

PiBlue™ Phosphate Assay Kits (POPB-500)

DESCRIPTION

The PiBlue™ Phosphate Assay Kit is based on a proprietary formulation of the malachite green dye. The PiBlue™ reagent forms a blue colored complex with free orthophosphate. The rapid color formation from the reaction can be conveniently measured on a spectrophotometer (600 - 660 nm) or on a plate reader. The non-radioactive colorimetric assay kits have been optimized to offer superior sensitivity and prolonged shelf life. The assay is simple and fast, involving a single addition step for phosphate determination. Assays can be performed in tubes, cuvettes or multi-well plates. The assays can be conveniently executed in 96-well plates for high-throughput screening of enzyme inhibitors.

KEY FEATURES

Reagent very stable. Due to our innovative formulation, no precipitation of reagent occurs. Therefore no filtration of reagent is needed prior to assays, as is often required with other commercial kits.

High sensitivity and wide detection range: detection of as little of 20 pmoles of phosphate and useful range between 0.4 μ M and 50 μ M phosphate.

Fast and convenient: single reagent "mix-and-measure" assay allows quantitation of free phosphate within 30 minutes.

Compatible with routine laboratory and HTS formats: assays can be performed in tubes, cuvettes or microplates, on spectrophotometers and plate readers.

Robust and amenable to HTS: Z' factors of 0.7 to 0.9 are observed in 96-well plates. Can be readily automated on HTS liquid handling systems.

APPLICATIONS

Phosphatase Assays: liberation of phosphate from peptide, protein or small molecule substrate.

Lipase Assays: liberation of phosphate from phospholipids

Nucleoside Triphosphatase Assays: liberation of phosphate from nucleoside triphosphates (ATP, GTP, TTP, CTP etc).

Quantitation of Phosphate in phospholipids, proteins and DNAs, etc.

Drug Discovery: high-throughput screen for phosphatase inhibitors.

KIT CONTENTS

Catalog #	Size (assays)	Reagent	Standard
POPB-500	500	50 mL	1 mL 1mM phosphate
POPB-HTS	10,000	1,000 mL	customized

Storage conditions. The kit is shipped at ambient temperature. The PiBlue™ Reagent and standard is stable for 12 months when stored at 4°C.

This protocol can be downloaded online at www.bioassaysys.com.

Precautions: reagents are for research use only. This reagent contains 0.44 M sulfuric acid. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Procedure using 96-well plate:

Important: The reagent must be brought to room temperature and well shaken before use. The PiBlue™ reagent is highly sensitive to phosphate. It is important that all enzyme preparations and assay buffers not contain free phosphate. Lab detergents may contain high levels of phosphate. Make sure that lab wares are washed thoroughly with distilled water and free from contaminating phosphate.

1. Dilution of phosphate standards. Prepare a 1000 μ L 40 μ M phosphate Premix solution by mixing 40 μ L 1 mM phosphate standard with 960 μ L distilled water. Number the tubes. Prepare concentration standards by diluting the Premix as shown in the The phosphate concentrations in the tubes are given below.

Transfer 50 μ L diluted standard in duplicate to wells in a clear-bottom 96-well plate. Store diluted standard at 4°C for future use.

No	Premix + H ₂ O	Final Vol (μ L)	Phosphate Conc (μ M)	pmoles Phosphate in 50 μ L
1	200 μ L + 0 μ L	200	40	2,000
2	160 μ L + 40 μ L	200	32	1,600
3	120 μ L + 80 μ L	200	24	1,200
4	80 μ L + 120 μ L	200	16	800
5	60 μ L + 140 μ L	200	12	600
6	40 μ L + 160 μ L	200	8	400
7	20 μ L + 180 μ L	200	4	200
8	0 μ L + 200 μ L	200	0	0

2. Transfer 50 μ L test sample (e.g. enzyme reaction) in duplicate into wells of the microplate. In the case of enzyme reactions, the reaction may be terminated by either adding a specific inhibitor, or can be stopped directly by the addition of the PiBlue™ Reagent. Reaction buffer can be added as a blank control for the samples.

3. Add 100 μ L of the PiBlue™ Reagent to each well. Mix by tapping the plate.

4. Incubate for 30 min at room temperature for color development.

5. Measure absorbance at 620 nm (600 nm - 660nm) on a plate reader.

Procedure using Cuvette:

For cuvette assays, add 800 μ L Reagent to 400 μ L sample and standards. Perform the assay as described for the microplate assay.

GENERAL CONSIDERATIONS

Incubation time. The chromogenic reaction is completed within 30 min.

Precipitation may occur at high concentrations of phosphate (>100 μ M), or in the presence of high concentrations of e.g. proteins and metals. If precipitation occurs, dilute samples in distilled water and repeat the assay.

Enzyme reaction buffer. Because any exogenous free phosphate would interfere with the assay, it is important to ensure that the protein preparation, the reaction buffer and lab wares employed in the assay should not contain free phosphate. This can be conveniently checked by adding the Reagent to the buffer and measuring the color formation.

DATA ANALYSIS

Plot pmoles phosphate versus OD_{620nm} for the standard curve. Use linear regression analysis to determine amount of free phosphate in the test samples.

LITERATURE

High-throughput Screening

1. Rumsfeld J, Ziegelbauer K, Spaltmann F (2000). High-throughput assay for inorganic pyrophosphatases using the cytosolic enzymes of *Saccharomyces cerevisiae* and human as an example. *Protein Expr Purif.* 18(3):303-9.

2. Cogan EB, Birrell GB, Griffith OH (1999). A robotics-based automated assay for inorganic and organic phosphates. *Anal Biochem.* 271:29-35.

3. Ng DH, Harder KW, Clark-Lewis I, Jirik F, Johnson P (1995). Non-radioactive method to measure CD45 protein tyrosine phosphatase activity isolated directly from cells. *J Immunol Methods.* 179(2):177-85.

4. Fisher DK, Higgins TJ (1994). A sensitive, high-volume, colorimetric assay for protein phosphatases. *Pharm Res.* 11(5):759-63.

Assays for phosphatases, lipases/phospholipids, nucleoside triphosphatases and phosphate in proteins and DNAs.

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5. Harder KW, Owen P, Wong LK, Aebersold R, Clark-Lewis I, Jirik FR (1994). Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase beta (HPTP beta) using synthetic phosphopeptides. *Biochem J*. 298 (Pt 2):395-401.
 6. Queiroz-Claret C, Meunier JC (1993). Staining technique for phosphatases in polyacrylamide gels. *Anal Biochem*. 209(2):228-31.
 7. Geladopoulos TP, Sotiroidis TG, Evangelopoulos AE (1991). A malachite green colorimetric assay for protein phosphatase activity. *Anal Biochem*. 192(1):112-6.
 8. Samizo K, Ishikawa R, Nakamura A, Kohama K (2001). A highly sensitive method for measurement of myosin ATPase activity by reversed-phase high-performance liquid chromatography. *Anal Biochem*. 293:212-5.
 9. Hackney DD, Jiang W (2001). Assays for kinesin microtubule-stimulated ATPase activity. *Methods Mol Biol*. 164: 65-71.
 10. Cen X, Huang Y, Wang R, Chen Z, Wu Z (1998). Simultaneous assay of Ca(2+)-ATPase and Na+, K(+)-ATPase activities of osteoblast rat by malachite green colorimetric method. *Hua Xi Yi Ke Da Xue Xue Bao*. 29(4):427-30.
 11. Mahuren JD, Coburn SP, Slominski A, Wortsman J (2001). Microassay of phosphate provides a general method for measuring the activity of phosphatases using physiological, nonchromogenic substrates such as lysophosphatidic acid. *Anal Biochem*. 298(2):241-5.
 12. Cala SE (1999). Determination of a putative phosphate-containing peptide in calreticulin. *Biochem Biophys Res Commun*. 259(2):233-8.
 13. Gibson NJ, Newton CR, Little S (1997). A colorimetric assay for phosphate to measure amplicon accumulation in polymerase chain reaction. *Anal Biochem*. 254(1):18-22.
 14. Ekman P, Jager O (1993). Quantification of subnanomolar amounts of phosphate bound to seryl and threonyl residues in phosphoproteins using alkaline hydrolysis and malachite green. *Anal Biochem*. 214:138-41.
 15. Lu X, Pearson A, Lunec J (2003). The MYCN oncoprotein as a drug development target. *Cancer Lett*. 197(1-2):125-30.
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16. Ohyama T, Matsubara C, Takamura K (1996). Sensitive densitometry for the determination of platelet-activating factor and other phospholipids in human tears. *Analyst*. 121(12):1943-7.
 17. Matsubara C, Ohyama T, Takamura K (1994). Densitometric quantitation of platelet activating factor and other phospholipids in human saliva using enzyme reaction on a silica plate. *Yakugaku Zasshi*. 114(9):681-90.
 18. Kirchgesser M, Dahlmann N (1990). A colorimetric assay for the determination of acid nucleoside triphosphatase activity. *J Clin Chem Clin Biochem*. 28(6):407-11.
 19. D'Angelo E, Crutchfield J, Vandiviere M (2001). Rapid, sensitive, microscale determination of phosphate in water and soil. *J Environ Qual*. 30(6):2206-2209.

TECHNICAL NOTES

The PiBlue™ Phosphate Assay Kits have been optimized and formulated to provide a sensitive, convenient and robust determination of free phosphate liberated from enzyme reactions and natural sources. Key features of the kits are as follows:

Reagent very stable. Due to our innovative formulation, no precipitation of reagent occurs. Therefore no filtration of reagent is needed prior to assays, as is often required with other commercial kits.

Safe. Non-radioactive assay.

High sensitivity and wide detection range: detection of as little of 20 pmoles of phosphate and useful linear range is between 0.4 μ M and 50 μ M phosphate.

Fast and convenient: single reagent "mix-and-measure" assay allows quantitation of free phosphate within 30 minutes.

Compatible with routine laboratory and HTS formats: assays can be performed in tubes or microplates, on spectrophotometers and plate readers.

Robust and amenable to HTS: Z' factors of 0.7 to 0.9 are observed in 96-well plates. Can be readily automated on HTS liquid handling systems.

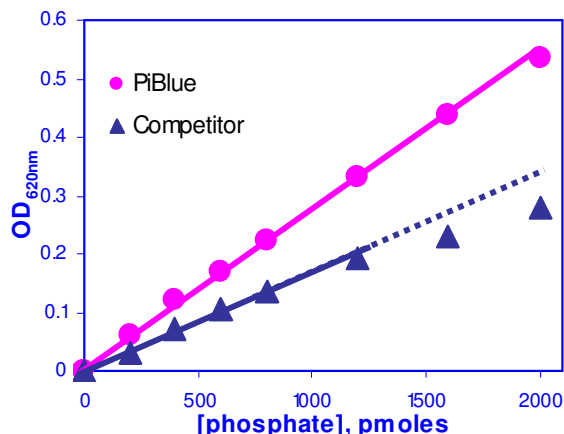


Figure 1. Phosphate standard curve in 96-well plate assay. 100 μ L PiBlue™ Reagent was added to 50 μ L phosphate standard. After 30 minute incubation, the OD at 620 nm was measured on a plate reader. Useful detection range was 0.4 μ M to 50 μ M phosphate. The detection limit estimated from blank control values was 20 pmoles. Coefficient of variance was generally below 6%. Z' factor was > 0.7. The same assay was performed using a competitor's product.