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A versatile spectrophotometric protein tyrosine phosphatase assay based on 3-nitrophosphotyrosine containing substrates

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ABSTRACT

A versatile assay for protein tyrosine phosphatases (PTP) employing 3-nitrophosphotyrosine containing peptidic substrates is described. These therapeutically important phosphatases feature in signal transduction pathways. The assay involves spectrophotometric detection of 3-nitrotyrosine production from 3-nitrophosphotyrosine containing peptidic substrates, which are accepted by many PTPs. Compared to conventional chromogenic phosphate derivatives, the more realistic peptidic substrates allow evaluating substrate specificity. The assay's applicability is demonstrated by determining kinetic parameters for several PTP-substrate combinations and inhibitor evaluation, as well as detection of PTP activity in lysates. The convenient new assay may assist further adoption of PTPs in drug development.

Phosphatases and kinases together regulate the vital phosphorylation posttranslational modification, involved in many cellular processes. Aberrant phosphorylation levels are implicated in diseases such as cancer and diabetes [1–4]. Both enzyme families are actively pursued in drug development [5–11]. However, whereas for kinases well-established assays are available to gauge both affinity and selectivity of potential interfering compounds, in the phosphatase field an urgent need for versatile assays still exists [10,11]. The most commonly used phosphatase assay involves simple chromogenic and fluorogenic phosphate esters, e.g., *para*-nitrophenyl phosphate. However for *protein* phosphatases, these are poor mimics of the natural phosphopeptide substrates. This may impact biological conclusions and definitely prohibits substrate specificity evaluation. Since phosphatases are generally promiscuous, the ability to evaluate multiple substrates is very attractive, for instance, to investigate selective inhibition.

Alternative approaches involve measuring inorganic phosphate (P_i) production, e.g., using malachite green [12,13]. However, these assays only allow endpoint readout and determining kinetic time courses is tedious. One alternative assay which alleviates this was described by Webb [14] and is commercially available. It involves a coupled assay where P_i produced is used as a substrate by a different enzyme leading to conversion of a chromogenic substance. Although phosphate production can be measured in real time, the complex biochemistry may make data interpretation more difficult. Furthermore, all P_i assays are sensitive to the presence of bulk phosphate, e.g., in the buffer or in biological matrices such as cell lysates.

Here we describe a convenient, versatile spectrophotometric assay which allows determination of protein tyrosine phosphatase (PTP)[1] activity in real time using peptidic substrates. Its 96-well

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[1] *Abbreviations used:* PTP, protein tyrosine phosphatase; k_{cat}, turnover number for enzymatic reactions; v_{ini}, initial velocity at the linear phase of enzyme reactions; IC_{50}, inhibitor concentration required to reduce enzyme activity by 50%; HEK, human embryonic kidney; WT, wild type; DEP1, density enhanced phosphatase 1 (receptor PTP type J); GLEPP1, global epithelial protein 1 (receptor PTP type O); LAR, leukocyte antigen related tyrosine phosphatase (receptor PTP type F); SHP2, Src homology 2 domain containing PTP 2 (nonreceptor PTP type 11); DTT, dithiothreitol; abbreviations for substrates (CADH2, CSK, EGFR, INSR, LCK, MBP, PAXI, PDPK1, cRET, SIGLEC2, STAT1, STAT3, and ZAP70) are listed in the supporting information.

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format allows evaluation of, e.g., substrate selectivity and kinetic parameters for PTP–substrate interactions, as well as inhibitor activity. Furthermore, it is compatible with complex biological matrices such as cell lysates.

The assay is based on our recent discovery that all PTPs investigated so far readily accept substrates incorporating a newly developed 3-nitrophosphotyrosine building block [15]. These substrates are converted by PTPs into the corresponding 3-nitrotyrosine peptides which have an absorption spectrum similar to commonly used *para*-nitrophenol and can be detected spectrophotometrically (Fig. 1). The required 3-nitrophosphotyrosine building block, as well as substrate peptides incorporating it, are conveniently accessible. Furthermore, the substrates can be stored for prolonged periods (at least a year) at −20 °C and are stable under assay conditions. We previously described an antibody-based microarray assay employing 3-nitrophosphotyrosine substrates and demonstrated that results obtained are similar to those of the corresponding phosphotyrosine peptides [15]. Our observation that substituents are tolerated at the 3-position is supported by a different reported approach where 3-fluoromethylphosphotyrosine containing substrates were recognized by PTPs [16].

Interestingly, a recent paper [17] described an alternative detection methodology for 3-nitrotyrosine residues by chemical reduction of the nitro group, reaction with salicylaldehyde and complexation of aluminium. The resulting complex is fluorescent. Although the ability to monitor PTP activity in real time would be lost, the fluorescent readout should enhance sensitivity at very low enzyme/substrate concentrations compared to absorption-based detection.

The PTP enzyme family includes PTP1B, which has received considerable drug development attention for treatment of diabetes type 2 [18], and the versatile assay presented here may be valuable for determining the selectivity of potential inhibitors. Furthermore, it may enable further investigation of other PTPs, for example, cancer targets GLEPP1 [19] and PTPγ [20].

Materials and methods

General

All reagents were used as supplied. All phosphatase experiments were carried out in a phosphate buffer, hereafter named PTP-buffer: 25 mM phosphate, pH 7.4, modified with 50 mM NaCl, 5 mM EDTA, and 1 mM DTT. All reagents for preparation of this buffer were purchased from Sigma Aldrich (St. Louis, MO, USA). All experiments were carried out in a roundbottom 96-well plate (Corning Life Sciences, Corning NY, USA). EnzChek P_i assay kit (Life Technologies, Paisley, UK) was used according to the supplier's protocols. PTPs were expressed and isolated as described earlier [15]. HEK293 cell line lysates were prepared as described earlier [15]. Both protocols are included in the Supporting information. Substrate peptides containing the 3-nitrophosphotyrosine residue were synthesized and purified to homogeneity as described earlier [15]. Spectrophotometric determination was carried out on a μQuant plate reader (Bio-Tek, Winooski VT, USA). Synthetic procedures, HPLC chromatograms, and MS spectra for all substrate peptides and curves for all PTP activity experiments and the NSC87877 inhibition experiment are included in the Supporting information.

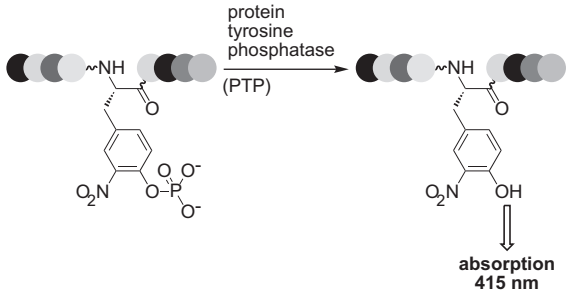

Fig.1. General principle of the spectrophotometric PTP assay using the 3-nitrophosphotyrosine building block.

PTP activity

As reference, serial dilutions of 3-nitrotyrosine in PTP-buffer were used (100 μL per well, starting from a 1–2 mM stock solution, the highest concentration was equal to the stock, followed by a 2-fold dilution with PTP-buffer, etc.). To each sample well were added serial dilutions of the substrate of interest in water (80 μL, starting from a 1–1.5 mM stock solution, highest final assay substrate concentration was equal to 0.8 times the stock concentration, followed by a 2-fold dilution with PTP-buffer, etc.), a stock solution of the enzyme of interest (10 μL, typical final assay enzyme concentration 10^{-1}–10^{-2} μM), and 10× strength PTP-buffer (10 μL). The plate was immediately placed in a spectrophotometer and absorption readings at 415 nm were acquired every minute for 45 min. The resulting progress curves were plotted and the slope at the initial linear phase of the reaction calculated (v_{ini}). Through nonlinear regression of the Michaelis-Menten equation on a plot of the resulting v_{ini} values against substrate concentration, K_m and k_{cat} were determined. Enzyme stock concentrations were measured using the Pierce micro BCA assay (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocols.

Vanadate inhibition

As reference, serial dilutions of 3-nitrotyrosine in PTP-buffer were used (80 μL per well, starting from a 1.8 mM stock solution, the highest concentration was equal to the stock, followed by a 2-fold dilution with PTP-buffer, etc.). To each sample well were added a 0.76 mM stock solution of the ZAP70 substrate peptide in PTP-buffer (40 μL, leading to a final assay substrate concentration of 0.38 mM), a stock solution of PTPγ (20 μL, stock concentration 3.2 μM, leading to a final assay concentration of 0.4 μM), and serial dilutions of sodium *ortho*-vanadate (Sigma Aldrich, St. Louis MO, USA) in PTP-buffer (20 μL, starting from a 6.5 mM stock solution, the highest final assay inhibitor concentration was equal to 0.25 times the stock concentration, followed by a 4-fold dilution with PTP-buffer, etc.). The plate was held at room temperature for 10 min after which the absorption at 415 nm was determined spectrophotometrically. From the absorption data product concentrations were determined and nonlinear regression on a plot of product concentration against log [inhibitor] was used to calculate the IC_{50}.

NSC87877 inhibition

As reference, serial dilutions of 3-nitrotyrosine in PTP-buffer were used (100 μL per well, starting from a 1.1 mM stock solution, the highest concentration was equal to the stock, followed by a 2-fold dilution with PTP-buffer, etc.). To each sample well were added a 1.1 mM stock solution of the STAT3 substrate peptide in PTP-buffer (80 μL leading to a final assay substrate concentration of 0.88 mM), serial dilutions of a 2.0 mM stock solution of NSC87877 [21] (Millipore, Billerica MA, USA) in PTP-buffer (10 μL, the highest final assay inhibitor concentration was equal to 0.1 times the stock concentration, followed by a 10-fold dilution with PTP-buffer, etc.) and a 0.75 μM stock solution of PTP1B in PTP-buffer (10 μL, leading to a final assay concentration of 0.075 μM).

The plate was immediately placed in a spectrophotometer and absorption readings at 415 nm acquired every minute for 45 min. The resulting progress curves were plotted and the slope at the initial linear phase of the reaction calculated (v_{ini}). From a plot of v_{ini} against log [inhibitor], the IC_{50} was determined by nonlinear regression.

PTP activity in HEK293 cell lysates

As reference, serial dilutions of 3-nitrotyrosine in PTP-buffer were used (60 μL per well, starting from a 2.5 mM stock solution, the highest concentration was equal to the stock, followed by a 2-fold dilution with PTP-buffer, etc.). To each sample well were added a 1.5 mM stock solution of the INSR substrate peptide in PTP-buffer (50 μL, leading to a final assay concentration of 1.25 mM) and the lysate of interest (10 μL corresponding to 10 μg total protein). The plate was immediately placed in a spectrophotometer and absorption readings at 415 nm were acquired every minute for 45 min. The resulting progress curves were plotted and the slope at the initial linear phase of the reaction calculated (v_{ini}). The total protein content of all lysates was determined using the Pierce micro BCA assay (Thermo Scientific, Rockford IL, USA) according to the manufacturer's protocols.

Results and discussion

Assay setup

First, the absorption maximum was determined by measuring spectra of serial dilutions of 3-nitrotyrosine in PTP-buffer in a 96-well plate. As Fig. 2A shows, an absorption maximum at 415 nm was observed which is the same as 4-nitrophenol. Additionally, absorption spectra were measured for nonphosphorylated 3-nitrotyrosine peptide STAT3, which also had an absorption maximum at 415 nm (Supporting information). The molar absorptivity (ε) of both compounds was highly similar (approximately $3.5 \times 10^3\ M^{-1}\ cm^{-1}$). The slightly lower value for the STAT3 sample may be explained by the presence of water in the peptide which would lead to a lower actual concentration and therefore higher molar absorptivity. The absorption was dependent on concentration and on evaluation a linear relationship between absorption at 415 nm and concentration was observed across at least 3 orders of magnitude.

PTP activity and profiling

Several therapeutically relevant combinations of 3-nitrophosphotyrosine containing substrate peptides and PTPs were evaluated in the new spectrophotometric assay (details on all substrates are included in the Supporting information). In all cases experiments were carried out in triplicate and serial dilutions (7 different concentrations) of a stock substrate solution were used to obtain curves of substrate concentration against v_{ini} (see, for example, Fig. 2B). From the resulting data the relevant kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were calculated by nonlinear regression (Table 1). For these enzyme–substrate combinations K_m values were in the 10^1–10^2 μM range and k_{cat} values were in the 10^0–$10^1\ s^{-1}$ range. Values for substrate specificity parameter k_{cat}/K_m were in the 10^{-3}–$10^{-1}\ \mu M^{-1}\ s^{-1}$ range. Although reported values for kinetic parameters of PTPs remain scarce, the data conform with typically observed ranges, i.e., 10^1–10^3 μM for K_m of PTPs with peptidic substrates, 10^0–$10^1\ s^{-1}$ both for k_{cat} of PTPs with *para*-nitrophenyl phosphate and for SHP2 with peptidic substrates, and 10^{-4}–$10^{-2}\ \mu M^{-1}\ s^{-1}$ for k_{cat}/K_m of SHP2 with peptidic substrates [22–25].

These experiments also serve as a demonstration of the power of the 96-well setup employed here. With one plate 5 different substrates and/or enzymes could be kinetically characterized in triplicate, with negative controls (PTP-buffer and enzyme without substrate) and a concentration series of 3-nitrotyrosine in PTP-buffer for calibration.

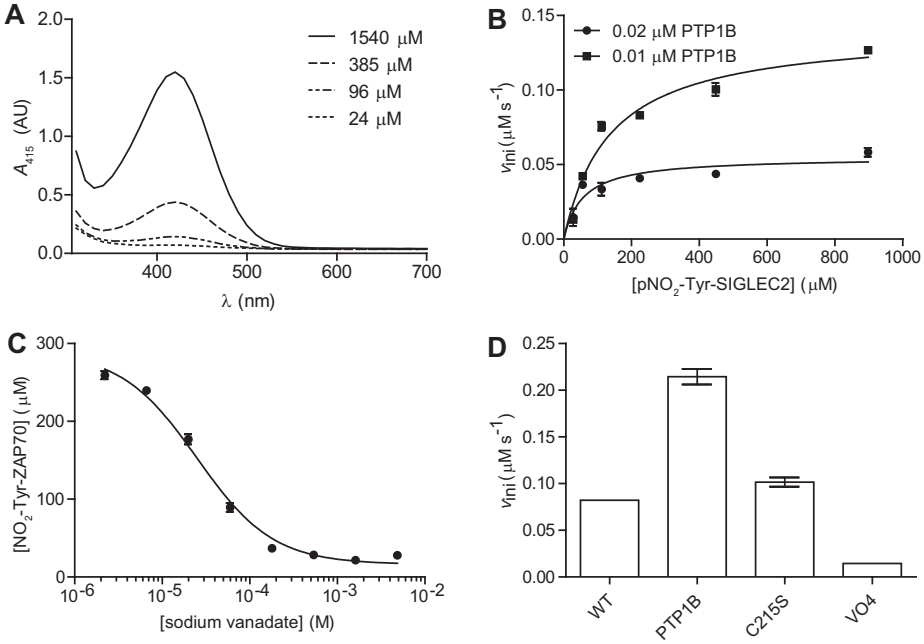

Fig.2. Sample data for the spectrophotometric PTP assay. All data points are averages of three experiments, errors expressed as SE. (A) Absorption spectra at different concentrations of 3-nitrotyrosine showing the maximum at 415 nm. (B) Initial velocities (v_{ini}) at different substrate concentrations for PTP1B with a 3-nitrophosphotyrosine peptide substrate derived from SIGLEC2. (C) Inhibition of PTPγ by different concentrations of sodium *ortho*-vanadate using a 3-nitrophosphotyrosine peptide substrate derived from ZAP70. (D) Initial velocities (v_{ini}) for HEK293 lysates (WT, nontransfected; PTP1B, transfected with PTP1B; C215S, transfected with inactive PTP1B; VO4, nontransfected in the presence of 250 μM vanadate) using a 3-nitrophosphotyrosine peptide substrate derived from INSR.

Table 1
Kinetic parameters for various PTP–substrate combinations.

Phosphatase	Substrate	K_m (mM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)[a]
PTPγ	ZAP70	0.34 ± 0.03	5.3 ± 0.3	0.015 ± 0.001
		(0.20 ± 0.01)	(11 ± 1)	(0.052 ± 0.007)
		[0.126 ± 0.08]	[6.8 ± 0.3]	[0.058 ± 0.009]
DEP1	INSR	0.27 ± 0.04	6.8 ± 0.4	0.026 ± 0.004
GLEPP1	STAT3	0.14 ± 0.03	2.1 ± 0.3	0.15 ± 0.02
		(0.21 ± 0.03)	(1.1 ± 0.1)	(0.54 ± 0.08)
		[0.13 ± 0.02]	[0.92 ± 0.07]	[0.7 ± 0.1]
PTPκ	LCK	0.11 ± 0.02	8.9 ± 0.7	0.08 ± 0.01
		(0.17 ± 0.07)	(14 ± 1)	(0.09 ± 0.01)
		[0.35 ± 0.09]	[20 ± 3]	[0.051 ± 0.008]
LAR	CSK	0.33 ± 0.06	1.7 ± 0.2	0.0050 ± 0.0009
PTP1B	STAT3	0.82 ± 0.09	20 ± 2	0.024 ± 0.002
GLEPP1	PDPK1	0.16 ± 0.04	32 ± 3	0.20 ± 0.05
PTP1B	SIGLEC2	0.12 ± 0.02	4.3 ± 0.3	0.037 ± 0.006

[a] Numbers between parentheses are measured by the Enzchek phosphate assay for the corresponding phosphotyrosine containing substrate peptide [15]. Numbers between square brackets were measured by the Enzchek phosphate assay for the 3-nitrophosphotyrosine peptide [15]. All data obtained with the new assay are triplicates and errors are represented as SE.

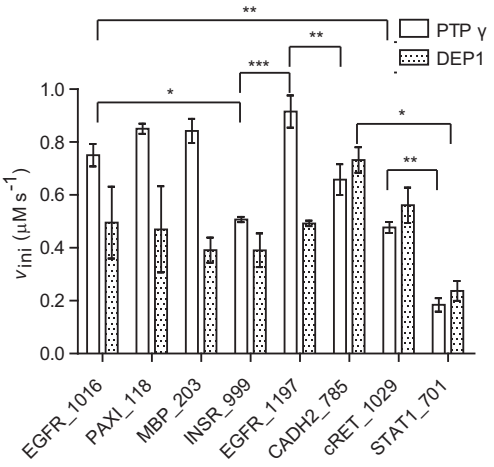

Fig.3. Profiling of PTPγ and DEP1 using eight 3-nitrophosphotyrosine peptide substrates. All data points are averages of three experiments, with error bars denoting SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

It should be noted that in order to generate suitable curves for Michaelis-Menten analysis a relatively high stock substrate concentration (1–2 mM) was applied, leading to appreciable consumption of precious 3-nitrophosphotyrosine peptide substrate. However, from the data generated in these experiments it is clear that substrate concentrations down to approximately 20–50 μM are sufficient to generate useful progress curves. In order to determine the enzyme demands of the assay, time courses were acquired for serial dilutions of the GLEPP1 enzyme with a fixed concentration of the PDPK1 substrate peptide (graphs included in the Supporting information). At an enzyme concentration of 0.042 μM a significant signal increase in time could still be determined. Similar concentrations were used successfully in several of the experiments presented here. Assay sensitivity may be increased by applying commercially available narrow-well microtiter plates which require smaller volumes for similar path lengths and therefore absorption ranges. Furthermore, an alternative fluorescence-based detection method exists [17] as noted earlier. This is potentially more sensitive, and therefore lowers substrate and enzyme requirements.

For comparison, several substrate–enzyme combinations studied here have been evaluated using the established, commercial Enzchek P_i assay as well [15]. Both 3-nitrophosphotyrosine substrates and the corresponding phosphotyrosine substrates were used in this reference assay. Similar results were obtained as for the spectrophotometric assay described here, and no differences were observed between 3-nitrophosphotyrosine substrates and the corresponding phosphotyrosine substrates (Table 1). This is particularly significant for the k_{cat}/K_m parameter which is an indicator for substrate specificity.

In addition, substrate specificity profiles were generated for two PTPs, the relatively promiscuous DEP1 and the more selective PTPγ [26]. These PTPs are promising cancer targets [20,27]. The resulting profiles are shown in Fig. 3. As expected, the relatively aspecific PTP DEP1 displayed similar activity against most substrates whereas PTPγ gave significant differences in dephosphorylation activity for different substrates. Such profiles are essential in the development of selective compounds interfering with PTPs. The most commonly used assays of PTP activity are based on simple chromogenic phosphate esters such as *para*-nitrophenyl phosphate, which obviously prohibit determining substrate preferences of PTPs, apart from being structurally different from the natural peptidic PTP substrates. In contrast, our new assay conveniently allows substrate specificity evaluation. In addition, since 3-nitrophosphotyrosine peptides can be conveniently prepared using established synthetic protocols, more substrates may be evaluated, leading to increasingly discriminating substrate specificity profiles.

Inhibition by vanadate and NSC87877

Two phosphatase inhibitors were studied since it is expected that PTP inhibitor evaluation will be a major application of the spectrophotometric assay in a drug development setting. First, sodium *ortho*-vanadate, a well-known nonselective inhibitor of phosphatases, was applied. As summarized in Fig. 2C, the expected sigmoidal curve was obtained on adding serial dilutions of vanadate to a fixed concentration of 3-nitrophosphotyrosine substrate ZAP70 with PTPγ. From this curve, an IC_{50} of 25 ± 3 μM was determined. This IC_{50} is reasonable for experiments carried out in a buffer containing DTT [28]. In an analogous experiment an IC_{50} of 70 ± 9 μM was determined for known inhibitor NSC87877 [21] with PTP1B and a 3-nitrophosphotyrosine peptide derived from STAT3. Since NSC87877 absorbs at 415 nm as well, a blank (inhibitor dissolved in PTP-buffer) was measured for each inhibitor concentration and subtracted from all corresponding sample absorptions prior to calculation of product concentrations used in the time courses. Absorption changes after subtraction were still sufficiently significant for determining reliable time courses. An IC_{50} of 1.7 ± 0.4 μM was reported for NSC87877 with PTP1B [21], which is an order of magnitude lower than the value obtained in our spectrophotometric assay. However, since in the original study a different, simple phosphate ester substrate was used at a much lower substrate concentration (20 μM vs 880 μM used here), the IC_{50} determined here is compatible with previous data.

PTP activity in HEK293 lysates

An important advantage of our assay is its insensitivity to background phosphate. All experiments described here were performed in a phosphate buffer, which we have previously found is a preferred choice for PTP activity measurements [15]. This is also important for experiments involving complex biological matrices such as cell line lysates, where P_i will be present irrespective of PTP activity. As a demonstration, PTP activity was evaluated with our spectrophotometric assay in a number of HEK293 cell line lysates (Fig. 2D). The insulin receptor (INSR) peptide was used as a substrate for all experiments. First, a lysate obtained from WT

HEK293 cells was evaluated and indeed PTP-catalyzed hydrolysis of the INSR substrate was observed (Fig. 2D, marked WT). Then, the protein phosphatase activity was investigated in lysates of HEK293 cells transiently transfected with either active PTP1B or inactive PTP1B mutant C215S [29,30]. The lysate overexpressing active protein phosphatase PTP1B showed an increase in dephosphorylating activity of the INSR substrate (Fig. 2D, marked PTP1B. This was expected since it is a known PTP1B substrate [31]. Conversely, in the lysate of HEK293 cells overexpressing the inactive C215S mutant of PTP1B dephosphorylation of the INSR substrate was again reduced to the level observed for WT HEK293 cells (Fig. 2D, marked C215S). Finally, the lysate prepared from WT HEK293 cells was spiked with 100 μM of nonselective phosphatase inhibitor sodium *ortho*-vanadate, resulting in significantly decreased dephosphorylation of the INSR substrate (Fig. 2D, marked VO4). The residual phosphatase activity observed was expected given the IC_{50} of 25 μM determined with a recombinant phosphatase as described above.

Although in the present experiments only one substrate peptide was studied, the 96-well format allows evaluating PTP activity in lysates on multiple substrates. Given the diabetes drug development interest in protein phosphatase PTP1B [18] as well as its promiscuity [26], pinpointing the substrate specificity of PTP1B in realistic biological settings may be of considerable value.

In summary, it is expected that the versatile spectrophotometric assay described here is a valuable addition to the currently available techniques, because of its real-time nature, more realistic substrates, and insensitivity to background phosphate. In particular, the new assay may assist in further adoption of PTPs as drug development targets.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.11.023.

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