# Chapter 18

# From Phosphoproteome to Modeling of Plant Signaling Pathways

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## Abstract

Quantitative proteomic experiments in recent years became almost routine in many aspects of biology. Particularly the quantification of peptides and corresponding phosphorylated counterparts from a single experiment is highly important for understanding of dynamics of signaling pathways. We developed an analytical method to quantify phosphopeptides (pP) in relation to the quantity of the corresponding non-phosphorylated parent peptides (P). We used mixed-mode solid-phase extraction to purify total peptides from tryptic digest and separated them from most of the phosphorous-containing compounds (e.g., phospholipids, nucleotides) which enhances pP enrichment on $TiO_2$ beads. Phosphoproteomic data derived with this designed method allows quantifying pP/P stoichiometry, and qualifying experimental data for mathematical modeling.

**Key words** Phosphopeptide enrichment, Mixed-mode solid-phase extraction, Metal oxide affinity chromatography, Mathematical modeling

## 1 Introduction

Mathematical modelingand dynamic simulation of signal transduction pathways is an important topic in systems biology [1, 2]. One of the purposes of the dynamic modeling in plant physiology is to evaluate the degree of involvement of different signaling pathways in plant responses to external perturbations [3, 4] or to explain phenotypic appearances of plant mutants. Protein phosphorylation is one of the fastest posttranslational modifications (PTM) that is an intrinsic mechanism of the signal transduction in some signaling pathways (e.g., MAPK cascades). Phosphorylation of signaling proteins traced in time allows revealing involvement of corresponding pathways into adaptive responses [5, 6]. Signaling pathways are organized in cascades of counteracting (e.g., cyclic) irreversible reactions [7], which generate and amplify the cellular signal (Fig. 1). Phosphorylation (by kinases)/de-phosphorylation (by phosphatases) of substrate-proteins is an elemental event in many signal transduction pathways [1, 3, 7]. Normally, proteins in

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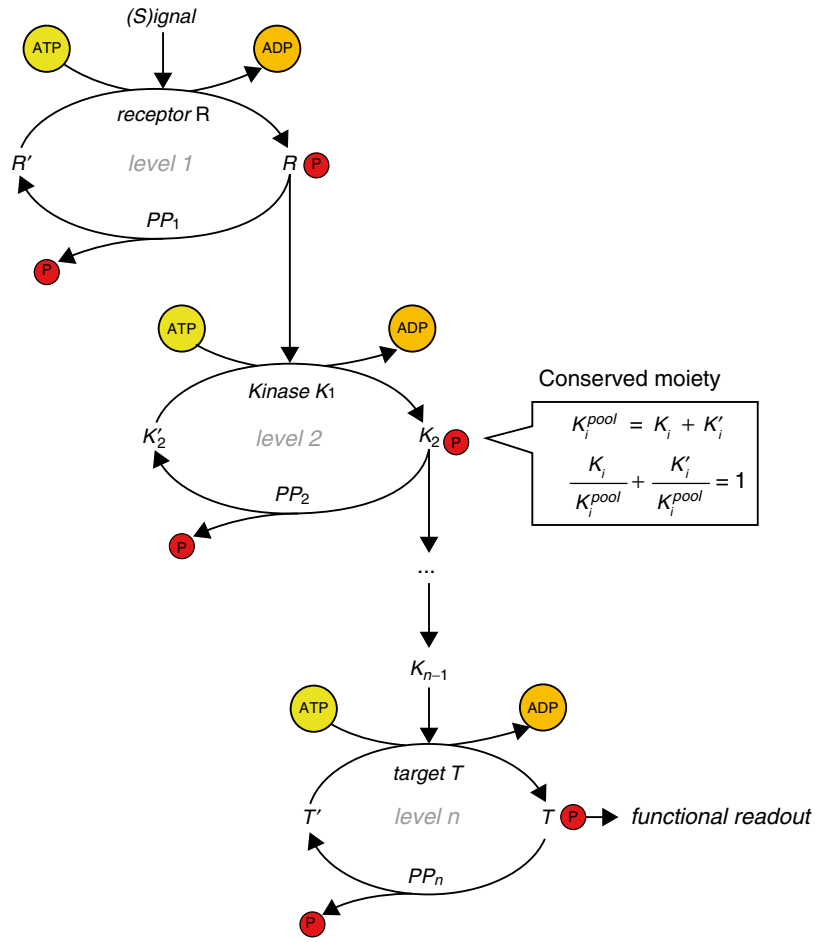


**Fig. 1** A simplified hypothetical scheme of a signal (*S*) transfer from receptor (*R*) to target (*T*) through a linear signal transduction pathway consisting of different kinases (*K*). At each cascade level, only two interconvertable forms are shown: active and inactive (marked by ′). The mechanism of activation is through phosphorylation by means of group transfer from phosphate-donor (e.g., ATP, GTP). The active form of a species from a preceding level activates species at a subsequent level. *PPi*—*i*th phosphatase. The figure was compiled from [1, 7]

cyclic reactions function under assumption of a conserved moiety (Fig. 1): a total pool of a protein remains unchanged in short time, but its degree of phosphorylation shows a fast response to the environmental stimuli. Therefore, it is crucial to measure the total pool of the signaling proteins as well as the degree of their phosphorylation, which, when measured in a time course, can provide one with sufficient information for parameter estimation of mathematical models based on kinetic expressions of individual reactions.

### 1.1 MS-Based Proteomics

Shotgun mass spectrometry (MS)-based proteomics offers a unique opportunity for identification and quantification of thousands of peptides (P) in a single analysis, and in combination with

computational methods provides information on expression of complete proteome of a research object at a given state [8, 9]. The shotgun MS-based proteomics also allows identifying most PTMs of proteins, such as phosphorylation, acetylation, methylation, glycosylation, and ubiquitination [10, 11], which drive signaling pathways, and their dynamics can be used for decoding signaling networks. The common workflow of sample preparation for MS-based proteomics relies on different analytical techniques: enzymatic protein digest, sample fractionation based on various chromatographic techniques, sample enrichment or desalting by solid-phase extraction (SPE), etc. [12]. Since the fraction of phosphopeptides is relatively low (<1 %) regarding to total peptides in trypsinized protein digests, usually an enrichment step is required for their confident identification and quantification [12–14].

### 1.2 Total Peptide Purification

The classical approach to purify total peptides from complex digest mixes exploits hydrophobic interactions between side chains of hydrophobic amino acids and $C_{18}$-groups of reverse-phase SPE sorbent [15]. However, this approach results in co-purification of a variety of nonpolar components, such as lipids, pigments, or sterols. This often is particularly problematic when working with strongly pigmented plant tissues. At low pH (e.g., <3.0) the zwitterion of a peptide becomes fully protonated (i.e., a weak cation or weak base) which allows the use of strong cation exchanger (SCX) for their purification, but it also results in co-purification of charged impurities such as nucleotides. Thus, a combination of the reverse-phase and SCX modes provides enhanced peptide purification and additionally a better removal of nonpolar and charged impurities. Oasis® MCX (mixed-mode cation-exchange and reversed-phase sorbent) provides such dual modes of retention by (1) strong sulfonic-acid-cation exchanger and (2) reversed-phase interactions in combination with hydrophilic interactions on a single organic co-polymer. The sorbent is highly selective and sensitive for extraction of basic compounds from acidified biological matrices (such as plasma, urine, bile, and pigmented plant tissue) and demonstrates very good capacity for the peptide purification from tryptic protein digests. Sample preparation based on use of mixed-mode solid phases provides superior removal of nearly all phospholipids and weaker acids, achieving double-positive effects by (1) eliminating phosphorous-containing compounds (phospholipids, nucleic acids, etc.) that strongly interfere with phosphopeptide enrichment and (2) eliminating the major sources of matrix effects (e.g., neutrals), a known case of ion suppression, loss of sensitivity, and inaccurate quantification by liquid chromatography with a tandem mass analyzer (LC-MS/MS).

### 1.3 Phosphopeptide (pP) Enrichment

The typical pP enrichment protocols rely on several unit operations, such as total peptide purification/desalting after the digest, pP enrichment, and further desalting, in order to provide salt-free samples for the LC-MS/MS analysis. Usually, all desalting steps are

carried out on $C_{18}$ Stop-and-go-extraction tips (StageTips) [15]. Most common pP enrichment protocols are based on metal oxide affinity chromatography (MOAC) on titanium, aluminum, and zirconium oxides [5, 13, 16]. The MOAC of pP is based on the affinity of phosphates to metals, but this interaction is interfered by non-phosphorylated acidic peptides which also show affinity for the metals [14]. Therefore, enhancers of the phosphopeptide selectivity on MOAC are widely used (e.g., lactic or β-hydroxypropanoic acids [16], 2,5-dihydroxybenzoic or phthalic acids [17]). The enhancers reduce unspecific binding of non-phosphorylated acidic peptides, in this sense giving preferences for pP to bind to the metal oxides. Obviously, the second desalting step after the use of enhancers is extremely important, but it leads to an additional loss of phosphopeptides.

In some research, the phosphoprotein enrichment [18, 19] is used prior to the digest followed by the phosphopeptide enrichment procedure to increase yield of phosphopeptides. However, in the context of our approach it is meaningless since non-phosphorylated cognates will be removed.

Other phosphorous-containing compounds (phospholipids, nucleotides, etc.) also compete with pP for the metal centers to bind. Therefore, we have chosen a strategy of eliminating the phosphorous-containing compounds on the level of total peptide purification/desalting (mixed-mode SPE) to give the advantages for the pP at the enrichment step on conventional $TiO_2$ without enhancers. Our method describes pP enrichment from a microsomal fraction [20] of roots of wild-type *Arabidopsis thaliana* (Fig. 2).

### 1.4 Peptide (P) and Phosphopeptide (pP) Quantification

There are different methods of peptide quantification in quantitative proteomics, which implement either absolute or relative peptide quantifications. The quantification techniques are based on the use of stable isotope labeling (e.g., SILAC), isobaric labeling (e.g., TMT, iTRAQ), isotope-coded affinity tag (ICAT), internal peptide standards [21], and label-free quantification (e.g., iBAQ). The workflow we developed is based on label-free relative peptide quantification (iBAQ by MaxQuant [22]) before and after pP enrichment, using the amount (literally a mass in μg) of total peptides and its loss during pP enrichment for the iBAQ value normalization (Fig. 2). This approach allows us to estimate the degree of phosphorylation of the signaling protein pool (*see* **Note 1**).

However, peptides and their phosphorylated counterparts have significant differences in ionization/detection efficiency (so-called flyability) [23]. Determination of flyability ratio for a particular pP/P pair (based on the technical replicates and conserved moiety assumption) allows to correct the signal intensities of the corresponding species and to quantify the absolute phosphorylation stoichiometry in each obtained pP/P pair [23]. This aspect also has to be taken into account for the final quantification.

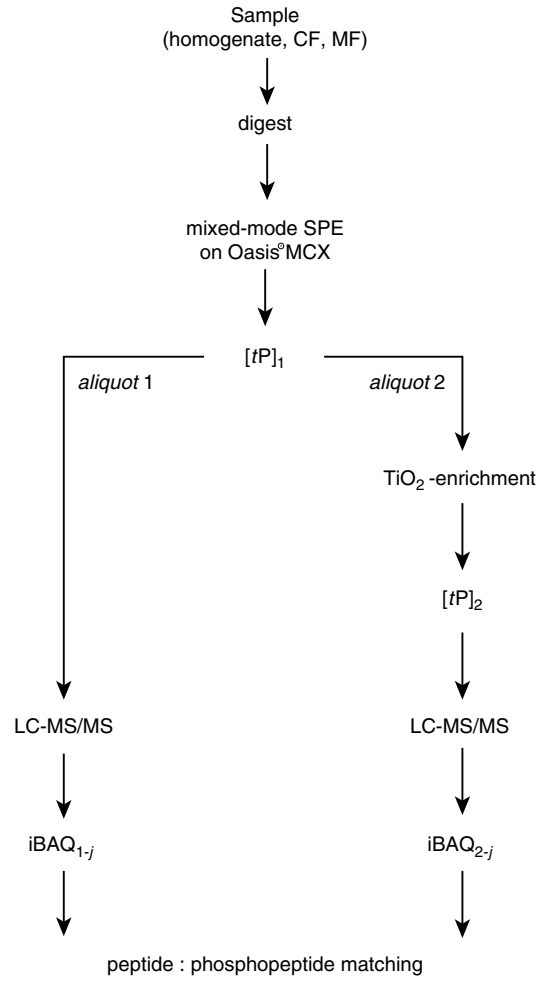


**Fig. 2** Workflow of label-free phosphopeptide/peptide ratio quantification based on $TiO_2$ enrichment of phosphopeptides. Refer to **Note 1** for the calculation algorithm and notations. *CF* cytosolic fraction, *MF* microsomal fraction, *SPE* solid-phase extraction, *tP* total peptide concentration, *iBAQ* intensity-based absolute quantification is used for label-free quantitation (for further details please refer to MaxQuant manual instructions)

## 2 Materials

Prepare all solutions using double-deionized ultrapure water (0.055 μS/cm; *see* **Note 2**) and analytical grade reagents. Use pipette tips and microcentrifuge tubes made from low-binding-capacity plastics to minimize peptide loss by adsorption to the plastic. Take maximum care to avoid keratin contamination. Prepare and store all reagents at room temperature (unless indicated otherwise).

### 2.1 Protein Quantification

1. Protein quantification reagents according to manual instruction for use of NanoOrange® protein quantification kit (Molecular Probe).
2. Bovine serum albumin (BSA) standard solutions in water, 1 mL of 0, 0.1, 0.3, 0.6, 1, 3, 6, 10, 30, 60, 100, 300, 600, 1000, 1500, 2000 μg/mL each.
3. BSA standard solutions in UTU buffer, 1 mL of 0, 0.1, 0.3, 0.6, 1, 3, 6, 10, 30, 60, 100, 300, 600, 1000, 1500, 2000 μg/mL each.
4. BSA standard solutions in loading buffer for LC-MS/MS, 1 mL of 0, 0.1, 0.3, 0.6, 1, 3, 6, 10, 30, 60, 100, 300, 600, 1000, 1500, 2000 μg/mL each.

### 2.2 Total Protein Extraction

1. Hammer and aluminum foil.
2. Liquid nitrogen.
3. Miracloth® filter paper (Merk Millipore).
4. Potter® homogenizer (10 mL; VWR).
5. Homogenization buffer: 330 mM sucrose, 100 mM KCl, 1 mM EDTA, 50 mM Tris–HCl adjusted with MES to pH 7.5, 6.5 mM dithiothreitol (DTT; add freshly). Protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 2 (Sigma), and phosphatase inhibitor cocktail 3 (Sigma) were added from stock solutions in 50 μL each/10 mL of the ice-cold homogenization buffer just before use.
6. UTU-buffer: 6 M Urea, 2 M thiourea, 50 mM Tris–HCl, pH 8.0.
7. Ultrasound bath (e.g., Ultrasonic Cleaner USC 300TH, VWR).
8. High-speed refrigerated benchtop centrifuge for max. speed 65,000 × *g* (e.g., Sigma 3-30KS).

### 2.3 In-Solution Digest

1. Reduction buffer: 6.5 mM (or 1 μg/μL; w/v) DTT in water.
2. Alkylation buffer: 27 mM (or 5 μg/μL; w/v) iodoacetamide in water.
3. 10 mM Tris–HCl, pH 8.0.
4. Lysyl endopeptidase (LysC) stock solution: 0.5 μg/μL (Promega).
5. Trypsin stock solution: 0.5 μg/μL (Promega).
6. 2 % Trifluoroacetic acid (TFA) in water (v/v).

### 2.4 Peptide Purification

1. Oasis® MCX 1 cc Vac Cartridge, 30 mg per Cartridge, 30 μm particle size (Waters).
2. 100 % Methanol.

3. Water.
4. 2 % Formic acid in water (v/v).
5. 5 % Ammonium hydroxide in 80 % methanol (pH 11.0) (v/v).
6. Thermo-Strips and Caps, 8 × 0.2 mL (ThermoScientific).

### 2.5 $C_8$-$TiO_2$-StageTip Column Preparation

The 200 µL $C_8$-StageTips are commercially available (e.g., ThermoScientific; or elsewhere) or can be in-house manufactured according to [15, 24].

1. $C_8$-StageTip: 200 µL Pipet tip packed with two $C_8$ disks.
2. Titanium dioxide ($TiO_2$) beads: Titansphere® beads (5–10 µm particle size, 100 Å pore size, spherical particle shape, $TiO_2$ crystals; GL-Sciences). Store under dry conditions to keep specificity against phosphopeptides (*see* **Note 3**).
3. 100 % Methanol.

### 2.6 Phosphopeptide Enrichment on $C_8$-$TiO_2$-StageTip

1. 5 % Acetonitrile, 0.2 % TFA (pH 2.0) in water (v/v).
2. 80 % Acetonitrile, 0.2 % TFA (pH 2.0) in water (v/v).
3. 5 % Ammonium hydroxide (pH 11.0) in water (v/v).
4. 5 % Piperidin in water (v/v).
5. 20 % Phosphoric acid in water (v/v).

### 2.7 $C_{18}$-StageTip Column Preparation

The 200 µL $C_{18}$-StageTips are commercially available (e.g., ThermoScientific; or elsewhere) or can be in-house manufactured according to [15, 24].

1. $C_{18}$-StageTip: 200 µL Pipet tip packed with two $C_{18}$ disks.
2. 80 % Acetonitrile, 0.2 % TFA.
3. 0.5 % Acetic acid in water (v/v).
4. 80 % Acetonitrile, 0.5 % acetic acid (v/v).
5. 5 % Acetonitrile, 0.2 % TFA (v/v).

### 2.8 LC-MS/MS

#### 2.8.1 Liquid Chromatography

1. Chromatographic system: Easy-nLC 1000 (ThermoScientific).
2. Column: EASY-Spray column, PepMap® RSLC, C18 (ThermoScientific); particle size 2 µm; pore size 100 Å; column dimensions 75 µm × 50 cm (I.D. × L).
3. Loading buffer: 5 % Acetonitrile, 0.2 % TFA, pH 2.0.
4. Buffer A: 0.5 % Acetic acid, pH 2.0.
5. Buffer B: 0.5 % Acetic acid, 80 % acetonitrile, pH 2.0.

#### 2.8.2 Mass Spectrometry Equipment

1. Mass analyzer: Q Exactive Plus (ThermoScientific).

## 3 Methods

### 3.1 Protein Quantification

1. Use 3 μL of proteins/ peptides contenting solution for quantification, which is performed according to the manual instruction for use of NanoOrange® protein quantification kit (*see* **Note 4**). Choose the BSA standard (in water, in UTU buffer, or in loading buffer for LC-MS/MS) in accordance with the matrix of the protein/peptide-contenting solution (*see* **Note 5**).

### 3.2 Total Protein Extraction

This protocol is adapted from [20]. All protein extraction steps must be done on ice.

1. Weigh root samples (*g* of fresh weight; gFW), wrap them individually in aluminum foil, and freeze them immediately in liquid nitrogen (*see* **Note 6**).
2. Break the frozen samples into small pieces with the hammer while keeping them wrapped in aluminum foil. Make sure that the samples are constantly frozen after harvesting to avoid rapid dephosphorylation.
3. Transfer the cell material into ice-cold homogenization buffer in a ratio of 5 mL homogenization buffer per 1 gFW.
4. Resuspend the cell material thoroughly by gentle stirring or shaking to get rid of clots.
5. Grind the sample manually in a Potter® homogenizer on ice, 50 smooth strokes per 7 mL sample.
6. Filter the homogenate through four layers of Miracloth® to get rid of cell wall material and tissue debris.
7. Centrifuge the homogenate at $7.5 \times 10^3 \times g$ for 15 min at 4 °C to get rid of unbroken cells and organelles.
8. Collect the supernatant.
9. Centrifuge the supernatant at $48 \times 10^3 \times g$ for 80 min at 4 °C to precipitate microsomal vesicles.
10. Collect the supernatant which represents the cytosolic fraction (i.e., water-soluble proteins, can be used for other experiments).
11. Resuspend the pellet, which represents the microsomal fraction (i.e., endomembranes and membrane-associated proteins), in 500 μL of ice-cold UTU-buffer.
12. Rigorously vortex the suspension and ultra-sonicate it for approximately 30 s.
13. Quantify total protein content in the microsomal fraction using NanoOrange ® protein quantification kit and BSA standards in UTU buffer.

### 3.3 In-Solution Protein Digest

1. Reduction: Add 1 μL of the reduction buffer per each 50 μg of the total protein and incubate for 30 min at 25 °C and 260 rpm.
2. Alkylation: Add 1 μL of alkylation buffer per each 50 μg of the total protein and incubate for 20 min at 25 °C in the dark and 260 rpm.
3. Digest 1: Add 0.5 μL of LysC stock solution per each 50 μg of the total protein and incubate for 3 h at 37 °C and 260 rpm.
4. Dilution: Dilute the sample by fivefold with 10 mM Tris–HCl, pH 8.0 (*see* **Note 7**).
5. Digest 2: Add 1 μL of trypsin stock solution per each 50 μg of the total protein and incubate overnight at 37 °C and 260 rpm.
6. Stop the digest: Acidify the digest to 0.2 % TFA final concentration (add 1/10 volume of 2 % TFA to reach pH 2.0).
7. Spin the sample on centrifuge at $20 \times 10^3 \times g$, 3 min, at room temperature to pellet any insoluble materials.

### 3.4 Peptide Purification

1. Conditioning: Condition the MCX cartridge with 1 mL of 100 % methanol (*see* **Note 8**).
2. Equilibrate the MCX cartridge with 1 mL of water.
3. Sample loading: Load the digest to the conditioned MCX cartridge (*see* **Note 9**). Collect the flow-through and re-load it two more times.
4. Wash 1: Wash the cartridge with 1 mL of 2 % formic acid (pH 2.0) to lock ionized compounds on MCX (*see* **Note 10**).
5. Wash 2: Wash the cartridge with 1 mL of 100 % methanol to remove interfering unionized weaker acids and neutrals. Completely expel the methanol from the cartridge.
6. Elution: Elute peptides from the cartridge with $5 \times 200$ μL of 5 % $NH_4OH$ in 80 % methanol (pH 11) (*see* **Note 11**). The final total volume is 1000 μL.
7. Split the eluate into 15 % ("aliquot 1") and 85 % ("aliquot 2") of the sample volume (*see* Fig. 2).
8. Dry both aliquots of the eluate to complete dryness in a vacuum centrifuge (10 mbar, $235 \times g$, rotor temperature 37 °C, e.g., Christ® RVC 2–25 CD plus).
9. Redissolve the dried "aliquot 1" in 50 μL of LC-MS/MS loading buffer (e.g., 5 % acetonitrile, 0.2 % TFA).
10. Rigorously vortex the sample, sonicate it for 30 s, and centrifuge at $2 \times 10^3 \times g$ for 5 min.
11. Transfer the supernatant of "aliquot 1" into fresh micro-tubes (0.2 mL).
12. Quantify total peptide content in "aliquot 1" using NanoOrange®protein quantification kit and BSA standards in

loading buffer for LC-MS/MS. This is $[Peptides]_1$ according to notations at Fig. 2.

13. Reserve the "aliquot 1" for the further LC-MS/MS analysis.

### 3.5 $C_8$-$TiO_2$-StageTip Column Preparation

1. Activation of $TiO_2$ beads: Activate $TiO_2$ beads at 130 °C for 30 min prior to use (*see* **Note 3**).
2. Preparing $TiO_2$ bead stock suspension: Weigh 25 mg of $TiO_2$ beads, resuspend it in 500 μL of 100 % methanol, and vortex the suspension well.
3. Loading of $TiO_2$ bead stock suspension: Load 20 μL of the stock suspension (overall 1 mg; *see* **Note 12**) on top of the $C_8$ disk in the 200 μL $C_8$-StageTip (*see* **Note 13**).
4. Let the suspension settle down under the gravity force in order to distribute the beads evenly.
5. Spin (*see* **Note 14**) the $C_8$-$TiO_2$-StageTip to force the solution through.

### 3.6 Phosphopeptide Enrichment on $C_8$-$TiO_2$-StageTip

1. Redissolve the dried "aliquot 2" in 50 μL of 80 % acetonitrile and 0.2 % TFA.
2. Rigorously vortex the sample, sonicate it for 30 s, and centrifuge at $2 \times 10^3 \times g$ for 5 min.
3. Insert the 200 μL $C_8$-$TiO_2$-StageTip into a spin adapter and place it in a fresh microcentrifuge tube.
4. $C_8$-$TiO_2$-StageTip conditioning: Load 100 μL of 80 % acetonitrile and 0.2 % TFA to the $C_8$-$TiO_2$-StageTip and spin it to force the solution through.
5. Replace the waste microcentrifuge tube with a fresh tube.
6. Sample loading: Load the sample (**step 1**) onto conditioned $C_8$-$TiO_2$-StageTip (**step 4**) and spin it to force the sample through.
7. Sample reloading: Collect the flow-through, reload the sample again, and then spin it to force the sample through. Repeat this step twice.
8. Wash: Load 100 μL of 5 % acetonitrile and 0.2 % TFA to the $C_8$-$TiO_2$-StageTip and then spin it to force the sample through into waste microcentrifuge tube.
9. Add 50 μL of 20 % phosphoric acid into a fresh microcentrifuge tube where the phosphopeptides will be eluted in.
10. Elution 1: Elute the phosphopeptides with 50 μL of 5 % $NH_4OH$ (pH 11.0) from the 200 μL $C_8$-$TiO_2$-StageTip into a microcentrifuge tube with 20 % phosphoric acid (**step 10**) (*see* **Note 11**).
11. Elution 2: Elute the remaining phosphopeptides with 50 μL of 5 % piperidine from the $C_8$-$TiO_2$-StageTip into the same tube. The final volume of the eluate is 150 μL.

### 3.7 Desalting on $C_{18}$-StageTip

1. Conditioning of $C_{18}$-StageTips: Load 100 µL of 80 % acetonitrile and 0.2 % TFA to the $C_{18}$-StageTip and spin it to force the solution through into a waste microcentrifuge tube.
2. Load $2 \times 100$ µL of 0.5 % acetic acid to the $C_{18}$-StageTip and spin it to force the solution through into a waste microcentrifuge tube.
3. Sample loading: Load the sample (Subheading 3.6, **step 11**) onto pre-conditioned $C_{18}$-StageTip and spin it to force the solution through into a waste microcentrifuge tube.
4. Washing: Load $2 \times 100$ µL of 0.5 % acetic acid to the C18-StageTip and spin it to force the solution through into a waste microcentrifuge tube.
5. Elution: Elute the phosphopeptide enriched fraction by $2 \times 20$ µL of 80 % acetonitrile and 0.2 % TFA into fresh microcentrifuge tube.
6. Spin down the eluate to dryness (10 mbar, $235 \times g$, rotor temperature 37 °C, e.g., Christ® RVC 2–25 CD plus).
7. Redissolve the phosphopeptides in 50 µL of loading buffer for LC-MS/MS (i.e., 5 % acetonitrile, 0.2 % TFA).
8. Rigorously vortex the sample, sonicate it for 30 s, and centrifuge at $2 \times 10^3 \times g$ for 5 min.
9. Transfer the supernatant of "aliquot 2" into a fresh micro-tube (0.2 mL).
10. Quantify the total peptide content in the sample using NanoOrange®protein quantification kit and BSA standards in loading buffer for LC-MS/MS. This is $[Peptides]_2$ according to notations at Fig. 2.

### 3.8 LC-MS/MS

#### 3.8.1 Liquid Chromatography

1. Injection volume: 1–5 µL to achieve at least 2 µg of overall column load with the total peptides.
2. Flow rate: 250 nL/min.
3. Gradient %B: 0 min 5 %, 200 min 35 %, 240 min 60 %, 242 min 90 %, 257 min 90 %, 258 min 5 %, 263 min 5 %.
4. Operation column temperature: 50 °C.
5. Operation pressure: 500 bar.

#### 3.8.2 Mass Spectrometry

1. Polarity: Positive.
2. Full MS: Resolution 70,000 (at $m/z = 200$ Th); AGC target 1e6; maximum IT 20 ms; scan range 300–1600 $m/z$.
3. dd-$MS^2$: Resolution 17,500 (at $m/z = 200$ Th); AGC target 1e5; maximum IT 120 ms; TopN 5; isolation window 2.2 m/z; scan range 200–2000 $m/z$; NCE 25.
4. dd-Settings: Underfill ratio 0.1 %; dynamic exclusion 40 s.

### 3.9 Data Employment

This method is mainly designated for quantification of stoichiometry in pairs of phosphorylated peptide and its corresponding unmodified cognate, i.e., to measure phosphorylation stoichiometry. However, this method can also be applied for search/screen of gross phosphorylation sites or qualitative assessment of phosphorylation of proteins from certain signaling pathways, without detecting the unmodified cognates.

Stimulus response (i.e., dynamic perturbation) experimental approaches are widely used in systems biology to provoke dynamic responses of the studied system. The phosphorylation stoichiometry of signaling proteins is a state variable in mathematical models of the conserved moieties or cascade reactions of the signaling pathways (Fig. 1). Steady-state phosphorylation stoichiometry and its time-dependent dynamics in response to a perturbation event allow parameter estimation of the kinetic equations that describe the corresponding cascades in signaling pathways [1]. Measurements of steady-state phosphorylation stoichiometry under different signal strength allow quantification of local and global response coefficients of the signaling pathway, if the kinetic properties of the reaction cascades are known [7]. This type of modeling can be performed either in package programs like MATLAB (The MathWorks) and MATHEMATICA (Wolfram Research) or in specialized software like Simmune [25].

## 4 Notes

1. Please refer to Fig. 2 for the notations associated with corresponding workflow steps. The loss of peptides' amounts ($l$) during phosphopeptide enrichment can be estimated as

$$l = \frac{[tP]_1}{[tP]_2} \tag{1}$$

where $[tP]i$—concentration of total peptides before (1) and after (2) enrichment (μg/mL). The peptide quantification must be accurate; therefore please refer to Subheading 3.1.

The amount (i.e., mass in μg) of the injected peptides ($mi$) for LC-MS/MS analysis can be calculated as

$$m_i = x_i \times [tP]_i \tag{2}$$

where $xi$—injection volume used for the LC-MS/MS analysis ($i = 1,2$) (μL).

The mass-specific content of individual peptide ($Pj$) and its phosphorylated species ($pPj$) can be estimated from its label-freequantification ($i$BAQ$i,j$) and normalized per corresponding $mi$ ($j = N$):

$$\begin{cases} P_j = \dfrac{i\mathrm{BAQ}_{1.j}}{m_1} \\ pP_j = \dfrac{i\mathrm{BAQ}_{2.j}}{m_2} \times l \end{cases} \tag{3}$$

where the correction factor $l$ is calculated in Eq. 1. The peptide content after phosphopeptide enrichment must be corrected with the loss of the total peptides mass (Eq. 1). The total pool of the particular peptide ($P_j^{\mathrm{pool}}$) consists of non-phosphorylated and phosphorylated species:

$$P_j^{\mathrm{pool}} = P_j + pP_j \tag{4}$$

and correspondingly a part of each species is

$$\frac{P_j}{P_j^{\mathrm{pool}}} + \frac{pP_j}{P_j^{\mathrm{pool}}} = 1 \tag{5}$$

2. Hereinafter designated just as water.
3. The specificity of $TiO_2$ beads against phosphopeptides is reduced by water absorption when it is kept without desiccation. The specificity can be recovered by heating the beads in a drying oven at 130 °C for 30 min [16].
4. Accurate and highly specific quantification of protein/peptide is essential for this approach. We have selected NanoOrange® protein quantification kit (Molecular Probe) for this purpose, because it is very sensitive and specific to proteins/peptides, has a wide quantification range (0.1–2000 μg/mL), and is compatible with nucleic acids, reducing agents, and detergents.
5. UTU buffer or 5 % acetonitrile and 0.2 % TFA significantly quench the fluorescence and therefore they must be included into the solution matrix for compensation.
6. The frozen samples can be stored at –80 °C for further analysis.
7. The dilution step is required in order to get final 1.2 M urea, 0.4 M thiourea, and 10 mM Tris–HCl (pH 8.0), which is favorable for trypsin operation.
8. Do not let the cartridge dry; always expel one mobile phase with another.
9. The 1 cc cartridge from Waters has a load volume of a matrix with an analyst up to 50 mL. At this step, the sample is in 1.2 M urea, 0.4 thiourea, 10 mM Tris–HCl, and 0.2 % TFA, pH 2.0.
10. This step also removes undigested proteins and salts.
11. Phosphopeptides are not stable in alkaline conditions; therefore, in order to minimize the exposure time, it is advised to

dry the eluate immediately at vacuum centrifuge, as it is exemplified in Subheading 3.4, **steps 7** and **8**, or immediately neutralize the alkaline solution with strong acid as it is exemplified in Subheading 3.6, **steps 10** and **11**.

12. 1 mg $TiO_2$ beads per a single $C_8$-StageTip column have a binding capacity of ~100 μg of total peptides from *Arabidopsis* [16].
13. The choice of the $C_8$ material is based on the idea that the membrane disk is only used to retain the $TiO_2$ beads inside the tip, but the $C_8$-disk itself does not participate in the phosphopeptide enrichment. The $C_8$-StageTips can be stored at room temperature.
14. "Spin" hereinafter refers to centrifugation of a StageTip in a bench microcentrifuge (e.g., Mini Star Silverline, Galaxy Mini Centrifuge, VWR) at $2 \times 10^3 g$ (or $6 \times 10^3$ rpm) for 1 min at room temperature.

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