Simply Sensitive: Phosphate Detection Made Easy.

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ABSTRACT

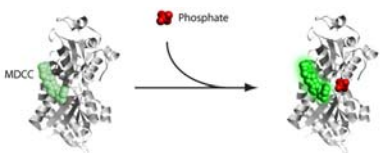
Numerous enzymatic reactions release inorganic phosphate (Pi) as a result of their activity. For example, phosphatases liberate phosphate from proteins as a way of regulating the activity of their targets, ATPase/GTPases hydrolyze NTP into NDP + Pi in order to generate energy to pump ions across a membrane, and helicases use ATP to open double stranded DNA. We describe here a simple tool that can be used to interrogate the activity of phosphate releasing enzymes. This tool is based on a bacterially-derived phosphate binding protein that has been modified with a fluorophore such that upon the binding of phosphate an increase in fluorescence is detected. Not only is the reagent simple to use, it can be used in conjunction with a variety of enzyme systems to give screening quality data, and is orders of magnitude more sensitive than the commonly used reagent, Malachite Green.

INTRODUCTION

Drug candidate screening typically requires complex assay systems that employ multiple enzymatic reactions to measure a response or need to have a different reagent for each target class. Along with this many of these systems suffer from lack of sensitivity which results in increased reagent usage. To this end we have developed the Phosphate Sensor (PV4406). This simple, versatile, and easy to use tool enables direct measurement of inorganic phosphate formation in picomolar quantities. This reagent is based on a bacterially-derived phosphate binding protein modified with a fluorophore such that upon binding of phosphate an increase in fluorescence is detected (figure 1) (1). The observed 6-10 fold increase in fluorescence can be used to interrogate a variety of enzymatic reactions that release phosphate. This system is orders of magnitude more sensitive than the commonly used phosphate detection system Malachite Green (such as the BIOMOL Green™ Reagent formulation (figure 2) and its single reagent system makes it much simpler than other assay formats.

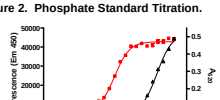
Here, we demonstrate how the Phosphate Sensor can be used with a variety of different target classes. Data gathered with Protein Tyrosine Phosphatase 1B (PTP1B) demonstrates the Phosphate Sensor is more sensitive than both BIOMOL Green™ Reagent and ProFluor® Tyrosine Phosphatase Assay, resulting in the reduction of both PTP1B and substrate used. In the Rho GTPase assay, the Phosphate Sensor can measure increase in activity of Rho GTPase by the addition of its associated GTPase Activating Protein (GAP). The Phosphate Sensor can also be configured to detect activity of a phosphodiesterase assay, illustrated by the Phosphodiesterase-5 titration and inhibitor titration presented.

Figure 1. Phosphate Senor Schematic.



Protein ribbon diagram of the Phosphate Senor with fluorescent dye indicated in green. Fluorescence intensity of the Phosphate Sensor increases 6-10 fold upon binding inorganic phosphate.

Figure 2. Phosphate Standard Titration.

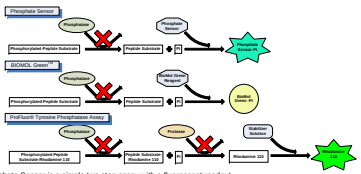


■ Phosphate Sensor ▲ Biomol Green™ Reagent (Malachite Green)

The Phosphate Sensor and BIOMOL Green™ Reagent were compared using a two-fold serial dilution of a phosphate standard.
The Phosphate Sensor is orders of magnitude more sensitive as compared to BIOMOL Green™ Reagent; EC_{50} values are 9.6 pM and 2200 pM respectively.

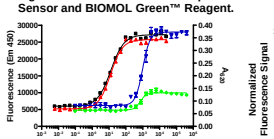
RESULTS

Figure 3. Phosphatase Assay Schematics.



The Phosphate Sensor is a simple two step assay with a fluorescent readout.
The BIOMOL Green™ Reagent is simple to use but lacks sensitivity and is an absorbance measurement.
The ProFluor® Tyrosine Phosphatase Assay requires the use of a protease which could be inhibited by test compounds (indicated by red X), adding complexity to the detection method.

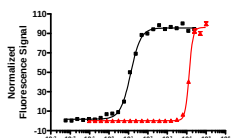
Figure 4. PTP1B titration - Phosphate Sensor and BIOMOL Green™ Reagent.



■ 50 µM substrate, Phosphate Sensor
▲ 10 µM substrate, Phosphate Sensor
▼ 50 µM substrate, Biomol Green™ Reagent
● 10 µM substrate, Biomol Green™ Reagent

Protein Tyrosine Phosphatase 1B (PTP1B, Calbiochem #539735) was 2-fold serial diluted and incubated with either 10 or 50 µM PTP1B Substrate (Calbiochem #539737) for 60 minutes. The Phosphate Sensor reaction was performed in 10 µL and inorganic phosphate was detected with the addition of 10 µL 1 µM Phosphate Sensor and read on a Safire²™; excitation 430(10) nm, emission 450(10) nm. The BIOMOL Green™ Reagent reaction was carried out in 50 µL with a 100 µL addition of BIOMOL Green™ Reagent and the plate was read on a Safire™ in absorbance mode at 620 nm.
The Phosphate Sensor is more sensitive than BIOMOL Green™ Reagent with EC_{50} values of 12 pM and 990 pM respectively. Additionally, improved sensitivity of the Phosphate Sensor enables the experiment to be performed with 1/5th the amount of substrate with no impact to assay window.

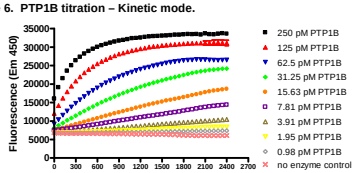
Figure 5. PTP1B titration - Phosphate Sensor and ProFluor® Assay.



■ 5 µM PTP1B subs, Phosphate Sensor
▲ 5 µM R110 subs, ProFluor® Assay

PTP1B was 2-fold serial diluted and incubated with 5 µM substrate for 60 minutes. The Phosphate Sensor data was performed as described earlier using PTP1B substrate. The 10 µL ProFluor® Tyrosine Phosphatase Assay was conducted using 5 µM PTPase R110 Substrate; this was followed by a 5 µL addition of Protease Solution and a 30 minute incubation. Then 5 µL of Stabilizer Solution was added and read on a Safire²™, excitation 485 (10) nm and emission 530 (10) nm.
The Phosphate Sensor is orders of magnitude more sensitive than the ProFluor® Assay. EC_{50} values are 12 pM and 13 nM respectively.

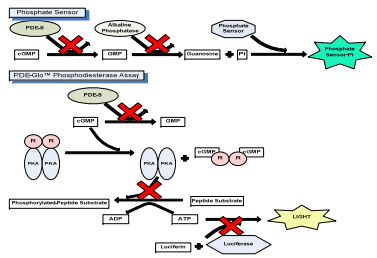
Figure 6. PTP1B titration – Kinetic mode.



■ 250 pM PTP1B
■ 125 pM PTP1B
▼ 62.5 pM PTP1B
◆ 31.25 pM PTP1B
● 15.63 pM PTP1B
□ 7.81 pM PTP1B
△ 3.91 pM PTP1B
▽ 1.95 pM PTP1B
◇ 0.98 pM PTP1B
× no enzyme control

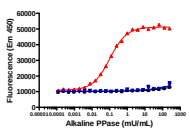
The PTP1B assay was run as described previously and read in kinetic mode, collecting data every minute for a total of 40 minutes.
The ability of the Phosphate Sensor to directly measure the amount of inorganic phosphate present allows for real time detection of its release. This is a unique feature of the Phosphate Sensor and can be used to determine enzymatic rates.

Figure 7. Phosphodiesterase Assay Schematic.



The Phosphate Sensor can be configured to detect phosphodiesterase activity through coupling with a hydrolase enzyme like alkaline phosphatase.
The PDE-Glo™ Phosphodiesterase Assay measures phosphodiesterase activity by coupling though PKA and Luciferase, adding complexity to the method. Test compounds could inhibit PDE-5, PKA, or luciferase as indicated by the red X's.

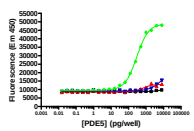
Figure 8. Alkaline Phosphatase titration.



■ no nucleotide ▲ 5 µM GMP ▼ 5 µM cGMP

Alkaline phosphatase (Calbiochem #524545) was 2-fold serial diluted and incubated with 5 µM GMP, cGMP or no nucleotide for 60 minutes in 10 µL. 10 µL of 1 µM Phosphate Sensor and read immediately after addition as described earlier.
Signal above background is only observed when GMP is hydrolyzed into gaunosine and inorganic phosphate.

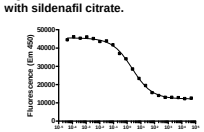
Figure 9. Human PDE5 titration.



■ no cGMP, no Alk. PPase ▼ no cGMP, 10 mU/mL Alk. PPase
▲ 5 µM cGMP, no Alk. PPase ◆ 5 µM cGMP, 10 mU/mL Alk. PPase

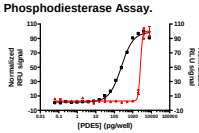
Human Phosphodiesterase-5 (PDE-5 SignalChem #P93-31G) was 2-fold serial diluted and incubated in the presence and absence of 5 µM cGMP and alkaline phosphatase for 60 minutes in 10 µL. 10 µL of 1 µM Phosphate Sensor was added and read as described earlier.
The PDE-5 generates a sigmoidal curve with an EC_{50} of 290 pg/well. Background signals from the alkaline phosphatase or cGMP are negligible.

Figure 10. Inhibition of Human PDE5 with sildenafil citrate.



Sildenafil citrate (Tocris #3784) was 3-fold serial diluted and incubated with 0.8 nM PDE-5 in 7.5 µL for 10 min. 2.5 µL of 20 µM cGMP and 40 mU/µL alkaline phosphatase was added to start the reaction and incubated for 60 minutes. 10 µL of 1 µM Phosphate Sensor was added and read as described earlier.
An IC_{50} value of 2.3 nM was obtained which agrees with the reported value of 3.7 nM (2).

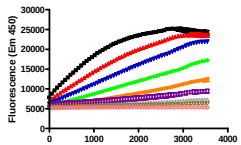
Figure 11. PDE-5 Titration - Phosphate Sensor and PDE-Glo™ Phosphodiesterase Assay.



■ Phosphate Sensor ▲ PDE-Glo™ Assay

Human Phosphodiesterase-5 (PDE-5 SignalChem #P93-31G) was 2-fold serial diluted and assayed using the Phosphate Sensor as described earlier and the PDE-Glo™ Phosphodiesterase Assay. The PDE-Glo™ Assay protocol includes the PDE-5 reaction steps, addition of a PDE-Glo™ Termination Buffer, a PDE-Glo™ Detection Solution, and the Kinase-Glo® Reagent. The plate was read on a BMG LabTech PHERAStar *Plus* in Luminescence mode.
The Phosphate Sensor is about 10 fold more sensitive than the PDE-Glo™ Phosphodiesterase Assay. EC_{50} values are 240 pg/well and 2.6 ng/well respectively.

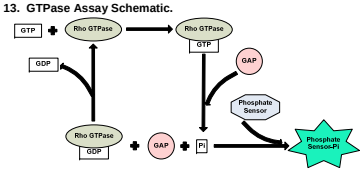
Figure 12. PDE-5 Titration – Kinetic Mode.



■ 4 ng PDE5
▲ 2 ng PDE5
▼ 1 ng PDE5
◆ 0.5 ng PDE5
● 0.25 ng PDE5
□ 0.125 ng PDE5
△ 0.063 ng PDE5
▽ 0.031 ng PDE5
◇ 0.016 ng PDE5

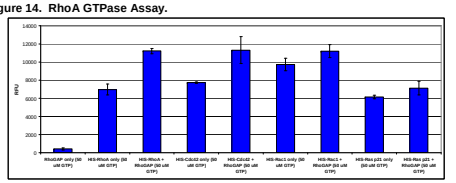
Human Phosphodiesterase-5 reaction was carried out as described previously except that the 10 µL of 1 µM Phosphate Sensor was added immediately and read every minute over a 60 minute incubation period.
The ability of the Phosphate Sensor to gather kinetic data is unique for the measurement of inorganic phosphate.

Figure 13. GTPase Assay Schematic.



The Phosphate Sensor can be configured to measure the activity of GTPase enzymes such as RhoA. As RhoA is stimulated by its associated GTPase Activating Protein (GAP), hydrolysis of GTP to GDP and inorganic phosphate increases. The Phosphate Sensor can be used to measure this hydrolysis event.

Figure 14. RhoA GTPase Assay.



GTPase assays were conducted using 4.75 µM GTPase with and without 5.2 µM Rho-GAP in the presence of 50 µM GTP in 10 µL total volume, and were incubated at 37°C for 2 hours. 10 µL of 1 µM Phosphate Sensor with 20 mM EDTA was added and the plate was read as described previously.
As expected Rho-GAP stimulated His-RhoA and His-Cdc42; marginal activation was observed for His-Rac1 and His-Ras p21.

CONCLUSIONS

The Phosphate Sensor is a simple assay system to directly measure the amount of inorganic phosphate generated in an enzymatic reaction.

The Phosphate Sensor is very sensitive; detecting picomolar quantities of inorganic phosphate.

The Phosphate Sensor is versatile and can be used for a variety of target classes; phosphatases, phosphodiesterases, GTPases, and ATPases.

REFERENCES and Useful Weblinks

(1) Brune, M., Hunter, J. L., Corrie, J. E. T., and Webb, M. R. Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry, 1994* 33, 8262-8271.
(2) Mitsi A. Blount, Alfreda Beasley, Roya Zoraghi, Konjeti R. Sekhar,Emmanuel P. Bessay, Sharron H. Francis, and Jackie D. Binding of Tritiated Sildenafil, Tadalafil, or Vardenafil to the Phosphodiesterase-5 Catalytic Site Displays Potency, Specificity, Heterogeneity, and cGMP Stimulation. *Molecular Pharmacology*, 2004, 66, 1, 144-152.
For More information about the Phosphate Sensor, please visit: www.invitrogen.com.

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