PhosphoWorks™ Fluorimetric Phosphate Assay Kit

Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 21660 (125 Assays), 21660B (1,250 assays)	Keep in freezer and protect from light	Fluorescence microplate readers

Introduction

Cells utilize a wide variety of phosphate and polyphosphate esters as enzyme substrates, second messengers, membrane structural components and vital energy reservoirs. Phosphate is involved in many biological processes. The detection of many phosphoester–metabolizing enzymes is difficult because suitable substrates are not available. It is usually necessary to determine inorganic phosphate release using tedious colorimetric assays or radioisotope based methods.

This PhosphoWorks™ Fluorimetric Phosphate Assay Kit has been developed to measure the activity of any Pi-generating enzyme using our red fluorescent phosphate sensor. The sensitive detection of Pi is based on the change in the absorbance or fluorescence of the new phosphate sensor. It is an alternative to hazardous radioactive methods and other less sensitive colorimetric assays. Our kit provides all the essential reagents including phosphate sensor, phosphate standards, and assay buffer. The assay is shown to quantitate phosphate as low as 0.1 µM. It can be used to measure the kinetics of phosphate release from phosphatases by coupling the two enzymatic reactions. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format.

Kit Key Features

Dual Reading Modalities:	Can be monitored by using either a fluorescence reader (Ex/Em = 540/590 nm) or an absorbance reader (570 nm).
Universal:	Can be used for monitoring any biological processes that either generate or consume phosphate.
Continuous:	Easily adapted to automation without mixing or separation.
Non-Radioactive:	No special requirements for waste treatment.
Use of Native Substrates:	Substrates can be proteins, peptides, nucleotides, sugars, organic molecules or inorganic salts.

Kit Components

Components	**Amount**	
	Cat. # 21660	**Cat. # 21660B**
	125 assays (96-well) 250 assays (384-well)	1,250 assays (96-well) 2,500 assays (384-well)
Component A: Assay Buffer	1 Bottle (5 mL)	1 Bottle (50 mL)
Component B: Phosphate Sensor	1 vial (lyophilized powder)	1 vial (lyophilized powder)
Component C: 1 mM KH_2PO_4	1 vial (1 mL)	1 vial (1 mL)

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare test samples (40 µL) along with serially diluted phosphate standards (40 µL) from 1 mM KH_2PO_4 → Add equal volume of Assay Buffer (40 µL) → Add Phosphate Sensor working solution (20 µL) → Incubate at room temperature for 15 minutes to 1 hour → Monitor fluorescence intensity at Ex/Em = 540/590 nm

Note: To achieve the best results, it's strongly recommended to use the black plates.

1. Prepare Phosphate Sensor working solution:

1.1 Thaw one of each kit component at room temperature before use.

1.2 Make 125X Phosphate Sensor stock solution: Add 20 µL of DMSO (For Cat. # 21660) or 200 µL of DMSO (for Cat # 21660B) into Phosphate Sensor (Component B) to make 125X Phosphate Sensor stock solution.

1.3 Make Phosphate Sensor working solution: Transfer 20 µL of 125X Phosphate Sensor stock solution (from Step 1.2) into 2.5 mL of sterile H_2O, and mix well
Note 1: Avoid direct exposure of Phosphate Sensor (Component B) to light.
Note 2: Aliquot and store the unused Assay Buffer (Component A) and Phosphate Sensor stock solution (from Step 1.2) at -20 °C. Avoid repeated freeze/thaw cycles and potential Pi contamination.
Note 3: Due to the high sensitivity of this assay to Pi, it is extremely important to use Pi-free laboratory ware and reagents.

2. Prepare serially diluted phosphate standards and test samples:

2.1 Prepare phosphate standard: 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. Then take 200 µL of 50 µM phosphate standard solution to perform 1:2 serial dilutions to get 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µM serially diluted phosphate standards.

2.2 Add phosphate containing samples and serially diluted phosphate standards into a solid black 96-well microplate according to Tables 1 and 2.

Table 1 Layout of phosphate standards and test samples in a solid black 96-well microplate

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

Note: PS=Phosphate Standard, BL=Blank Control, TS=Test Sample.

Table 2 Reagent composition for each well

Phosphate Standards	**Blank Control**	**Test Sample**
Serial Dilutions*: 40 µL	H_2O or Buffer: 40 µL	40 µL

Note: *Add the serial dilutions of phosphate standard from 0.1 µM to 50 µM into wells from PS1 to PS7.

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3. Run PhosphoWorks™ fluorimetric phosphate assay:

Warning: *Run the phosphate assay at pH 6.5 to 7.4.*

3.1 Add 40 µL of Assay Buffer (Component A) and 20 µL of Phosphate Sensor working solution (from Step 1.3) into each well of phosphate standard, blank control, and test samples (see Step 2.2) to make the total phosphate assay volume of 100 µL/well.
Note: For a 384-well plate, add 20 µL of sample, 20 µL of Assay Buffer (Component A) and 10 µL of Phosphate Sensor working solution (from Step 1.3) into each well.

3.2 Incubate the reaction mixture at room temperature for 15 minutes to 1 hour.

3.3 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 nm.

Data Analysis

The fluorescence reading in blank wells (with H_2O or buffer only) is used as a control, and is subtracted from the values of those wells with the phosphate standards and test samples. A phosphate standard curve is shown in Figure 1. Calculate the phosphate concentrations of the samples according to the phosphate standard curve.

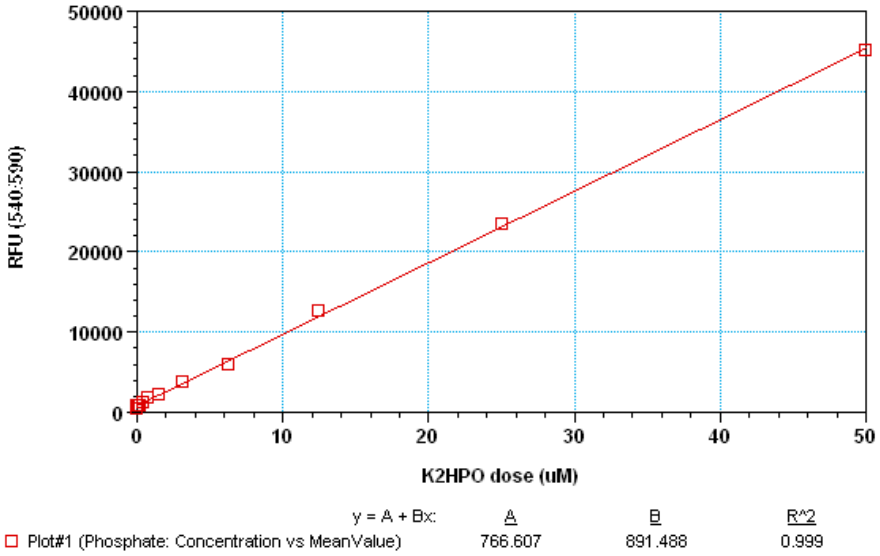

Figure1. Phosphate dose response was measured with the PhosphoWorks™ Fluorimetric Phosphate Assay Kit on a solid black 96-well plate using a Novostar microplate reader (BMG Labtech). As low as 0.1 µM phosphate can be detected with 1 hour incubation.
Note 1: The phosphate standard curve is used to calibrate the variation of different instruments and different batches of experiments.
Note 2: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

References

1. Webb MR, Hunter JL. (1992) Interaction of GTPase-activating protein with p21ras, measured using a continuous assay for inorganic phosphate release. Biochem J, 287 (Pt 2), 555.
2. Webb MR. (1992) A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc Natl Acad Sci U S A, 89, 4884.