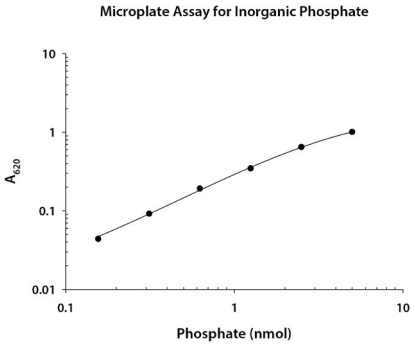
CALCULATION OF RESULTS

Plot nmol phosphate vs. A_{620}. Average the readings for each phosphate standard and sample and subtract the average blank optical density. Create a standard curve by using linear regression or a computer generated four parameter logistic (4-PL) curve fit. Use the regression line to calculate unknown values from the standard curve.

TYPICAL DATA

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.



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APPENDIX

The following buffers[1] did not interfere at concentrations ≤ 100 mM: Tris-HCl pH 9.0, HEPES pH 7.5, MOPS pH 7.0, Imidizole pH 7.0, and MES pH 5.0.

The following detergents and common reagents were tested for interference in the phosphate assay. The effects occurred at concentrations above those listed.

Detergents[2]	Level	Effect
Triton™ X-100	0.3%	Increased Blank
Tween® 20	0.1%	Reduced Sensitivity
NP-40 Alternative	1%	None
CHAPS	1%	None
SDS	≤ 0.01%	Increased Blank
Deoxycholate	≤ 0.01%	Precipitates, Increased Blank
Common Reagents[2]	**Level**	**Effect**
Glycerol	5%	Reduced Sensitivity
DMSO	10%	Reduced Sensitivity
Ethanol	25%	Reduced Sensitivity
BSA	0.03 mg/mL	Reduced Sensitivity
EDTA	10 mM	None
Dithiothreitol	3 mM	Reduced Sensitivity
β-mercaptoethanol	10 mM	None
Na_3VO_4	1 mM	Reduced Sensitivity
NaF	10 mM	None
NaCl	100 mM	None
KCl	100 mM	None
$CaCl_2$	10 mM	None

[1]Tested using the microplate assay protocol with or without 1 nmol phosphate (KH_2PO_4).

[2]Tested using the microplate assay protocol in 25 mM Tris-HCl, pH 7.5, with or without 1 nmol phosphate (KH_2PO_4).

Malachite Green Phosphate Detection Kit

Catalog Number: DY996

INTENDED USE

This Malachite Green Phosphate Detection Kit employs a simple, sensitive, reproducible, and non-radioactive method for measuring inorganic phosphate in aqueous solutions. The assay is based on the malachite green-molybdate binding reaction (1), and has a wide variety of applications, including the measurement of protein and lipid phosphorylation and phosphatase activity (2-5). The protocol presented is for the assay of phosphate in a 96-well microplate format. The Malachite Green Phosphate Detection Kit can also be run using 1 mL cuvettes or 384-well plates suitable for high-throughput screening.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at room temperature.
Do not use past kit expiration date.

DESCRIPTION	PART #	DESCRIPTION	STORAGE OF OPENED MATERIAL
Malachite Green Reagent A	895855	5 vials (3 mL/vial) of ammonium molybdate in 3 M sulfuric acid.	Store at room temperature.*
Malachite Green Reagent B	895856	5 vials (3mL/vial) of malachite green oxalate and polyvinyl alcohol.	
Phosphate Standard	892809	1 mL of 1 M phosphate (KH_2PO_4).	Store at room temperature for up to 6 months after initial use*

* Provided this is within the expiration date of the kit.

SOLUTIONS REQUIRED

Assay Buffer - The composition of the Assay Buffer will vary with the user's application and must be optimized for each sample type. Refer to the Appendix for a list of tested interfering and compatible compounds. Prepare fresh buffer as needed.

MATERIALS REQUIRED

- Microplate reader or spectrophotometer capable of measuring absorbance at 620 nm.
- Microplate or spectrophotometer cuvettes.
- Eppendorf microcentrifuge tubes.
- Pipettes and pipette tips.

PRECAUTIONS

The Malachite Green Reagent A, Malachite Green Reagent B, and Phosphate Standard supplied with this kit are acidic solutions. Wear eye, hand, face, and clothing protection when using these materials.

MICROPLATE ASSAY PROTOCOL

Note: *An initial 1:100 dilution of the 1M phosphate standard in Assay Buffer is needed before preparing the standard curve. To avoid precipitation of the standard, dilute it to 10 mM (1:100) with divalent cation-free buffer before making dilutions into buffers containing these ions.*

1. Prepare a six point standard curve using 2-fold serial dilutions with a high standard of 100 µM. Dilutions should be made in Assay Buffer.
2. Add 50 µL of sample, phosphate standard or blank (Assay Buffer) per well. If desired, the assay volume may be increased by adding proportionately larger volumes of sample, phosphate standard, blank, Malachite Green Reagent A, and Malachite Green Reagent B.
3. Add 10 µL of Malachite Green Reagent A to each well. Mix thoroughly and incubate for 10 minutes at room temperature.
4. Add 10 µL of Malachite Green Reagent B to each well. Mix thoroughly and incubate for 20 minutes at room temperature. **Note:** *For best inter-assay consistency, read plates at a fixed time after adding Malachite Green Reagent B.*
5. Determine the optical density of each well using a microplate reader set to 620 nm.

Microplate Assay Phosphate Standard Dilution		
Phosphate Standard	**Phosphate (µM)**	**nmol/well**
1	100	5
2	50	2.5
3	25	1.25
4	12.5	0.625
5	6.25	0.312
6	3.12	0.156
Blank	0	0

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TECHNICAL HINTS & LIMITATIONS

- For best inter-assay consistency, read plates at a fixed time after adding Malachite Green Reagent B.
- It is recommended that all phosphate standards and samples be assayed in duplicate or triplicate.
- This Malachite Green Phosphate Detection Kit should not be used beyond the expiration date on the label.
- Malachite Green is a highly sensitive phosphate detection method. Many soaps and dish detergents contain phosphate and will leave a residue that will increase the background absorbance of the assay. Containers should be rinsed extensively with distilled or deionized water before use.
- Cell and tissue extracts/lysates also contain phosphates from the breakdown of nucleic acids, lipids, etc. Extraneous free phosphate should be removed from samples to be analyzed. Two commonly used methods of removing phosphate from samples are desalting columns and immunoprecipitation capture of the phosphate-generating enzymes being studied.
- Divalent cations such as calcium, magnesium, and manganese form phosphate salts that have low water solubility.
- If a precipitate forms, check water purity, and consider using water from a different source.
- Sample dilution may be required because high concentrations of phosphate in the sample can cause precipitation.
- To reduce high assay background, dilution may be required with samples containing greater than 100 µM ATP.
- If color development is low, there may be insufficient phosphate present in the sample. Prepare several dilutions of the sample to determine optimal sample concentration for the assay.
- Malachite Green measures only inorganic, water-soluble phosphate. To measure protein- or lipid- bound phosphate, the samples must be hydrolyzed. This can be achieved by heating the samples with NaOH for proteins or perchloric acid for lipids. After hydrolysis, the samples must be neutralized before phosphate measurements are performed. A protocol example is listed below.

Protein Hydrolysis and Microplate Assay Protocol

- Add 25 mL of 4 M NaOH to 50 µL of sample.
- Heat at 100 °C for 30 minutes.
- Cool to room temperature and centrifuge briefly.
- Add 25 µL of 4 M HCl to the sample.
- Transfer the sample to a microplate well.
- Add 20 µL of Malachite Green Reagent A and incubate for 10 minutes at room temperature.
- Add 20 µL of Malachite Green Reagent B and incubate for 20 minutes at room temperature.
- Read the absorbance at 620 nm.

Lipid Hydrolysis and Microplate Assay Protocol

- See Reference # 4.