APPLICATION NOTE
Phosphodiesterase Activity: A Phophate Sensor Application

Simple, Sensitive Detection of Protein Phosphodiesterase 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 by coupling to an alkaline phosphatase against a commonly used coupled luciferase assay, examined responses for human phosphodiesterase A (PTE5A) in a titration, in real-time kinetic mode, and with a sildenafil citrate inhibitor. Phosphate Sensor is orders of magnitude more sensitive than the coupled luciferase method, faster and simpler to use than other competitor methods, and uniquely qualified for determining enzymatic rates.

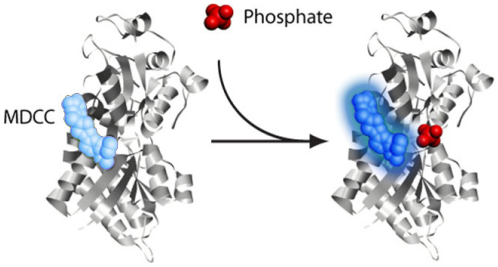

Figure 1. Phosphate Sensor assay principle. The protein ribbon diagram illustrates the modified phosphate-binding protein with the MDCC fluorophore (shown in blue). Upon binding inorganic phosphate, fluorescence of Phosphate Sensor increases approximately 6- to 8-fold and can be measured in real time.

Second messenger systems that involve an increase or decrease of cyclic nucleotides (cAMP or cGMP) mediate intracellular signal transduction. Nucleotide degradation regulates this process and is mediated through the action of phosphodiesterase (PDE) enzymes, which also play an integral role in a number of disorders including erectile dysfunction [1], asthma [2], and chronic obstructive pulmonary disease [2], as well as schizophrenia, bipolar disorder, and major depression [3]. As research identifies additional potential drug targets, methodologies for measuring activity become vital.

We describe here the use and optimization of a simple, flexible reagent for measurement of phosphodiesterase activity through coupling it with a phosphatase. Phosphate Sensor is a phosphate-binding protein modified with a fluorophore [4]. As the sensor binds free inorganic phosphate (P_i), fluorescence intensity increases (Figures 1 and 2). This simple direct measurement of released phosphate is not dependent on a specific substrate or enzyme, making it 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).*

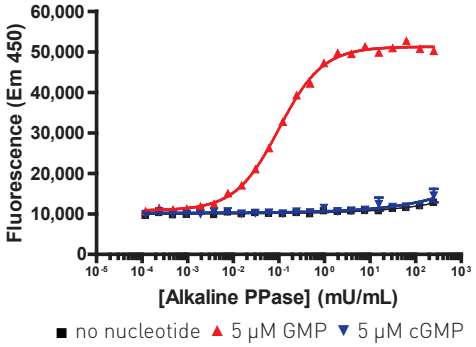

Figure 3. Alkaline phosphatase titration. A two-fold serial dilution of alkaline phosphatase was incubated for 60 minutes with no nucleotide, 5 µM GMP, or 5 µM cGMP. While alkaline phosphatase had no affect on cGMP, signal above background was observed when GMP hydrolyzed into guanosine and inorganic phosphate.

Experimental Procedures

Materials and methods

Phosphodiesterase 5A (PDE5A; SignalChem P/N P93-31G), cGMP (Sigma P/N G6129), GMP (Sigma P/N G8377), alkaline phosphatase (Calbiochem P/N 524545), sildenafil citrate (Tocris P/N 3784), PDE-Glo™ Phosphodiesterase Assay (Promega P/N V1361), 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich P/N I5879), and Phosphate Sensor (Invitrogen P/N PV4406), were used in these experiments. The PDE enzymatic reaction buffer consisted of 50 mM Tris pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 0.01% Triton® X-100, and 0.5 mM DTT. The 1X termination buffer for the PDE-Glo™ reaction consisted of 100 mM IBMX in 100% DMSO. The Phosphate Sensor detection buffer consisted of 20 mM Tris pH 7.6 and 0.05% Triton® X-100. All assays were performed in black 384-well low-volume, round-bottom, non-treated plates (Corning P/N 3677), except for the "Competitor A" assays, which were performed in white 384-well low volume, round bottom, non-treated plates (Corning P/N 3674).

Optimization of alkaline phosphatase concentration

The first step of the comparison process was optimization of the alkaline phosphatase read-out reaction. A two-fold dilution series of alkaline phosphatase ranging from 250 mU/mL to 0.00012 mU/mL was run in a final 10 µL reaction volume and incubated for 60 minutes with no nucleotide, 5 µM GMP, or 5 µM cGMP. After the reaction, 10 µL of 2X (1 µM) Phosphate Sensor in Phosphate Sensor detection buffer was added to a final volume of 20 µL and concentration of 0.5 µM. The plate was mixed and read immediately on a Tecan Safire2™ microplate reader at excitation 430 nm (10) and emission 450 nm (10) (Figure 3).

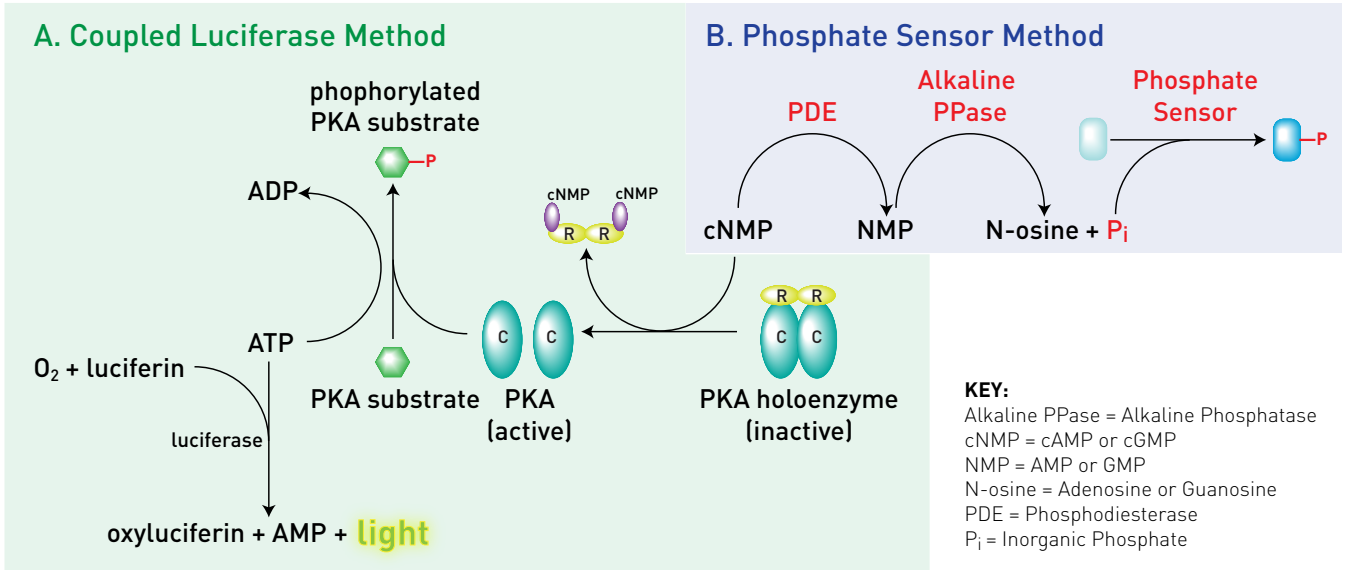

Figure 2. Comparison of a coupled luciferase assay to the Phosphate Sensor method. (A) The Promega PDE-Glo™ assay involves coupled reactions that measure phosphodiesterase activity by following the activation of protein kinase A (PKA) from holoenzyme and assessing the amount of ATP depleted with Kinase-Glo® Reagent in a luciferase reaction. (B) By coupling Phosphate Sensor with a hydrolase enzyme like alkaline phosphatase (alkaline PPase), the Phosphate Sensor method detects phosphodiesterase activity in fewer steps.

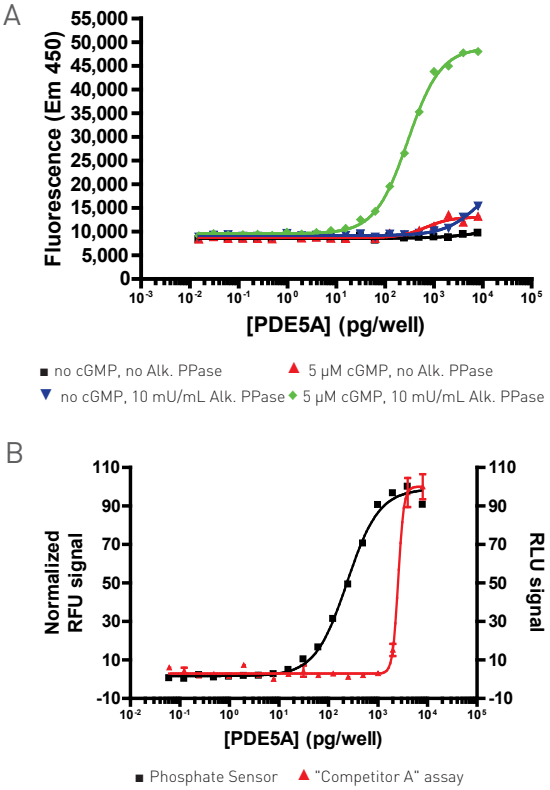

Figure 4. Human PDE5A titrations. (A) Human PDE5A titration using the Phosphate Sensor assay. The PDE5A generates a sigmoidal curve with an EC_{50} of 290 pg/well. As expected, PDE5A activity was only observed in the presence of both cGMP and alkaline phosphatase. Background signals from the alkaline phosphatase or cGMP are negligible. (B) The Phosphate Sensor assay as compared to "Competitor A" assay.

Human PDE5A titration

In the second step of the evaluation, a direct comparison of Phosphate Sensor to a competitor technology was determined via a human phosphodiesterase 5A (PDE5A) titration. For Phosphate Sensor, a two-fold serial dilution of PDE5A was made in PDE enzymatic reaction buffer and incubated for 60 minutes in the presence or absence of 5 µM cGMP and 10 mU/mL alkaline phosphatase. The PDE reaction was performed in 10 µL and inorganic phosphate was detected by adding 10 µL of 2X (1 µM) Phosphate Sensor to a final volume of 20 µL and concentration of 0.5 µM. The plate was mixed and read as previously described. For the PDE-Glo™ technology, which relies on a multiple enzymatic cascade before readout, the PDE5A reaction was run according to the user guide supplied with the kit. In short, a two-fold serial dilution of PDE5A in PDE-Glo™ reaction buffer was made, incubated for 60 minutes with 5 µM cGMP in 5 µL, and followed by the addition of 2.5 µL of a 1X termination buffer with the IBMX inhibitor and incubated for 10 minutes. Next, 2.5 µL of protein kinase a (PKA) was added in a 1X detection buffer and incubated for 20 minutes. Finally, 10 µL of Kinase-Glo® reagent was added and incubated for 10 minutes in a final reaction volume of 20 µL before reading luminescence in the plate on a BMG LabTech PHERAStar *Plus* microplate reader (Figure 4). The enzymatic steps and assay setups for the two methods compared are shown in Figure 2 and Table 1 shows the comparison of these two assay setups.

Kinetic enzyme reaction

In the third portion of this comparison, Phosphate Sensor was utilized to analyze a PDE5A reaction in real time kinetic-mode. The PDE5A assay was run as described previously except that after addition of cGMP and alkaline phosphatase, there was no incubation period. Rather, the 2X Phosphate Sensor was added immediately and the plate was read in kinetic mode, collecting data every minute for a total of 60 minutes (Figure 5).

	Phosphate Sensor Method	**PDE-Glo™ Method**
detection	fluorophore	multiple enzymatic cascade
assay format	384-well	384-well
final assay volume	20 µL	20 µL
assay time	60 minutes	100 minutes
inhibitable steps	2 (PDE5A and alkaline PPase reactions)	3 (PDE5A, PKA, and luciferase reactions)
steps	3	5
protocol	1. Add 5 µL of 2X PDE5A 2. Add 5 µL of 2X cGMP and 2X alkaline PPase, incubate 60 minutes at RT 3. Add 10 µL of 2X Phosphate Sensor, read plate immediately	1. Add 2.5 µL of 2X PDE5A 2. Add 2.5 µL of 2X cGMP, incubate 60 minutes at RT 3. Add 2.5 µL IBMX in 1X termination buffer 4. Add 2.5 µL PKA in 1X detection buffer 5. Add 10 µL Kinase-Glo® reagent

Table 1. Comparison of PDE5A Assays between Phosphate Sensor and a competitor method. The Phosphate Sensor Assay requires fewer steps (also see Figure 5) in a shorter total assay time than the multiple enzymatic cascade technology. The Phosphate Sensor assay also has fewer "inhibitable" steps.

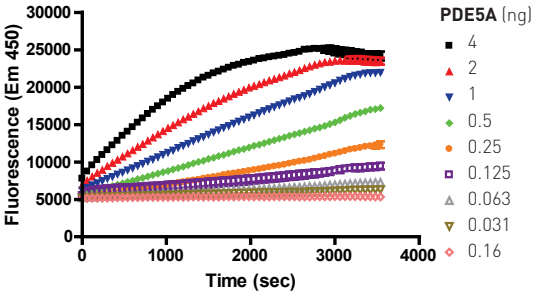

Figure 5. PDE5A titration in kinetic mode using Phosphate Sensor. The ability of Phosphate Sensor to directly measure the amount of inorganic phosphate allows for real-time detection of its release. Because other competitor technologies cannot do this, determining enzymatic rates is a unique feature of the Phosphate Sensor technology.

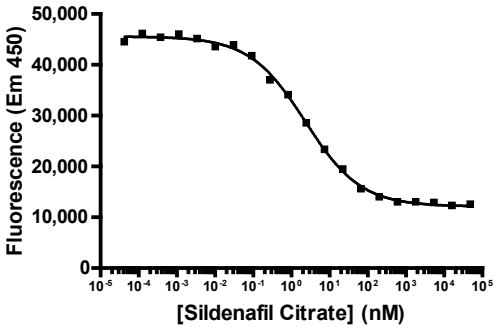

Figure 6. Inhibition of human PDE5A activity with sildenafil citrate using Phosphate Sensor. The IC_{50} value of 2.3 nM obtained agrees with the reported value of 3.7 nM [5].

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).

Inhibition of PDE5A activity using sildenafil citrate

In the final step of this comparison, Phosphate Sensor was used to analyze inhibition of PDE5A activity by sildenafil citrate. The assay was run as previously described, except that the order of addition for the PDE5A reaction was modified. First, a 3-fold serial dilution of sildenafil citrate (2.5 µL of 4X concentrate) was made and incubated with 5 µL of 2X (1.6 nM) PDE5A (total of 7.5 µL) for 10 minutes. Then, 2.5 µL of 4X (20 µM) cGMP and 4X (40 mU/mL) alkaline phosphatase was added to start the reaction and incubated for 60 minutes. Last, 10 µL of 2X (1 µM) Phosphate Sensor was added and read as described earlier (Figure 6). The final concentration of PDE5A was 0.8 nM per reaction.

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 phosphodiesterase (PDE), protein phosphatase, ATPase, GTPase, and other activity assays.

References

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5. Blount MA, Beasley A, et al. *Mol Pharmacology* 66: 144-152.

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Product Description	Catalog Number	Size
Phosphate Sensor	PV4406	10 nmol
Phosphate Sensor	PV4407	100 nmol
DTT, 1M	P2325	1 ml

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