beads, Binding Buffer, Washing Buffer, Elution Buffer)

Magnet for microcentrifuge tubes (Fisher Scientific)

Multi-channel pipettor

Vortex shaker

12 x 32 mm autosampler vial (National Scientific, catalog #C4000-87)

HPLC A buffer (99% water, 0.9% ACN, 0.1% FA)

HPLC B buffer (100% ACN)

C₁₈ ZipTip (Millipore, ZTC18S096, 96 count)

ZipTip binding and wash buffer (0.1% trifluoroacetic acid (TFA))

ZipTip elution buffer (40% ACN/0.1% TFA)

Pico-Frit packed C₁₈ columns: 75µm ID x 15cm length (New Objective, PF7515-150H002-3P)

High resolution/high mass accuracy mass spectrometer: ThermoFisherScientific (LTQ-Orbitrap XL, Velos Pro Orbitrap, Velos Elite Orbitrap, qExactive), Waters Xevo, AB/Sciex 5600, Agilent QTOF 6500 series, Bruker microTOF, etc.

Nanoflow HPLC: ThermoFisherScientific EASY-nLC, Waters NanoAcquity, Eksigent NanoLC Ultra, Bruker Nanoflow-LC, etc.

Method

Cell lysis

- 1 Prepare cells to make sure they are in log-phase. Lyse a sufficient amount of cells ($\sim 10^7$) in ~ 5 mL of Lysis buffer with protease/phosphatase inhibitors to produce at least 10 mg of protein

NOTE: For lysis of frozen tissue (use ~ 100 mg to produce 10 mg of protein), grind it using a liquid nitrogen stainless steel mortar with ceramic pestle until it is a powder and let liquefy at 4°C and then immediately add lysis buffer containing protease/phosphatase inhibitors.
- 2 Incubate lysate for 45 minutes at 4°C with gentle rocking in a 15 mL conical tube
- 3 Centrifuge 15 mL tube with lysate for 20 minutes at full speed (14,000g) at 4°C to remove cell debris, keep supernatant

- 4 Take a small aliquot and determine the protein concentration using the Bradford assay
- 5 Transfer to a new tube and dilute the protein lysate to ~2 mg/mL (5 mL total) and keep at 4°C

Immunoprecipitation

- 6 Add ~8–10 µg of antibody or enough to clear the lysate of the protein of interest (varies across different antibodies) and incubate the solution on a rotator from 2 hr - overnight at 4°C
- 7 Wash protein A (or G) agarose beads with 1 mL of Lysis buffer by agitating 5X, centrifuge at 2,500g, discard buffer, repeat 3X and make a 1:1 slurry, at 4°C
- 8 Add 80µL of the beads slurry (40 µL pure beads) to the lysate - antibody solution and incubate for 2 hr on a rotator at 4°C
- 9 Centrifuge at 2,500g for 2 minutes at 4°C, remove supernatant completely
- 10 Wash the protein-antibody-beads complex with 1 mL of Lysis buffer by rocking for 3 minutes at 4°C
- 11 Centrifuge with 2,500g for 2 minutes at 4°C, repeat STEPS 10–11 3X
- 12 Elute the proteins from beads by adding ~80 µL of 1X SDS sample buffer (twice the vol of pure beads) for 5 minutes at 95°C.

NOTE: Proteins can also be eluted from bait protein/antibody complex using competition with a peptide (e.g. FLAG or HA for tagged bait proteins), small molecule or pH change in some cases instead of SDS sample buffer.

SDS-PAGE (preferred for protein purity)

- 13 Load sample (in 1X SDS sample buffer) on a SDS-PAGE gradient or fixed percentage polyacrylamide gel appropriate for purifying the specific MW of the protein of interest.
- 14 Run mini SDS-PAGE at ~120 V for ~1 hr or until the solvent dye front reaches the bottom of the gel for optimal resolution
- 15 Stain the gel with coomassie blue stain for 1 hr at RT and destain the gel overnight at 4°C with at least 5 different solvent changes in the first 2 hr
- 16 Excise the protein gel band(s) of interest, put them in 1.5 mL plain microcentrifuge tube and wash with 150–200 µL of 50% ACN/50% water for 15 minutes and discard supernatant, repeat 1X. The gel sample can be stored in same tube, moist (not submerged), and frozen at –20°C or below.

In-gel digestion—Carrying out in-gel enzymatic digestion (Li *et al.*, 1997) from SDS-PAGE allows for low cost and efficient processing method of samples that removes salts and/or detergents that are part of the lysis buffer and interfere with mass spectrometry analyses. It allows for both sample fractionation and separation and provides protein molecular weight information. The disadvantages are the potential loss of material incurred by the additional sample manipulation steps introduced by loading samples onto gels and potential losses during peptide extraction from gels after tryptic digestion, however, these drawbacks are relatively minor.

- 17 Dry the gel bands in a SpeedVac concentrator for ~15 minutes with no heat.

- 18 Reduce the gel with a volume sufficient to cover the gel pieces with 10 mM DTT in 100 mM NH_4HCO_3 at 56°C for 30 minutes
- 19 Add 100% ACN to shrink the gel pieces (same volume like in STEP 18) for ~5 minutes, RT.
- 20 Remove liquid and add 55 mM IAA in 100 mM NH_4HCO_3 to the gel with the same volume that was used for reduction (STEP 18) and keep in the dark (closed drawer), for 30 minutes at RT.
- 21 Wash gel pieces (volumes as needed) with 100 mM NH_4HCO_3 for 10 minutes at RT. Discard buffer and shrink the gel piece with 100% ACN for ~5 minutes, RT
- 22 Remove all liquid and swell with same volume as in STEP 18 of 100 mM NH_4HCO_3 for ~5 minutes, RT
- 23 Remove all liquid and dehydrate/shrink with the same volume of 100% ACN as in STEP 18 for 5 minutes at RT.
- 24 Remove all liquid and completely dry gel in SpeedVac with no heat for ~15 minutes
- 25 On ice, add a volume of 25 ng/ μL trypsin (195 μL 50 mM NH_4HCO_3 + 5 μL of 1 $\mu\text{g}/\mu\text{L}$ TPCK modified trypsin in 50 mM acetic acid) according to the following guideline based on gel size: [Small = 14 μL , Medium = 18 μL , Large = 22 μL]
NOTE: Different enzymes can be used separately in addition to trypsin in STEP 25 such as chymotrypsin, AspN or GluC for increasing the protein's amino acid coverage.)
- 26 Incubate 15 minutes on ice, then add an additional volume of 50 mM NH_4HCO_3 (enough to completely cover gel slice)
- 27 Digest overnight (>12 hr) at 37°C in a shaking thermal incubator
- 28 Add 30–45 μL (or as needed) 20 mM NH_4HCO_3 (depending on the gel size, gel must be covered) and incubate for 10 minutes at 37°C with shaking. Spin down in benchtop microcentrifuge for 30 seconds and collect supernatant and add to a plain 1.5 mL microcentrifuge tube.
- 29 Add ~50–75 μL (or as needed) of 2% formic acid/40% acetonitrile and incubate for 15 minutes at 37°C with shaking. Spin down in benchtop microcentrifuge for 30 seconds and add supernatant to first extraction.
- 30 Partially dry down in SpeedVac with no heat to a final volume of 10 μL .

Alternative Protocol 1: Precipitation (for IP elution with peptide or small molecule)

Acetone precipitation cleans protein samples of both salts and detergents (ThermoScientific, 2009). Use only when proteins are eluted by competition without IgG contamination since excess IgG will suppress phosphopeptides of interest.

- 1 Cool the required volume of acetone to –20°C in freezer.
- 2 Place protein sample in acetone-compatible tube.
- 3 Add four times the sample volume of cold (–20°C) acetone to the tube.
- 4 Vortex tube and incubate for 60 minutes at –20°C.
- 5 Centrifuge 10 minutes at 14,000g, 4°C.

- 6 Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
- 7 Repeat STEPS 3–6 1X
- 8 Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes. Do not over-dry pellet, or it may not dissolve properly.

In-solution digestion (precipitated protein)

- 9 Add 20 μ L of 10 mM DTT solution (same for gel digests) to the sample pellet and incubate for 30 minutes at 56°C.
- 10 Add 20 μ L of 55 mM IAA solution (same for gel digests) and incubate for 30 minutes at RT in dark.
- 11 Dilute sample to 200 μ L with 50 mM ammonium bicarbonate solution pH~8.3 (same for gel digests).
- 12 Add 2.0 micrograms (μ g) of trypsin (2 μ L of the 1 μ g/ μ L TPCK modified trypsin solution from STEP 25)
- 13 Incubate overnight at 37°C with shaking.
- 14 The next day, add 20 μ L of 5% TFA to stop digest and the sample is ready for clean-up. Take 1–2 μ L to check pH ~3.5 (pH paper is sufficient)
- 15 Concentrate sample in SpeedVac to a final volume of 10 μ L

Purification and Concentration of solution digest

- 16 Prepare C₁₈ ZipTip by aspirating/expelling 20 μ L of 100% ACN, discard washes, repeat 1X
- 17 Aspirate/expel 20 μ L of 0.1% TFA/40% ACN, discard washes, repeat 2X
- 18 Equilibrate ZipTip by aspirating/expelling 20 μ L of 0.1% TFA, discard washes, repeat 3X
- 19 Load acidified peptide digest onto ZipTip, by aspirating/expelling the sample solution 5X, save flow-through
- 20 Wash loaded sample with 20 μ L 0.1% TFA buffer by aspirating/expelling, discard washes, repeat 4X
- 21 Elute with 10 μ L of 0.1% TFA/40% ACN by aspirating/expelling the same solution 5X into a new 0.5 mL microcentrifuge tube or 12 x 32 mm autosampler vial
- 22 Add 40 μ L HPLC A buffer, dry down in SpeedVac to 10 μ L final volume

Tandem mass spectrometry (LC-MS/MS)

- 23 Inject a 3–5 μ L aliquot (depending upon amount of starting material) by microcapillary reversed-phase liquid chromatography/tandem mass spectrometry (LC-MS/MS) (IMPORTANT: Do not inject more than half of the sample in case of a system failure)
- 24 An HPLC system capable of nanoliter flow rates is needed. This can be a split or splitless system. Use a C₁₈ packed analytical column with typical dimensions of 75 μ m ID x 15 cm length at a flow rate of ~250 nL/min for optimal sensitivity.

NOTE: packed analytical columns can be purchased commercially or empty columns can be purchased and self-packed with C₁₈ material (Lee, 2001)

- 25 Set up method for data-dependent acquisition (DDA) or “shotgun” mode over a 90 minute LC-MS/MS acquisition using an appropriate high resolution/high mass accuracy tandem mass spectrometer. It is typical to collect a single MS spectrum followed by 5–10 MS/MS spectra per DDA cycle with a 120 second exclusion window and a 2.5 *m/z* MS/MS isolation window.

NOTE: One can set the mass spectrometer to include specific *m/z* values representing phosphopeptides of interest to increase the sensitivity of detection through “inclusion lists” or fixed targeted scan events (Egan *et al.*; Yang *et al.*; Dibble *et al.*, 2009).

- 26 The nano-LC gradient conditions should be the following: Initial conditions 2% B; 2% B to 38% B over 90 minutes; 38% B to 95% B over 2 minutes; hold at 95% B for 4 minutes; 95% B to 2% B over 1 minute; hold at 2% B for 12 minutes to re-equilibrate column.

NOTE: One can optionally also use in-line micro filters and/or trap columns to help further purify samples and extend analytical column life.

Database searching and phosphorylation site identification

- 27 The raw MS/MS fragmentation data must be processed using database search engine software such as commercially available Sequest (ThermoFisherScientific) or Mascot (Matrix Science, Ltd.) at <http://www.matrixscience.com/>. These software programs contain algorithms to extract MS/MS datafiles for querying protein databases such as the annotated all-species Swiss-Prot (<http://www.uniprot.org/downloads>) or species specific databases from IPI (<http://www.ebi.ac.uk/IPI/>). Alternatively, one can use freeware search engines such as XTandem! (<http://www.thegpm.org/tandem/>) or Andromeda within MaxQuant software environment (<http://www.maxquant.org/>).
- 28 Once downloaded, it is recommended that the protein database is also set up as a decoy database. This can be either a database of reversed sequences or random sequences and is usually concatenated with the target (forward) database. This allows one to calculate a false discovery rate (FDR) (Elias *et al.*, 2005) in order to statistically evaluate the search results.
- 29 In the search engine, use the following parameters:
 - a. Enzyme: Trypsin (cleavage at C-term of Lys (K)/Arg (R))
 - b. Precursor mass tolerance: ≤ 15 ppm for a well-calibrated mass spectrometer
 - c. Differential modifications: oxidation +15.9949 on Met (M); phosphorylation +79.9663 on Ser (S)/Thr (T)/Tyr (Y)
 - d. Fragment ion tolerance: ≤ 15 ppm for high mass accuracy MS/MS (HCD, TOF, Orbitrap) and ~ 0.08 Da for low mass accuracy MS/MS (ion trap CID, quadrupole CID)

NOTE: Alternatively, one can use a single entry database with no enzyme specificity. This is useful when enzymes other than trypsin or combinations of enzymes were used for digestion of the protein; however, more rigorous validation is required.

Interpretation of Phosphorylation sites—Phosphorylation results in a peptide ion with a +80 Da mass increase compared to the unmodified peptide for each phosphorylated Ser, Thr, or Tyr residue. In addition, site localization can be achieved on a peptide through the fragmentation pattern whereby fragment ions starting at the modification site will reflect the +80 Da shift. Increased confidence in phosphopeptide assignments can be gained by considering specific spectral features, for example, the facile neutral loss of phosphoric acid (−98 Da) from Ser and Thr phosphorylated peptides upon fragmentation with (Hunter and Games, 1994; Asara and Allison, 1999) Tyr phosphorylation tends to show a prominent loss of metaphosphoric acid (−80 Da). While this can help indicate the presence of a phosphate group, it can occasionally adversely affect the ability of search engines to identify phosphopeptides with sufficient scores due to the reduction in sequence specific fragment ions as they are transferred to the neutral loss (Boersema *et al.*, 2009). This loss is more prominent during fragmentation with ion traps than with collision cell mass analyzers.

- 30 Set threshold search engine scores so that the FDR is $\leq 1.0\%$. This assures that quality spectra and true phosphopeptide matches are accepted in the analysis.
- 31 For critically important phosphorylation sites, view the MS/MS spectrum to verify that the **b**- (fragment ions from the N-terminus of the peptide) and **y**- ions (fragment ions from C-terminus) are consistent with the predicted sequence and modification site from the search engine.
- 32 Software is available for creating amino acid coverage maps including ProteinReport in Proteomics Browser Software (ThermoFisherScientific or <http://www.mcb.harvard.edu/microchem/>) and Scaffold PTM Software (Proteome Software, Inc.) as shown in Figure 2.

NOTE: One can crudely assess the stoichiometry of a phosphorylation site by comparing the number of sequence events (dark green lines) for the phosphorylated (containing magenta highlight, Figure 2) peptide versus the non-phosphorylated peptide (Gwinn *et al.*, 2008).
- 33 Software is also available to perform site localization of phosphorylation using probabilistic algorithms such as ASCORE (Beausoleil *et al.*, 2006) within ScaffoldPTM software (Proteome Software, Inc. or at <http://ascore.med.harvard.edu/>) and MaxQuant (Cox and Mann, 2008; Cox *et al.*, 2009) at <http://maxquant.org/>.

Support Protocol 1: Phosphopeptide Enrichment

Affinity based enrichment phosphopeptides with immobilized metal affinity chromatography (IMAC) can also be used to enhance the number of identified phosphopeptides in an analysis. Typically, one can use commercially available reagents that include Fe^{3+} , Ga^{3+} or titanium dioxide (TiO_2) (Larsen *et al.*, 2005). TiO_2 has lower affinity for multi-phosphopeptides and works better for enrichment of monophosphorylated peptides while IMAC has a higher efficiency for the recovery of multi-phosphorylated peptides (Thingholm *et al.*, 2006). Sometimes, TiO_2 can also contain nonspecific binding from acidic non-phosphorylated peptides (contain D and E residues). This can be significantly reduced by including 2,5-dihydroxybenzoic acid (DHB), phthalic acid, or glycolic acid and high concentrations of TFA in the loading buffer (Thingholm *et al.*, 2008; Villen and Gygi, 2008)

For enrichment of phosphopeptides several commercial kits are available including IMAC (PHOS-Select Iron affinity gel, SIGMA) and TiO_2 (Phos-Trap kit, Perkin-Elmer or TiO_2 SpinTips, Protea Biosciences). We suggest that half of the sample peptide mixture be run directly by LC-MS/MS and the second half of the sample can be used for phosphopeptide enrichment using a support protocol below.

OPTION 1: Protea TiO₂ SpinTips Sample Prep Kit (Protea Biosciences)**Preparation**

- 1 Ensure that the packing material is at the bottom of the tip by gently tapping the tip to displace any packing material sticking to the top of the red cap.
- 2 Place SpinTip adaptor onto a 2 mL centrifuge tube.
- 3 Wash the SpinTip to wet the packing material by adding 100 μ L TiO₂ Reconstitution and Wash 1 Solution to the top of the SpinTip using a micropipette.
- 4 Centrifuge the system at 4,000g for 3–5 minutes. Discard eluent and repeat STEPS 3–4.

Binding

- 5 Reconstitute ~5–20 μ L of the peptide sample in ~200 μ L TiO₂ Reconstitution and Wash 1 Solution and sonicate until the sample is completely dissolved.
- 6 Load 200 μ L of the sample solution by adding it to the top of the SpinTip and centrifuging the system at 4,000g for 3–5 minutes, RT.
- 7 Wash the sample to elute salts and other loosely-bound components by adding 100 μ L TiO₂ Reconstitution and Wash 1 Solution to the top of the SpinTip.
- 8 Centrifuge the system at 4,000g for 3–5 minutes. Discard eluent and repeat STEPS 7–8.

Elution

- 9 Transfer the SpinTip to a new, clean 2 mL centrifuge tube to collect the sample during elution.
- 10 Elute the sample by adding 100 μ L TiO₂ Elution Solution to the top of the SpinTip. Centrifuge the system at 4,000g for 3–5 minutes. Repeat the SpinTip sample elution 2X using the same elution solution.
- 11 Partially dry the solution to ~ 5 μ L in SpeedVac and then add 100 μ L of HPLC A buffer and transfer to a 12x32 mm autosampler vial
- 12 Partially dry to 5 μ L and inject onto LC-MS/MS system.

OPTION 2: Phos-Trap TiO₂ phosphopeptide enrichment kit (PerkinElmer)**Preparation**

- 1 Mix the vial of magnetic beads so that the beads are evenly suspended in solution. Take 20 μ L from vial of magnetic beads and 180 μ L of HPLC grade water for each experiment. For example, for 3 experiments, take 60 μ L of beads with a 1 mL pipet since beads can clog the 200 μ L pipets and add 540 μ L of water and mix in an appropriately sized tube.
- 2 Dispense 200 μ L of bead solution into each 1.5 mL microcentrifuge tube.
- 3 Place on a magnet to catch the beads and remove the liquid without removing any beads.
- 4 Wash beads with 200 μ L of binding buffer. Add buffer “off” the magnet and lightly shake to suspend the beads evenly in solution for 1 minute.
- 5 Put tray (tube) on magnet to remove liquid without removing beads, repeat 2X.

Binding and washing

- 6 Dilute purified peptide sample 1:10 in binding buffer but do not exceed 150 μ L in total volume. Partially SpeedVac the sample prior to dilution if necessary.
- 7 Add the sample solution to the beads and incubate for 60 minutes while shaking continuously to keep the beads suspended in solution.
- 8 Put 1.5 mL micro centrifuge tube on magnet and remove liquid (not beads) from the tubes and transfer to a tube labeled 'TiO₂ flow through'. These peptide mixtures can be analyzed in the mass spectrometer if one is interested in non-phosphorylated peptides.
- 9 Wash the beads with 200 μ L of binding buffer for 1 minute, put tube on magnet and remove liquid, repeat 3X.
- 10 Wash the beads with 200 μ L of wash buffer for 1 minute, put tube on magnet and remove liquid.

Elution

- 11 Elute the phosphopeptides by adding 35 μ L of elution buffer to beads and incubate for 15 minutes with continuous shaking.
- 12 Place tube on a magnet to catch the beads, aspirate solution but DO NOT draw up any beads and add the elution containing the sample to a 12x32 mm autosampler vial.
- 13 Add 50 μ L of A buffer and SpeedVac to 5 μ L
- 14 Inject entire 5 μ L volume onto LC-MS/MS system

Anticipated Results

Figure 2 shows the typical results that one should obtain from an experiment whereby a protein was immunoprecipitated from cell lysate, purified by SDS-PAGE, excised, digested with trypsin and analyzed by microcapillary LC-MS/MS. In order to enhance the detection of phosphorylation sites, one can use phosphopeptide enrichment methods such as metal affinity with TiO₂ and/or IMAC. Figure 3A shows an example of a MS/MS spectrum acquired using CID for a pSer-containing peptide displaying the typical neutral loss of phosphoric acid observed by most Ser/Thr phosphopeptides. Figure 3B shows the computational results determining the site localization of a phosphopeptide. Software such as ASCORE (<http://ascore.med.harvard.edu/> or Scaffold PTM software), GraphMod (Proteomics Browser Software) and MaxQuant (<http://maxquant.org/>) are capable of determining the accurate phospho site in a given peptide.

Time Consideration per sample

Protein IP

- Lysis and immunoprecipitation: 2 hr to overnight (12–16 hr)
- Digestion (in-gel or in-solution): overnight (12–18 hr)
- Optional: ZipTip purification: 1 hr
- Optional: TiO₂ Phosphopeptide enrichment: 2–3 hr
- LC-MS/MS: 2–3 hr
- Data analysis 1–3 hr

– Total time: 2–3 days

Basic Protocol 2: Global Phosphorylation Analysis (Ser/Thr/Tyr)

In addition to determining the phosphorylation sites from purified proteins or protein complexes, it is possible to perform global phosphorylation site profiling directly from whole cell extracts and tissue sources (Olsen *et al.*; Kirkpatrick *et al.*, 2005). Using the procedures to follow, researchers have discovered up to 36,000 *in vivo* phosphorylation sites from various mouse organ tissues though most global phosphorylation studies result in several thousand phosphorylation sites detected (Olsen *et al.*; Beausoleil *et al.*, 2004; Oppermann *et al.*, 2009; Pan *et al.*, 2009). While these analyses are very time-consuming, require milligrams of material, offline strong cation exchange (SCX) peptide fractionation and a dozen 2-hour LC-MS/MS runs, one can achieve hundreds to thousands of phosphopeptide identifications from a biological sample. In general, the ratio of threonine, serine and tyrosine protein phosphorylation is approximately 90%, 10% and 0.05%, respectively (Hunter and Sefton, 1980). We describe a protocol for determining Ser/Thr/Tyr phosphopeptide identifications on a global scale as well as a protocol for isolating just the Tyr phosphopeptides from cell and tissue extracts. Figure 4 shows a flowchart outlining the sequential steps used in the following protocol for identifying potentially thousands of phosphorylation sites.

Materials for Basic Protocol 2

Urea buffer (8 M Urea, 1.5 M Thiourea, 20 mM HEPES pH=8.0)

Protease/phosphatase inhibitors (Basic Protocol 1)

45 mM DTT (Mix 180 μ L of 1.25 M DTT (19.25 g/100 mL) in 5 mL HPLC grade water, add to sample in ~1/10 dilution (e.g. 0.5 mL DTT in 5 mL sample))

110 mM IAA (Dissolve 209 mg IAA in 10 mL HPLC grade water, add to sample in ~1/10 dilution)

Sequencing grade modified Trypsin, 1 mg/vial (Worthington, LS02123)

Trifluoroacetic acid (TFA) 20%, 10%, 1%, 0.1% solutions (vol/vol)

Sep-Pak C₁₈ cartridges 6cc/500 mg (Waters, WAT036790) for P-TYR IP method

Sep-Pak C₁₈ cartridges 3cc/50 mg (Waters, WAT054960) for SCX method

Sep-Pak Elution Buffer (0.1% TFA/40% ACN)

250- μ L pipette tips for StageTip preparation

IMAC (PHOS-Select Iron affinity gel, SIGMA # P9740)

IMAC binding buffer (40% ACN (vol/vol), 25 mM FA, H₂O)

IMAC elution buffer A (50 mM K₂HPO₄/NH₄OH, pH 10.0)

IMAC elution buffer B (500 mM K₂HPO₄, pH=7)

Formic acid (1%, 10% solutions (vol/vol))

Empore 3M C₁₈ material

Cutter device (Hamilton, Needle Kel-F hub (KF), point style 3, gauge 16, # 90516; plunger assembly N, RN, LT, LTN for model 1702 (25 μ L), # 1122-01)

Strong cation exchange (SCX) Column: PolySULFOETHYL A 250 x 9.4 mm; 5 μ m pore size; 200Å (PolyLC, 259-SE0502)

SCX buffer A (7 mM KH_2PO_4 , pH=2.65, 30% ACN (vol/vol))

SCX buffer B (7 mM KH_2PO_4 , 350 mM KCl, pH=2.65, 30% ACN (vol/vol))

Note for SCX buffers: Organic solvents affect the pH reading, the pH adjustments for buffers A and B should be performed before the addition of ACN.

StageTip and Sep-Pak Binding and Wash Buffer (0.1% TFA (vol/vol) in H_2O)

StageTip Elution Buffer (40% ACN (vol/vol), 0.5% HA (vol/vol) in H_2O)

Cell Lysis of cells and/or tissues

- 1 Prepare cells to make sure they are in log-phase.

Note: One can stimulate the cells with FCS to increase the general phosphorylation signaling or treat with specific growth factors and/or drugs to activate/inhibit a particular signaling pathway.

- 2 Lyse enough cells ($\sim 10^7$ – 10^8) or tissue (~ 150 – 200 mg) to produce ~ 15 – 20 mg of protein in ~ 5 – 10 mL of Urea lysis buffer with protease/phosphatase inhibitors.

NOTE: For lysis of frozen tissue, grind it using a liquid nitrogen stainless steel mortar with ceramic pestle until it is a powder and let liquefy at 4°C and then immediately add Urea lysis buffer containing protease/phosphatase inhibitors.

- 3 Aspirate/expel the lysate several times with a 1 mL pipette
- 4 Sonicate for 1 minute at 4°C , repeat 2X
- 5 Centrifuge 15 mL tube with lysate for 5 minutes at full speed at 15°C to remove cell debris, keep supernatant
- 6 Take a small aliquot and determine the protein concentration using the Bradford assay

Digestion

- 7 Add 1/10 of the solution volume of 45 mM DTT to the protein lysis solution to make a 4.5 mM DTT solution and incubate it for 30 minutes at 56°C .
- 8 Add the same amount of 110 mM IAA and incubate it for 30 minutes at RT in the dark to make an 11.0 mM IAA solution.
- 9 Dilute sample 1:5 with water to final concentration of 1.6 M urea and a final pH=8.0
- 10 Dissolve trypsin in 1 mL of 50 mM acetic acid to the final concentration of 1 $\mu\text{g}/\mu\text{L}$ in trypsin vial
- 11 Add trypsin solution to the lysate (~ 75 :1 substrate/protease ratio)
- 12 Incubate the solution overnight at 37°C on a thermal incubator (~ 450 rpm).
- 13 The next day stop the protease reaction by adding 20% TFA to 1/20 of the digestion solution volume to a final concentration of 1% TFA
- 14 Let it stand for 10 minutes at RT
- 15 Clear the sample of debris by centrifuging for 10 minutes 14,000g ($r = 300$ mm: 2100 rpm) at 15°C , keep the supernatant (digested peptides)

Purification

Samples first need to be purified prior to subsequent pTyr IP or SCX fractionation to eliminate interference

- 16 Check pH of cleared sample to be sure it is between pH=2.0–3.0
- 17 Pre-wet 6cc/500mg C₁₈ Sep-Pak with 3 mL of ACN
- 18 Wash with 3 mL of 0.1% TFA/40% ACN
- 19 Wash with 3 mL of 0.1% TFA, discard washes, repeat 2X
- 20 Load peptide digestion sample (STEP 15) onto Sep-Pak
- 21 Wash column with 3 mL of 0.1% TFA, discard washes, repeat 2X
- 22 Elute peptides with 1 mL of 0.1% TFA/40% ACN, repeat 2X and combine eluate for 3 mL total volume.
- 23 Divide sample into two microcentrifuge tubes. Dry down partially in SpeedVac with no heat to ~200–400 μ L, combine both samples into one 1.5 mL microcentrifuge tube.
- 24 Dry down completely to a pellet in a SpeedVac with no heat

Strong Cation Exchange (SCX) (peptide fractionation)

This approach fractionates the digested peptides according to their solution charge using strong cation exchange (SCX) chromatography. Phosphopeptide enrichment with IMAC is then used after peptide separation prior to LC-MS/MS.

In strong cation exchange (SCX)-chromatography, trypsinized peptides are eluted from the column according to their solution charge state. Most phosphorylated peptides at pH=3 carry 1+ or 2+ charges since the peptide charge is reduced by 1 for each negatively charged phosphate group (Olsen and Mann, 2004; Villen and Gygi, 2008). Most non-phosphorylated tryptic peptides carry 2+ and 3+ charges in solution. However, other factors including hydrophobicity also play a role with SCX separation. In addition, attention should be paid to multi-phosphorylated peptides containing a low or neutral net charge because these peptides are unable to bind to the stationary phase and elute in the flow-through fraction. SCX separation should take place using a HPLC system capable of 1 mL/min flow-rates offline from the LC-MS/MS system. This procedure is frequently referred to as multidimensional protein identification technology (MUDPIT) (Washburn *et al.*, 2001; Wolters *et al.*, 2001).

HPLC

- 25 Degas SCX A and B buffers, purge SCX HPLC pumps, clean 1 mL sample loop and equilibrate column with 1% B for 14 minutes at 1 mL/min.
- 26 Load a blank sample containing only SCX A buffer onto sample loop (system in load position) to prepare the HPLC system for subsequent runs
- 27 Inject the blank sample (system in inject position) and run HPLC method at a flow rate of 1 mL/min over a PolySULFOETHYL A SCX column using the following gradient conditions:

1%–30% B: 40 minutes, 30%–50% B in 1 minute, 50%–100% B in 5 minutes, 100% B for 5 minutes, 100%–1% B for 1 minute, 1% B for 14 minutes to re-equilibrate column

- 28 Dilute dried, desalted pellet from STEP 24 in 950 μ L of SCX A buffer, vortex to completely dissolve
- 29 Inject 950 μ L of sample onto 1 mL sample loop and run SCX method as in STEPS 26–27

Fraction collection

- 30 Collect ~ 3–4 minutes (3–4 mL) fractions from the elution starting at time 0' for a total of ~12 fractions in separate 15 mL conical tubes
- 31 Concentrate fractions to dryness in SpeedVac with no heat

Purification of salts

- 32 Dissolve dried peptide pellet in 500 μ L of 0.1% TFA
- 33 Check pH of cleared sample with pH paper to be sure it is between 2.0–3.0 using 5 μ L from each of the 12 fractions
- 34 Pre-wet 3cc/50 mg capacity C₁₈ Sep-Pak with 1 mL of ACN (Prepare 1 Sep-Pak per fraction)
- 35 Wash Sep-Pak with 1 mL of 0.1% TFA/40% ACN
- 36 Wash Sep-Pak with 1 mL of 0.1% TFA, repeat 2X
- 37 Load peptide digestion sample from STEP 32 onto prepared Sep-Pak
- 38 Wash sample on Sep-Pak with 1 mL of 0.1% TFA, repeat 2X
- 39 Elute peptides with 333 μ L of Sep-Pak Elution Buffer in 1.5 mL microcentrifuge tube, repeat 2X with fresh elution buffer and combine for 1 mL total volume
- 40 Dry peptide sample completely to a pellet in a SpeedVac using no heat

Phosphopeptide Enrichment

Since there are no good quality antibodies available to this date for the enrichment of general phosphoserine (pSer) and/or phosphothreonine (pThr) residues, there are two possible strategies for enriching all phosphorylation sites from a complex cell and/or tissue lysate using immobilized metal affinity chromatography (IMAC) using iron as well as TiO₂ (see Support Protocol 1).

PHOS-Select IMAC (Sigma)

Preparation

- 41 Prepare 100 μ L of IMAC beads by washing them with 1 mL of IMAC binding buffer, turning over the vial 5X to re-suspend all beads and spinning at 2,500g to remove the liquid. Repeat 3X and prepare 50% slurry in the same buffer.
- 42 Prepare twelve 0.5 mL microcentrifuge tubes and place 10 μ L of IMAC beads slurry into each. Cutting the end of the pipet tip to a wider opening facilitates pipetting of beads.

Binding

- 43 Dissolve each dried peptide fraction obtained from STEP 40 in 120 μ L of IMAC-binding buffer and transfer to the IMAC beads to a total volume of 130 μ L.
- 44 Incubate peptides on beads for 60 minutes at RT with vigorous shaking.

- 45 During this time, prepare twelve StageTips by cutting two disks of Empore 3M C₁₈ material with the cutter device and packing into 250-μL pipette tips (Rappsilber *et al.*, 2007).
- 46 Wash and equilibrate packed StageTips by passing through 20 μL of MeOH followed by 20 μL of 40% ACN/0.5% HA and then 2X with 20 μL of 1% FA. For convenience and increased throughput, one can use a centrifuge by holding the StageTips within a 2-mL Eppendorf tube with the top part of a 500 μL Eppendorf body as an adaptor, limiting spinning speed to 2,000g and time to the minimum to get the liquid passed through.
- 47 After the 60-minute incubation in STEP 44, transfer IMAC beads to the top of the StageTips and spin down. The beads will get retained on the StageTip and the solution will pass through. As the buffer contains 40% ACN, nonphosphorylated peptides, which are not retained in the IMAC resin, will not be retained by the C₁₈ material. These peptide mixtures can be collected and analyzed as well in the mass spectrometer, if desired.
- 48 Wash with 50 μL of IMAC binding buffer. Repeat 1X.
- 49 Wash with 40 μL of 1% FA. This step allows equilibrating the StageTip C₁₈.
- 50 Wash with 70 μL of IMAC Elution Buffer B, repeat 2X.
At this point, phosphopeptides are eluted from IMAC resin and retained on the C₁₈ material.
- 51 Wash with 40 μL of 1% FA to remove phosphate salts.
- 58 Elute phosphopeptides from StageTips into 12x32 mm autosampler vials for MS analysis with 40 μL of 40% ACN/0.5% HA.
- 59 Dry down the samples from STEP 47 (nonphosphorylated peptides) and STEP 58 (phosphopeptides) with SpeedVac or lyophilizer.
- 60 Resolubilize the dried samples with 15–20 μL of HPLC A buffer and inject 2–10 μL depending upon sample quantity onto the LC-MS/MS system.
- 61 Run the mass spectrometer according Basic Protocol 1: STEPS 24–31 with the following change: LC-MS/MS gradient and acquisition time of ~120 minutes.

NOTE: For complex samples in Basic Protocols 2 and 3, the LC-MS/MS gradient and acquisition time should be increased from 60–90 minutes to ~120 minutes per sample to increase peptide/protein coverage.

Alternative Protocol 1: SDS-PAGE Protein Fractionation

This protocol involves fractionating proteins from a whole cell extract according to their molecular weight using a SDS-PAGE gel followed by in-gel digestion and subsequent IMAC or TiO₂ phosphopeptide enrichment and LC-MS/MS analysis. This is sometimes called GeLC-MS (Chang *et al.*, 2007).

(Several steps have been abbreviated since SDS-PAGE is described In Basic Protocol 1)

1. Lyse the material in Lysis and IP-buffer containing protease/phosphatase inhibitors for 45 minutes rocking at 4°C, centrifuge for 20 minutes at full speed at 4°C, keep supernatant
2. Load samples by SDS-PAGE according to the capacity of the mini-gel (fixed or gradient) and run completely to bottom of gel.

3. Stain with coomassie and destain overnight. The stain is mostly used here as a lane and protein amount indicator and not to resolve bands.
4. Cut gel into ~10–12 equal sections and place gel pieces in 1.5 mL centrifuge tubes.
5. Follow the above method for in-gel digestion (Basic Protocol 1: STEPS 17–30) and dry to a final volume of ~10 μ L.
6. For phosphopeptide enrichment of gel digested fractions, follow either Support Protocol 1 (TiO₂) or Basic Protocol 2 (IMAC) to prepare sample for LC-MS/MS.
7. Run the mass spectrometer according Basic Protocol 1: STEPS 24–31 with the following change: LC-MS/MS gradient and acquisition time of ~120 minutes.

Time Consideration per sample

Global pThr/pSer/pTyr

- Lysis and digestion: overnight (12–18 hr)
- Sep-Pak purification: 6 hr to overnight including dry-down step
- SCX HPLC run: 2 hr
- Sep-Pak purification of salted peptides: 6 hr to overnight with dry-down step
- IMAC/TiO₂ phosphopeptide enrichment: ~2–3 hr
- StageTip peptide purification: ~ 2–3 hr
- LC-MS/MS analysis for 12 samples: ~28–39 hr
- Data analysis for 12 runs: 8–12 hr
- Total time: ~7 days

Basic Protocol 3: Phosphotyrosine (pTyr) Site Identification

Although phosphotyrosine appears for only 0.05% of cellular protein phosphorylation it is the critical key player in a lot of signaling events starting from receptor tyrosine kinases. In addition, 20% of the kinome represents protein tyrosine kinases and reveals the importance for tyrosine phosphorylation events (Manning *et al.*, 2002). Due to the larger size of the modified tyrosine residue it is more qualified for developing antibodies for purification of proteins or peptides from complex cellular extracts containing pTyr residues (Rush *et al.*, 2005).

Materials for Basic Protocol 3

Phosphotyrosine P-Tyr-100 mouse antibody (mAb), sepharose conjugated (Cell Signaling Technology, # 9419)

P-Tyr-100 Elution buffer: 0.15% TFA solution (vol/vol)

1 M Tris (not pH adjusted)

Preparation—Before the pTyr immunoprecipitation can be performed, the solution must be desalted and the pH must be adjusted to neutral

- 1 Add 1.4 mL Lysis and IP-buffer (Basic Protocol 1) to digested and purified sample pellet (STEP 24, Basic Protocol 2), sonicate in 4°C water bath
- 2 Check pH, if less than pH=5, add 1 M Tris (not pH adjusted) dropwise until the solution is in the 6.0–7.5 pH range

- 3 Centrifuge 5 minutes at 1,800g (r = 60 mm: 5000 rpm) keep supernatant, cool on ice

Binding to pTyr Ab

- 4 Using 15–20 mg of total protein prior to digestion, add ~80 µg of P-TYR-100 mAB (#9419, Cell Signaling Technology) bead solution
- 5 Incubate for 2 hr on tube rotator at 4°C
- 6 Centrifuge with 2,500g for 1 minute at 4°C
- 7 Remove supernatant completely with gel loading tips to avoid removal of beads
- 8 Wash beads with 1 mL Lysis and IP-buffer, agitate by inverting five times, centrifuge at 2,500g for 1 minute at 4°C, discard buffer, repeat 1X
- 9 Wash beads with 1 mL HPLC grade water (4°C) to remove the detergents, agitate by inverting 5X, centrifuge at 2,500g for 1 minute, discard wash buffer, repeat 4X

Elution

- 10 Elute with 55 µL of P-Tyr-100 Elution buffer (0.15% TFA), tap bottom of tube several times to disrupt bead packing, incubate 10 minutes at RT
- 11 Tap again and centrifuge for few seconds at 2,500g, keep supernatant
- 12 Add 45 µL of 0.15% TFA tap bottom several times, and centrifuge for a few seconds at 2,500g, keep supernatant (with gel loading tip) and combine with previous elution (STEP 11) for a total of 100 µL

Purification and Concentration

- 13 Centrifuge for 1 minute at 2,500g to remove potential remaining agarose beads in tube
- 14 Divide sample in two equal parts
- 15 Prepare C₁₈ ZipTip: cut off the tip of a 200 µL tip, attach to top of ZipTip, it should fit tightly into upper ring of ZipTip.
- 16 Prepare ZipTip by aspirating/expelling 40 µL of 100% ACN, discard washes, repeat 1X
- 17 Aspirate/expel 40 µL of 0.1% TFA/40% ACN, discard washes, repeat 2X
- 18 Equilibrate ZipTip by aspirating/expelling 40 µL of 0.1% TFA, discard washes, repeat 3X
- 19 Place tip in 1st half of the sample, aspirate/expel 10X in sample tube (do not discard)
- 20 Place tip in 2nd half of the sample, aspirate/expel 10X in sample tube (do not discard)
- 21 Wash the ZipTip with 55 µL of 0.1% TFA, repeat 2X
- 22 Elute the peptides with 15 µL of 0.1% TFA/40% ACN but aspirate/expel 5X prior to final elution into a new 12 x 32 mm autosampler vial
- 23 Add 35 µL 0.1% TFA to the peptide elution (50 µL total vol), partially dry to 10 µL

IMPORTANT: Do not dry completely or peptides may be irreversibly lost

- 24** Inject 5 μ L of sample onto LC-MS/MS system with a 2–3 hour nano-LC gradient (save half of the sample in case of a system failure).

P-Tyr IP

- Lysis and digestion: overnight (12–18 hr)
- Sep-Pak purification: 6 hr to overnight including dry-down step
- Immunoprecipitation: 2 hr–4hr
- ZipTip Purification: 1.5 hr
- LC-MS/MS: 2–3 hr
- Data analysis: 2–3 hr
 - Total time: ~3 days

COMMENTARY

Background Information

Currently, outside of the very low throughput of site-specific antibodies, mass spectrometry is the only known and validated technology for identifying and in some cases quantifying individual sites of phosphorylation on proteins. Mass spectrometry is routinely used by researchers in the fields of molecular and cellular biology. While mass spectrometers are expensive and sophisticated pieces of equipment, many times the work is performed either within a core facility or in collaboration with an analytical laboratory specializing in the mass spectrometry field. While high resolution mass spectrometers are being acquired in some biology laboratories, it is not yet widespread to the cost of the equipment and maintenance required for optimal performance. The protocols above are designed to aid both the biologist who is getting into the mass spectrometry field or already possesses a state-of-the-art mass spectrometer and a reference for collaborators of biologists already in the mass spectrometry field to optimize strategies for identifying sites of phosphorylation from biological sources. Much of the work involved in this protocol is bench level work with cells and proteins and that is generally handled very well by the biologist. Mass spectrometry level expertise comes into play for acquisition of phosphopeptide data and subsequent processing and interpretation of the acquired data. However, software has improved to the point where trained technicians can interpret the data using statistics. Additional bioinformatics analyses of the data were not described in this protocol and require expertise in those fields.

Critical Parameters and Troubleshooting

Digestion failed	<ul style="list-style-type: none"> • Check for successful digestion, take aliquot before and after digestion and run on gel to verify proteolysis. • Check for pH before adding trypsin for digestion. It should be ~ pH=8.3. • Dilute solution digestion 1:5 with water before adding trypsin
No peptide peaks detected by LC-MS/MS	<ul style="list-style-type: none"> • Be sure peptide solution is acidic and purify peptides via Sep-Pak, StageTip or ZipTip with 0.1% TFA several times before loading the lysate • Detergents or other chemical contaminations left in the sample can prevent successful MS detection, wash IP with low-salt and detergent-free buffer pure before elution

<p>Peaks in LC/MS chromatogram but no peptide identifications</p>	<ul style="list-style-type: none"> Partially dry down samples in SpeedVac to remove organic solvents that can prevent binding to reverse-phase columns High salt concentrations may be left in the sample => filter sample through C18 Sep-Pak, StageTip or ZipTip for small volumes Mass spectrometer may be out of calibration MS/MS acquisition not working properly Be sure that the protein database used for searching contains the species of interest and the enzyme used in digestion was selected during the search Be sure the differential modification for phosphorylation of S/T/Y is enabled during the database search
<p>No phosphorylation sites detected</p>	<ul style="list-style-type: none"> Add inhibitors to lysis buffer Lyse on 4°C Check pH before adding antibody, should be between pH 5–9. Wash the TiO₂/IMAC beads with binding buffer before adding the sample
<p>High levels of contaminating proteins (gel based)</p>	<ul style="list-style-type: none"> Work clean, rinse everything including trays, tweezers, gloves, razors, etc. with HPLC grade water Digest the gel sections in a clean and dust free environment (e.g. in laminar flow hood)

Anticipated Results—An example of a typical result of global phosphoproteomics analysis from a K562 cancer cell lysate is shown in Figure 5A, C using SCX, IMAC and tandem mass spectrometry, the approach produced ~ 2000 phosphorylation sites from 10 mg of starting material (Breitkopf *et al.*). Figure 5A and B show typical examples of a SCX chromatogram for peptide separation as well as a typical coomassie stained SDS-PAGE gel from a cell extract for protein fractionation. Figure 5C shows the ratio of Ser, Thr and Tyr phosphorylation across two different experiments from 12 IMAC purified SCX fractions and 24 LC-MS/MS experiments.

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Single protein/protein complex analysis **Figure 1.**

Flowchart describing the sequential steps for identifying phosphorylation sites from single proteins or immunopurified simple protein complexes using tandem mass spectrometry. Describes an optional protocol for enriching phosphopeptides from digestion mixtures.

Figure 2.

A) Example of a SDS-PAGE mini gel purification of protein(s) from different biological conditions via immunoprecipitation (IP). **B)** Amino acid coverage map showing the tryptic peptides sequenced by LC-MS/MS in dark green and the detected phosphorylation sites highlighted in magenta. Light green highlights oxidation, an in vitro processing artifact. Ideally, for successful phosphopeptide mapping of a protein, amino acid coverage should exceed ~80%. Phosphopeptides can be enriched by using TiO_2 or IMAC and additional proteolytic enzymes can be used for digestion to increase amino acid coverage.

Figure 3.

A) Example of a MS/MS fragmentation spectrum of the phosphorylated peptide sequence GpSPEFPGMVTDQGSR at the first serine residue. Notice the dominant neutral loss of phosphoric acid from the precursor ion and sequence specific fragment ions, including phosphate losses. **B)** Software such as GraphMod or ASCORE can be used to help identify the site specificity in a phosphopeptide. In this example for the phosphopeptide sequence KIPSTEDINK, the first S residue is the correct modification site. This is especially useful when adjacent or multiple STY residues are present on a phosphopeptide.

Figure 4.

Flowchart describing the sequential steps for identifying global phosphorylation sites from cell and tissue lysate using fractionation, phosphopeptide enrichment and tandem mass spectrometry. Describes method for identification of Ser/Thr/Tyr phosphorylation as well as pTyr isolation.

Figure 5.

Example of the typical results of global phosphorylation site identification from **A**) SCX peptide fractionation followed by IMAC phosphopeptide enrichment and subsequent LC-MS/MS analysis or **B**) SDS-PAGE protein fractionation followed by trypsin digestion, TiO_2 phosphopeptide enrichment and LC-MS/MS. **C**) Data acquired by tandem mass spectrometry is searched against protein databases and results are validated to a false discovery rate (FDR) $\leq 1\%$.