



Malachite Green Phosphate Assay Kit

Catalog Number KA1331

96 assays

Version: 08

Intended for research use only

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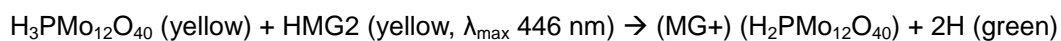
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Introduction

Principle of the Assay

Malachite Green Phosphate Assay Kit provides a fast, reproducible, and non-radioactive method for measuring inorganic free phosphate in aqueous solutions. This simple assay method is based on the complex formed between malachite green molybdate and free orthophosphate under acidic conditions.^{1,2}



The formation of the green molybdophosphoric acid complex measured at 620-640 nm is directly related to the free organic phosphate concentration.² Applications for this assay include quantification of phosphorylation and phosphate release from protein phosphatase substrates.³ This assay measures only inorganic free phosphate; lipid-bound or protein-bound phosphates must first be hydrolyzed and neutralized prior to measurement. Overall, this assay is a reliable and suitable means of detecting and quantifying minimal amounts of inorganic free phosphate in acidic environments and is amenable to high-throughput screening applications.⁴ The assay is formatted to low-volume 96-well plates, but could easily be modified for use in 96-well, 384-well, or cuvette-based assays.

General Information

Materials Supplied

List of component

Item	Quantity/Size
MG Phosphate Standard (1 M)	100 μ L
MG Blue Solution	1.8 mL x 2
MG Acidic Solution	600 μ L x 2
96-Well Solid Plate (low volume)	2 plates
96-Well Cover Sheet	2 covers

Storage Instruction

This kit will perform as specified if stored at temperatures outline in the table below, and used before the expiration date indicated on the outside of the box.

Item	Storage
MG Phosphate Standard (1M)	Room Temperature
MG Blue Solution	4°C (in the Dark)
MG Acidic Solution	4°C (in the Dark)
96-Well Solid Plate (low volume)	Room Temperature
96-Well Cover Sheet	Room Temperature

Materials Required but Not Supplied

- ✓ A plate reader capable of measuring absorbance between 620 nm and 650 nm.
- ✓ Adjustable pipettes and a repeating pipettor.
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable.
- ✓ Assay Buffer - Assay Buffer is not supplied in the Malachite Green Phosphate Assay Kit as the buffer will be highly dependent upon the protein phosphatase used in the assay. Refer to the Interference Chart (Table 2) for a complete list of tested interfering and compatible compounds. Prepare fresh buffer as needed.

Precautions for Use

- Please read these instructions carefully before beginning this assay.

- **Warning**
This product is for research use only. Not for human or veterinary diagnostic or therapeutic use.

- **General Information**
 - ✓ The kit components may be stored at room temperature prior to use. For long term storage, we recommend 4°C.
 - ✓ It is not necessary to use all the wells on the plate at one time.
 - ✓ It is recommended that a standard curve should be run at least in duplicate every time.
 - ✓ For each assay it is recommended that two blanks (Blk) be used.

- **Pipetting Hints**
 - ✓ It is recommended that an adjustable pipette be used to deliver reagents to the wells. This saves time and helps to maintain more precise times of incubation.
 - ✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
 - ✓ Do not expose the pipette tip to the reagent(s) already in the well.

- **Safety Data**
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet.

Assay Protocol

Reagent Preparation

- Standard Curve Preparation

Dilute the 1 M MG Phosphate Standard 1:100 by adding 10 μL to 990 μL Assay Buffer (or water). *Note: Assay Buffer is not included in the Malachite Green Phosphate Assay Kit as the assay buffer used will be highly dependent upon the protein phosphatase being used. Do NOT use a phosphate-based Assay Buffer in the Malachite Green Phosphate Assay. UltraPure water may be used in place of Assay Buffer. The concentration of this solution (tube D1, Bulk Standard) will be 10 mM. Store at 4°C; this standard will be stable for one day.*

To prepare the standard curve for use: Obtain eight clean test tubes and number them #D2 and #D3 (dilution tubes) and #S1 through #S6 (standard tubes). Aliquot 990 μL Assay Buffer (or water) to tube #D2 and 500 μL to tubes D3 and S1 through S6. Transfer 10 μL of the Bulk Standard (tube #D1, 10 mM) to tube #D2 and mix thoroughly; the phosphate concentration will be 100 μM . Transfer 500 μL of #D2 into #D3 and mix thoroughly; the phosphate concentration will be 50 μM . Serially dilute the standards by removing 500 μL from tube #D3 and placing in tube #S1; mix thoroughly. Next, remove 500 μL from tube #S1 and place it into tube #S2; mix thoroughly. Repeat this process for tubes #S3- S5, leaving #S6 as the blank. These diluted standards should not be stored for more than 24 hours for use in the assay.

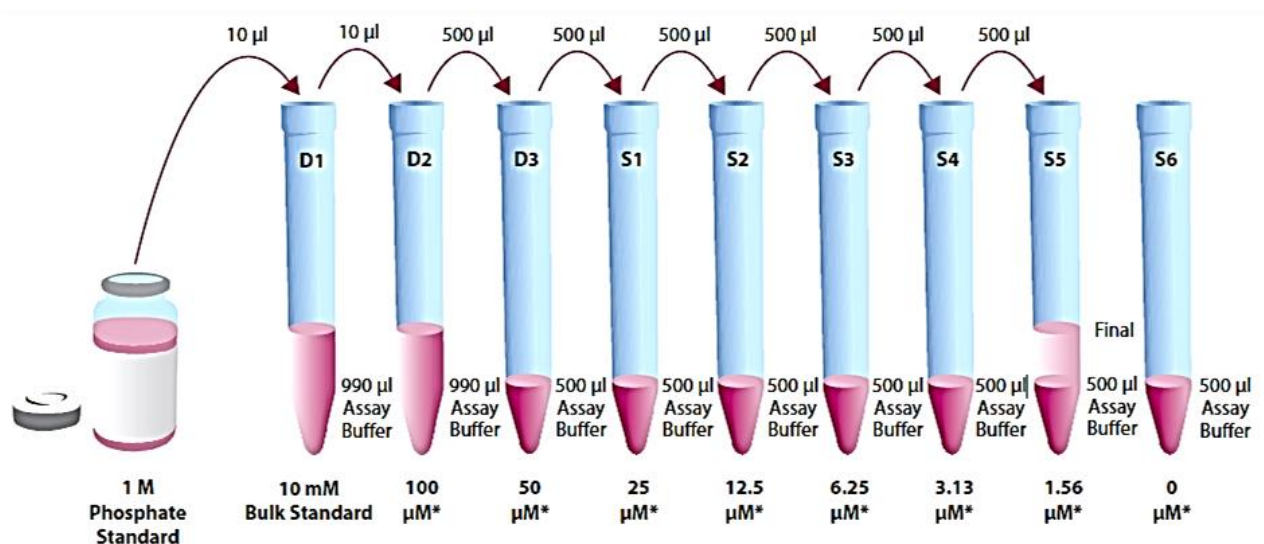


Figure 1. Phosphate standard curve dilutions

*Corresponding nmoles/50 μ L values can be found in Table 1. *Note: UltraPure water may be used in place of Assay Buffer.*

Phosphate Standard	Phosphate μ M Concentration	nmoles/50 μ L
S1	25	1.25
S2	12.5	0.63
S3	6.25	0.31
S4	3.13	0.16
S5	1.56	0.08
S6 (Blank)	0	0

Table 1. Phosphate standard concentrations

Assay Procedure

- **Plate Set Up**

There is no specific pattern for using the wells on the plate. A typical layout of Phosphate Standards and samples to be measured in duplicate is given in Plate Layout. We suggest you record the contents of each well in the template sheet provided.
- **Performing the Assay**
 1. Prepare the six point standard curve (see Figure 1).
 2. Apply 50 μ L of the Phosphate Standards (vial #S1 through S5), samples, and blank (vial #S6; Assay Buffer or UltraPure water) to each well. *Note: The assay volume may be increased or decreased by adding proportionately larger or smaller volumes of sample, MG Acidic Solution, and MG Blue Solution. The standard curve solutions should have the same final volume as the samples. Depending on the Assay Buffer, the D3 dilution (2.5 nmol/50 μ L) can be in the linear range of the Malachite Green Assay. You may want to include this dilution in the standard curve.*
 3. Add 5 μ L of MG Acidic Solution to each well. Mix by gently tapping, and incubate for 10 minutes at room temperature.
 4. Add 15 μ L of MG Blue Solution to each well. Mix by gently tapping, and incubate for 20 minutes at room temperature.
 5. Determine the absorbance of each well using a microplate reader set to 620 nm.

Data Analysis

Calculation of Results

Subtract the blank (Assay Buffer or UltraPure water) from each of the standards and samples. Plot the average absorbance of each phosphate standard as nmol versus A_{620} . Create a standard curve and perform linear regression. Use the regression line to solve for sample concentrations.

The standard curves presented here are examples of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use these ones to determine the values of your samples. Depending on the development conditions, the purity of the water and the Assay Buffer used, your results could differ from the data presented below.

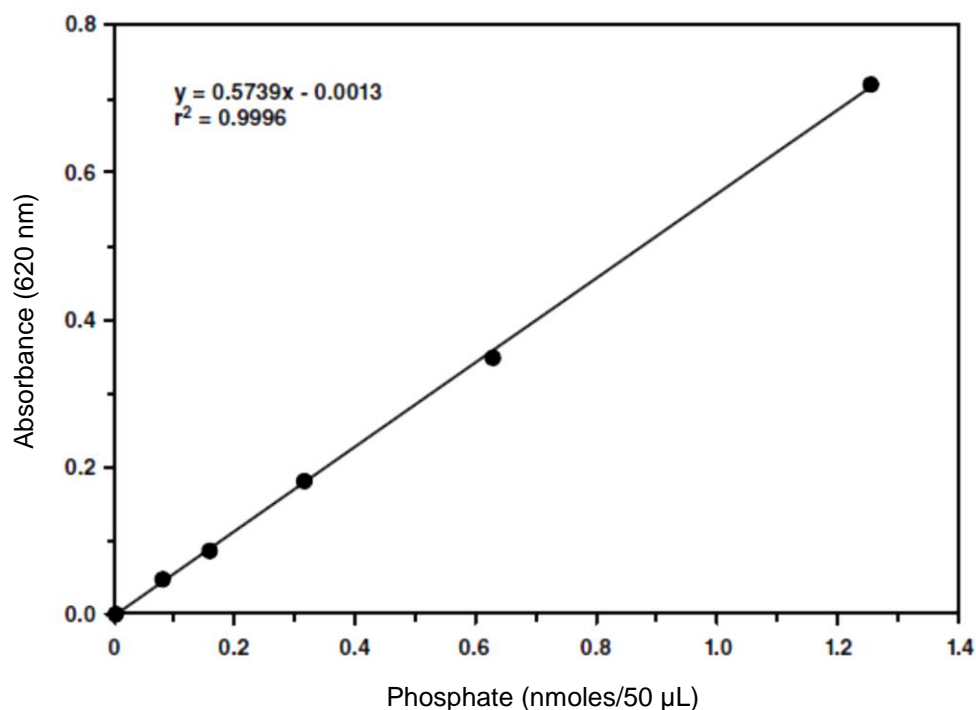


Figure 2. Typical standard curve in an Assay Buffer

Note: The Phosphate Standards #S1-S6 were diluted in Assay Buffer (50 mM HEPES, 1 mM DTT, 1 mM EDTA, 0.05% NP-40 with 0.1 mg/mL BSA) and run in triplicate (50 µL/well).

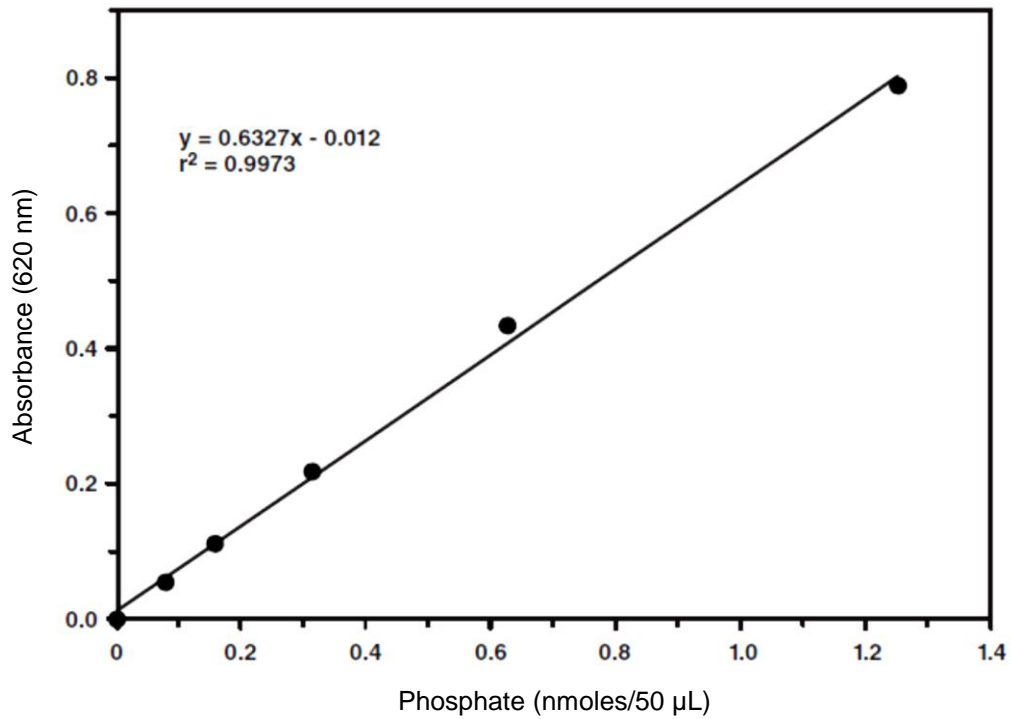


Figure 3. Typical standard curve in Milli-Q water

Note: The Phosphate Standards #S1-S6 were diluted in fresh Milli-Q water and run in triplicate (50 µL/well).

Performance Characteristics

- Interferences

		Concentration	Effect
Common Detergents	Triton X-100	0.1%	Increased blank, Decreased signal
	Polysorbate 20	0.1%	None
		1%	Increased signal
	NP-40	1%	Increased signal
		≥3%	Not recommended
	SDS	0.01%	Increased signal
Common Reagents	Glycerol	3%	None
		≥10%	Not recommended
	DMSO	10%	None
	Ethanol	10%	Increased signal
	Methanol	10%	None
	BSA	0.3 mg/mL	Decreased signal
	EDTA	10 mM	Increased signal
	Dithiothreitol (DTT)	3 mM	None
	β-Mercaptoethanol	10 mM	None
	Na ₃ VO ₄	1 mM	Increased signal
	NaF	10 mM	None
	NaCl	100 mM	None
	KCl	100 mM	Decreased signal
	CaCl ₂	10 mM	None

Table 2. Common Detergents and Reagents and their effect in the Malachite Green Assay.

Reagents were tested in the presence of 0, 0.08, 0.16, 0.31, 0.63, 1.25, and 2.5 nmol phosphate and compared to the Standard Curve diluted in UltraPure MilliQ water. Assay volumes were 50 µL prior to the addition MG Acidic and MG Blue solutions.

Resources

Troubleshooting

Problem	Recommended Solutions
High background	The malachite green assay is very sensitive; soaps and detergents may cause high background; all containers that come into contact with any solutions used in the assay should be triple washed with distilled water prior to use; be sure to add reagents in the correct order; using fresh Milli-Q water to prepare the standard curves and buffers has been shown to decrease background; consult Table 2 to determine if the Assay Buffer may interfere.
Precipitation	Divalent cations (Magnesium, Copper, Zinc, and Calcium) can form phosphate salts and have low water solubility; to avoid precipitation, dilute the phosphate standard to 10 mM with cation-free buffer before making dilutions into buffer; check the concentration of the purified protein and substrate (consider investigating other concentrations); if the standard curve or blank samples (Assay Buffer only) have precipitate, check the Assay Buffer components and reference Table 2 to determine if the Assay Buffer contents may interfere with the assay; high concentrations of phosphate in the sample can also cause precipitation. Dilute the sample and rerun the assay.
High signal in all wells	Check to make sure the standard curve was properly made (See Table 1). Use a fresh source of Milli-Q water that is free of phosphate to prepare any dilutions. Residual soaps and detergents will cause high background (be sure that containers that come into contact with any solutions used in the assay are thoroughly rinsed prior to use).
High signal in sample wells	If the standard curve gives reasonable values and the experimental samples give high signal, revisit the protein used in the assay. The Malachite Green Assay is designed to be used with purified proteins (check purity of the protein that is being assayed). Phosphates in buffers can increase the signal (check that the purified protein is in a suitable Assay Buffer that does not contain phosphates). Be sure to include proper controls with each assay. Revisit the amount of pure protein in the assay (saturating amounts of protein can cause precipitation of sample in the wells, resulting in high signal).
Weak signal in sample wells	Increase the amount of purified protein and/or substrate, or increase the incubation time with the enzyme prior to addition of MG Acidic Solution. Make sure the optimal incubation temperature for the protein assayed is being used. Prepare fresh Assay Buffer using UltraPure Milli-Q water.

References

1. D'Angelo, E., Crutchfield, J., Vandiviere, M. Rapid, sensitive, microscale determination of phosphate in water and soil. *J. Environ. Qual.* 30, 2206-2209 (2001).
2. O'Toole, M., Lau, K. T., Shepherd, R., et al. Determination of phosphate using a highly sensitive paired emitter-detector diode photometric flow detector. *Analytica Chimica Acta* 597, 290-294 (2007).
3. Maehama, T., Taylor, G. S., Slama, J. T., et al. A sensitive assay for phosphoinositide phosphatases. *Anal. Biochem.* 279, 248-250 (2000).
4. Attin, T., Becker, K., Hannig, C., et al. Suitability of a malachite green procedure to detect minimal amounts of phosphate dissolved in acidic solutions. *Clinical Oral Investigations* 9(3), 203-207 (2005).

Plate Layout

	1	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6		
A	2	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6		
B	3								
C	4								
D	5								
E	6								
F	7								
G	8								
H	9								
	10								
	11								
	12								