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ab219938 Phosphate Assay Kit (Colorimetric - UV absorption)

For the rapid, sensitive and accurate measurement of Phosphate in various samples.

This product is for research use only and is not intended for diagnostic use.

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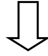
1. Overview

Phosphate Assay Kit (Colorimetric - UV absorption) (ab219938) has been developed for measuring the activity of any phosphate-generating enzyme such as ATPases or phosphatases. In the presence of inorganic phosphate, the MESG reagent is converted to 2-amino-6-mercapto-7-methlpurine by purine nucleoside phosphorylase with absorption wavelength shift to red. This feature has been used to develop our convenient MESG phosphate assay kit, an alternative to hazardous radioactive methods. The MESG substrate gives an absorbance increase at OD 360 nm on phosphorylysis at pH 6.5-8.5. The assay is shown to quantitate phosphate at the final concentration as low as 0.2 µM in cell lysates or biological fluids.

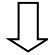
Phosphate is involved in many biological processes. For example, phosphatases, ATPases and several other enzymes catalyze biochemical reactions in which inorganic phosphate is released from a phosphoester substrate. The detection of many phosphoester–metabolizing enzymes is difficult because suitable substrates are not available. It is usually necessary to determine inorganic phosphate release using tedious colorimetric assays or radioisotope based methods.

2. Protocol Summary

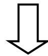
Standard curve preparation



Sample preparation



Add reaction mix and incubate at RT for 30 minutes



Measure absorbance at OD360 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- If applicable, please refer to the current Safety Data Sheet (SDS) provided with this product for safety, handling, and disposal information. The most up to date and current versions are available on our website www.abcam.com.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Assay Buffer	10 mL	-20°C	-20°C
MESG Substrate (10 mg, lyophilized)	1 vial	-20°C	-20°C
Purine Nucleoside Phosphorylase (1 mg, lyophilized)	1 vial	-20°C	-20°C
1 mM KH_2PO_4	1 mL	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

– Microplate reader capable of measuring absorbance at OD 360 nm
– Double-distilled water (ddH_2O)
– 50 mM HEPES Buffer pH 7
– Pipettes and pipette tips, including multi-channel pipette
– Assorted glassware for the preparation of reagents and buffer solutions
– Tubes for the preparation of reagents and buffer solutions
– Clear 96 well UV-transparent plate with flat bottom
– Dounce homogenizer (if using tissue)
– (Optional) Mammalian Cell Lysis Buffer 5X (ab179835)
– (Optional) Protease Inhibitors: we recommend Protease Inhibitor Cocktail II (ab201116) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail

Δ **Note:** due to the high sensitivity of this assay to inorganic phosphate, it is extremely important to use phosphate-free laboratory ware and reagents. We recommend using disposable plastic labware for all sample and reagent preparation to avoid contamination.

8. Technical Hints

- **This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer (10 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 MESG Substrate (10 mg):

Reconstitute the MESG Substrate in 500 µL of Assay Buffer to generate a MESG Substrate Solution. Mix well by vortexing.

Δ **Note:** this amount is enough for 2 x 96 well plate. If you are not going run 2 full plates, aliquot substrate and store unused solution at -20°C.

9.3 Purine Nucleoside Phosphorylase (1 mg):

Reconstitute Purine Nucleoside Phosphorylase (PNP) in 100 µL of ddH_2O solution to generate a Purine Nucleoside Phosphorylase Solution. Mix well by vortexing. Aliquot PNP so that you have enough volume to perform the desired number of assays.

9.4 1 mM KH_2PO_4 (1 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C. Aliquot so that you have enough volume to perform the desired number of assays.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 50 µM phosphate standard by diluting 50 µL of the 1mM KH_2PO_4 solution (Step 9.4) into 950 µL of ddH_2O. Mix well by pipetting up and down.

10.2 Using the 50 µM mM KH_2PO_4, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard#	Sample to dilute	Volume standard in well (µL)	ddH_2O (µL)	End conc Phosphate in well
1	50 µM	400	0	50 µM
2	Std #1	200	200	25 µM
3	Std #2	200	200	12.5 µM
4	Std #3	200	200	6.25 µM
5	Std #4	200	200	3.125 µM
6	Std #5	200	200	1.56 µM
7	Std #6	200	200	0.78 µM
8 (blank)	0	0	400	0 µM

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- If using, add protease inhibitors to sample buffer immediately prior use.

11.1 Cell lysates:

11.1.1 Harvest the number of cells necessary for each assay (initial recommendation: 2-5 x 10^5 cells).
11.1.2 Wash cells with cold HEPES or ddH_2O.
11.1.3 Resuspend or scrape cells in 100 µL cold HEPES.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Centrifuge 5 minutes at 4°C at 13,000 x*g* in a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Keep on ice.

11.2 Tissue lysates:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation: 20 mg).
11.2.2 Wash tissue with cold HEPES or ddH_2O.
11.2.3 Homogenize tissue in 400 µL cold HEPES using a Dounce homogenizer.
11.2.4 Centrifuge homogenate at 2,500 rpm for 5-10 minutes at 4°C.
11.2.5 Transfer supernatant to a new tube.
11.2.6 Keep sample on ice.

11.3 Plasma, Serum and Urine (and other biological fluids):

Samples can be used directly or diluted in HEPES for testing. No sample pre-treatment is required.

Δ **Note**: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- The protocol describe in this section is for 1 x 96-well plate. To perform the assay in a 384-wp, scale down volumes by half.

12.1 Reaction wells set up (UV-transparent plate):

- Blank control = 50 µL HEPES or ddH_2O.
- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with HEPES).

12.2 Phosphate Assay Solution:

12.2.1 Add 500 µL of MESG Substrate Solution (Step 9.2) and 100 µL of Purine Nucleoside Phosphorylase Solution (Step 9.3) into the bottle of Assay Buffer (10 mL) to prepare Phosphate Assay Solution. Mix well, and keep on ice.

Δ **Note**: Phosphate Assay Solution is stable for at least 4 hours on ice. Do not freeze the assay solution to use in another assay.

Δ **Note**: This amount if for 2 x 96 well plates. If you are only running 1 x 96 plate, prepare half of the amount described

12.3 Run Phosphate Assay:

12.3.1 Add 50 µL of Phosphate Assay Solution into each well. Mix thoroughly by pipetting up and down (total volume: 100 µL/well).

12.3.2 Incubate reaction at room temperature for 30 minutes.

12.4 Measurement:

12.4.1 Monitor the absorbance with a microplate reader or spectrophotometer at 360 nm.

Δ **Note**: for cuvette assay that requires a total volume larger than 100 µL, multiply the volume of sample and assay reagent before measuring the absorption.

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1 Average the duplicate reading for each standard and sample.
13.2 Subtract the mean absorbance value of the blank (Standard #8) from all standard and sample readings. This is the corrected absorbance.
13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of Phosphate.
13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
13.5 Apply the corrected sample OD reading to the standard curve to get Phosphate (B) amount in the sample wells.
13.6 Concentration of Phosphate (µM) in the test samples is calculated as:

$$Phosphate\ concentration = \frac{B}{V} * D$$

Where:

B = amount of Phosphate in the sample well calculated from standard curve (µM).

V = sample volume added in the sample wells (µL).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

14. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

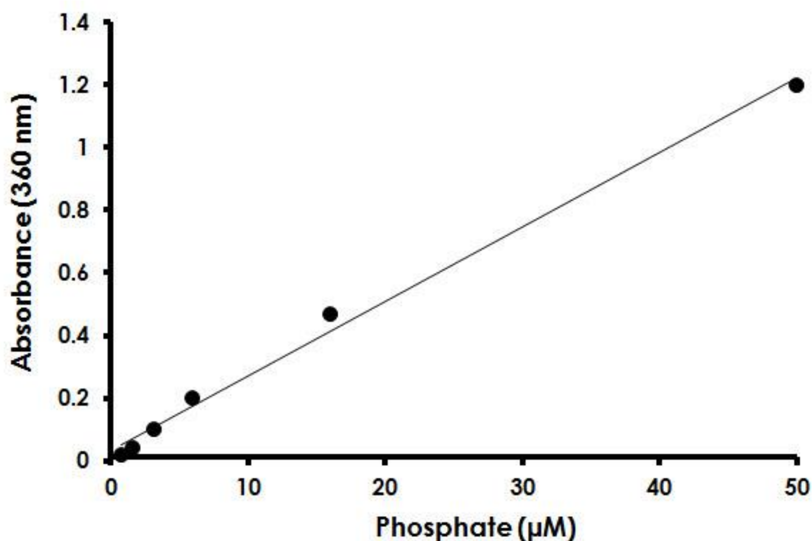


Figure 1. Typical Phosphate standard calibration curve. Phosphate dose response was measured on a 96 well UV plate using a SpectraMax Plus microplate reader (Molecular Devices). As low as 0.2 µM phosphate can be detected with 30 minutes incubation.

15. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare reagents and aliquot; get equipment ready.
- Prepare Phosphate standard dilution [50-0.78-50 µM].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL) and samples (50 µL).
- Prepare Phosphate Assay Solution: 500 µL MESG Substrate Solution +100 µL Purine Nucleoside Phosphorylase Solution + 10 mL Assay Buffer.
- Add 50 µL of Assay solution into each well.
- Incubate plate at RT for 30 minutes.
- Monitor the absorbance with a microplate reader at OD 360 nm.

16. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:

– Phosphate: use phosphate-free solutions and disposable labware.

18. Notes

Technical Support

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