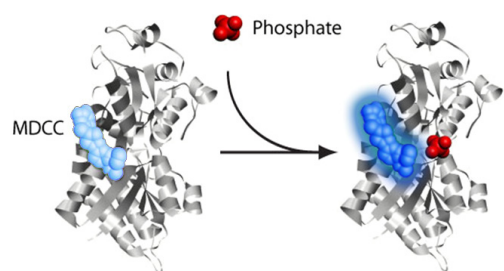


# Phosphate Sensor for sensitive detection of GTPase activity

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 measured GTPase activity by performing kinetic analyses of RhoA and cdc42 GTPases. Phosphate Sensor is 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.

Signal transduction between cells requires the signal to traverse the membrane to activate cellular responses. G proteins, which utilize the hydrolysis of GTP activity to disperse this message inside the cell, mediate these actions. These GTPases play key roles in a wide variety of cellular activities, including cell growth, differentiation, secretion, and protein trafficking [1] and a central role in cellular functions implicates them in cancer [2], neurological disorders [3], and cardiovascular disease [4]. As additional potential drug targets arise, methodologies for measuring activity become vital.

We describe here the use and optimization of a simple, flexible reagent for measurement of GTPase activity. Phosphate Sensor is a phosphate-binding protein modified with a fluorophore [5]. As the sensor binds free inorganic phosphate, fluorescence intensity increases (Figures 1 and 2). 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.

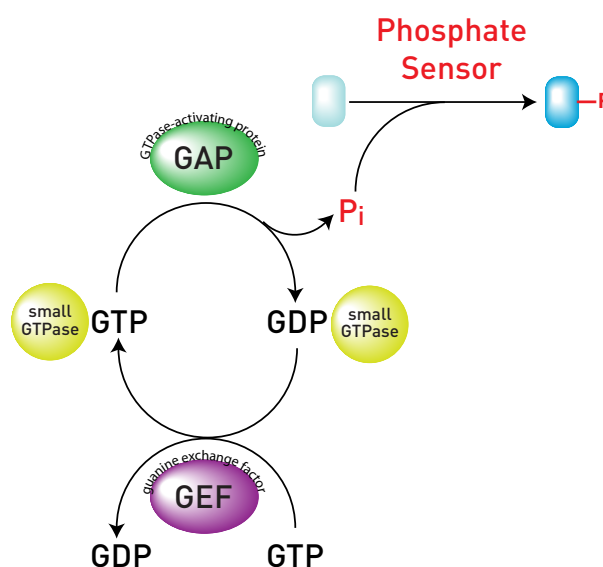
# Phosphate Sensor for sensitive detection of GTPase activity

**Note:** Phosphate is common in biological materials, buffers, and reagents (including GTP), as well as on plastics and glassware. Care should be taken to minimize contamination from these sources when using Phosphate Sensor. GTP sources that contain a significant amount of free phosphate ( $GTP + P_i$ ) should not be used. Instead, an ultra-pure quality GTP 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).

## Experimental Procedures

### Materials and methods

HIS-RhoA (WT, human, recombinant) [Cytoskeleton P/N RH01], HIS-cdc42 (WT, human, recombinant) [Cytoskeleton P/N CD01], p50 RhoGAP (catalytic domain, human, recombinant) [Cytoskeleton P/N GAS01], HIS-hDbs (RhoGEF, DH/PH domain, human, recombinant) [Cytoskeleton P/N GE01], GTP (Sigma P/N G8877), and Phosphate Sensor (Invitrogen P/N PV4406) were used in these experiments. The GTPase reaction buffer consisted of 50 mM Tris pH 7.6, 10 mM  $MgCl_2$ , 100 mM NaCl, 1 mM EDTA, 0.01% Triton<sup>®</sup> X-100, and 1 mM DTT. The Phosphate Sensor detection buffer consisted of 20 mM Tris pH 7.6 and 0.05% Triton<sup>®</sup> X-100. All assays were performed in black 384-well low-volume, round bottom, non-treated plates (Corning P/N 3677).



### Figure 2. GTPase activity of small G proteins and Phosphate Sensor detection.

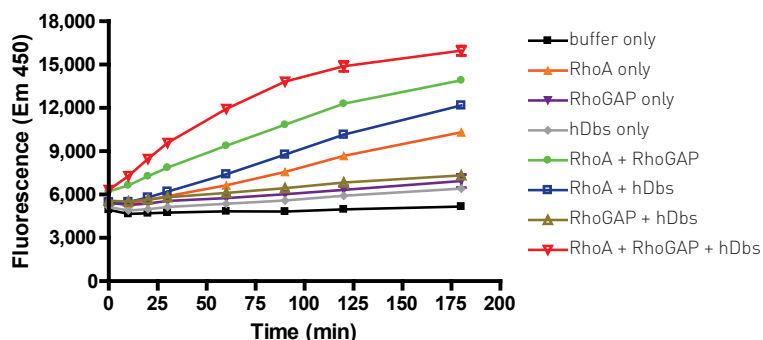
The GTPase activity of small G proteins is limited by the endogenous GTPase activity and the GDP dissociation rate. The endogenous GTPase activity can be enhanced by the addition of a suitable GTPase-activating protein (GAP) and the GDP dissociation rate can be enhanced by addition of a suitable guanine exchange factor (GEF) protein. Phosphate Sensor can be used to measure inorganic phosphate released by the GTPase reaction.

### Kinetic analysis of RhoA GTPase activity

The first evaluation of Phosphate Sensor was an analysis of GAP and GEF stimulation of RhoA GTPase activity in kinetic mode. The 10  $\mu$ L assays were run with 5  $\mu$ L of GTPase reaction buffer or 2X concentrated protein in GTPase reaction buffer followed by addition of 5  $\mu$ L of reaction buffer or 2X concentrated (8  $\mu$ M) GTP in reaction buffer to start the reactions. The final assay concentrations were 1  $\mu$ M HIS-RhoA, 0.5  $\mu$ M p50 RhoGAP,

## Phosphate Sensor for sensitive detection of GTPase activity

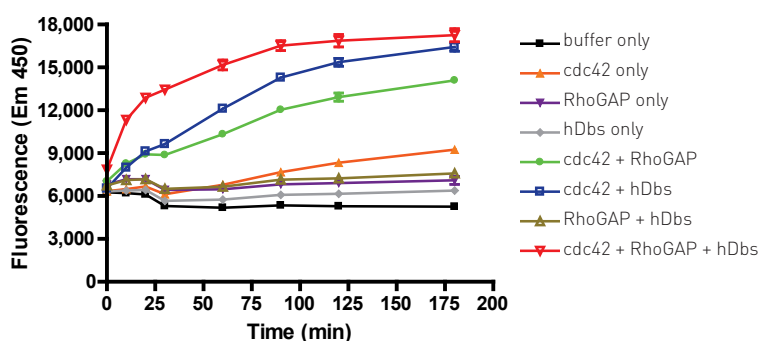
0.5  $\mu\text{M}$  HIS-hDbs, and 4  $\mu\text{M}$  GTP. After the addition of GTP, inorganic phosphate detection was initiated by the immediate addition of 10  $\mu\text{L}$  2X Phosphate Sensor (1  $\mu\text{M}$ ) in Phosphate Sensor detection buffer to 0.5  $\mu\text{M}$  Phosphate Sensor in a volume of 20  $\mu\text{L}$  (see Table 1). The plate was mixed, incubated at room temperature and read on a Tecan Safire2™ microplate reader at excitation 430 (10) nm and emission 450 (10) nm at 0, 10, 20, 30, 60, 90, 120, and 180 minutes (Figure 3).



**Figure 3. Detection of RhoGAP and hDbs (RhoGEF) stimulation of RhoA GTPase activity using Phosphate Sensor.** RhoGAP only, hDbs (RhoGEF) only, or the combination of RhoGAP and hDbs did not stimulate activity as shown by negligible signals above background (buffer only). RhoA alone showed some intrinsic GTPase activity, which was enhanced by the addition of RhoGAP or hDbs and further stimulated by the addition of both RhoGAP and hDbs.

### *Kinetic analysis of cdc42 GTPase activity*

The second evaluation of Phosphate Sensor was an analysis of GAP and GEF stimulation of *cdc42* GTPase activity in kinetic mode. The assay was run as previously described except that a final concentration of 1  $\mu\text{M}$  *cdc42* was utilized instead of RhoA and the plate was read at 0, 10, 20, 30, 60, 90, 120, and 180 minutes (Figure 4).



**Figure 4. Detection of RhoGAP and hDbs (RhoGEF) stimulation of *cdc42* GTPase activity using Phosphate Sensor.** Similar to the previous experiment, RhoGAP alone, hDbs (RhoGEF) alone, or the combination of RhoGAP and hDbs gave negligible signal above the buffer only background. Only very little intrinsic *cdc42* GTPase activity was seen in *cdc42* alone. While activity was greatly enhanced by the addition of RhoGAP or hDbs, kinetic stimulation was achieved by the addition of both RhoGAP and hDbs.

## Phosphate Sensor Method

<i>detection</i>	fluorophore
<i>assay format</i>	384-well
<i>final assay volume</i>	20 $\mu$ L
<i>kinetic format</i>	yes
<i>steps</i>	3
<i>protocol</i>	1. Add 5 $\mu$ L of reaction buffer with or without 2X concentrated protein 2. Add 5 $\mu$ L of GTP in reaction buffer 3. Add 10 $\mu$ L of 2X Phosphate Sensor, read plate immediately

**Table 1. The Phosphate Sensor method.** The Phosphate Sensor assay requires just 3 steps and has the unique ability to read the reaction in “kinetic-mode”.

### 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  $\mu$ M 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  $-80^{\circ}\text{C}$ ) and PNPase (Sigma P/N N8264) to 500 U/mL (dispensed into small aliquots to avoid freeze/thaw cycles and stored at  $-80^{\circ}\text{C}$ ).

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

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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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