PhosphoWorks™ Fluorimetric Phosphate Assay Kit *Red Fluorescence*

Catalog number: 21660, 21661
Unit size: 125 Tests, 1250 Tests

Component	Storage	Amount	
		Cat No. 21660	Cat No. 21661
Component A: Assay Buffer	Freeze (<-15 °C)	1 bottle (5 mL)	10 Bottles
Component B: Phosphate Sensor	Freeze (<-15 °C), Minimize light exposure	1 vial (lyophilized powder)	10 vials (lyophilized powder)
Component C: 1 mM KH2PO4	Refrigerate (2-8 °C), Minimize light exposure	1 vial (1 mL)	1 vial (1 mL)

OVERVIEW

Cells utilize a wide variety of phosphate (Pi) and polyphosphate esters as enzyme substrates, second messengers, membrane structural components and vital energy reservoirs. Phosphate is involved in many biological processes. For example, phosphatases, ATPases and several other enzymes catalyze biochemical reactions in which inorganic phosphate is released from a phosphoester substrate. Detection of many phosphoester-metabolizing enzymes is difficult because suitable substrates are not available. It usually has been necessary to determine inorganic phosphate release using tedious colorimetric assays or radioisotope-based methods. This PhosphoWorks™ Fluorimetric Phosphate Assay Kit has been developed for measuring the activity of any Pi-generating enzyme using our red fluorescent phosphate sensor. The kit provides sensitive detection of Pi, an alternative to hazardous radioactive methods and other less sensitive colorimetric assays. The measurement of Pi is based on the change in the absorbance and fluorescence of our new phosphate sensor. Our kit provides all the essential reagents including phosphate sensor, phosphate standards and assay buffer. The assay is shown to quantitate phosphate in solution at concentrations at least down to 0.1 µM. It can be used to measure the kinetics of phosphate release from phosphatases (such as GTPases and ATPases) by coupling the two enzymatic reactions. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required.

AT A GLANCE

Protocol summary

1. Prepare test samples or Phosphate standards (40 µL)
2. Add equal volume of Assay Buffer (40 µL)
3. Add Phosphate Sensor working solution (20 µL)
4. Incubate at room temperature for 15 to 60 minutes
5. Monitor fluorescence intensity at Ex/Em = 540/590 nm (Cutoff = 570 nm)

Important To achieve the best results, it's strongly recommended to use the black plates. Thaw one of each kit component at room temperature before starting the experiment.

KEY PARAMETERS

Instrument:	Fluorescence microplate reader
Excitation:	540 nm
Emission:	590 nm
Cutoff:	570 nm
Recommended plate:	Solid black

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.

1. Phosphate Sensor stock solution (125X):
Add 20 µL of DMSO into Phosphate Sensor (Component B) to make 125X Phosphate Sensor stock solution.

PREPARATION OF STANDARD SOLUTION

Phosphate standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/21660

Add 50 µL of 1 mM KH_2PO_4 (Component C) into 950 µL of deionized water or enzyme reaction buffer to get a 50 µM Phosphate standard solution (PS7). Take 50 µM Phosphate standard solution (PS7) and perform 1:2 serial dilutions to get serially diluted Phosphate standards (PS6 - PS1) with deionized H_2O or enzyme reaction buffer.

PREPARATION OF WORKING SOLUTION

Add 20 µL of 125X Phosphate Sensor stock solution into 2.5 mL of sterile H_2O and mix well to make Phosphate Sensor working solution. Avoid potential Pi contamination.

Note Avoid direct exposure of Phosphate Sensor (Component B) to light. Due to the high sensitivity of this assay to Pi, it is extremely important to use Pi-free laboratory ware and reagents.

SAMPLE EXPERIMENTAL PROTOCOL

Run the phosphate assay at pH 6.5 to 7.4

Table 1. Layout of Phosphate standards and test samples in a solid black 96-well microplate. PS=Phosphate Standard (PS1 - PS7, 0.78 to 50 µM), BL=Blank Control, TS=Test Sample.

BL	BL	TS	TS
PS1	PS1	...	...
PS2	PS2	...	...
PS3	PS3		
PS4	PS4		
PS5	PS5		
PS6	PS6		
PS7	PS7		

Table 2. Reagent composition for each well.

Well	Volume	Reagent
PS1 - PS7	40 µL	Serial Dilutions (0.78 to 50 µM)
BL	40 µL	H_2O or Enzyme Reaction Buffer
TS	40 µL	test sample

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1. Prepare Phosphate standards (PS), blank controls (BL), and test samples (TS) according to the layout provided in Tables 1 and 2. For a 384-well plate, use 20 µL of reagent per well instead of 40 µL.

2. Add 40 µL of Assay Buffer (Component A) and 20 µL of Phosphate Sensor working solution to each well of Phosphate standard, blank control, and test samples to make the total Phosphate assay volume of 100 µL/well. For a 384-well plate, add 20 µL of Assay Buffer (Component A) and 10 µL of Phosphate Sensor working solution into each well instead, for a total volume of 50 µL/well.

3. Incubate the reaction mixture at room temperature for 15 to 60 minutes.

4. Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 nm (Cutoff = 570 nm).

EXAMPLE DATA ANALYSIS AND FIGURES

The reading (RFU) obtained from the blank standard well is used as a negative control. Subtract this value from the other standards' readings to obtain the base-line corrected values. Then, plot the standards' readings to obtain a standard curve and equation. This equation can be used to calculate Pi samples. We recommend using the Online Linear Regression Calculator which can be found at:

https://www.aatbio.com/tools/linear-logarithmic-semi-log-regression-online-calculator

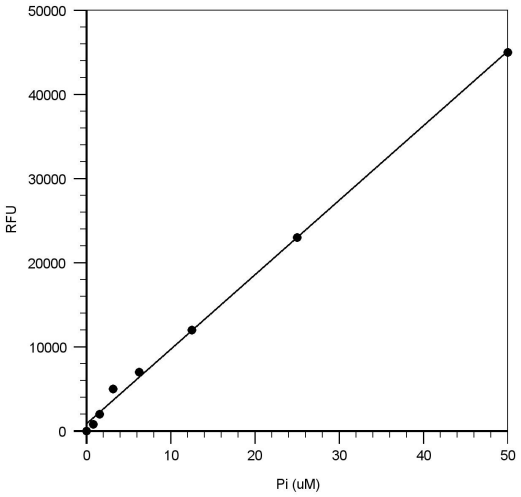

Figure 1. Phosphate dose response was measured with PhosphoWorks™ Fluorimetric Phosphate Assay Kit on a solid black 96-well plate using a Novostar microplate reader (BMG Labtech).

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