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PhosphoWorks™ MESG Phosphate Assay Kit

UV Absorption

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: #21659 (200 Assays)	Keep in freezer and avoid light	Absorbance microplate reader or spectrophotometer

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 radioisotope-based methods. This PhosphoWorks™ MESG Phosphate Assay Kit has been developed for measuring the activity of any Pi-generating enzyme using MESG reagent. 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 absorbance of MESG by phosphate. In the presence of inorganic phosphate MESG is converted to 2-amino-6-mercapto-7-methlpurine by purine nucleoside phosphorylase (EC 2.4.2.1) with absorption wavelength shift to red. This feature has been used to develop our convenient MESG phosphate assay kit. Our kit provides all the essential reagents including MESG, phosphorylase and reaction buffer. The MESG substrate gives an absorbance increase at 360 nm on phosphorylysis at pH 6.5-8.5, and at pH 7.6 the change in extinction coefficient is 11,000 $M^{-1}cm^{-1}$. The assay is shown to quantitate phosphate in solution at concentrations at least down to 2 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

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.
Convenient:	Formulated to have minimal hands-on time.
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**
Component A: Assay Buffer	1 Bottle (10 mL)
Component B: MESG Substrate	1 Vial (lyophilized powder)
Component C: Purine Nucleoside Phosphorylase (PNP)	1 Vial (lyophilized powder)
Component D: 1 mM KH_2PO_4	1 Vial (1 mL)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare test samples (50 µL) and phosphate standards (50 µL) from Component D → Add assay solution (50 µL) → Incubate at room temperature for 30 min → Read Absorbance at 360 nm

1. Prepare assay reagents:

1.1 Thaw all the four components at room temperature before use.

1.2 Prepare Component B Solution: Add 500 µL of Assay Buffer (Component A) to Component B (MESG Substrate). Mix well by vortex to give Component B Solution.

1.3 Prepare Component C Solution: Add 100 µL of Assay Buffer (Component A) to Component C [(Purine Nucleoside Phosphorylase (PNP)]. Mix well by vortex to give Component C solution.

1.4 Prepare Assay Solution: Add whole volume of Component B Solution (from Step 1.2) and Component C Solution (from Step 1.3) to Component A (Assay Buffer) bottle, mix well to make the assay solution. Place it on ice.
Note 1: This Assay Solution is stable for at least 4 hours on ice. We do not recommend refreezing the assay solution for another assay.
Note 2. UV-transparent plates or cuvettes are required for achieving the desirable results.
Note 3. Due to the high sensitivity of this assay for Pi, it is extremely important to use Pi-free laboratory ware and reagents.

2. Prepare phosphate standards and test samples:

2.1 Prepare Phosphate Standards: Add 50 µL of 1 mM KH_2PO_4 (component D) in 950 µL of deionized water or enzyme reaction buffer to get 50 µM phosphate solution.

2.2 Take 200 µL of 50 µM phosphate solution to perform 1:2 serial dilutions to give 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µM phosphate solutions.

2.3 Add phosphate-containing test samples and phosphate standards into a 96-well clear UV-transparent microplate according to 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**
Serial dilution* (50 µL)	Phosphate-free water or buffer (50 µL)	50 µL

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

3. Run PhosphoWorks™ MESG phosphate assay:

3.1 Add 50 µL/well of Assay Solution (from Step 1.4) to the wells of phosphate standards, blank control, and test samples. Mix the reagents completely.
Note: For a 384-well plate, add 25 µL sample and 25 µL Assay Solution per well.

3.2 Incubate at room temperature for 30 min. Measure absorbance at 360 nm on a microplate reader or a spectrophotometer.
Note: For cuvette assay that requires the total volume larger than 100 µL, you can multiple the volume of sample and assay reagent proportionally before measuring the absorption.

Data Analysis

The absorption (OD reading) in blank wells (with water or buffer only) is used as a control, and is subtracted from the values for those wells with the phosphate standards and test samples. A typical set of data is shown in Figure 1 (phosphate standard curve). Calculate the phosphate concentration of the samples according to the phosphate standard curve.

Note: The phosphate standard curve is used to calibrate for the variation of different instruments and for different assay conditions.

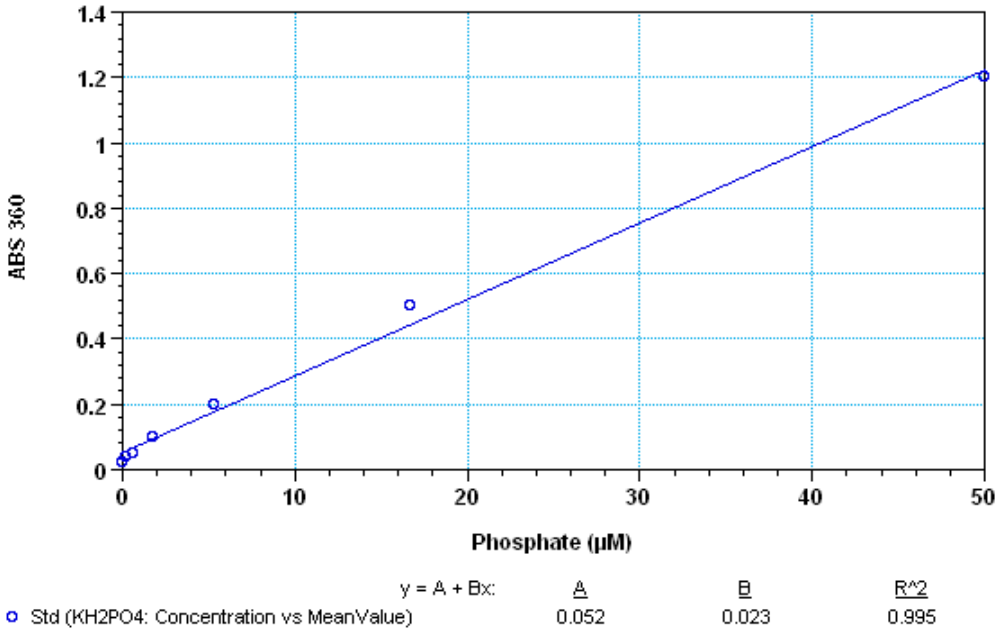

Figure1. Phosphate dose response on 96-well UV plate using a SpectraMax Plus microplate reader (Molecular Devices) measured with the PhosphoWorks™ MESG Phosphate Assay Kit*Colorimetric*. As low as 0.2 µM phosphate can be detected with 30 min incubation time.

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.