Malachite Green Phosphate Assay Kits (POMG-25H)

DESCRIPTION

The Malachite Green Phosphate Assay Kit is based on quantification of the green complex formed between Malachite Green, molybdate and 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 executed in tubes, cuvettes or multi-well plates. The assays can be conveniently performed in 96- and 384-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 1.6 pmoles of phosphate and useful range between 0.02 μM and 20 μM phosphate.
Fast and convenient: homogeneous "mix-and-measure" assay allows quantitation of free phosphate within 10 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 and 384-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
POMG-25H	2,500	50 mL	1 mL 1mM phosphate
POMG-HTS	>30,000	customized	customized

Storage conditions. The Malachite Green Reagent and standard is stable for one year when stored at 4℃.

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

Precautions: reagents are for research use only. 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 shaken well before use. Before each assay, it is important to check that all enzyme preparations and assay buffers do not contain free phosphate. This can be conveniently done by adding 20 μL of the Malachite Green Reagent to 80 μL sample solution. The blank OD values at 650 nm should be lower than 0.2. If the OD readings are higher than 0.2, check water phosphate level. Double distilled water usually have OD readings lower than 0.1. Lab detergents may contain high levels of phosphate. Make sure that lab wares are free from contaminating phosphate after thorough washes.*

1. Preparation of phosphate standards. Prepare a Premix solution (Tube 1) containing 40 μM phosphate by pipetting 12 μL 1 mM phosphate standard to 288 μL distilled water or enzyme reaction buffer. Number the tubes. Make a serial dilution of the phosphate standard solution provided in the kit as follows. Pipet 200 μL water or reaction buffer to each tube. Transfer 100 μL Premix solution to Tube 2 and mix well. Transfer 100 μL solution from Tube 2 to Tube 3 and mix well. Repeat this procedure to complete the serial dilution. The concentrations in the tubes are given below. Pipette 80 μL standard in duplicate into wells of a clear-bottom 96-well plate. Add blank controls containing water or reaction buffer only.

No	Serial dilution	Final vol (μL)	Phosphate Conc (μM)	Phosphate Conc (pmoles/80 μL)
1	200μL + 100μL	200	40.0	3,200
2	200μL + 100μL	200	13.3	1,066
3	200μL + 100μL	200	4.4	356
4	200μL + 100μL	200	1.5	119
5	200μL + 100μL	200	0.49	40
6	200μL + 100μL	200	0.16	13
7	200μL + 100μL	300	0.054	4.4
8	200μL	200	0	0

2. Transfer 80 μL test sample (e.g. enzyme reaction) into wells of the microplate. In the case of enzyme reactions, the reaction may be terminated by adding a specific inhibitor, or can be stopped directly by the addition of the Malachite Green Reagent.

3. Add 20μL of Malachite Green Reagent to each well. Mix gently by tapping the plate.

4. Incubate for 10 min at room temperature for color development.

5. Measure absorbance at 600 nm - 660nm on a plate reader. Reading is preferably done between 10 and 20 min.

For assays in 384-well plates, the procedures are the same, except that the volume of the standard and sample solution should be 40 μL and that of the Malachite Green Reagent should be 10 μL.

For cuvette assays, add 200 μL Reagent to 800 μL sample.

GENERAL CONSIDERATIONS

Incubation time. The chromogenic reaction is fast and is completed within 10 min. The signal is best read between 10 and 20 min.
Precipitation may occur at high concentrations of phosphate (>100 μM), or in the presence of high concentrations of e.g. proteins and metals. In this case, dilute samples in distilled water and repeat the assays.
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 Malachite Green Reagent to the buffer and measuring the color formation.

Liquid disposal. The Malachite Green Reagent contains 1M sulfuric acid. It is recommended that the waste liquid be neutralized with 1 N NaOH at about equal volume.

DATA ANALYSIS

Plot pmoles phosphate versus OD_{650nm} for the standard curve. Use non-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

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, Sotiroudis 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 colorimetic 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.

Clinical and General Diagnosis

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 Malachite Green Phosphate Assay kits have been optimized and formulated to provide a sensitive, convenient and robust quantitation 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 1.6 pmoles of phosphate and 0.02 µM to 40 µM phosphate.
Fast and convenient: homogeneous "mix-and-measure" assay allows quantitation of free phosphate within 20 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 and 384-well plates. Can be readily automated on HTS liquid handling systems.

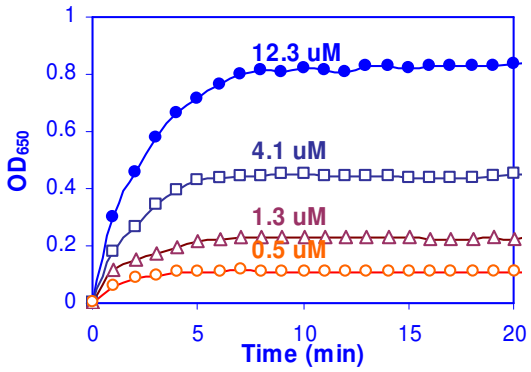

Figure 1. Kinetics of the chromogenic reaction. 80 µL of standard phosphate solution was transferred to a clear-bottom 96-well plate. 20 µL of the Malachite Green Reagent was added and OD at 650 nm was read every minute. The chromogenic reaction was completed within 10 minutes and the signal was stable between 10 and 20 minutes.

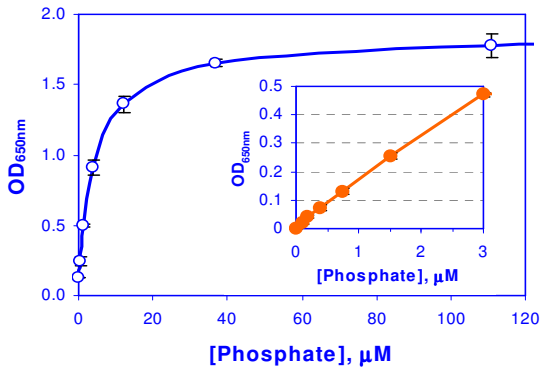

Figure 2. Phosphate standard curve. The reaction was set up as described in Figure 1. After 10 minute incubation, the OD at 650 nm was read. The reaction became saturated at phosphate concentration > 40 µM. Data are presented as mean ± SD (n = 3). Useful detection range was 0.02 to 20µM phosphate. Insert: Linear relationship between OD_{650nm} and phosphate concentration between 0 and 3 µM. Data are presented as mean ± SD (n = 3). The detection limit was estimated from blank values to be 1.6 pmoles. Coefficient of variance was generally below 5%. Z' factor was > 0.7.