3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Malachite Green Phosphate Assay Kit

Catalog Number **MAK307**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product 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.

This kit may be used to measure the liberation of free orthophosphate in the following 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.

The non-radioactive colorimetric assay kit has 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 multiwell plates. The assays can be conveniently performed in 96 and 384 well plates for high-throughput screening of enzyme inhibitors.

Key Features:

- Reagent is very stable: Due to the 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 0.02–40 μM phosphate.
- Fast and convenient: homogeneous “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 0.7–0.9 are observed in 96 and 384 well plates. Can be readily automated on HTS liquid handling systems.

Components
The kit is sufficient for 2,500 assays in 96 well plates.

Reagent A Catalog Number MAK307A	50 mL
Reagent B Catalog Number MAK307B	1 mL
1 mM Phosphate Standard Catalog Number MAK307C	1 mL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader.

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Working Reagent: Each 96 well plate assay requires 20 μL of Working Reagent. Prepare a sufficient volume of Working Reagent by mixing 100 volumes of Reagent A and 1 volume of Reagent B (e.g., 5 mL of Reagent A and 50 μL of Reagent B). Working Reagent is stable for at least 1 day at room temperature.
Notes: The reagent must be brought to room temperature 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 Working Reagent to 80 μL of sample solution.

The blank OD values at 620 nm should be <0.2. If the OD readings are >0.2, check water phosphate level. Double distilled water usually has OD readings <0.1. Lab detergents may contain high levels of phosphate. Make sure labware is free from contaminating phosphate after thorough washes.

Storage/Stability
The kit is shipped at ambient temperature. Store all components at 2–8 °C upon receiving.

Procedure
All samples and standards should be run in duplicate.

Assay Reaction
1. Preparation of phosphate standards. Prepare a Premix solution containing 40 μM phosphate by pipetting 40 μL of the 1 mM phosphate standard into 960 μL of ultrapure water or enzyme reaction buffer. Number the tubes (see Figure 1).

Figure 1.
Preparation of Standards

#	Premix + water	Final Vol (μL)	Phosphate Conc (μM)	pmoles Phosphate in 50 μL
1	200 μL + 0 μL	200	40	2,000
2	160 μL + 40 μL	200	32	1,600
3	120 μL + 80 μL	200	24	1,200
4	80 μL + 120 μL	200	16	800
5	60 μL + 140 μL	200	12	600
6	40 μL + 160 μL	200	8	400
7	20 μL + 180 μL	200	4	200
8	0 μL + 200 μL	200	0	0

2. Transfer 80 μL of test samples into separate wells of the plate.

Notes: 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 Working Reagent. Dilution of reaction mixture may be necessary prior to the assay.

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

Precipitation may occur at high concentrations of phosphate (>100 μM), or in the presence of high concentrations of proteins and metals. If precipitation occurs, perform a serial dilution of sample with ultrapure water, run the assay, and determine the dilution factor from wells with no precipitation. Repeat assays using diluted samples.

For ATPase or GTPase assays, the ATP or GTP concentration should be <0.25 mM. If the reaction mixture contains >0.25 mM ATP or GTP, dilute samples with ultrapure water. For example, if the ATPase reaction contained 1 mM ATP, at the end of reaction, dilute reaction mixture 4-fold with water prior to the assay.

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

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

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

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

Results

Calculation

Plot $OD_{620\ nm}$ versus phosphate standard concentrations. Determine sample phosphate concentrations from the standard curve.

References

1. Guérette, D. et al., Molecular evolution of type VI intermediate filament proteins. BMC Evolutionary Biology 2007, **7**, 164 (2007).
2. Green, M.L. et al., Ethylene glycol induces hyperoxaluria without metabolic acidosis in rats. Am. J. Physiol. Renal Physiol., **289**, F536–F543 (2005).
3. Saran, D. et al., Multiple-turnover thio-ATP hydrolase and phospho-enzyme intermediate formation activities catalyzed by an RNA enzyme. Nucleic Acids Research, **34**(11), 3201–3208 (2006).
4. Adkins, M.W. et al., Chromatin disassembly from the PHO5 promoter Is essential for the recruitment of the general transcription machinery and coactivators. Mol. Cell. Biol., **27**, 6372–6382 (2007).

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mix before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

SJ,JAC,MAM 03/16-1