# Rapid, Sensitive Detection of Protein Phosphatase Activity

## Measure real-time signal differences in the picomole range with the Phosphate Sensor method

*Described in this application is a method utilizing the Life Technologies Phosphate Sensor, a simple tool to interrogate the activity of phosphate-releasing enzymes. The assays described detect the increase of fluorescence intensity when free inorganic phosphate binds to a bacterially derived phosphate-binding protein modified with a fluorophore. To evaluate the Phosphate Sensor methods, we compared detection to a commonly used malachite green reagent and a protease-coupled fluorescent assay, examined titrations of PTP1B protein tyrosine phosphatase, and analyzed a PTP1B reaction in real-time kinetic mode. Phosphate Sensor is orders of magnitude more sensitive than malachite green, faster and simpler to use than other competitor methods, and uniquely qualified for determining enzymatic rates.*

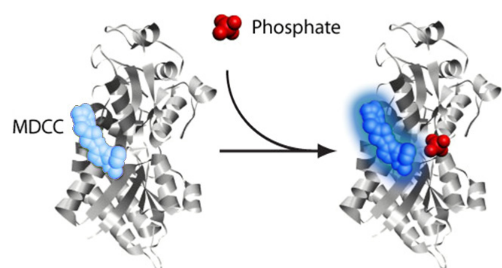


**Figure 1. Phosphate Sensor assay principle.** The protein ribbon diagram illustrates the modified phosphate-binding protein with the MDCC fluorophore (shown in blue). Upon binding inorganic phosphate, fluorescence of Phosphate Sensor increases approximately 6- to 8-fold and can be measured in real time.

Numerous enzymatic reactions release inorganic phosphate ($P_i$). For example, protein phosphatases liberate phosphate from proteins as a way of regulating the activity of their targets, NTPases (ATPase, GTPase) hydrolyze NTP into NDP plus $P_i$ to generate the energy needed to pump ions across a membrane, and helicases use ATP to open double-stranded DNA.

Protein phosphatases play an integral role in the phosphorylation/dephosphorylation regulatory mechanism used by a variety of biological pathways to increase or decrease the output of the pathway. This implicates protein phosphatases in a variety of disorders, such as tumor growth [1, 2], neuronal development and cognition [3, 4] and diabetes [5]. Research about their activity continues to uncover the diverse range of their target substrates, including tyrosines, serines/threonines, lipids, and others. Whenever a potential drug target is discovered, tools to enable high-throughput screening and identification of small molecule modulators are needed.

Here we describe the use and optimization of Phosphate Sensor, a simple, flexible toolbox reagent that allows measurement of protein phosphatase activity. Phosphate Sensor is a phosphate-binding protein modified with a fluorophore [6]. As the sensor binds free $P_i$, fluorescence intensity increases, allowing direct measurement of the released phosphate (Figure 1). Detection is not dependent on a specific substrate or enzyme class, making this detection method amenable to almost any target of interest.



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***Note:*** *Phosphate is common in biological materials, buffers, and reagents, as well as on plastics and glassware. Care should be taken to minimize contamination from these sources when using Phosphate Sensor. Using the phosphate mop (Box 1) in control samples can rule out phosphate contamination problems or help identify sources. Care also should be used when selecting a microplate as some surface-coated plates contain significant amounts of phosphate. We recommend using Corning 384-well uncoated plates (P/N 3677).*

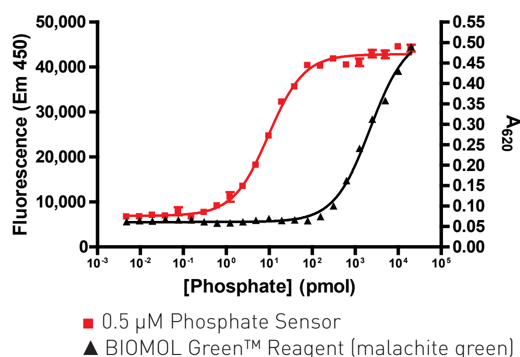



**Figure 2. Comparison of the sensitivity of Phosphate Sensor versus malachite green.** Methods comparing Phosphate Sensor and BIOMOL Green™ (malachite green) reagents were completed using a two-fold serial dilution of a phosphate standard. Compared to the 2,200 pmol $EC_{50}$ value of the malachite green reagent, detection with the Phosphate Sensor method is orders of magnitude more sensitive resulting in a 9.6 pmol $EC_{50}$.

## Experimental Procedures

### *Materials and methods*

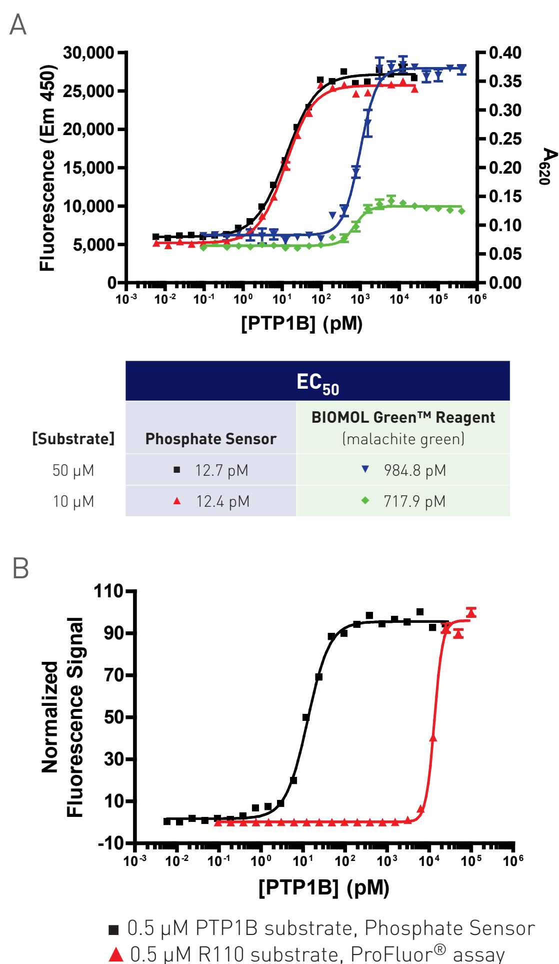
Protein tyrosine phosphatase 1B (PTP1B; Calbiochem P/N 539735), PTP1B substrate (Calbiochem P/N 539737), Phosphate Sensor (Invitrogen P/N PV4406), BIOMOL Green™ Reagent (Enzo® Life Sciences, Inc. P/N BML-AK111-0250), and ProFluor® Tyrosine Phosphatase Assay (Promega Corporation P/N V1280) were used in the reactions. The phosphatase enzymatic reaction buffer consisted of 50 mM Tris pH 7.6, 25 mM NaCl, 0.01% Triton® X-100, and 0.5 mM DTT. The Phosphate Sensor detection buffer consisted of 20 mM Tris pH 7.6 and 0.05% Triton® X-100. All assays were performed in 384-well, low-volume, round-bottom, non-treated plates (Corning P/N 3677), except for the malachite green reagent experiments, which were performed in 96-well, clear plates (Costar P/N 3599).

### *Phosphate standard titration*

The first step of the comparison process was a direct detection of phosphate with Phosphate Sensor and BIOMOL Green™ (malachite green) reagents, using a two-fold (23-point) dilution series of phosphate standard, with final reaction amounts ranging from 20,000 pmol to 0.0048 pmol phosphate. For the Phosphate Sensor method, a 10 µL standard titration series was added to the 384-well plate in nanopure water, followed by addition of 10 µL of 2X (1 µM) Phosphate Sensor in Phosphate Sensor detection buffer. Immediately after mixing, the plate was read on a Tecan® Safire2™ microplate reader at excitation 430 (10) nm and emission 450 (10) nm. For the malachite green comparison, a 50 µL standard titration series was added to the 96-well plate in nanopure water, followed by addition of 100 µL of the BIOMOL Green™ reagent to a final volume of 150 µL. After incubating the plate for 20 minutes, absorbance at 620 nm was read on the Tecan® Safire microplate reader (Figure 2).

| | Phosphate Sensor Method | BIOMOL Green™ Method | ProFluor® Method |
|---|---|---|---|
| *detection* | fluorophore | malachite green | protease-coupled fluorescence |
| *assay format* | 384-well | 96-well | 384-well |
| *final assay volume* | 20 µL | 150 µL | 20 µL |
| *assay time* | 60 minutes | 80 minutes | 90 minutes |
| *inhibitable steps* | 1 (PTP1B reaction) | 1 (PTP1B reaction) | 2 (PTP1B & protease reactions) |
| *steps* | 3 | 3 | 4 |
| *protocol* | 1. Add 5 µL of 2X PTP1B<br>2. Add 5 µL of 2X PTP1B substrate, incubate 60 minutes at RT<br>3. Add 10 µL of 2X Phosphate Sensor, read plate immediately | 1. Add 25 µL of 2X PTP1B<br>2. Add 25 µL of 2X PTP1B substrate, incubate 60 minutes at RT<br>3. Add 100 µL malachite green, incubate 20 minutes at RT, read plate | 1. Add 5 µL of 2X PTP1B<br>2. Add 5 µL of 2X R110 substrate, incubate minutes at RT<br>3. Add 5 µL protease solution, incubate 30 minutes at RT<br>4. Add 5 µL stabilizer reagent, read plate |

**Table 1. Comparison of PTP1B Assays between Phosphate Sensor and competitor methods.** The Phosphate Sensor Assay requires the fewest steps (also see Figure 5) in the shortest total assay time, when compared to malachite green (BIOMOL Green™ Reagent) and protease-coupled fluorescence (ProFluor® Tyrosine Phosphatase Assay) technologies.




| [Substrate] | $EC_{50}$ Phosphate Sensor | $EC_{50}$ BIOMOL Green™ Reagent (malachite green) |
|---|---|---|
| 50 μM | ■ 12.7 pM | ▼ 984.8 pM |
| 10 μM | ▲ 12.4 pM | ◆ 717.9 pM |

**Figure 3. Titrations of PTP1B tyrosine phosphatase.** (A) Phosphate Sensor is more sensitive than the BIOMOL Green™ Reagent (see $EC_{50}$ values in table above). Improved sensitivity of Phosphate Sensor enables the use of significantly less substrate (one-fifth or 20%) for each experiment with no impact to assay window. Assay window is calculated by dividing the maximal signal observed by the minimal signal or no PTP1B wells. (B) Phosphate Sensor, with an $EC_{50}$ value of 12 pM, is also orders of magnitude more sensitive than the protease-coupled flourescence ProFluor® assay, with an $EC_{50}$ value of 13,000 pM.

*Enzyme titration*

The second evaluation performed was a direct comparison of the Phosphate Sensor method to malachite green (BIOMOL Green™ Reagent) and protease-coupled fluorescent (ProFluor® Tyrosine Phosphatase Assay) technologies by titration of PTP1B protein tyrosine phosphatase. A comparison of the three separate assay setups is shown in Table 1 and the data in Figure 3.

For Phosphate Sensor, a two-fold serial dilution of PTP1B into phosphatase enzymatic reaction buffer in a 384-well plate was incubated for 60 minutes at room temperature with either 10 or 50 μM PTP1B substrate. Inorganic phosphate released from the 10-μL phosphatase reaction was detected by Phosphate Sensor after mixing the plate and reading as described above.

For the malachite green method using BIOMOL Green™ Reagent, the same phosphatase enzymatic reaction buffer and PTP1B substrate concentrations were utilized. The 50-μL phosphatase reaction in a 96-well plate, incubated for 60 minutes at room temperature, was treated with malachite green, incubated, and read as previously described.

For the protease-coupled fluorescent ProFluor® assay comparison, the Phosphate Sensor assay was run as previously described, except that the final 1X PTP1B substrate concentration was decreased to 5 μM, and the phosphatase reaction was run according to the user guide supplied with the kit. This involved a two-fold serial dilution of PTP1B in 1X reaction buffer B and a 60-minute incubation with 5 μM R110 substrate. Because ProFluor® assay technology relies on a coupled enzymatic assay format, 5 μL of a protease solution in 1X termination buffer and $Na_3VO_4$ was added to the 10-μL phosphatase reaction. Following a 30-minute incubation, 5 μL of a stabilizer reagent in termination buffer with $Na_3VO_4$ was added to a final reaction volume of 20 μL. The plate was read on a Tecan Safire2™ microplate reader at excitation 485 (10) nm and emission 530 (10) nm.

*Kinetic enzyme reaction*

The final evaluation performed with Phosphate Sensor was to analyze a PTP1B reaction in real-time kinetic mode. The PTP1B assay was run as described previously except the incubation period after the addition of the substrate was omitted. Rather, the 2X Phosphate Sensor reagent was added immediately and the plate was read in kinetic mode, collecting data every minute for a total of 40 minutes (Figure 4).

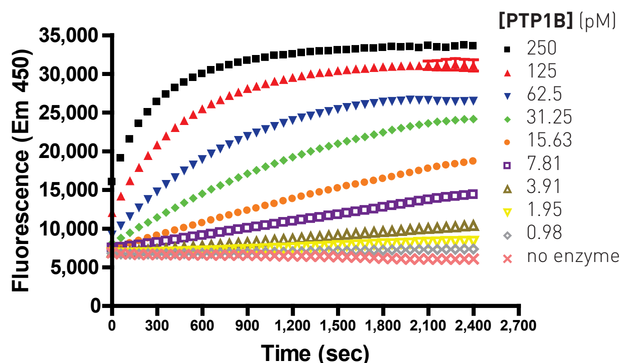



**Figure 4 ►. PTP1B titration in kinetic mode using the Phosphate Sensor.** The Phosphate Sensor method directly measures the amount of inorganic phosphate present, allowing real-time detection of $P_i$ release. Because other competitor technologies cannot do this, determining enzymatic rates is a unique feature of the Phosphate Sensor technology.

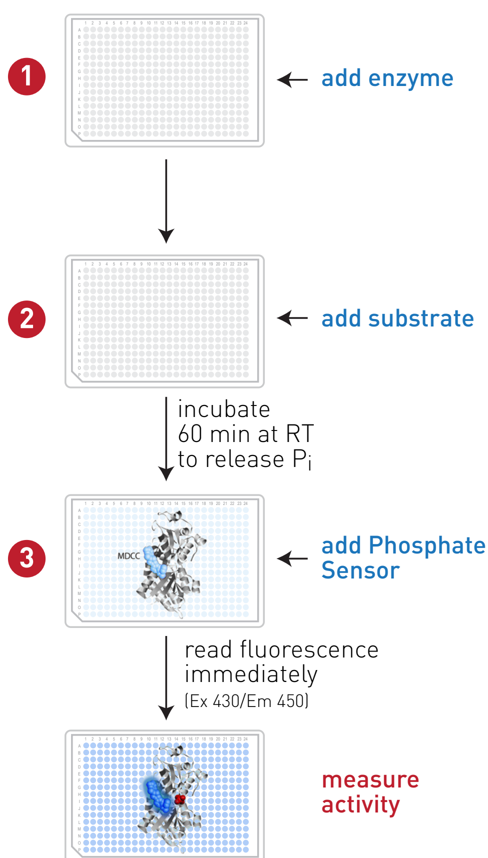



**Figure 5. Quick protocol**

## Box 1. The phosphate mop.

The phosphate mop, comprised of 7-methyl guanosine (7-MEG) and purine nucleoside phosphorylase (PNPase), can be used to sequester potentially contaminating inorganic phosphate that may be present in experimental solutions or materials in the form of ribose-1-phosphate [7].

For typical applications, 200 µM 7-MEG and 0.1 to 1.0 U/mL PNPase are used. Water is used to dissolve 7-MEG (Sigma P/N M0627) to a 30 mM stock solution (stored at –80°C) and PNPase (Sigma P/N N8264) to 500 U/mL (dispensed into small aliquots to avoid freeze/thaw cycles and stored at –80°C).

## Conclusions

Phosphate Sensor is a simple assay system used to directly measure the amount of inorganic phosphate generated in an enzymatic reaction. This tool can also be configured to perform kinetic reads, which allows users the unique ability to measure enzymatic rates.

Phosphate Sensor is very sensitive, detecting picomole quantities of inorganic phosphate in protein phosphatase, phosphodiesterase (PDE), ATPase, GTPase, and other activity assays.

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| Product Description | Catalog Number | Size |
|---|---|---|
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| Phosphate Sensor | PV4407 | 100 nmol |
| DTT, 1M | P2325 | 1 ml |


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