APPLICATION NOTE
ATPase Activity: A Phophate Sensor Application

Phosphate Sensor for sensitive detection of ATPase 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 of ATPase activity against a commonly used multiple enzyme luciferase assay, in enzyme titration assays with two kinesins, examined responses for kinesin heavy chain in real-time kinetic mode, and the effects of monastral inhibitor on Eg5. Phosphate Sensor, which offers sensitivity comparable to the coupled luciferase method, is faster and simpler to use than other competitor methods, and uniquely qualified for determining enzymatic rates in kinetic runs.

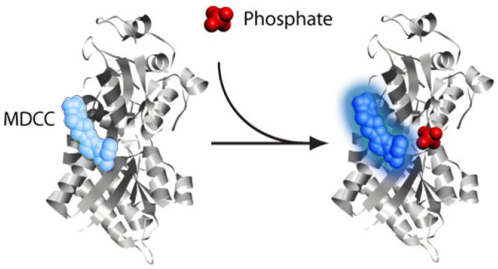

Figure 1. Phosphate Sensor assay principle. The protein ribbon diagram illustrates the modified phosphate-binding protein with the MDCC fluorophore (shown in blue). Upon binding inorganic phosphate, fluorescence of Phosphate Sensor increases approximately 6- to 8-fold and can be measured in real time.

The movement of materials into cells and from one location to another within cells is important to normal cellular functions. Many of these activities require proteins to either change configuration or shift location, requiring an energy input. Typically this energy comes from the hydrolysis of ATP by ATPases, which perform many different functions, including proton pumping and cargo transportation. These enzymes also play an integral role in Parkinson's Disease [1], cancer [2,3], and other disorders. As additional potential drug targets are identified, methodologies for measuring activity become vital.

We describe here the use and optimization of a simple, flexible reagent for measurement of ATPase activity. Phosphate Sensor is a phosphate-binding protein modified with a fluorophore [4]. As the sensor binds free inorganic phosphate, fluorescence intensity increases (Figures 1 and 3). This simple direct measurement of the release of 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 (including ATP), as well as on plastics and glassware. Care should be taken to minimize contamination from these sources when using Phosphate Sensor. ATP sources that contain a significant amount of free phosphate (ATP + P_i) should not be used. Instead, an ultra-pure quality ATP should be used whenever using Phosphate Sensor detection. 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).*

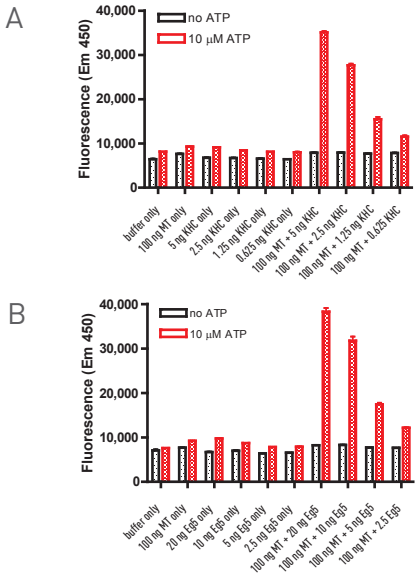

Figure 2. Kinesin endpoint enzyme titrations using Phosphate Sensor. Titrations in the presence or absence of 10 µM ATP were performed with KHC (kinesin heavy chain) motor domain (A) and Eg5 motor domain (B) in the presence and absence of of microtubules (MT). No ATPase activity was seen with KHC or Eg5 in the absence of microtubules or in the presence of only microtubules (no ATP) but was observed in the presence of microtubules and ATP, as expected.

Experimental Procedures

Materials and methods

Pre-formed microtubules (MT) (Cytoskeleton P/N MT002), taxol (used to inhibit microtubule substrate depolymerization, thus promoting stabilization) (Cytoskeleton P/N TXD01), kinesin heavy chain (KHC) motor domain protein (human, recombinant) (Cytoskeleton P/N KR01), kinesin Eg5 motor domain protein (human, recombinant) (Cytoskeleton P/N EG01), kinesin reaction buffer (Cytoskeleton P/N KRB01), the compound monastrol (Enzo P/N BML-GR322)), ADP-Glo™ Kinase Assay (Promega P/N V9101), and Phosphate Sensor (Invitrogen P/N PV4406), were used in these experiments. The kinesin reaction buffer consisted of 15 mM PIPES pH 7.0 and 5 mM $MgCl_2$. 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 ADP-Glo™ assays, which were performed in white 384-well low volume, round bottom, non-treated plates (Corning P/N 3674).

Enzyme titration

The first step of the comparison process was an endpoint enzyme titration assay utilizing Phosphate Sensor to analyze KHC and Eg5 activity. The assays were run by making additions to the plate. First, 5 µL of reaction buffer supplemented with 20 µM of taxol was added. Next, 5 µL of taxol (20 µM) stabilized microtubules at 20 ng/µL (to 100 ng microtubules per well) or equivalent volume of buffer only for wells without microtubules was added. Finally, 2.5 µL of a 4-point ,2-fold serial titration of KHC (final concentrations of 5, 2.5, 1.25, and 0.625 ng/well) or Eg5 (final concentrations of 20, 10, 5, and 2.5 ng/well) in reaction buffer without taxol or an equivalent volume of buffer only was added. To start the ATPase reactions, 2.5 µL of reaction buffer without taxol or 60 µM ATP in reaction buffer without taxol was added to a final concentration of 10 µM ATP. The 15 µL ATPase reaction was allowed to proceed for 40 minutes at room temperature before adding 5 µL of 4X (2 µM) Phosphate Sensor to detect inorganic phosphate in a final reaction volume of 20 µL and final concentration of Phosphate Sensor 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 2).

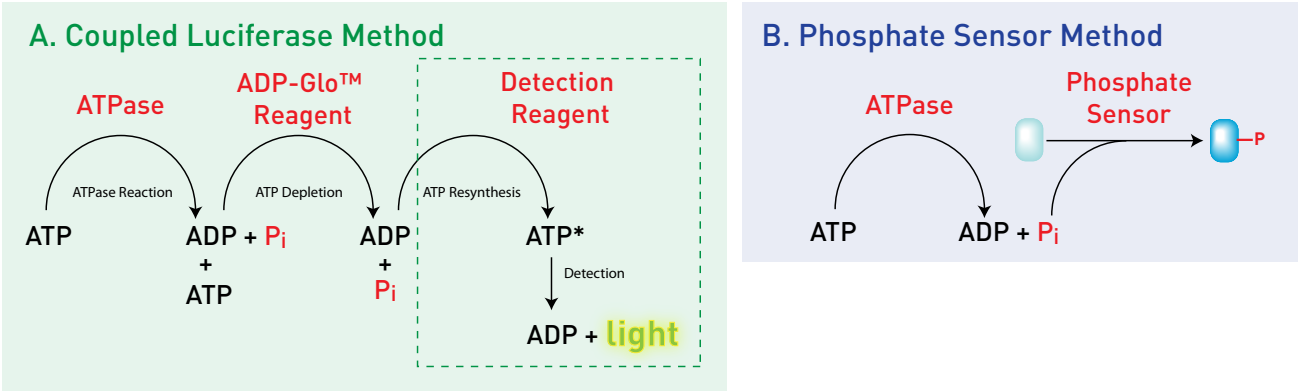

Figure 3. Comparison of a coupled luciferase assay to the Phosphate Sensor method. (A) For the coupled luciferase assay, ADP-Glo™ Reagent is added to terminate the ATPase reaction and deplete the remaining ATP before adding the kinase detection reagent that resynthesizes ATP from ADP and provides the luciferase reagents needed to produce a luminescent signal. (B) The Phosphate Sensor method directly detects ATPase activity in fewer steps.

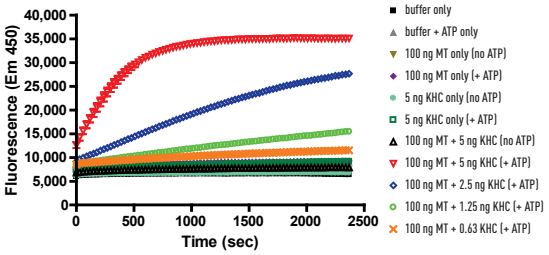

Figure 4. Microtubule-activated KHC motor domain ATPase activity in kinetic mode using Phosphate Sensor. Phosphate Sensor can directly measure the amount of inorganic phosphate present and has the unique capacity to measure real-time detection of P_i release and determine enzymatic rates.

Kinetic enzyme reaction

The second step of the comparison process utilized Phosphate Sensor to analyze a microtubule-activated KHC motor domain ATPase reaction in real-time kinetic-mode. The KHC assay was run as described previously except that after addition of ATP, there was no incubation period. Rather, 4X Phosphate Sensor was added immediately and the plate was read in kinetic mode, collecting data every minute for a total of 40 minutes (Figure 4).

Phosphate Sensor comparison to competitor technology

The third step of the comparison process involved a direct comparison of Phosphate Sensor to the ADP-Glo™ assay detection technology. A two-fold dilution series of KHC, ranging from 10 to 0.078 ng/well was used. For Phosphate Sensor, the assay was set up and run as previously described except that the ATPase reaction was run for 60 minutes at room temperature. For the competitor technology, the ADP-Glo™ assay was run in plates according to the user guide supplied with the kit and the protocol summarized in Table 1. First, 1.25 µL of reaction buffer supplemented with 20 µM taxol was added. Then 1.25 µL of buffer only or 80 ng/µL taxol-stabilized microtubules in 20 µM taxol to 100 ng microtubules per well was added. Next 1.25 µL of buffer only without taxol or KHC in an 8-point, 2-fold serial dilution in the reaction buffer without taxol to 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.078 ng/well was added. Finally, 1.25 µL of buffer only without taxol or 4X (40 µM) ATP in reaction buffer without taxol) was added to a final concentration of 10 µM ATP to start the ATPase reactions in an assay volume of 5 µL, which were allowed to proceed for 60 minutes at room temperature. After the ATPase reactions, 5 µL of ADP-Glo™ reagent

	Phosphate Sensor Method	ADP-Glo™ Method
detection	fluorophore	coupled luminescence
assay format	384-well	384-well
final assay volume	20 µL	20 µL
assay time	60 minutes	130 minutes
inhibitable steps	1 (KHC ATPase reaction)	4 (KHC ATPase, ATP depletion, ADP detection, and luciferase reactions)
kinetic read ability?	yes	no
steps	5	6
protocol	1. Add 5 µL of reaction buffer (+ taxol) 2. Add 5 µL of MT (20 ng/µL) to 100 ng per well (in reaction buffer + taxol) 3. Add 2.5 µL of KHC (final concentrations from 10 to 0.078 ng per well) in reaction buffer 4. Add 2.5 µL of ATP (60 µM) in reaction buffer, incubate 60 minutes at RT 5. Add 10 µL of 2X Phosphate Sensor, read plate immediately	1. Add 1.25 µL of reaction buffer (+ taxol) 2. Add 1.25 µL of MT (20 ng/µL) to 100 ng per well (in reaction buffer + taxol) 3. Add 1.25 µL of KHC (final concentrations from 10 to 0.078 ng per well) in reaction buffer 4. Add 1.25 µL of ATP (40 µM) in reaction buffer, incubate 60 minutes at RT 5. Add 5 µL of ADP-Glo™ reagent, incubate 40 minutes at RT 6. Add 10 µL of kinase detection reagent, incubate 30 minutes at RT

Table 1. Comparison of microtubule-activated KHC ATPase assays between Phosphate Sensor and competitor methods. The Phosphate Sensor assay requires fewer steps in less than half the total assay time. The Phosphate Sensor assay also has fewer "inhibitable" steps and has the ability to read the reaction in "kinetic-mode".

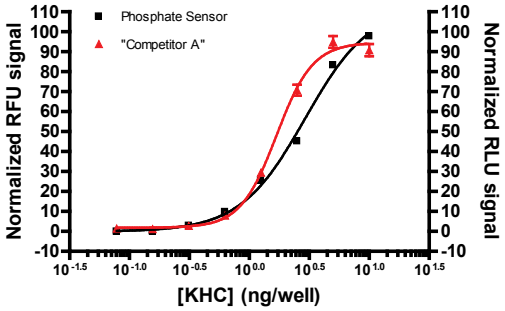

Figure 5. Microtubule-activated KHC ATPase activity in a 60-minute endpoint assay. Phosphate Sensor (EC_{50} = 2.9 ng/well) is comparable to the assay using ADP-Glo™ reagents (EC_{50} = 1.7 ng/well). Compared to the competitor's multiple-step enzyme process, the Phosphate Sensor assay has fewer steps and fewer "inhibitable" steps, a shorter total assay time, and the ability to read reactions in "kinetic-mode".

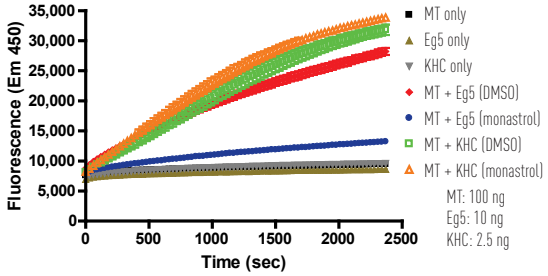

Figure 6. Microtubule-activated KHC and Eg5 ATPase activity in kinetic mode. Monastrol inhibited Eg5 activity at a much slower rate than the DMSO-only control, but did not affect KHC activity.

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).

was added and allowed to incubate for 40 minutes at room temperature. To detect activity, 10 µL of kinase detection reagent was added and the final reaction volume of 20 µL was incubated for 30 minutes at room temperature. The plate was read on BMG LabTech PHERAStar Plus in Luminescence mode (Figure 5). A comparison of the two separate assay setups can be seen in Table 1.

Inhibition of Eg5 activity using monastrol

In the final step of the comparison, Phosphate Sensor was used to analyze the inhibition of microtubule-activated Eg5 activity by the compound monastrol in kinetic mode. Monastrol is a specific Eg5 inhibitor and should have no activity against other kinesins, such as KHC. The assay was run as previously described in kinetic mode, except that included with the initial 5 µL addition of reaction buffer with taxol, either DMSO only or 3X concentrated (300 µM) monastrol in DMSO was included. The plate was read in kinetic-mode as described earlier (Figure 6).

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

References

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4. Brune M, Hunter JL, et al (1994) *Biochemistry* 33: 8262–8271.

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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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