PhosphoWorks™ Fluorimetric Phosphate Assay Kit

Red Fluorescencec

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: #21660 (125 Assays), #21660B (2,500 assays)	Keep in freezer and avoid 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. For example, phosphatases, ATPases and several other enzymes catalyze biochemical reactions in which inorganic phosphate (Pi) 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 radioisotopebased 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.

Kit Key Features

Dual Reading Modalities:	Can be monitored by either fluorescence (Ex =540 nm and Em = 590 nm) or absorption (570 nm).
Universal:	Can be used for monitoring any biological processes that either generate or consume phosphate.
Continuous:	Easily adapted to automation with no mixing or separation protocols.
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	**#21660** 125 assays (96-well) 250 assays (384-well)	**#21660B** 2,500 assays (96-well) 5,000 assays (384-well)
Component A: Assay Buffer	1 Bottle (5 mL)	2 Bottles (50 mL/bottle)
Component B: Phosphate Sensor	1 Vial (lyophilized powder)	2 Vials (lyophilized powder)
Component C: 1 mM KH_2PO_4	1 Vial (100 μL)	1 Vial (1 mL)

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Assay Protocol for One 96-Well Plate

Brief Summary

Prepare test samples (40 µL) along with phosphate standard dilutions (40 µL) from Component C → Add equal volume of Component A (40 µL)→ Add reconstituted Component B (20 µL) → Incubate at room temperature for 15 min to 1 hr→ Read fluorescence using Ex/Em = 540/590 nm

We strongly recommend that black plates be used for achieving the best results

1. **Prepare assay reagents:**
   1.1 Thaw 1 vial each of all the three components at room temperature before use.

   1.2 ***For 21660***, add 20 µL of DMSO into Component B (Phosphate Sensor), then transfer the whole content into 2.5 mL ddH_2O. Mixed well.

   1.3 ***For 21660B***, add 200 µL of DMSO into Component B (Phosphate Sensor), then transfer the whole content into 25 mL dd H_2O. Mixed well.
   Note 1. Avoid exposing Component B to light directly.
   Note2. Aliquot and store the unused Components A and B (DMSO stock) at -20°C, avoid freeze/thaw cycles and potential Pi contamination.
   Note3. Due to the high sensitivity of this assay for Pi, it is extremely important to use Pi-free laboratory ware and reagents.

2. **Prepare samples:**
   2.1 Prepare phosphate standard: Add 50 µL of 1mM phosphate standard (Component C) in 950 µL of deionized water or enzyme reaction buffer to get 50 µM phosphate solution. Then take 200 µL of 50 µM phosphate solution to perform 1:2 serial dilutions to get 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µM phosphate solutions.

   2.2 Add phosphate containing samples and phosphate standards into a 96-well black based on Tables 1 and 2.

Table 1. Layout of phosphate standard and test samples in a clear 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 Standard	**Blank Control**	**Test Sample**
Serially dilution* 40 µL	H_2O or buffer 40 µL	40 µL

Note: *Add the serially diluted phosphate from 0.1 µM to 50 µM into wells PS1 to PS7.

3. **Run PhosphoWorks™ fluorimetric phosphate assay:**

Warning: *The phosphate assay should be run at pH from 6.5 to 7.4.*

3.1 Add 40 µL of component A and 20 µL of reconstituted Component B (from step 1.2 or 1.3) to each well of the phosphate standard, blank control, and test samples (see step 2.2) so that the total phosphate assay volume is 100 µL/well.
Note: For a 384-well plate, add 20 µL sample, 20 µL Component A and 10 µL of reconstituted Component B per well.

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

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

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 for those wells with the phosphate standards and test samples. The typical data are in Figure 1 (phosphate standard curve). Calculate the phosphate concentration of the samples according to the phosphate standard curve.

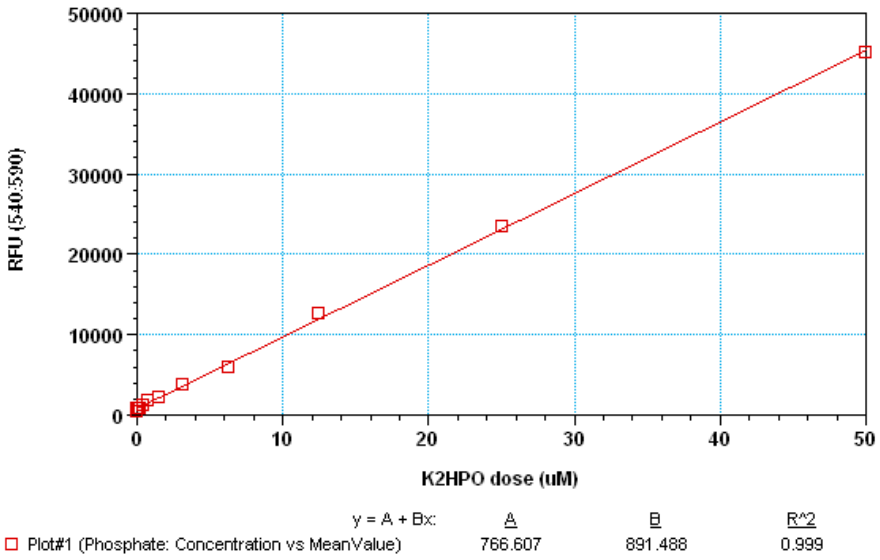

Figure1. Phosphate dose response on 96-well black plate using a Novostar microplate reader (BMG Labtech) measured with the PhosphoWorks™ Fluorimetric Phosphate Assay Kit. As low as 0.1 µM phosphate can be detected with 1 hr incubation time.

Note1: The phosphate standard curve is used to calibrate for the variation of different instruments and for different batches of experiments.
Note2: 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.

Warning: This kit is only sold for the end users. Neither resale nor transfer to a third party is allowed without written permission from ABD Bioquest. Chemical analysis of kit components is strictly prohibited. Please call us at 408-733-1055 or e-mail us at info@abdbioquest.com if you have any questions.