Chapter 13

Measuring Activity and Specificity of Protein Phosphatases

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Abstract

Reversible protein phosphorylation plays essential roles in coordinating cell division and many other biological processes. Cell cycle regulation by opposing kinase and protein phosphatase activities is often complex and major challenges exist in identifying the direct substrates of these enzymes and the specific sites at which they act. While cell cycle kinases are known to exhibit strict substrate specificities important for coordinating the complex events of cell division, phosphatases have only recently been recognized to exert similarly precise regulatory control over cell cycle events through timely dephosphorylation of specific substrates. The molecular determinants for substrate recognition by many phosphatases that function in cell division are still poorly delineated. To understand phosphatase specificity, it is critical to employ methods that monitor the dephosphorylation of individual phosphorylation sites on physiologically relevant substrates. Here, using the cell cycle phosphatase Cdc14 as an example, we describe two methods for studying phosphatase specificity, one using synthetic phosphopeptide substrates and the other using intact phosphoprotein substrates. These methods are useful for targeted characterization of small substrate sets and are also adaptable to large-scale applications for global specificity studies.

Key words Protein phosphatase, Protein kinase, Cell cycle, Protein dephosphorylation, Multisite phosphorylation, Mass spectrometry, Protein phosphatase assay, Cdc14 phosphatase assay, Phosphopeptide substrates, High-throughput phosphatase assay

1 Introduction

Mitotic kinases coordinate orderly progression through the cell division cycle by phosphorylating specific substrates at precisely the right time and location. The regulation and substrate specificity of protein kinases are fundamental components of the cell cycle control system. Protein phosphatases, which catalyze removal of phosphate groups deposited by kinases, have historically been thought of as mostly unregulated enzymes whose purpose is to restore a basal phosphorylation state upon kinase inactivation [1]. It is now clear that mitotic phosphatases are precisely regulated enzymes that act at specific times toward specific substrates to help coordinate cell cycle progression and checkpoint responses [2–5].

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Understanding how phosphatases function in cell cycle control requires knowledge of the determinants for substrate selection and the identities of their direct substrates. Most cell cycle control proteins are regulated by multiple phosphorylation sites, sometimes controlled by multiple kinases and phosphatases [6, 7]. This regulatory complexity requires analytical methods capable of detecting and quantifying individual phosphorylation sites on physiologically relevant substrate proteins.

Many methods for monitoring protein phosphatase activity toward protein substrates report on the global phosphorylation status without providing site-specific information. This includes detecting release or loss of radioactive [^{32}P]-containing phosphate from protein substrates, a change in phosphorylation-induced gel mobility shift, and colorimetric detection of released free phosphate using malachite green dye [8–11]. Other methods use nonphysiological substrates like *p*-nitrophenyl phosphate or 6,8-difluoro-4-methylumbelliferyl phosphate in colorimetric or fluorometric assays or employ [^{32}P]-labeled artificial protein substrates, such as myelin basic protein. The use of nonphysiological substrates may have been influenced by the previously held view that protein phosphatases exhibit little substrate specificity.

Cdc14 phosphatases are members of the dual-specificity phosphatase subfamily of the protein tyrosine phosphatases [12, 13]. Despite their evolutionary and mechanistic relationship to tyrosine phosphatases, Cdc14 enzymes are highly selective for phosphoserine substrates [14]. They play roles in counteracting cyclin-dependent kinase (Cdk) phosphorylation during the cell division cycle [15–17] and exhibit a strong preference in vitro for the consensus sequence pSer-Pro-x-Lys/Arg [18], representing a subset of the Ser/Thr-Pro sites targeted by Cdks. The strong intrinsic selectivity of Cdc14 for a subset of Cdk sites deposited during the cell cycle was revealed using the methods described herein, highlighting the power of analytical methods that monitor individual phosphorylation sites.

We describe two protocols for measuring Cdc14 phosphatase activity on individual phosphorylation sites that have proven to be important in establishing the authenticity of candidate substrates [14, 18, 19] and are generally useful for studying phosphatase substrate specificity. The first uses synthetic phosphopeptides, measures the release of phosphate spectrophotometrically, and is easily adapted to high-throughput format for screening libraries of phosphatase peptide substrates, inhibitors, or activators. The second uses mass spectrometry (MS) to measure dephosphorylation at multiple individual sites on intact protein substrates. With yeast Cdc14, we found that the specificity observed with phosphopeptide substrates in vitro existed with intact proteins in cells [14, 18], indicating that these phosphopeptides mimic natural targets. However, many protein phosphatases recognize substrate features distal

from the sites of phosphorylation; thus phosphopeptides are not always useful analogs of physiological targets. The phosphopeptide assay is convenient, relatively inexpensive, and easy to implement with standard lab equipment. The MS assay requires more advanced instrumentation and is more technically challenging, but offers the advantage of studying single, or complex, mixtures of physiological protein substrates.

2 Materials

Prepare all solutions using ultrapure water. Store all reagents at room temperature unless otherwise noted. Solvents and liquid reagents should be of HPLC grade. Low-protein-binding microtubes should be used to minimize peptide/protein loss by adsorption.

2.1 Phosphopeptide Dephosphorylation Assay

1. Phosphopeptide stocks: Lyophilized synthetic phosphopeptides (*see* **Note 1**).
2. Phosphate standard solution: 10 mM K_2HPO_4 (or Na_2HPO_4) in water. Serially dilute to generate a set of concentrations between 0 and 40 μM (e.g., 4, 8, 16, 24, 32, and 40 μM) for creating standard curves. Use either water (for determining phosphopeptide concentration) or phosphatase reaction buffer (for enzyme assays) for the dilutions.
3. Phosphate detection reagent: BIOMOL GREEN™ (Enzo Life Sciences), store at 4 °C. Remove the appropriate volume required for an assay and allow it to reach room temperature (*see* **Note 2**).
4. 1 g Sep-Pak® C18 columns (Waters Corporation).
5. C18 mobile-phase solvents: Prepare 15 ml each of 5, 10, 20, 50, and 95 % acetonitrile (ACN) solutions, each containing 0.1 % trifluoroacetic acid (TFA).
6. Methanol.
7. α-Cyano-4-hydroxycinnamic acid: 5 mg/ml in 75 % ACN, 0.1 % TFA.
8. 50 mm borosilicate glass tubes.
9. Ashing reagent: 10 % $MgNO_3 \cdot 6H_2O$ in 95 % ethanol.
10. Phosphatase reaction buffer: 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 % β-mercaptoethanol (BME).
11. Purified recombinant Cdc14: Procedures for purification of recombinant Cdc14 fused to N-terminal 6×-histidine, glutathione S-transferase, or other affinity tags have been published (*see* ref. 14). We store purified Cdc14 in 25 mM Tris–HCl pH 7.5, 300 mM NaCl, 40 % glycerol, 2 mM EDTA, and 0.1 % BME in small aliquots at −80 °C. Working aliquots

are kept at –20 °C for up to 2 weeks. Dilute to 10× final concentration in phosphatase reaction buffer each day prior to use and keep on ice.

12. Clear plastic 96-well microplates suitable for measuring absorbance at visible wavelengths and/or semi-micro volume disposable cuvettes.
13. 15 ml conical tubes.

2.2 Intact Protein Dephosphorylation Assay

1. Kinase buffer: 10 mM HEPES pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 10 % glycerol (*see* **Note 3**).
2. Phosphatase reaction buffer: 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 % BME.
3. Adenosine trisphosphate (ATP): 100 mM in 25 mM HEPES pH 7.5. Store at –20 °C.
4. 4× SDS sample buffer: 40 % glycerol, 240 mM Tris–HCl pH 6.8, 8 % SDS, 0.04 % bromophenol blue, 4 % BME.
5. Coomassie brilliant blue R-250: 0.05 % (w/v) in 25 % methanol, 10 % acetic acid.
6. Destain: 10 % acetic acid.
7. 100 mM ammonium bicarbonate (ABC) in water (must be made fresh).
8. Trypsin solution: 20 ng/μl proteomics-grade porcine trypsin in 100 mM ABC (*see* **Note 4**).
9. ACN.
10. Wash buffer: 50 mM ABC, 50 % ACN. Prepare by mixing 100 mM ABC and ACN at 1:1.
11. HPLC solvent A: 0.1 % formic acid.
12. HPLC solvent B: 95 % ACN, 0.1 % formic acid.
13. Purified proteins (*see* **Note 5**):
    (a) Cyclin-dependent kinase 1 (Cdk1) or other kinase.
    (b) Cdc14 or other phosphatase.
    (c) Protein substrate(s): Affinity-purified and retained on affinity beads.

3 Methods

3.1 Phoshopeptide Dephosphorylation Assay

Steps 1–9 are needed only for crude synthetic peptide stocks (*see* **Note 6**).

3.1.1 Substrate Preparation

1. Resuspend crude phosphopeptide in 2 ml of 0.1 % TFA (*see* **Note 7**). Remove and save ~3 μl pre-purification sample for MS analysis.

2. Condition a dry 1 g Sep-Pak® column with 3 ml methanol and then 3 ml 100 % ACN/0.1 % TFA (*see* **Note 8**). Drain the column by gravity flow for each step. Equilibrate column 2× with 3 ml 0.1 % TFA.
3. Apply peptide solution to the column and collect the flow through as one fraction in a 15 ml conical tube.
4. Wash column 2× with 3 ml 0.1 % TFA. Save the flow-through for MS analysis.
5. Step elution: Apply 3 ml 5 % ACN/0.1 % TFA to column and collect 1 ml fractions. Repeat with 3 ml 10, 20, and 50 % ACN/0.1 % TFA. There should be 12 total elution fractions.
6. Strip column 2× with 3 ml 100 % ACN/0.1 % TFA, collecting 1.5 ml fractions for analysis (*see* **Note 9**).
7. Analyze the elution fractions, starting peptide solution, and wash fractions by MALDI-TOF MS to find the desired phosphopeptide. A sample (typically 0.5 μl) of each fraction is spotted on the MALDI plate, and overlaid with 0.5 μl α-cyano-4-hydroxycinnamic acid solution (*see* **Note 10**).
8. Pool fractions containing the target peptide peak (based on MS intensity) and dry in a lyophilizer or centrifugal vacuum concentrator. Typically, the bulk of the peptide will be found in two to three fractions.
9. Resuspend phosphopeptide in water to the desired approximate concentration based on weight (make sure that it is fully soluble (*see* **Note 7**)—we usually start with 20 mM).
10. Add 50 μl of each phosphate standard solution (from above) or 50 μl of water to a 50 mm borosilicate glass tube (providing 0, 0.2, 0.4, 0.8, 1.2, 1.6, and 2 nmol phosphate). In triplicate, add two different volumes of purified phosphopeptide solution to separate tubes to achieve nmol amounts within this same range.
11. Add 25 μl ashing reagent to each tube. Mix and evaporate to dryness (an oven at ~50 °C works well).
12. Using a Bunsen burner, carefully heat the bottom of each tube until the brown fumes disappear and a white residue appears. Avoid prolonged heating, as black particles will form.
13. Allow tubes to cool in a rack and then dissolve the white residue in phosphate detection reagent (*see* **Note 11**).
14. Incubate for 20 min at room temperature and measure the absorbance at 640 nm in clean, phosphate-free cuvettes or microplate. The sample containing only water is used as a blank (*see* **Note 12**).

15. Create a standard curve of absorbance versus phosphate concentration and use it to calculate the concentration of the phosphopeptide solution.
16. Store phosphopeptide stocks in small aliquots at –80 °C to minimize freeze-thaw cycles. Prepare working stocks by diluting in phosphatase reaction buffer immediately prior to use. Prepare only the volume required for use each day.

3.1.2 Assay

1. Dispense 40 µl phosphatase reaction buffer per well in a 96-well plate and add 5 µl of phosphopeptide per well to achieve the desired final concentration. For Cdc14 endpoint assays we typically use substrates at 100 µM (*see* **Note 13**).
2. Add 5 µl of 10× Cdc14 working stock to each reaction well, mix thoroughly but carefully with a pipette to avoid creating bubbles, and incubate plate at 30 °C for 30 min (*see* **Note 14**).
3. Dispense 50 µl of each phosphate standard prepared above in reaction buffer into wells of the 96-well plate. Include one well with just 50 µl reaction buffer.
4. Add 100 µl of phosphate detection reagent to all wells to stop reaction and initiate color development. Incubate for 20 min at room temperature, and measure the absorbance at 640 nm in a plate reader.
5. Create a standard curve (as in Subheading 3.1.1, **step 15**) to calculate reaction rates from the absorbance values.

3.2 Intact Protein Dephosphorylation Assay

Keep proteins ice cold except where other incubation temperatures are noted.

3.2.1 Substrate Preparation (See **Note 15**)

1. Starting with affinity-purified substrate protein immobilized on the affinity matrix, equilibrate approximately 100 µg substrate by rinsing twice with several volumes kinase buffer, pelleting the resin at 1000 × *g* for 2 min, and discarding the supernatant (*see* **Note 16**).
2. Add 1 bead volume kinase buffer and ATP to 1 mM. Mix gently to resuspend the substrate-bound resin. Mix gently.
3. Add purified Cdk1 to the protein substrate resin, mix gently, and incubate suspension at 30 °C for 30 min (*see* **Note 17**).
4. Pellet the resin by centrifugation at 1000 × *g* for 2 min and discard the supernatant.
5. Wash the resin three times with at least 3 resin volumes of phosphatase reaction buffer to remove the kinase. Gently mix the solution each time and centrifuge at 1000 × *g* for 2 min. Carefully remove the supernatant and discard.
6. Optional: It is recommended to evaluate phosphorylation either by MS, using the procedure described below for the phosphatase assay, or by an alternative method such as Phos-Tag™ SDS-PAGE mobility shift.

3.2.2 Assay

The phosphatase assay can be conducted with the substrate still bound to the affinity resin, or after the substrate has been eluted. Here, we describe our procedure for assaying recombinant glutathione S-transferase-tagged substrates bound to glutathione-agarose resin (*see* **Note 18**).

1. Resuspend the resin with bound phosphorylated substrate in 4 volumes phosphatase reaction buffer.
2. Divide the resin into four equal aliquots (or additional aliquots if more time points are desired).
3. Add 4× SDS sample buffer to one aliquot and heat at 95 °C for 5 min. This will serve as the reference time = 0 sample.
4. Add Cdc14 to the remaining fractions at a final concentration of 100 nM and mix thoroughly by gentle agitation (*see* **Note 19**).
5. Incubate the reactions at 30 °C. Add 4× SDS sample buffer to one aliquot every 10 min (or other suitable time intervals) and heat immediately at 95 °C for 5 min (*see* **Note 20**).
6. Pellet the resin at 1000 × *g* for 2 min and transfer each supernatant to a low-bind microfuge tube.
7. Subject each sample to SDS-PAGE and stain with Coomassie blue. Destain thoroughly and store in ultrapure water prior to processing for MS analysis.

3.2.3 Peptide Preparation for MS Analysis

1. Excise bands of interest from gels using a clean razor blade. Chop each into roughly a half dozen smaller pieces and transfer to a low-bind microfuge tube (*see* **Note 21**).
2. Add 100 µl of wash buffer and incubate for 30 min, vortexing occasionally. Remove wash buffer and repeat until gel pieces are destained.
3. Add 500 µl ACN and incubate until gel pieces are dehydrated and appear small and white.
4. Remove and discard ACN. Air-dry gel pieces for 10 min.
5. Add enough trypsin solution to cover the gel pieces (typically 30–40 µl) and wait for 30 min to allow rehydration. Add additional trypsin solution to completely submerge gel pieces if necessary. Incubate overnight at 37 °C.
6. Add ACN (2× trypsin solution volume used) and incubate for 15 min at room temperature, vortexing occasionally. Transfer liquid to a new low-bind microfuge tube.
7. Repeat peptide extraction by rehydrating gel slices with 40 µl water for 10 min, adding 80 µl of ACN, incubating for 15 min, and pooling liquid with the first extraction.
8. Dry extracted peptides in a centrifugal vacuum concentrator or freeze and dry by lyophilization.

3.2.4 Liquid Chromatography-Mass Spectrometry (LC-MS) Acquisition and Analysis

The following protocol should be generally applicable for a wide variety of nanospray LC-MS systems.

1. Resuspend the dried peptides in a small volume of HPLC solvent A to a concentration suitable for MS analysis (typically ~0.2 μg/μl). Excess material may be stored at −80 °C (*see* **Note 22**).
2. Inject 1 μg (typically 5 μl) onto a C18 trap column and wash with HPLC solvent A.
3. Resolve the sample with a linear gradient from 5 to 40 % HPLC solvent B through a microcapillary C18 column and a nanoelectrospray emitter tip at a flow rate of ~300 nl/min (*see* **Note 23**).
4. Acquire MS survey and data-dependent MS/MS fragmentation scans during the entire linear gradient (*see* **Note 24**).
5. After data acquisition, peptides must be identified and quantified. Here we describe a procedure for identification of peptides using the Mascot database search engine and manual quantification of changes in abundance across time points. However, other options will work equally well (*see* **Note 25**).
6. Mascot is accessed from www.matrixscience.com. Parameter settings may vary depending on the instrument and other factors. The standard parameter settings that we use for peptide identification with the Mascot MS/MS Ions Search algorithm are presented in Table 1 (*see* **Note 26** for more detailed information on search settings).
7. Once peptides have been identified, the extracted ion current (XIC) for all phosphopeptides and for several non-phosphorylated "standard" peptides (minimum 4) must be obtained. An XIC simply represents the HPLC trace for a specific mass/charge value during the LC-MS experiment (*see* Fig. 1). The software from most instrument vendors includes functions for manually generating XICs for ions of interest. Alternatively, some freely available software packages, including MaxQuant (www.maxquant.org), will generate XICs automatically for all identified peptides (*see* **Note 27**). The procedure we describe is suitable for analysis of singly phosphorylated peptides. Analysis of peptides with multiple phosphorylation sites is more complicated.
8. The raw phosphopeptide XIC signals for each time point must be corrected using the non-phosphorylated standard peptides to account for sample-to-sample signal variation. We have used different approaches for this, but the simplest to implement is the following (*see* Fig. 2): (1) Normalize the XIC signals for all non-phosphorylated standard peptides and phosphopeptides, such that time = 0 is set to 1 and all other time points represent a fraction of the time = 0 signal. (2) Average these normalized values from the standard peptides to obtain a set of correction

Table 1
Mascot MS/MS ions search settings

Parameter	Setting
Database(s)	SwissProt
Enzyme	Trypsin
Allow up to # missed cleavages	2
Taxonomy	Select appropriate species
Fixed modifications	None, unless optional alkylation has been performed
Variable modifications	Oxidation (M), Phospho (ST), and/or Phospho (Y)
Peptide tolerance (ppm)	±10
MS/MS tolerance (Da)	±0.25
Peptide charge	+2, +3, and +4
Mass value	Monoisotopic
Data file	Locate and upload raw data file
Data format	Mascot accepts MGF, DTA, ASC, PKL, PKS, Sciex API III, XML, and mzML file formats
Instrument	Select type of instrument used

All parameters not listed can be left at their default settings

factors, one for each time point (the value for time = 0 will be 1). (3) Divide the normalized phosphopeptide values by the appropriate correction factor to obtain adjusted fractional phosphopeptide signals. With a robust instrument and carefully prepared samples, the corrections should be minimal (*see* **Note 28**).

9. Finally, generate dephosphorylation plots of the adjusted phosphopeptide signals. Time = 0 will be 1 and the other time points will represent the fraction of phosphopeptide remaining (*see* Fig. 2). The plots can generally be fit with an exponential decay equation to quantitatively compare rates of dephosphorylation.

4 Notes

1. Custom phosphopeptides are readily obtained commercially. Phosphopeptide length as well as the position of the phosphorylation site can vary according to need or preference. For Cdc14, we typically select a sequence of ~16 amino acids centered on the phosphorylated residue, or a sequence of ten amino acids with the phosphorylated residue at position 3. Both versions seem to yield similar results. Examples of phosphopeptide substrates used extensively for characterization

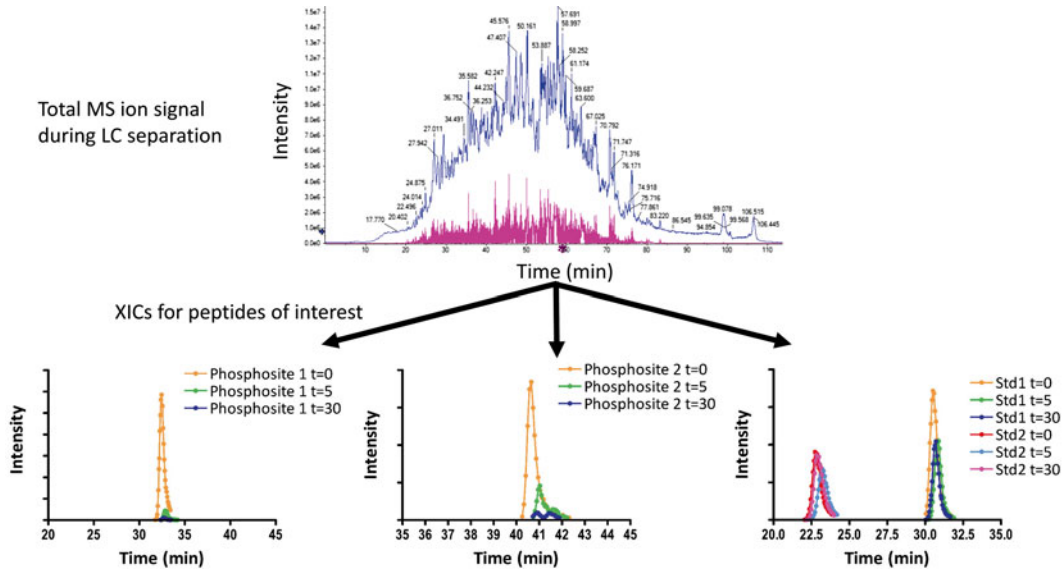

Fig. 1 Extraction of MS ion signals from LC-MS data. Raw LC-MS files are a composite of a broad range of measured mass/charge signals over the time course of an HPLC separation (*top plot, blue trace*). From this raw data, the mass/charge signal for specific peptides of interest, in this case two phosphopeptides (phosphosites 1 and 2) and two unphosphorylated standard peptides (Std1 and Std2), must be extracted (*bottom three plots*). Such plots are termed extracted ion currents, or XICs, and are obtained using software tools typically supplied with MS instruments. They require only the measured mass/charge value of the peptides of interest, obtained from the database search used to identify the peptides present

of budding yeast Cdc14 are VKGNELR**S**PSKRRSQI and MI**S**PSKKRTIL derived from the Acm1 protein [14]. Peptides are synthesized with an amide group at the C-terminus.

2. Other formulations of the malachite green/ammonium molybdate dye are commercially available as well. Vendor-specific instructions for use of other reagents should be followed. In addition, ammonium molybdate and malachite green reagents can be made and used as described [20].
3. The kinase buffer is optimized for budding yeast cyclin-dependent kinase 1 (Cdc28-Clb2 complex). The kinase buffer may need to be altered if other purified kinases are being used to create phosphoprotein substrates.
4. In our experience, unused trypsin solution can be stored in aliquots at −80 °C with minimal loss of activity. Alternatively, for long-term storage, resuspend trypsin at 100 ng/μl in 1 mM HCl and store in aliquots at −20 °C. Adjust pH and concentration with fresh 100 mM ABC prior to use.
5. Budding yeast Cdk1 can be purified as described [21]. Alternative protocols for Cdk1 purification from budding yeast or other species should be suitable but have not been evaluated. Cdc14 can be purified as described in Subheading 2.1, **item 11**, for the peptide assay. Substrates, either recombinant

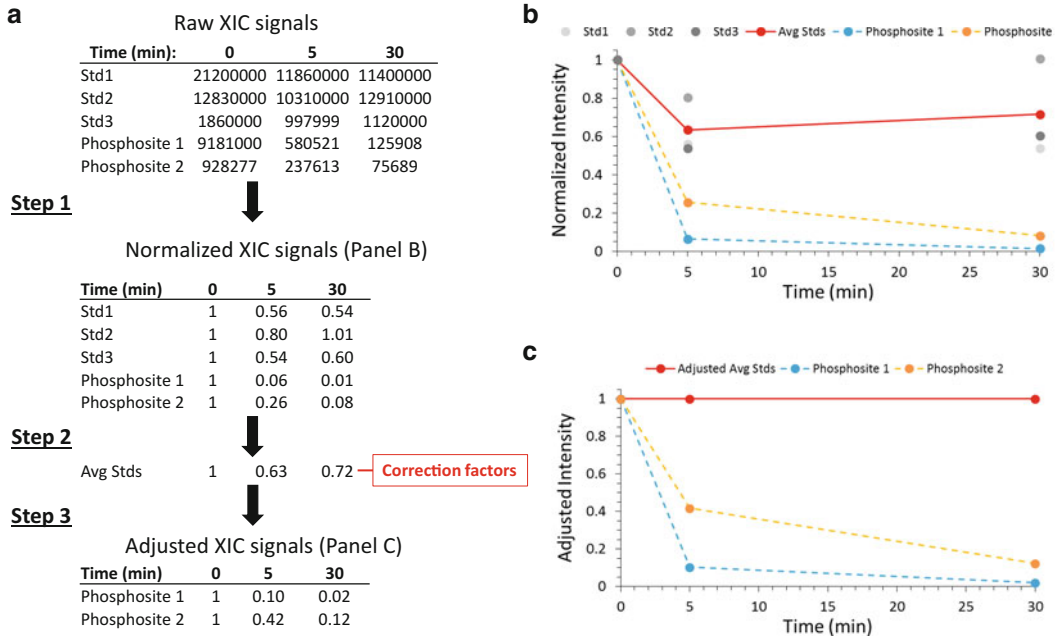

Fig. 2 Generation of dephosphorylation plots and normalization of XIC signals. Integration of XIC peaks shown in Fig. 1 provides quantitative values for plotting as a function of time during the phosphatase assay (*top table in panel* a) but these values must be adjusted first to correct for variation in sample processing, instrument performance, and other factors. In step 1 the raw XIC values for each peptide have been normalized by dividing each by the value at time = 0. This makes the first time point equal to 1 and the other time points a fraction relative to the first time point. A plot of these normalized values is shown in *panel* b. Notice that the signal for the standards varies between time points. In step 2 the normalized XIC values for just the standard peptides are averaged (*red line in panel* b) to create a set of correction factors, one for each time point. In step 3 the normalized phosphopeptide XIC signals are divided by the correction factor from the corresponding time point to generate the final adjusted XIC values that are plotted in *panel* c. Notice that the effect of this procedure is to correct the average standard peptide signals to 1, satisfying the assumption that the average abundance of these unphosphorylated standard peptides should be constant over time (*red line in panel* c). With additional data points, the adjusted phosphopeptide plots can be fit with an exponential decay equation to calculate rates of dephosphorylation

or from the native source, should be affinity purified and retained/stored on the affinity matrix until use. We commonly use a glutathione S-transferase fusion for affinity purification of substrate proteins expressed in *E. coli*. For recombinant substrates, affinity resin should be washed and resuspended in kinase buffer. For native substrates that are already phosphorylated, proteins can be left on the matrix or eluted into phosphatase reaction buffer or other suitable storage buffer.

6. If phosphopeptides are synthesized commercially, many companies also provide purification for an additional cost, eliminating the need for **steps 1–9**, Subheading 3.2.1. To achieve higher purity, conventional reverse-phase HPLC with a C18 column and ACN gradient elution can be employed as an alternative to the procedure described here.

7. Peptide solubility varies greatly. If solubility is a problem, lower concentrations or different solution conditions may be needed for the resuspension step.
8. We use 1 g Sep-Pak® 6 cc Vac cartridges (Waters catalog #186004621) for crude phosphopeptides up to 50 mg (a typical prep is 10 mg of crude phosphopeptide). A variety of Sep-Pak® C18 sizes are available to accommodate different peptide amounts.
9. Sep-Pak® columns can be regenerated and stored for repeated use by washing 3× with 3 ml methanol and then air-drying the column.
10. If MALDI-TOF or other MS analysis is not readily available, peptides should be purchased pre-purified. This routine MS analysis can often be provided by a core facility.
11. For measurements in semi-micro plastic cuvettes use 500–1000 µl. For 96-well plates use 100–200 µl.
12. Malachite green dye has a broad absorbance peak; thus any wavelength from 600 to 680 nm can be used.
13. The assay volume can be scaled down for use in high-throughput analyses in 384-well plates or scaled up for use with individual plastic cuvettes and a conventional spectrophotometer. Endpoint assays at a single substrate concentration below the K_M are an effective way to compare catalytic efficiency of many different substrates. Alternatively, k_{cat} and K_M values can be determined by measuring the reaction rate across a range of substrate concentrations.
14. We use a final concentration of 50–100 nM Cdc14 when optimal substrates are being assayed. Higher concentrations are needed for less efficient substrates. For other phosphatases, optimal enzyme concentrations as well as reaction times and temperatures may differ from those employed here. The amount of substrate consumed in reactions must be less than 10 % of the initial value.
15. This procedure is optimized for cyclin-dependent kinase 1 (Cdk1) and Cdc14 and may require modification to accommodate other protein kinases and phosphatases.
16. It is useful to obtain an estimate of the amount of substrate protein isolated on the affinity matrix. The most practical approach is to elute the substrate from a small, defined fraction of the matrix and perform a Bradford or BCA protein assay using commercially available reagents.
17. The goal is to achieve stoichiometric modification of substrate phosphorylation sites, and therefore the kinase concentration should be high and the incubation time may need to be adjusted. The stoichiometry of phosphorylation can usually be

estimated from the LC-MS data simply by comparing the signal intensity of phosphorylated and unphosphorylated peptide species.

18. For antibody-based affinity captures, it may be advantageous to elute substrates from the resin (e.g., by competition with antigenic peptide) prior to the phosphatase reaction, particularly if the substrate protein might co-migrate on SDS-PAGE with one of the antibody chains. The reducing agent required in the phosphatase reaction buffer may cause dissociation of antibody disulfide bonds leading to high levels of free antibody chains on SDS-PAGE in the subsequent steps.
19. We use 100 nM Cdc14 for substrates containing phosphosites efficiently targeted by Cdc14. The concentration of Cdc14 may need to be increased or decreased in accordance with the protein's efficacy as a substrate.
20. The appropriate incubation time will vary depending on the substrate, the substrate concentration, and the enzyme concentration and must be optimized.
21. Use gloves and a clean, dust-free environment when handling and processing gels to minimize keratin contamination. Use dedicated gel plates and staining trays cleaned only with isopropanol, and avoid handling them without gloves. Additional information on in-gel digest procedures, including optional cysteine reduction and alkylation steps, can be found here [22].
22. Peptide concentration can be difficult to measure accurately. To avoid consuming part of the sample we estimate the amount of protein from the Coomassie blue-stained gel band and assume 100 % protein digestion and 100 % peptide recovery from the in-gel digest.
23. We typically use a 60-min gradient, but shorter times may be sufficient for analysis of peptides derived from single proteins. In cases where a larger number of protein substrates are evaluated simultaneously, gradient length can be increased to enhance the number of peptides detected.
24. Specific method parameters will vary depending on the instrument. This includes flow rate, number and duration of MS/MS acquisitions per cycle, fragmentation settings, electrospray voltages, and dynamic exclusion window.
25. Use of Mascot with large LC-MS data files requires a license. Any database search program capable of identifying peptides and modified peptides should be suitable and several commonly used and freely accessible options exist. More detailed descriptions of the procedure for manual quantitation of phosphopeptide signals have been published elsewhere [14, 18, 21].

26. Notes on Mascot search parameter settings: Database—any database that contains the sequence of the protein(s) being analyzed will work. Enzyme—we have described the procedure for preparing samples by digestion with trypsin, but other site-specific endoproteinases can be used as well, particularly if specific phosphorylation sites of interest are not detected using trypsin. It is necessary to select the enzyme that was used to prepare the peptide samples. Fixed modifications—if the optional alkylation of cysteines has been performed with iodoacetamide during the in-gel digestion then "carbamidomethyl (C)" must be selected as a fixed modification. Variable modifications—oxidation (M) is not essential to select. However, in our experience, peptides containing methionine are almost always detected in both reduced and oxidized states. Peptide and MS/MS tolerance—these settings depend on both the type of mass analyzer and the quality of the calibration. The indicated values are our standard settings for a quadrupole-TOF instrument. Other analyzers may require very different tolerance settings. For example, Orbitraps may accommodate a more stringent peptide tolerance whereas conventional ion traps will require much less stringent peptide and MS/MS tolerances. The mass accuracy of an instrument can easily be determined using a collection of peptide standards. Peptide charge—this setting is overridden when the charge state of precursor peptides is provided in the LC-MS data file, as it almost always will be. Therefore this setting can usually be ignored. Mass value—this should always be set to monoisotopic unless a low-resolution analyzer is used that is not able to distinguish individual peptide isotope peaks. Data format—for instruments that generate raw data files in a proprietary format not supported by Mascot, conversion to one of these supported formats is required. Software is generally provided by instrument vendors for file conversion to nonproprietary formats and freely available tools exist as well.
27. Generation of an XIC for a peptide requires only its measured mass/charge value. Because multiple ions could have similar mass/charge values (this will become more frequent with increasing sample complexity), it is important that the XIC peaks used for quantitative analysis be matched to an MS/MS spectrum that identifies the peptide. An MS/MS spectrum identifying the peptide from at least one of the samples defines the peptide's LC retention time. The integrated XIC peak should match this retention time. MaxQuant will perform both the peptide identification step using the Andromeda search algorithm and the generation of integrated XIC values for all identified peptides. Information on use of MaxQuant has been published [23, 24] and is available from the MaxQuant website, www.maxquant.org.

28. The more non-phosphorylated peptides used to determine correction factors for each time point the better. We use a minimum of four. Try to select peptides that give a strong MS signal, do not have missed protease cleavage sites, and lack methionine and cysteine. Discard any peptide whose profile deviates dramatically from the average peptide profile.

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