ab65622
Phosphate Assay Kit
(Colorimetric)

Instructions for use:

For quantitative measurement of Phosphate in a variety of biological samples.

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

Version 5 Last Updated 03 March 2016
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1. BACKGROUND

Phosphate Assay Kit (Colorimetric) (ab65622) provides an easy, quick and sensitive method to assess phosphate over a wide range of concentrations in a variety of samples. The assay utilizes a proprietary formulation of malachite green and ammonium molybdate which forms a chromogenic complex with phosphate ion giving an intense absorption band around 650 nm.

Phosphate is one of the most important of the inorganic ions in biological systems. It functions in a variety of roles. One of the most important roles is as a molecular switch, turning enzyme activity on and off through the mediation of the various protein kinases and phosphatases in biological systems. Phosphate is also of great importance in mineralization processes and is a primary stimulus of algal blooms frequently found in bodies of fresh water, due to run-off from areas of high fertilizer use.

Phosphate concentrations between 1 µM and 1 mM, with a lower limit of detection of approximately 0.1 nmol, can be directly determined. The Phosphate Colorimetric Assay Kit provides 500 assays using microtiter plates.
INTRODUCTION

2. ASSAY SUMMARY

- Sample Preparation
- Standard Curve Preparation
- Add Phosphate Reagent
- Measure Optical Density (OD 650 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.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- 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.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at room temperature 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

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Reagent</td>
<td>15 mL</td>
<td>RT</td>
<td>RT</td>
</tr>
<tr>
<td>10 mM Phosphate Standard</td>
<td>500 µL</td>
<td>RT</td>
<td>RT</td>
</tr>
</tbody>
</table>
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 650 nm
- TBS
- MilliQ water or other type of double distilled water (ddH\textsubscript{2}O)
- 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
- 96 well plate with clear flat bottom
- Tris Buffer or any generic lysis buffer (such as RIPA) – for sample preparation
- 1.0 mm glass beads/ Dounce homogenizer (if using tissue)
- Sonicator (if using cells)
- (Optional) Protease inhibitors: we recommend Protease Inhibitor Cocktail II (ab201116) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.

**NOTE:** many laboratory detergents contain high amounts of phosphates which can adhere to cleaned glassware. It is highly recommended to use disposable plastic labware for all sample, standards and reagents 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 which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure you have the right type of plate for your detection method of choice.

- 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. **Phosphate Reagent:**

Ready to use as supplied. Store at room temperature. **NOTE:** there may be small amount of precipitate visible which doesn’t affect the assay.

9.2. **Phosphate Standard (10 mM):**

Ready to use as supplied. Store at room temperature.
10. STANDARD PREPARATION

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

10.1. Prepare a 100 µM Phosphate Standard by diluting 10 µL of the 10mM Phosphate Standard in 990 µL of dH₂O.

10.2. Using 100 µM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Phosphate Standard (µL)</th>
<th>ddH₂O (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>Amount standard in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>600</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>570</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>640</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>610</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>480</td>
<td>200</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>150</td>
<td>450</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 200 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 complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Add protease inhibitors to sample buffer immediately prior use.

11.1. Cell (adherent or suspension) samples:

11.1.1. Harvest the amount of cells necessary for each assay (initial recommendation = 1 x 10^7 cells).
11.1.2. Wash cells with cold TBS.
11.1.3. Resuspend the cell pellet in 150 µL of Tris Buffer (or other general sample buffer) on ice.
11.1.4. Homogenize cells quickly by pipetting up and down a few times.
11.1.5. Sonicate cells for 50 seconds 3X at high setup (one cycle = 30 s sonication – 10 s break – 10 s sonication – 10 s break).
11.1.6. Centrifuge sample for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
11.1.7. Collect supernatant and transfer to a clean tube.
11.1.8. Keep on ice.
ASSAY PREPARATION

11.2. **Tissue samples:**

11.2.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 – 30 mg).

11.2.2. Wash tissue in cold TBS.

11.2.3. Resuspend tissue in 1 mL of Tris Buffer (or other general sample buffer).

11.2.4. Add glass beads to sample and homogenize for 1.5 min in the homogenizer. Repeat homogenization step. If using Dounce homogenizer, homogenize with 10 – 15 passes sitting on ice.

11.2.5. Centrifuge samples for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6. Collect supernatant and transfer to a clean tube.

11.2.7. Keep on ice.

11.3. **Serum and urine samples:**

Serum and urine samples can be tested directly by adding sample to the microplate wells. No sample pre-treatment is necessary.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (initial recommendation = 1:100 – 1:2,000).

**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 correct temperature prior to use.
- We recommend to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1. Plate Loading:

- Standard wells = 200 µL standard dilutions
- Sample wells = 1 - 200 µL samples (adjust volume to 200 µL/well with ddH₂O Assay Buffer).

12.2. Reaction Mix:

12.2.1. Add 30 µL of Phosphate Reagent (see section 9.1) to standard and sample wells.

12.2.2. Mix well.

12.3. Plate Measurement:

12.3.1. Incubate plate for 30 minutes at room temperature protected from light.

12.3.2. Measure output on a microplate reader at OD 650 nm.

**NOTE:** the color is stable for several hours.
OPTIONAL: PROTOCOL FOR 1 mL CUVETTES
Increase all reaction component 5X when using 1 mL cuvettes.
Total reaction mixture (1 mL) will contain:
- 0 – 25 nmol phosphate (0 – 500 µL)
- 150 µL Phosphate reagent
Top up volume to 1.0 mL with ddH$_2$O
Mix well and incubate at room temperature for 30 minutes protected from light.
Measure output at OD 650 nm on a cuvette spectrophotometer.
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 (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3. Plot standard curve readings and draw the line of the best fit to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.4. Concentration of phosphate in the test samples is calculated as:

\[
\text{Phosphate Concentration} = \left( \frac{B}{V} \right) \times D
\]

Where:

- B = amount of phosphate in the sample well calculated from standard curve (nmol).
- V = original sample volume added into the reaction well (µL).
- D = sample dilution factor.
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: Standard calibration curve. Graph shows mean of duplicates (± SD) with background readings subtracted.

Figure 2: Phosphate levels in tissue lysates of mouse leg and rat brain showing quantity (µmol) per mg of extracted protein. Protein concentration for samples varied between 9 – 14 mg/mL. Samples were diluted 400 – 4,000 fold.
**Figure 3:** Phosphate levels in cell culture lysates showing quantity (µmol) per mg of extracted protein. Samples containing $1.3 \times 10^7$ cells/mL were used. Samples were diluted 400 – 4,000 fold.

**Figure 4:** Phosphate levels in multiple biological fluids showing quantity (µmol) per mL of tested sample. Samples were diluted 100 – 1,000 fold.
15. QUICK ASSAY PROCEDURE

**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 standard (aliquot if necessary); get equipment ready.
- Prepare Phosphate Standard dilution [1 – 5 nmol/well].
- Prepare samples in optimal dilutions so that they fit standard curve readings.
- Set up plate in duplicate for standard (200 µL) and samples (200 µL).
- Add 30 µL of Phosphate Reagent to all wells.
- Mix and incubate plate at RT for 30 minutes protected from light.
- Measure output (OD 650 nm) on a microplate reader.

**OPTIONAL PROTOCOL FOR 1mL CUVETTES**

- Prepare standard and samples in duplicate (find optimal dilutions to fit standard curve readings); get equipment ready.
- Set up cuvettes for plate for standard (0 - 500 µL), samples (0 - 500 µL) and background control sample (0 - 500 µL) wells.
- Add 150 µL of Phosphate Reagent to all wells. Top up to 1 mL with ddH₂O.
- Mix and incubate plate at RT for 30 minutes protected from light.
- Measure output (OD 650 nm) on a cuvette spectrophotometer.
## 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimeters: Clear plates, Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use provided protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer (increase number of strokes); observe for lysis under microscope</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------------------------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a</td>
<td>Pipetting errors in standard or</td>
<td>Avoid pipetting small volumes and prepare a master mix whenever</td>
</tr>
<tr>
<td>linear pattern</td>
<td>reaction mix</td>
<td>possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td></td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>substances</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the</td>
<td>Concentrate/ Dilute sample so as to be in the linear range</td>
</tr>
<tr>
<td></td>
<td>linear range</td>
<td></td>
</tr>
</tbody>
</table>
17. INTERFERENCES

These chemicals or biological materials will cause interference in this assay causing compromised results or complete failure:

- Laboratory detergents - contain high levels of phosphates which can adhere to cleaned glassware. It is highly recommended to use disposable plastic labware for all samples, standards and reagents to avoid contamination.

18. FAQs

I would like to use salt water based media with approx. 17,000 ppm of chloride, will it inhibit the reaction or have any other adverse effects or interference the data?

Chloride in salt water samples will have no interference in the assay.

I would like to check the amount of phosphate uptake in the cells before and after certain treatment. Can I use this product for this purpose? What protocol do you recommend? Any special treatment for lysing the cell?

Yes, this kit can be used for this purpose. Use any generic lysis buffer (TBS is preferred to RIPA buffer) for making the extract. Use a Dounce homogenizer / sonicator for homogenization. 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.

Will the kit detect all \( \text{PO}_4^{3-} \), \( \text{H}_2\text{PO}_4^- \) and \( \text{HPO}_4^{2-} \)?

The kit will detect all the three types of \( \text{PO}_4 \) in the reaction.
I want to perform this assay and I’m worried about the influence of the precipitates on the OD reading. How should I proceed?

Any precipitate in the wells will interfere with the reading and that has to be avoided. As long as you are not pipetting in the precipitate and taking it into the analysis wells, it should be fine. The clear reagent can be used directly for the assay. You can also quickly spin your samples and take the supernatant only to avoid interference from the precipitate.

I would like to quantify the amount of phospholipid in liposome samples. The most frequent used method is to determine the content of phosphor in the sample, and thereby determine the amount of phospholipid. Can I use this product for this application? Would we be able to measure the phosphor amount directly on the intact phospholipids, or does the liposome suspension have to be pretreated in order liberate the phosphate as a free ion?

This assay measures inorganic phosphate, so you would need to release the phosphate enzymatically from your liposomes.
19. NOTES
UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaltlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaltlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China
Email: cn.technical@abcam.com | Tel: 400 628 6880 WeChat: abcam_cn

Asia Pacific
Email: hk.technical@abcam.com | Tel: +852 2603 6823

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

Australia
Email: au.technical@abcam.com | Tel: +61 (0)3 8652 1450

New Zealand
Email: nz.technical@abcam.com | Tel: +64 (0)9 909 7829

Singapore
Email: sg.technical@abcam.com | Tel: +65 6734 9242

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