# 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, and other processes. High-resolution/high mass accuracy mass spectrometers are used to identify protein phosphorylation sites due to their speed, sensitivity, selectivity, and throughput. The protocols described here focus 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 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 ($TiO_2$) 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, phosphopeptide enrichment by IMAC or $TiO_2$, and LC-MS/MS. Alternatively, the protein lysate can be fractionated by SDS-PAGE, followed by digestion, phosphopeptide enrichment, and LC-MS/MS. One can also immunoprecipitate only phosphotyrosine peptides using a pTyr antibody followed by LC-MS/MS. *Curr. Protoc. Mol. Biol.* 98:18.19.1-18.19.27. © 2012 by John Wiley & Sons, Inc.

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## INTRODUCTION

Phosphorylation of proteins plays an important role in cellular signaling events and metabolic processes, and is therefore the most studied post-translational modification (White, 2008; Choudhary and Mann, 2010; Zarei et al., 2011). 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 phosphoproteome of mammalian cells and tissues is complex and displays a wide dynamic range of varying concentration. To reduce the complexity of the human proteome 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., 2011). These methods utilize gel electrophoresis, ion-exchange chromatography, and microcapillary HPLC.

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



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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, 2010), peptide precursor ions are analyzed in the Orbitrap at high resolution/high mass accuracy at $\leq$1 to 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; or 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$25K, 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 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 systems with microcapillary columns that are capable of flow rates as low as $\sim$200 nl/min (e.g., EASY-nLC from ThermoFisherScientific; NanoAcquity from Waters; NanoLC Ultra from Eksigent; etc.). Tandem mass spectrometry (capable of fragmentation) 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), as well as freely available MaxQuant (Andromeda; Cox et al., 2011) and XTandem! (Falkner and Andrews, 2005), are commonly used to interrogate protein databases for peptide/protein/PTM identifications.

The protocols in this unit focus 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.

In order to comprehensively study the functional role of phosphorylation of 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 (Basic Protocol 1). 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 using an epitope tag such as FLAG, HA, or Myc, or through immunoprecipitation (IP; Bonifacino et al., 1999) with a suitable antibody. The next step involves protein purification using one of two methods. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is recommended for the case of antibody IP, followed by gel band excision and proteolytic digestion, followed by tandem mass spectrometry (LC-MS/MS). For epitope-tagged proteins, solution-based digestions are possible if detergent-free and low salt-containing buffers are used for protein elution (Alternate 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 ($TiO_2$; Support Protocols 1 and 2).

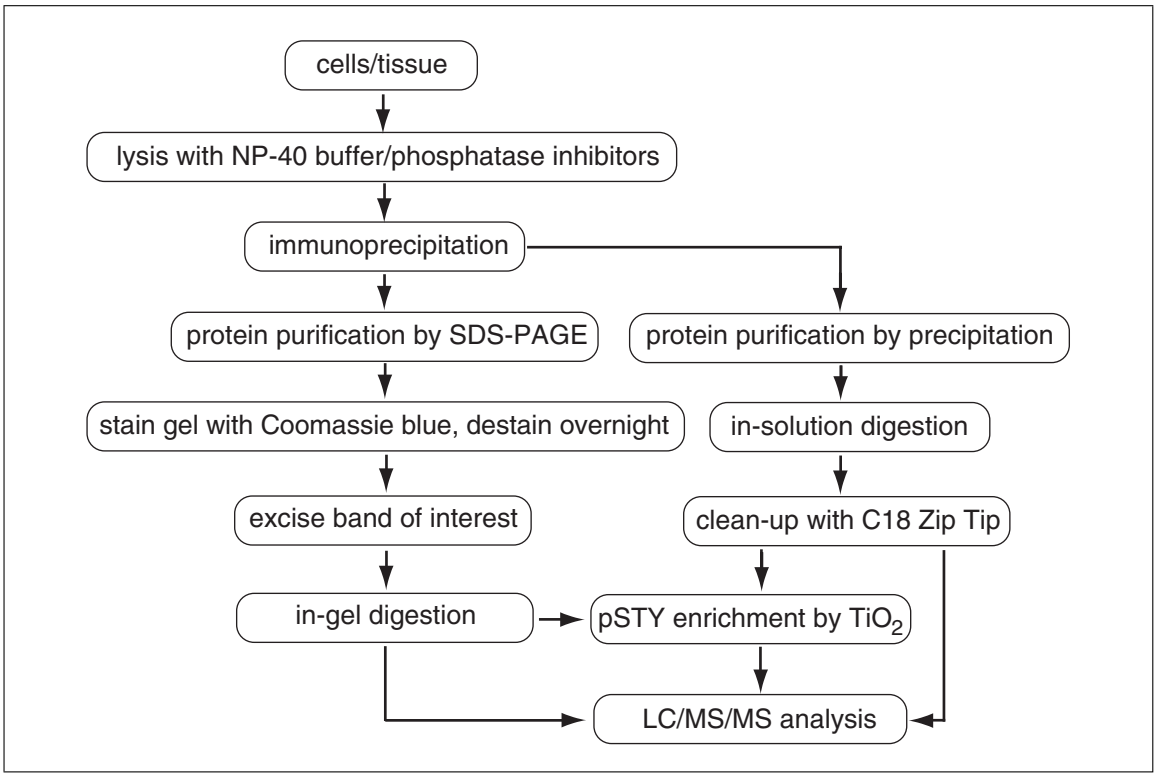


**Figure 18.19.1** Flowchart describing the sequential steps for identifying phosphorylation sites from single proteins or immunopurified simple protein complexes using tandem mass spectrometry. Describes an optional protocol for enriching phosphopeptides from digestion mixtures.

Figure 18.19.1 shows a flowchart describing the sequential steps used in the following protocol for identifying/mapping phosphorylation sites on single proteins and simple protein mixtures.

## SINGLE-PROTEIN (PROTEIN COMPLEX) PHOSPHORYLATION SITE MAPPING

***BASIC PROTOCOL 1***

The following protocol focuses on identifying and mapping phosphorylation sites from a single immunopurified protein or a protein complex. This is typically used when a researcher has a targeted question and is interested in mapping phosphorylation from a single protein of interest or its strongly bound interaction partners. Proteins are generally immunopurified, separated by SDS-PAGE, excised, digested with an enzyme such as trypsin, and analyzed by LC/MS/MS (Dibble et al., 2009; Soltoff et al, 2010; Tigno-Aranjuez et al., 2010; Egan et al., 2011; Lee et al., 2011) directly or with phosphopeptide enrichment (Soltoff et al., 2010). Results can then be compared across different biological conditions such drug treatments and/or stimuli to find differentially regulated phosphorylation sites on a protein of interest. This in contrast to global phosphorylation site analysis, where a researcher aims to identify all phosphorylation sites from a biological sample (Basic Protocol 2).

### *Materials*

Cells/tissue source (e.g., cell line, tumor, extracted bodily tissue)
Lysis/IP buffer (see recipe)
Liquid nitrogen (−196°C)
Bradford protein assay kit (BioRad; also see *UNIT 10.1A*)
Antibody targeting protein of interest

Protein A (GE Healthcare, cat. no. 17-0963-03) or G (GE Healthcare, cat. no. 17-0618-02) agarose beads

1× SDS sample buffer (see recipe)

SDS-PAGE Tris-glycine polyacrylamide minigels 10-well, 1-mm (10% fixed or 4% to 20% gradient; Lonza, cat. no. 58511)

Tris-glycine SDS running buffer (1× formulation: 25 mM Tris·Cl, 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)

50% (v/v) acetonitrile/$H_2O$ (LC-MS grade)

10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate ($NH_4HCO_3$)

100% acetonitrile

55 mM iodoacetamide (IAA) in 100 mM $NH_4HCO_3$

100 mM, 50 mM and 20 mM $NH_4HCO_3$

25 ng/μl trypsin (see recipe)

2% (v/v) formic acid/40% (v/v) acetonitrile

HPLC buffer A: 99% (v/v) $H_2O$, 0.9% acetonitrile, 0.1% formic acid

HPLC buffer B: 100% acetonitrile

Stainless steel mortar with ceramic pestle

Platform rocker with circular motion

15-ml conical polypropylene centrifuge tubes

Refrigerated centrifuge and microcentrifuge

End-over-end rotator

56° and 95°C heat block or water bath

Minigel SDS-PAGE apparatus (BioRad or other vendor) with power supply

37°C shaking incubator

Nanoflow HPLC: ThermoFisherScientific EASY-nLC, Waters NanoAcquity, Eksigent NanoLC Ultra (*http://www.eksigent.com/*), Bruker Nanoflow-LC, or equivalent

Pico-Frit packed $C_{18}$ columns: 75 μm ID × 15 cm length (New Objective, PF7515-150H002-3P; *http://www.newobjective.com/*)

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, or equivalent

Database search engine software: e.g., Sequest (ThermoFisherScientific) or Mascot (Matrix Science; *http://www.matrixscience.com/*)

Proteomics Browser Software (ThermoFisherScientific or *http://www.mcb.harvard.edu/microchem/*)

Scaffold PTM Software (Proteome Software, Inc.; *http://www.proteomesoftware.com/*)

Additional reagents and equipment for protein assay (*UNIT 10.1A*), SDS-PAGE (*UNIT 10.2A*), and staining of gels (*UNIT 10.6*),

### *Lyse cells*

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

   *For lysis of frozen tissue (use ~100 mg to produce 10 mg of protein), grind it using liquid nitrogen in a stainless steel mortar with a ceramic pestle until it is a powder, let liquify at 4°C, and then immediately add lysis buffer containing protease/phosphatase inhibitors.*

2. Incubate lysate for 45 min at 4°C with gentle rocking on a platform rocker in a 15-ml conical tube.
3. Centrifuge 15-ml tube with lysate for 20 min at 14,000 × *g*, 4°C, to remove cell debris. Keep supernatant.
4. Take a small aliquot of the supernatant and determine the protein concentration using the Bradford assay (also see *UNIT 10.1A*).
5. Transfer to a new tube, dilute the protein lysate with lysis/IP buffer (containing protease and phosphatase inhibitors) to ~2 mg/ml (5 ml total), and keep at 4°C.

***Perform immunoprecipitation***

6. Add ~8 to 10 µg of antibody, or enough to clear the lysate of the protein of interest (this amount will vary across different antibodies), and incubate the solution on an end-over-end rotator 2 hr to overnight at 4°C.
7. Wash 50 µl of protein A (or G) agarose beads per immunoprecipitation to be performed with 1 ml of lysis/IP buffer by agitating five times, centrifuging 3 min at 2500 × *g*, 4°C, and discarding buffer. Repeat wash three times, then make a 1:1 slurry in lysis/IP buffer, at 4°C
8. Add 80 µl of the bead slurry (40 µl pure beads) to the lysate-antibody solution and incubate for 2 hr on an end-over-end rotator at 4°C
9. Centrifuge 2 min at 2500 × *g*, 4°C, then remove supernatant completely.
10. Wash the protein–antibody beads complex with 1 ml of lysis/IP buffer by rocking for 3 min at 4°C.
11. Centrifuge 2 min at 2500 × *g*, 4°C, remove supernatant, then repeat the wash described in steps 10 to 11 three times.
12. Elute the proteins from beads by adding ~80 µl of 1× SDS sample buffer (twice the volume of pure beads) and incubating for 5 min at 95°C.

    *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.*

***Perform SDS-PAGE (preferred for obtaining protein of highest purity)***

13. Load sample (in 1× SDS sample buffer) on an SDS-PAGE gradient or fixed-percentage polyacrylamide minigel appropriate for purifying the specific molecular weight of the protein of interest.

    *UNIT 10.2A contains detailed protocols for SDS-PAGE.*

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 room temperature and destain the gel overnight at 4°C with at least five solvent changes in the first 2 hr.

    *Staining and destaining of gels is described in UNIT 10.6. Figure 18.19.2A shows a representative Coomassie-stained gel of immunoprecipitated samples.*

16. Excise the protein gel band(s) of interest, put each band in a 1.5-ml plain microcentrifuge tube, and wash with 150 to 200 µl of 50% acetonitrile/50% water for 15 min and discard supernatant. Repeat wash once.

    *The gel sample can be stored in same tube, moist (not submerged), and frozen at –20°C or below.*

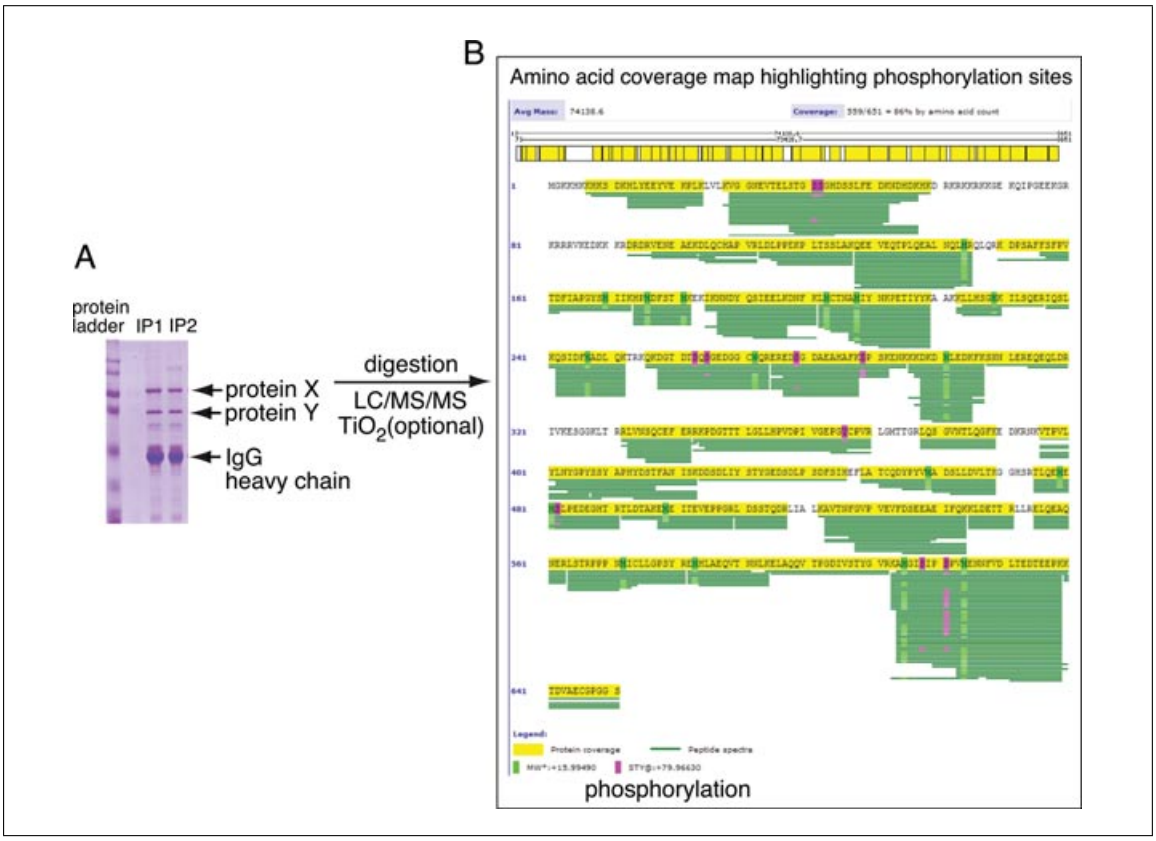


**Figure 18.19.2** (**A**) Example of an SDS-PAGE minigel purification of protein(s) from different biological conditions after immunoprecipitation (IP). (**B**) Amino acid coverage map showing the tryptic peptides sequenced by LC-MS/MS in dark green and the detected phosphorylation sites highlighted 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. For color version of figure go to *http://www.currentprotocols.com/protocol/mb1819*.

### *In-gel digestion*

Carrying out in-gel enzymatic digestion (Li et al., 1997) from SDS-PAGE is a low-cost and efficient method for processing 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 min with no heat.
18. Add a volume of 10 mM DTT in 100 mM $NH_4HCO_3$ sufficient to cover the gel pieces, and incubate at 56°C for 30 min, to reduce the gel.
19. Add 100% acetonitrile to shrink the gel pieces (same volume used in step 18) for ~5 min, room temperature.
20. Remove liquid, add 55 mM IAA in 100 mM $NH_4HCO_3$ to the gel at the same volume that was used for reduction (step 18), and keep in the dark (closed drawer) for 30 min at room temperature.

21. Wash gel pieces (volumes as needed) with 100 mM $NH_4HCO_3$ for 10 min at room temperature. Discard buffer and shrink the gel piece with 100% acetonitrile for ~5 min at room temperature.

22. Remove all liquid and swell with same volume as in step 18 of 100 mM $NH_4HCO_3$ for ~5 min at room temperature.

23. Remove all liquid and dehydrate/shrink with the same volume of 100% acetonitrile as in step 18 for 5 min at room temperature.

24. Remove all liquid and completely dry gel in SpeedVac with no heat for ~15 min

25. On ice, add a volume of 25 ng/μl trypsin according to the following guideline based on gel size: small, use 14 μl, medium, use 18 μl, large, use 22 μl.

    *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 min 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 to 45 μl (or as needed) of 20 mM $NH_4HCO_3$ (depending on the gel size, gel must be covered) and incubate for 10 min at 37°C with shaking. Microcentrifuge 30 sec at maximum speed, then transfer supernatant to a plain 1.5-ml microcentrifuge tube.

29. Add ~50 to 75 μl (or as needed) of 2% formic acid/40% acetonitrile and incubate for 15 min at 37°C with shaking. Microcentrifuge 30 sec and add supernatant to first extraction.

30. Partially dry down in SpeedVac with no heat to a final volume of 10 μl.

***Tandem mass spectrometry (LC-MS/MS)***

An HPLC system capable of nanoliter flow rates is needed for microcapillary reversed-phase liquid chromatography tandem mass spectrometry (LC-MS/MS). This can be a split or splitless system.

31. Equip the system with a $C_{18}$ packed analytical column with typical dimensions of 75 μm i.d. × 15 cm length at a flow rate of ~250 nl/min for optimal sensitivity.

    *Packed analytical columns can be purchased commerciall,y or empty columns can be purchased and self-packed with $C_{18}$ material (Lee, 2001).*

32. Set up a method for data-dependent acquisition (DDA) or "shotgun" mode over a 90-min 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 to 10 MS/MS spectra per DDA cycle with a 120-sec exclusion window and a 2.5 m/z MS/MS isolation window. The mass spectrometer can be configured to include specific m/z values representing phosphopeptides of interest to increase the sensitivity of detection through "inclusion lists" or fixed targeted scan events (Dibble et al., 2009; Egan et al., 2011; Yang et al., 2011).*

33. Inject a 3- to 5-μl aliquot (depending upon amount of starting material) of the sample onto the equilibrated column using the following conditions: initial conditions 2% HPLC buffer B; 2% B to 38% B over 90 min; 38% B to 95% B over 2 min; hold at 95% B for 4 min; 95% B to 2% B over 1 min; hold at 2% B for 12 min to re-equilibrate column.

IMPORTANT NOTE: *Do not inject more than half of the sample, in case of a system failure.*

*One can optionally also use in-line microfilters and/or trap columns to help further purify samples and extend the life of the analytical column.*

### *Database searching and phosphorylation site identification*

34. Process the raw MS/MS fragmentation data using database search engine software such as commercially available Sequest or Mascot.

    *These software programs contain algorithms to extract MS/MS data files 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 the MaxQuant software environment (http://www.maxquant.org/).*

35. Once downloaded, set up the protein database 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 and be assured that only correct peptide matches are carried forward in the final results.*

36. In the search engine, use the following parameters:

    a. Enzyme: Trypsin (cleavage at C-term of Lys (K)/Arg (R).
    b. Precursor mass tolerance: $\leq$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: $\leq$15 ppm for high mass accuracy MS/MS (HCD, TOF, Orbitrap) and $\sim$ 0.08 Da for low mass accuracy MS/MS (ion trap CID, quadrupole CID).

    *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.*

### *Interpret 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 (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.

37. 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.*

38. For critically important phosphorylation sites, view the MS/MS spectrum to verify that the **b–** ions (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.

39. Create amino acid coverage maps using software such as ProteinReport in Proteomics Browser Software and Scaffold PTM Software, as shown in Figure 18.19.2.

    *The stoichiometry of a phosphorylation site can be crudely assessed by comparing the number of sequence events (dark green lines) for the phosphorylated peptide (containing magenta highlight, Figure 18.19.2) versus the nonphosphorylated peptide (Gwinn et al., 2008). 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 http://ascore.med.harvard.edu/) and MaxQuant (Cox and Mann, 2008; Cox et al., 2009) at http://maxquant.org/.*

## ACETONE PRECIPITATION OF PROTEIN SAMPLES (FOR IP ELUTION WITH PEPTIDE OR SMALL MOLECULE)

***ALTERNATE PROTOCOL 1***

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

***Additional Materials** (also see Basic Protocol 1)*

Acetone
Protein sample
1 μg/μl TPCK-modified trypsin prepared in 50 mM acetic acid
5% (w/v) trifluoroacetic acid (TFA)
ZipTip binding and wash buffer: 0.1% (w/v) trifluoroacetic acid (TFA)
ZipTip elution buffer: 0.1% (w/v) trifluoroacetic acid/40% acetonitrile

Acetone-compatible tubes (polypropylene only)
pH paper
$C_{18}$ ZipTips (Millipore, cat. no. ZTC18S096)
0.5-ml microcentrifuge tubes or 12 × 32 mm autosampler vials (National Scientific, cat. no. C4000-87)

***Precipitate protein***

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 min at −20°C.
5. Centrifuge 10 min at 14,000 g, 4°C.
6. Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
7. Repeat steps 3 to 6 one more time.
8. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 min. Do not over-dry pellet, or it may not dissolve properly.

***In-solution digestion (precipitated protein)***

9. Add 20 µl of 10 mM DTT in 100 mM $NH_4HCO_3$ to the sample pellet from step 8 and incubate for 30 min at 56°C.
10. Add 20 µl of 55 mM IAA in 100 mM $NH_4HCO_3$ and incubate for 30 min at room temperature in the dark.
11. Dilute sample to 200 µl with 50 mM $NH_4HCO_3$, pH 8.3 (same for gel digests).
12. Add 2.0 µg of trypsin (i.e., 2 µl of 1 µg/µl TPCK modified trypsin prepared in 50 mM acetic acid).
13. Incubate overnight at 37°C with shaking.
14. The next day, add 20 µl of 5% TFA to stop digestion. Use 1 to 2 µl to check that the pH is ~3.5 (pH paper is sufficient).
15. Concentrate sample in a SpeedVac concentrator to a final volume of 10 µl.

***Purify and concentrate solution digest***

16. Prepare $C_{18}$ ZipTip by aspirating/expelling 20 µl of 100% acetonitrile. Discard washes and repeat one time.
17. Aspirate/expel 20 µl of ZipTip elution buffer (0.1% TFA/40% acetonitrile), discard washes, and repeat two times.
18. Equilibrate ZipTip by aspirating/expelling 20 µl of ZipTip binding/wash buffer (0.1% TFA), discard washes, and repeat three times.
19. Load acidified peptide digest (from step 15) onto ZipTip by aspirating/expelling the sample solution five times. Save the flowthrough.
20. Wash loaded sample with 20 µl of ZipTip binding/wash buffer (0.1% TFA) by aspirating/expelling the sample solution, discarding washes. Repeat four times.
21. Elute with 10 µl of ZipTip elution buffer (0.1% TFA/40% acetonitrile) by aspirating/expelling the same solution five times into a new 0.5-ml microcentrifuge tube or 12 × 32 mm autosampler vial.
22. Add 40 µl HPLC buffer A, then dry down in SpeedVac concentrator to 10 µl final volume.
23. Proceed to step 31 of Basic Protocol 1 for LC-MS/MS.

***SUPPORT PROTOCOL 1***

## PHOSPHOPEPTIDE ENRICHMENT USING PROTEA BIOSCIENCES $TiO_2$ SPIN TIPS

Affinity-based enrichment of phosphopeptides using immobilized metal affinity chromatography (IMAC) can 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 nonspecifically bind 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 $TiO_2$ ($TiO_2$ SpinTips, Protea Biosciences, described in this protocol; and Phos-Trap kit, Perkin-Elmer described in Support Protocol 2). It is recommended that half of the simple peptide mixture be analyzed directly by LC-MS/MS and the second half of the sample be used for phosphopeptide enrichment using Support Protocol 1 or 2.

***Additional Materials** (also see Basic Protocol 1)*

Peptide sample
Protea $TiO_2$ SpinTips Sample Prep Kit (Protea Biosciences) including:
  SpinTip adapers
  $TiO_2$ Reconstitution and Wash Solution 1
  $TiO_2$ Elution Solution
2-ml microcentrifuge tubes

***Prepare SpinTips***

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 adapter onto a 2-ml microcentrifuge tube.
3. Wash the SpinTip to wet the packing material by adding 100 µl $TiO_2$ Reconstitution and Wash Solution 1 to the top of the SpinTip using a repeating pipettor.
4. Centrifuge the system 3 to 5 min at 4000 × *g*, room temperature. Discard eluate and repeat steps 3 to 4.

***Binding***

5. Reconstitute ~5 to 20 µl of the peptide sample in ~200 µl $TiO_2$ Reconstitution and Wash Solution 1 and vortex until the sample is completely dissolved.
6. Load 200 µl of the sample solution by adding it to the top of the SpinTip, and centrifuge 3 to 5 min at 4000 × *g*, room temperature.
7. Wash the sample to elute salts and other loosely bound components by adding 100 µl $TiO_2$ Reconstitution and Wash Solution 1 to the top of the SpinTip.
8. Centrifuge 3 to 5 min at 4000 × *g*, room temperature. Discard eluate and repeat steps 7 to 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 µl $TiO_2$ Elution Solution to the top of the SpinTip. Centrifuge 3 to 5 min at 4000 × *g*, room temperature. Repeat the SpinTip sample elution two times using the same elution solution.
11. Partially dry the solution to ~5 µl in SpeedVac, then add 100 µl of HPLC buffer A and transfer to a 12 × 32–mm autosampler vial
12. Partially dry to 5 µl and inject onto LC-MS/MS system.

## PHOSPHOPEPTIDE ENRICHMENT USING THE PHOS-TRAP $TiO_2$ PHOSPHOPEPTIDE ENRICHMENT KIT

*SUPPORT PROTOCOL 2*

See introduction to Support Protocol 1 for considerations regarding both $TiO_2$ phosphopeptide enrichment procedures/kits.

***Materials***

Peptide sample
Phos-Trap $TiO_2$ phosphopeptide enrichment kit (PerkinElmer) including:
Magnetic beads
Binding buffer
Wash buffer
Elution buffer

Magnet (bench top, for microcentrifuge tubes)
12 × 32 mm autosampler vials (National Scientific, cat. no. C4000-87)

***Preparation***

1. Mix the vial of magnetic beads from the kit so that the beads are evenly suspended in solution. Take 20 µl from the vial of magnetic beads and 180 µl of HPLC-grade water for each experiment.

   *For example, for three experiments, take 60 µl of beads with a 1-ml (P-1000) pipet tip (beads can clog 200-µl pipet tips), add 540 µl of water, and mix in an appropriately sized tube.*

2. Dispense 200 µl of bead suspension per 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 µl of binding buffer (from kit). Add buffer "off" the magnet and lightly shake to suspend the beads evenly in solution for 1 min.
5. Put tray (tube) on magnet to remove liquid without removing beads; repeat two times.

***Binding and washing***

6. Dilute purified peptide sample 1:10 in binding buffer, but do not exceed 150 µl in total volume. Use a SpeedVac concentrator to partially evaporate the sample prior to dilution, if necessary.
7. Add the sample solution to the beads and incubate for 60 min while shaking continuously to keep the beads suspended in the solution.
8. Put tube on magnet, remove liquid (not beads) from the tubes, and transfer to a tube labeled "$TiO_2$ flowthrough."

   *These peptide mixtures can be analyzed in the mass spectrometer if one is interested in non-phosphorylated peptides.*

9. Wash the beads with 200 µl of binding buffer for 1 min, then put tube on magnet and remove liquid; repeat three times.
10. Wash the beads with 200 µl of wash buffer (from kit) for 1 min, then put tube on magnet and remove liquid.

***Elution***

11. Elute the phosphopeptides by adding 35 µl of elution buffer (from kit) to beads and incubate for 15 min 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 12 × 32–mm autosampler vial.

13. Add 50 µl of HPLC buffer A and concentrate using SpeedVac concentrator to 5 µl.

14. Inject entire 5-µl volume onto LC-MS/MS system.

## GLOBAL PHOSPHORYLATION ANALYSIS (Ser/Thr/Tyr)

***BASIC PROTOCOL 2***

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 (Kirkpatrick et al., 2005; Olsen et al., 2010). Using the procedures below, researchers have discovered up to 36,000 in vivo phosphorylation sites from various mouse organ tissues, although most global phosphorylation studies result in several thousand phosphorylation sites detected (Beausoleil et al., 2004; Oppermann et al., 2009; Pan et al., 2009; Olsen et al., 2010). While these analyses are very time-consuming and require milligrams of material, offline strong cation exchange (SCX) peptide fractionation, and a dozen 2-hr 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).

Below is 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 18.19.3 shows a flowchart outlining the sequential steps used in the following protocol for identifying potentially thousands of phosphorylation sites.

### *Materials*

Cells of interest, in log phase
Urea lysis buffer (see recipe)
Liquid nitrogen (−196°C)
Bradford protein assay kit (BioRad; also see *UNIT 10.1A*)
45 mM DTT: mix 180 µl of 1.25 M DTT (19.25 g/100 ml) with 5 ml HPLC-grade water; add to sample at ∼1/10 dilution (e.g., 0.5 ml DTT per 5-ml sample)
110 mM IAA: Dissolve 209 mg iodoacetamide (IAA) in 10 ml HPLC-grade water; add to sample at ∼1/10 dilution
Sequencing grade modified trypsin (100 µg/vial; Worthington, cat. no. LS02122)
50 mM ammonium bicarbonate ($NH_4$ $HCO_3$)
20%, 10%, 1%, 0.1% (v/v) trifluoroacetic acid (TFA)
0.1% TFA/40% acetonitrile
SCX buffer A: 7 mM $KH_2PO_4$, pH 2.65 in 30% (v/v) acetonitrile
SCX buffer B: 7 mM $KH_2PO_4$ /350 mM KCl, pH 2.65 in 30% (v/v) acetonitrile
IMAC (PHOS-Select Iron affinity gel, Sigma, cat. no. P9740)
IMAC binding buffer (40% acetonitrile (v/v), 25 mM formic acid, $H_2O$)
IMAC elution buffer A (50 mM $K_2HPO_4$/$NH_4OH$, pH 10.0)
IMAC elution buffer B (500 mM $K_2HPO_4$, pH 7)
Methanol
40% (v/v) acetonitrile/0.5% acetic acid
1% (v/v) formic acid

Stainless steel mortar with ceramic pestle
Bath sonicator
Centrifuge
56°C water bath or heat block
pH paper
Sep-Pak $C_{18}$ cartridges 6-cc/500 mg (Waters, WAT036790) for whole digested lysate

Strong cation exchange (SCX) column: PolySULFOETHYL A 250 × 9.4 mm; 5 μm pore size; 200 Å (PolyLC, 259-SE0502)

Nanoflow HPLC: ThermoFisherScientific EASY-nLC, Waters NanoAcquity, Eksigent NanoLC Ultra (*http://www.eksigent.com/*), Bruker Nanoflow-LC, or equivalent

1-ml sample loop

High-flow-rate HPLC (offline SCX fractionation): e.g., Agilent 1200, Michrom Paradigm MG4 (Bruker), Shimadzu Prominence

15-ml conical centrifuge tubes

Sep-Pak $C_{18}$ cartridges 3-cc/50 mg (Waters, WAT054960) for fractionated sample (after SCX); one cartridge for each fraction (12 SCX fractions = 12 Sep-Pak 3-cc/50-mg cartridges)

0.5-ml microcentrifuge tubes

Empore 3M $C_{18}$ material (*http://www.shop3m.com/*)

Cutter device (Hamilton, Needle Kel-F hub (KF), point style 3, gauge 16, cat. no. 90516; plunger assembly N, RN, LT, LTN for model 1702 (25 μl), cat. no. 1122-01

200-μl pipet tips for StageTip preparation

12 × 32 mm autosampler vials (National Scientific, cat. no. C4000-87)

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, or equivalent

Additional reagents and equipment for protein assay (*UNIT 10.1A*)

*NOTE:* For SCX buffers, organic solvents affect the pH reading. The pH adjustments for SCX buffers A and B (see above) should be performed before the addition of acetonitrile.

### *Lyse cells and/or tissues*

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

   *One can stimulate the cells with fetal bovine serum (FBS) to increase the general phosphorylation signaling, or treat with specific growth factors and/or drugs to activate/inhibit a particular signaling pathway.*

2. Lyse sufficient cells (∼$10^7$ to $10^8$) or tissue (∼150 to 200 mg) to produce ∼15 to 20 mg of protein in ∼5 to 10 ml of urea lysis buffer with protease/phosphatase inhibitors.

   *For lysis of frozen tissue, grind it using a liquid nitrogen stainless steel mortar with ceramic pestle until it is a powder, let liquify 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 (P-1000) pipet tip.

4. Sonicate for 1 min at 4°C using a bath sonicator on medium setting, repeat two times.

5. Centrifuge lysate in a 15-ml tube 5 min at 14,000 × *g*, 15°C to remove cell debris. Retain supernatant.

6. Determine the protein concentration in a small aliquot of the supernatant using the Bradford assay.

### *Digestion*

7. Add 1/10 of the solution volume of 45 mM DTT to the protein lysis solution to yield a 4.5 mM DTT solution. Incubate for 30 min at 56°C.

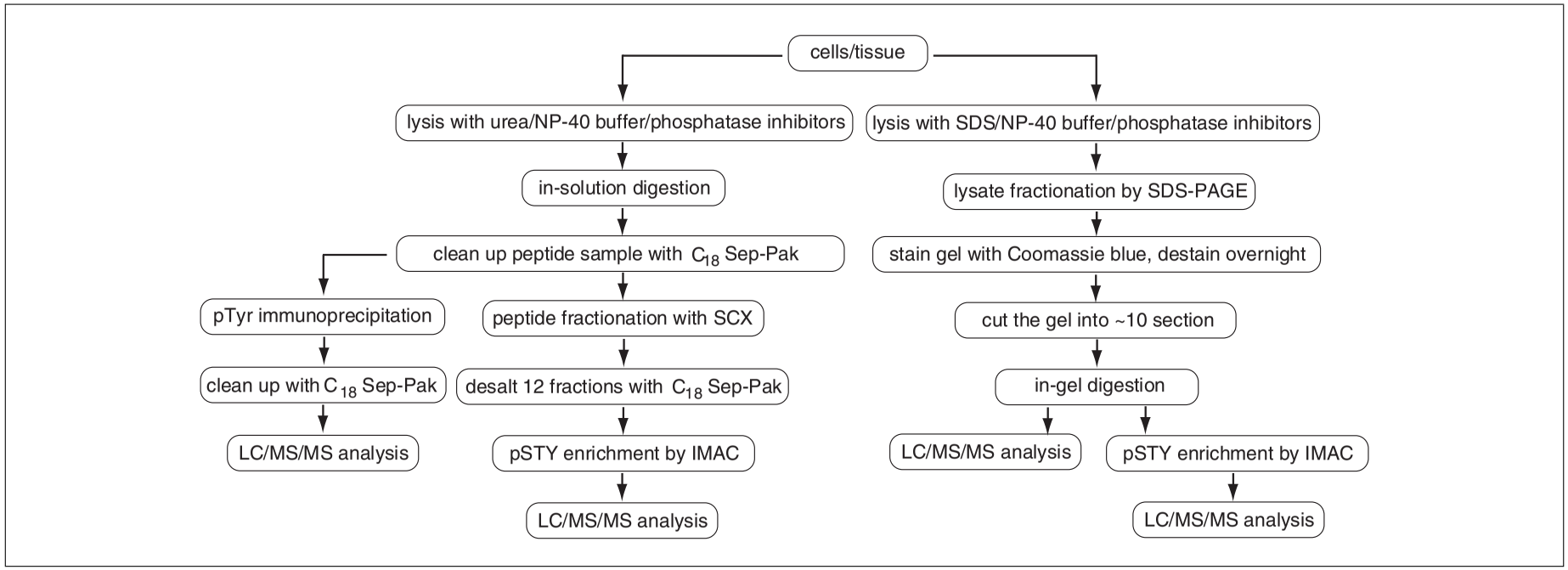


**Figure 18.19.3** Flowchart describing the sequential steps for identifying global phosphorylation sites from cell and tissue lysate using fractionation, phosphopeptide enrichment, and tandem mass spectrometry. Describes method for identification of Ser/Thr/Tyr phosphorylation as well as pTyr isolation.

8. Add the same amount of 110 mM IAA to make an 11.0 mM IAA solution. Incubate for 30 min at room temperature in the dark.
9. Dilute sample 1:5 with 50 mM $NH_4HCO_3$ to final concentration of 1.6 M urea and a final pH of 8.2.
10. Dissolve 200 μg trypsin in 200 μl of 50 mM $NH_4CO_3$ to a final concentration of 1 μg/μl in trypsin vial (two vials are needed).
11. Add 150 to 200 μl trypsin solution to the lysate (~100:1 substrate/protease ratio).
12. Incubate the solution overnight at 37°C in a shaking incubator (~450 rpm).
13. The next day, stop the protease reaction by adding 1/40 the solution volume of 20% TFA, for a final concentration of 0.5% TFA.
14. Let mixture stand for 10 min at room temperature.
15. Clear the sample of debris by centrifuging 10 min at 14,000 × *g*, 15°C. Retain the supernatant containing digested peptides.

***Purification***

Samples first need to be purified prior to subsequent pTyr IP (Basic Protocol 3) or SCX fractionation to optimize antibody binding and charge separation.

16. Check pH of cleared sample to be sure it is between 2.0 and 3.0.
17. Prewet 6-cc/500 mg $C_{18}$ Sep-Pak with 3 ml of acetonitrile.
18. Wash with 3 ml of 0.1% TFA/40% acetonitrile.
19. Wash with 3 ml of 0.1% TFA, then discard wash; repeat two times.
20. Load peptide digestion sample (from step 15) onto Sep-Pak.
21. Wash column with 3 ml of 0.1% TFA, then discard wash; repeat two times.
22. Elute peptides with 1 ml of 0.1% TFA/40% acetonitrile, repeat two times, and combine eluates for 3 ml total volume.
23. Divide sample into two microcentrifuge tubes. Dry down partially in SpeedVac with no heat to ~200 to 400 μl, then combine both samples into one 1.5-ml microcentrifuge tube.
24. Dry down completely to a pellet in a SpeedVac with no heat.

*At this point it is possible to proceed to Basic Protocol 3 for pTyr site identification. For SCX peptide fractionation, continue with steps 25 to 55, below.*

***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 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 nonphosphorylated tryptic peptides carry $2^+$ and $3^+$ charges in solution. However, other factors including hydrophobicity also play a role in 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 an HPLC system capable of 3 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 buffers A and B, purge SCX HPLC pumps, clean 1 ml sample loop and equilibrate column with 1% SCX buffer B for 14 min at 1 ml/min.
26. Load a blank sample containing only SCX buffer A 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 3 ml/min over a PolySULFOETHYL A SCX column using the following gradient conditions:

    1%–35% B: 40 min
    35%–50% B over 1 min
    50%–100% B over 5 min
    100% B for 5 min
    100%–1% B over 1 min
    1% B for 14 min to re-equilibrate column.

28. Dilute dried, desalted pellet from step 24 in 950 µl of SCX buffer A. Vortex to completely dissolve.
29. Inject 950 µl of sample onto a 1-ml sample loop and run SCX method as in steps 26 to 27.

***Fraction collection***

30. Collect 3 min (9 ml) fractions from the elution starting at time 0 min for a total of ~15 fractions in separate 15-ml conical tubes.
31. Concentrate fractions to dryness in SpeedVac concentrator with no heat.

***Purification of salts***

32. Dissolve dried peptide pellet in 500 µl of 0.1% TFA.
33. Check pH of cleared sample with pH paper to be sure it is between 2.0 and 3.0, using 5 µl from each of the 12 fractions.
34. Pre-wet 3-cc/50 mg capacity $C_{18}$ Sep-Pak with 1 ml of acetonitrile (prepare one Sep-Pak per fraction).
35. Wash Sep-Pak with 1 ml of 0.1% TFA/40% acetonitrile.
36. Wash Sep-Pak with 1 ml of 0.1% TFA; repeat two times.
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 two times.
39. Elute peptides with 333 µl of Sep-Pak Elution Buffer in 1.5-ml microcentrifuge tube; repeat two times with fresh elution buffer and combine for 1-ml total volume.
40. Dry peptide sample completely to a pellet in a SpeedVac concentrator using no heat.

***Phosphopeptide enrichment***

Since there are currently no good-quality antibodies available 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: immobilized metal affinity chromatography (IMAC) using iron (step 41), or $TiO_2$ enrichment (see Support Protocol 1).

***PHOS-Select IMAC (Sigma)***

*Preparation*

41. Prepare 100 µl of IMAC beads by washing them with 1 ml of IMAC binding buffer, inverting the vial five times to resuspend all beads, and centrifuging 3 min at 2500 × *g*, room temperature, to remove the liquid. Repeat three times and prepare 50% slurry in the same buffer.

42. Prepare twelve 0.5-ml microcentrifuge tubes and place 10 µl 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 µl of IMAC binding buffer, and transfer to the IMAC beads for a total volume of 130 µl.

44. Incubate peptides on beads for 60 min at room temperature with vigorous shaking.

45. During this time, prepare twelve StageTips by cutting two disks of Empore 3 M $C_{18}$ material with a cutter device from Hamilton and packing into 250-µl pipet tips (Rappsilber et al., 2007).

46. Wash and equilibrate packed StageTips by passing 20 µl of methanol through each of the tips, followed by 20 µl of 40% acetonitrile/0.5% acetic acid, and then two 20-µl aliquots of 1% formic acid.

    *For convenience and increased throughput, one can use a centrifuge by placing the StageTips within a 2-ml microcentrifuge tube with the top part of a 500-µl microcentrifuge tube body as an adapter, limiting spinning speed to 2000 × g and time to the minimum to get the liquid passed through.*

47. After the 60-min incubation in step 44, transfer IMAC beads to the top of the StageTips, and spin down by microcentrifuging briefly at 2500 × *g*.

    *The beads will be retained on the StageTip and the solution will pass through. As the buffer contains 40% acetonitrile, nonphosphorylated peptides, which are not retained in the IMAC resin, will not be retained by the $C_{18}$ material. These peptide mixtures can be collected and analyzed in the mass spectrometer, if desired.*

48. Add 50 µl IMAC binding buffer to the top of the StageTips, then microcentrifuge as in step 47 to remove liquid. Repeat once.

49. Wash once with 40 µl of 1% formic acid using the technique described in step 48.

    *This equilibrates the $C_{18}$ StageTip.*

50. Wash with 70 µl of IMAC Elution Buffer B. Repeat two times.

    *At this point, phosphopeptides are eluted from IMAC resin and retained on the $C_{18}$ material.*

51. Wash with 40 µl of 1% formic acid to remove phosphate salts.

52. Elute phosphopeptides from StageTips into 12 × 32–mm autosampler vials for MS analysis with 40 μl of 40% acetonitrile/0.5% acetic acid.

53. Dry down the samples from step 47 (nonphosphorylated peptides) and step 58 (phosphopeptides) with SpeedVac concentrator or lyophilizer.

54. Resolubilize the dried samples with 15 to 20 μl of HPLC buffer A, and inject 2 to 10 μl depending upon sample quantity onto the LC-MS/MS system.

55. Run the mass spectrometer as described in Basic Protocol 1, steps 31 to 33, with the following change: for complex samples in Basic Protocols 2 and 3, the LC-MS/MS gradient and acquisition time should be increased from 90 min to ∼120 min per sample to increase peptide/protein coverage.

## SDS-PAGE PROTEIN FRACTIONATION OF WHOLE-CELL EXTRACTS

***ALTERNATE PROTOCOL 2***

This protocol involves fractionating proteins from a whole-cell extract according to their molecular weight using SDS-PAGE followed by in-gel digestion and subsequent IMAC or $TiO_2$ 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.

For materials, see Basic Protocol 1.

1. Lyse the material in lysis/IP buffer containing protease/phosphatase inhibitors for 45 min with rocking at 4°C. Centrifuge for 20 min at full speed at 4°C. Retain supernatant.

2. Load samples onto denaturing polyacrylamide gels according to the capacity of the minigel (fixed or gradient) and electrophorese until the dye front runs completely to the bottom of the gel.

3. Stain with Coomassie and destain overnight.

   *The stain is mostly used here as a lane and protein amount indicator and is not intended to resolve bands.*

4. Cut gel into ∼10 to 12 equal sections and place each gel piece in a 1.5-ml centrifuge tube.

5. Follow Basic Protocol 1, steps 17 to 30, for in-gel digestion, and dry to a final volume of ∼10 μl.

6. For phosphopeptide enrichment of gel-digested fractions, follow either Support Protocol 1 ($TiO_2$) or Basic Protocol 2 (IMAC) to prepare sample for LC-MS/MS.

7. Run the mass spectrometer according to Basic Protocol 1, steps 31 to 33, with the following change: the LC-MS/MS gradient and acquisition time should be increased from 90 min to ∼120 min per sample to increase peptide/protein coverage.

## PHOSPHOTYROSINE (pTyr) SITE IDENTIFICATION

***BASIC PROTOCOL 3***

Although phosphotyrosine appears in only 0.05% of cellular protein phosphorylation, it is the critical key player in many signaling events starting from receptor tyrosine kinases. In addition, 20% of the kinome represents protein tyrosine kinases, revealing the importance of 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***

Digested and purified sample pellet (Basic Protocol 2, step 24)
Lysis/IP buffer (see recipe)
1 M Tris base (pH not adjusted)
Phosphotyrosine P-Tyr-100 mouse antibody (mAb), Sepharose conjugated (Cell Signaling Technology, cat. no. 9419)
P-Tyr-100 elution buffer: 0.15% (v/v) trifluoroacetic acid (TFA)
100% acetonitrile
0.1% (v/v) trifluoroacetic acid (TFA)/40% acetonitrile
0.1% (v/v) trifluoroacetic acid

Bath sonicator
End-over-end rotator
Gel-loading pipet tips
Refrigerated centrifuge
$C_{18}$ ZipTips (Millipore, cat. no. ZTC18S096)
Nanoflow HPLC: ThermoFisherScientific EASY-nLC, Waters NanoAcquity, Eksigent NanoLC Ultra (*http://www.eksigent.com/*), Bruker Nanoflow-LC, or equivalent

***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/IP buffer to digested and purified sample pellet from step 24 of Basic Protocol 2, then sonicate in 4°C water bath at medium setting, three times for 1 min each time.
2. Check pH. If less than pH 5, add 1 M Tris base dropwise until the solution is pH 6.0 to 7.5.
3. Centrifuge 5 min at 1,800 × *g*, 4°C. Keep supernatant, and place on ice.

***Bind to pTyr Ab***

4. Using 15 to 20 mg of total protein prior to digestion, add ∼80 μg of P-Tyr-100 mAb beads in suspension.
5. Incubate for 2 hr on an end-over-end rotator at 4°C.
6. Centrifuge 1 min at 2500 × *g*, 4°C.
7. Remove supernatant completely with gel-loading tips to avoid removal of beads.
8. Wash beads with 1 ml lysis/IP buffer, agitate by inverting five times, and centrifuge 1 min at 2,500 × *g*, 4°C. Discard supernatant. Repeat one more time.
9. Wash beads with 1 ml HPLC-grade water (4°C) to remove the detergents, agitate by inverting five times, then centrifuge 1 min at 2,500 × *g*, 4°C. Discard supernatant. Repeat four times.

***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, and incubate 10 min at room temperature.
11. Tap again and centrifuge for a few seconds at 2500 × *g*. Keep supernatant.
12. Add 45 μl of 0.15% TFA, tap bottom of tube several times, and centrifuge for a few seconds at 2,500 × *g*. Keep supernatant (with gel loading tip) and combine with previous elution (step 11) for a total of 100 μl.

***Purification and concentration***

13. Centrifuge 1 min at 2,500 × *g*, room temperature, to remove potential remaining agarose beads in tube.
14. Divide sample in two equal parts.
15. Prepare $C_{18}$ ZipTip by cutting off the small end of a 200-µl (P-200) pipet tip and attaching it to the top of a ZipTip; it should fit tightly into the upper ring of the ZipTip.
16. Prepare ZipTip by aspirating/expelling 40 µl of 100% acetonitrile through the tip. Discard wash. Repeat once.
17. Aspirate/expel 40 µl of 0.1% TFA/40% acetonitrile. Discard wash. Repeat two times.
18. Equilibrate ZipTip by aspirating/expelling 40 µl of 0.1% TFA. Discard wash. Repeat three times.
19. Place tip in the first half of the sample. Aspirate/expel ten times in sample tube (do not discard).
20. Place tip in second half of the sample. Aspirate/expel ten times in sample tube (do not discard).
21. Wash the ZipTip with 55 µl of 0.1% TFA. Repeat two times.
22. Elute the peptides with 15 µl of 0.1% TFA/40% acetonitrile, but aspirate/expel five times prior to final elution into a new 12 × 32 mm autosampler vial.
23. Add 35 µl 0.1% TFA to the peptide elution (50 µl total volume). Partially dry to 10 µl using SpeedVac concentrator with no heat.

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

24. Inject 5 µl of sample onto LC-MS/MS system with a 180 min nano-LC gradient. Save half of the sample in case of a system failure.

    *Use gradient from Basic Protocol 1, step 33, except increase the time going from 2% B to 38% B from 90 min to 180 min.*

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

***Lysis/IP buffer***

0.5% (v/v) NP-40
1% (v/v) Triton X-100
150 mM NaCl
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
1 mM EDTA
1 mM EGTA
Store the solution with the above ingredients up to 2 weeks at 4°C
Add protease and phosphatase inhibitors (see recipe) just before use

***Protease and phosphatase inhibitors***

1 mM of $Na_3VO_4$
1 mM aprotinin
1 mM leupeptin

*continued*

1 mM pepstatin
10 mM NaF
1 mM PMSF
2.5 mM sodium pyrophosphate
1 mM β-glycerophosphate
Do not store; use immediately at 4°C

***SDS sample buffer, 1×***

31.25 mM Tris·Cl, pH 6.8 (*APPENDIX 2*)
5% glycerol
1% (w/v) SDS
0.36 M 2-mercaptoethanol
0.0025% (v/v) bromphenol blue
Store up to 2 weeks at −20°C

***Trypsin, 25 ng/μl***

Prepare 1 μg/μl trypsin (TPCK-modified sequencing grade; Promega, cat. no. 9PIV5113) in 50 mM acetic acid. Combine 5 μl of the 1 μg/ml trypsin with 195 μl of 50 mM $NH_4HCO_3$. Store up to 72 hr at −20°C.

*The solution is only stable at –20°C at pH 3.0; if pH ≥ 8, it must be used immediately.*

***Urea lysis buffer***

8 M urea
2.5 M thiourea
20 mM HEPES, pH 8.0
Prepare fresh; do not store
Add protease and phosphatase inhibitors (see recipe) just before use

## COMMENTARY

### Background Information

Currently, apart from 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, often the work is performed either within a core facility or in collaboration with an analytical laboratory specializing in mass spectrometry. While high-resolution mass spectrometers are being acquired in some biology laboratories, they are not yet widespread, due to the cost of the equipment and maintenance required for optimal performance. The protocols described in this unit are designed to aid the biologist getting into the mass spectrometry field or already possessing a state-of-the art mass spectrometer, and to be a reference for collaborators of biologists already in the mass spectrometry field to optimize strategies for identifying sites of phosphorylation in biological samples. 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 are not described in this unit, and require expertise in those fields.

### Critical Parameters and Troubleshooting

Table 18.19.1 is a troubleshooting guide listing problems that might arise with the protocols in this unit and possible solutions to such problems.

### Anticipated Results

Figure 18.19.2 shows the typical results that can be obtained from an experiment where a protein was immunoprecipitated from

**Table 18.19.1** Troubleshooting Guide for Determining In Vivo Phosphorylation Sites Using Mass Spectrometry

| Problems | Possible solutions |
|---|---|
| Trypsin digestion failed | 1. Check for successful digestion; take aliquot before and after digestion and run on gel to verify proteolysis.<br>2. Check pH before adding trypsin for digestion; it should be ~8.3.<br>3. Dilute digestion solution 1:5 with water before adding trypsin. |
| No peptide peaks detected by LC-MS/MS | 1. 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.<br>2. Detergents or other chemical contaminants left in the sample can prevent successful MS detection. Wash immunoprecipitate with low-salt and detergent-free buffer before elution.<br>3. Partially dry down samples in SpeedVac to remove organic solvents that can prevent binding to reverse-phase columns.<br>4. High salt concentrations may be left in the sample. Filter sample through $C_{18}$ Sep-Pak, StageTip or ZipTip for small volumes. |
| Peaks in LC/MS chromatogram but no peptide identifications | 1. Mass spectrometer may be out of calibration.<br>2. MS/MS acquisition not working properly.<br>3. 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.<br>4. Be sure the differential modification for phosphorylation of S/T/Y is enabled during the database search. |
| No phosphorylation sites detected | 1. Add protease inhibitors to lysis buffer. Lyse cells at 4°C.<br>2. Check pH before adding antibody.<br>3. It should be between 5 and 9. Wash the $TiO_2$/IMAC beads with binding buffer before adding the sample. |
| High levels of contaminating proteins (gel-based) | 1. Rinse everything including trays, tweezers, gloves, razors, etc. with HPLC-grade water.<br>2. Digest the gel sections in a clean and dust-free environment (e.g., in laminar flow hood). |

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, it is useful to include phosphopeptide enrichment methods such as metal affinity with $TiO_2$ and/or IMAC. Figure 18.19.4A 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 18.19.4B 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 phosphorylatable site in a given peptide.

An example of a typical result of global phosphoproteomics analysis from a K562 cancer cell lysate is shown in Figure 18.19.5A,C. Using SCX, IMAC, and tandem mass spectrometry, the approach identified ~2000 phosphorylation sites from 10 mg of starting material (Breitkopf et al., 2010). Figure 18.19.5, panels A and B, show typical examples of an SCX chromatogram for peptide separation, as well as a typical Coomassie-stained SDS-PAGE gel from a fractionated cell extract. Figure 18.19.5C 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.

## Time Considerations

***Protein IP (Basic Protocol 1)***

Lysis and immunoprecipitation: 2 hr to overnight (12 to 16 hr)

Digestion (in-gel or in-solution): overnight (12 to 18 hr)

Optional: ZipTip purification: 1 hr

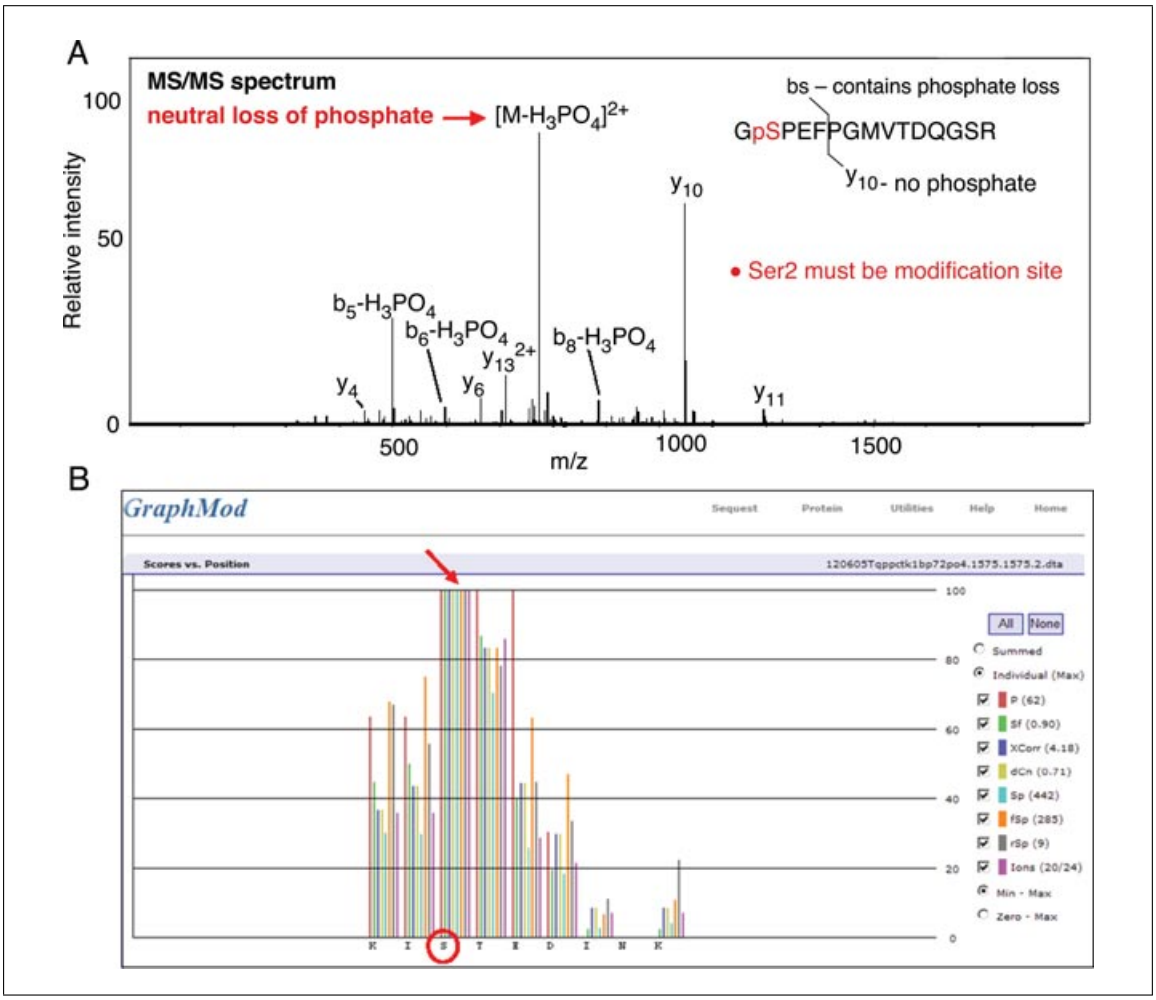


**Figure 18.19.4** (**A**) Example of an MS/MS fragmentation spectrum of the phosphorylated peptide sequence G**pS**PEFPGMVTDQGSR at the first serine residue. Notice the dominant neutral loss of phosphoric acid from the precursor ion and sequence-specific fragment ions, including phosphate losses. (**B**) 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 KI**pS**TEDINK, the first S residue is the correct modification site. This is especially useful when adjacent or multiple STY residues are present on a phosphopeptide.

Optional: $TiO_2$ phosphopeptide enrichment: 2 to 3 hr
LC-MS/MS: 2 to 3 hr
Data analysis 1 to 3 hr
Total time: 2-3 days.

***Global pThr/pSer/pTyr (Basic Protocol 2)***

Lysis and digestion: overnight (12 to 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/$TiO_2$ phosphopeptide enrichment: ~2 to 3 hr
StageTip peptide purification: ~ 2 to 3 hr
LC-MS/MS analysis for 12 samples: ~28 to 39 hr
Data analysis for 12 runs: 8 to 12 hr
Total time: ~7 days.

***P-Tyr IP (Basic Protocol 3)***

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

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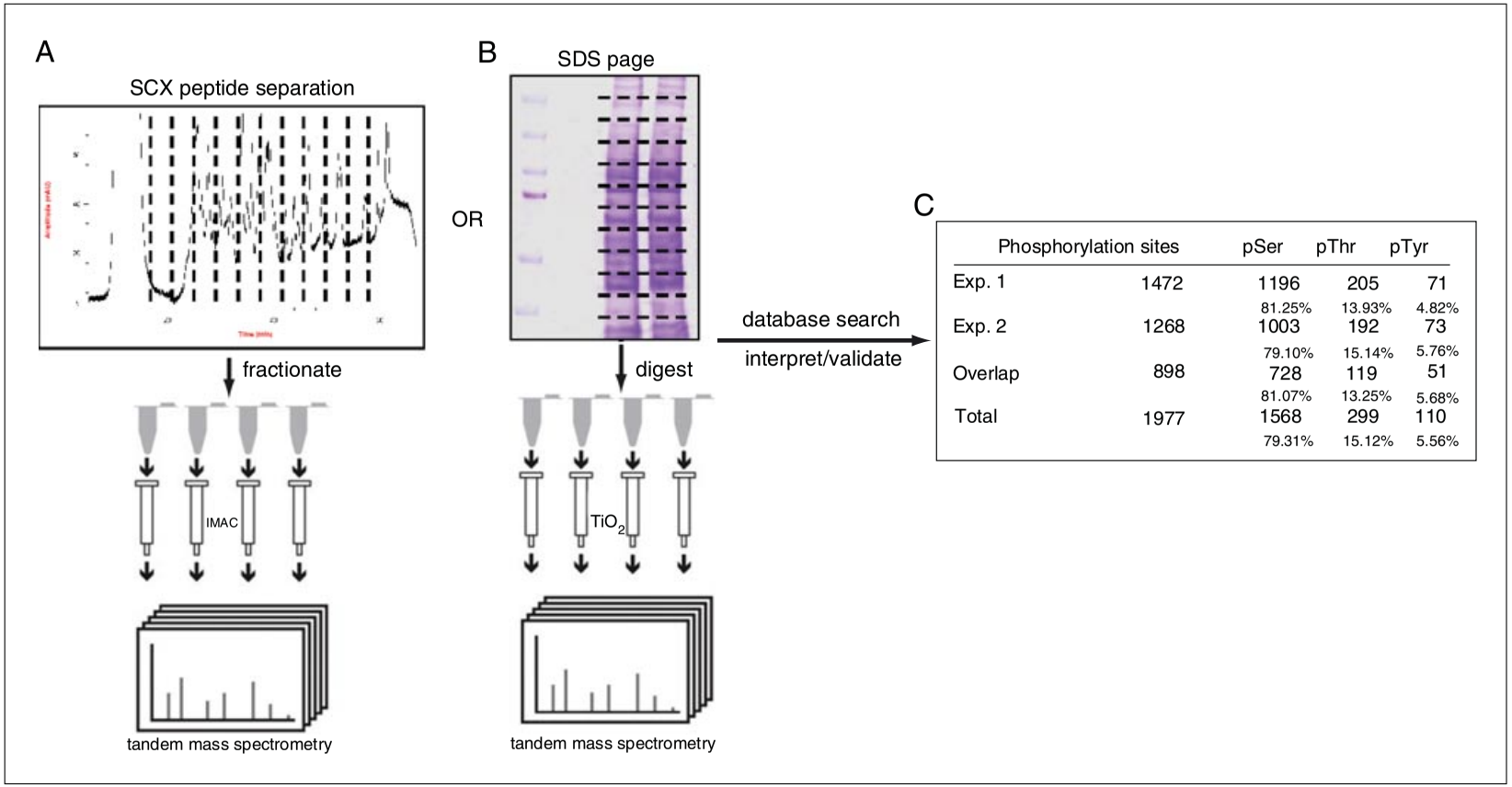


| Phosphorylation sites | | pSer | pThr | pTyr |
|---|---|---|---|---|
| Exp. 1 | 1472 | 1196 | 205 | 71 |
| | | 81.25% | 13.93% | 4.82% |
| Exp. 2 | 1268 | 1003 | 192 | 73 |
| | | 79.10% | 15.14% | 5.76% |
| Overlap | 898 | 728 | 119 | 51 |
| | | 81.07% | 13.25% | 5.68% |
| Total | 1977 | 1568 | 299 | 110 |
| | | 79.31% | 15.12% | 5.56% |

**Figure 18.19.5** 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%.

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