Malachite Green Phosphate Assay Kit

Item No. 10009325

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Materials Supplied

Item Number	Item	Quantity/Size	Storage
10011141	MG Phosphate Standard (1M)	1 vial/100 µl	Room Temperature
10011142	MG Blue Solution	2 vials/1.8 ml	4°C (in the Dark)
10011143	MG Acidic Solution	2 vials/600 µl	4°C (in the Dark)
10011144	96-Well Solid Plate (low volume)	2 plates	Room Temperature
400012	96-Well Cover Sheet	2 covers	Room Temperature

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at temperatures outlined in the Materials Supplied, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 620 nm and 650 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. 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, on page 12) for a complete list of tested interfering and compatible compounds. Prepare fresh buffer as needed.

INTRODUCTION

About This Assay

Cayman's Malachite Green 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]

$$H_3PMo_{12}O_{40}\ (\text{yellow}) + \text{HMG2}\ (\text{yellow},\ \lambda_{max}\ 446\ \text{nm}) \longrightarrow (\text{MG+})\ (H_2PMo_{12}O\text{-}40) + 2H\ (\text{green})$$

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.

ASSAY PROTOCOL

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 Figure 1, below. We suggest you record the contents of each well on the template sheet provided (see page 15).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	3	3	11	11	19	19	27	27	35	35
B	S2	S2	4	4	12	12	20	20	28	28	36	36
C	S3	S3	5	5	13	13	21	21	29	29	37	37
D	S4	S4	6	6	14	14	22	22	30	30	38	38
E	S5	S5	7	7	15	15	23	23	31	31	39	39
F	S6	S6	8	8	16	16	24	24	32	32	40	40
G	1	1	9	9	17	17	25	25	33	33	41	41
H	2	2	10	10	18	18	26	26	34	34	42	42

S1-S6 - Standards
1-42 - Samples

Figure 1. Sample plate format

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.

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.

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.

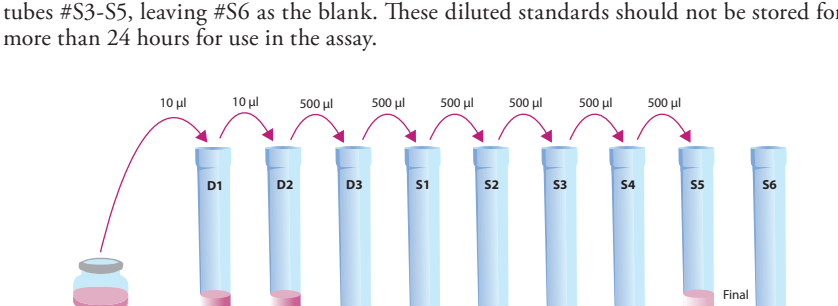


Figure 2. Phosphate standard curve dilutions

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

Phosphate Standard	Phosphate µM Concentration	nmoles/50 µl
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

Performing the Assay

1. Prepare the six point standard curve (see Figure 2 on page 8).
2. Apply 50 µl 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 µl) 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 µl of MG Acidic Solution to each well. Mix by gently tapping, and incubate for 10 minutes at room temperature.
4. Add 15 µl 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.

ANALYSIS

Calculations

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.

Performance Characteristics

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.

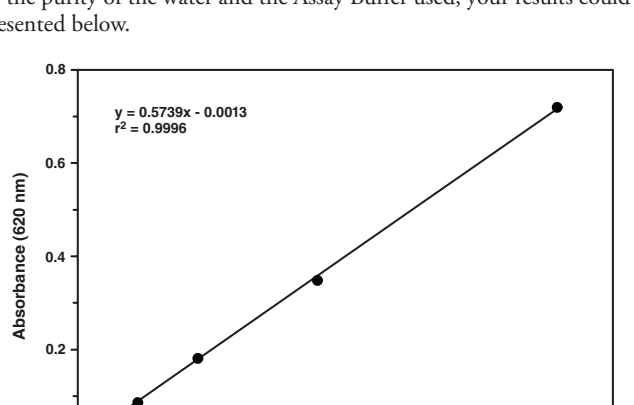


Figure 3. Typical standard curve in an Assay Buffer

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).

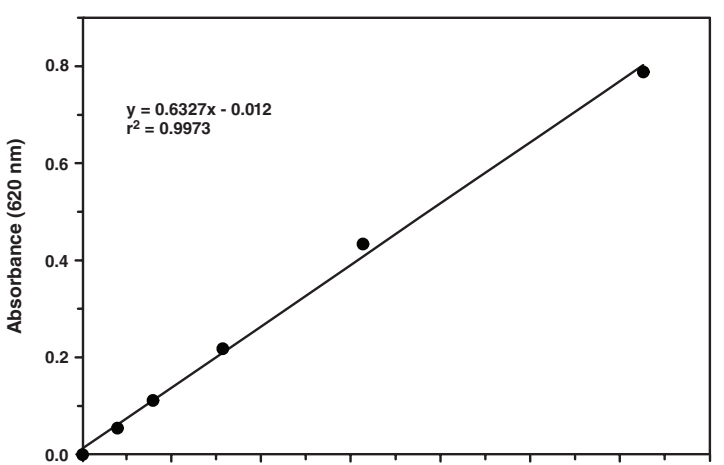


Figure 4. Typical standard curve in Milli-Q water

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

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_3VO_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.

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, on page 9). 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).

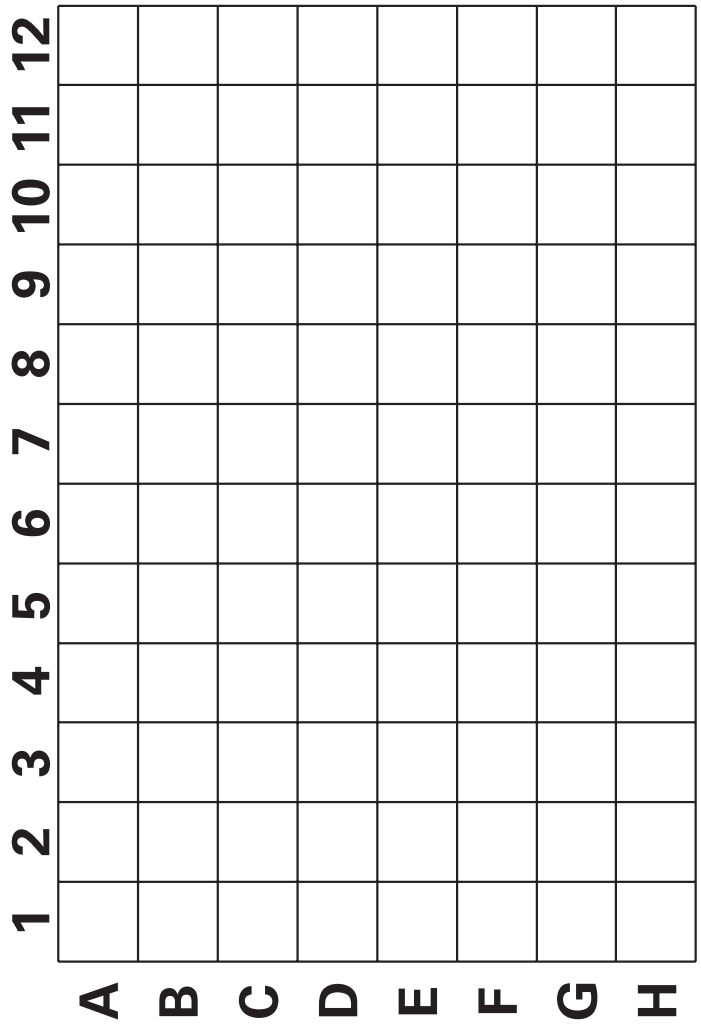
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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