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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.

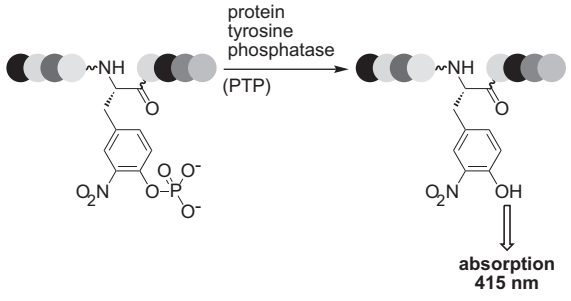


**Fig.1.** General principle of the spectrophotometric PTP assay using the 3-nitrophosphotyrosine building block.

### 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).