ab83369

Alkaline Phosphatase Assay Kit (Colorimetric)

For the rapid, sensitive and accurate measurement of Alkaline Phosphatase (ALP) activity in various samples.

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

Alkaline Phosphatase Assay Kit (Colorimetric) (ab83369) is a highly sensitive, simple, direct and HTS-ready colorimetric assay designed to measure alkaline phosphatase (ALP) activity in serum and other biological samples.

This kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow (ODmax= 405 nm) when dephosphorylated by ALP. It can detect 10-250 µU ALP in samples. It contains 10 substrate tablets providing convenience for multiple usages.

Alkaline phosphatase (ALP, EC 3.1.3.1) catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate. Changes in alkaline phosphatase level and activity are associated with various disease states in the liver and bone.
2. Protocol Summary

Standard curve preparation

↓

Sample preparation

↓

Add ρNPP to samples

↓

Add ALP enzyme solution to samples and standard

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Incubate for 60 minutes at 25°C

↓

Measure absorbance (OD405 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 handled 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 -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.

△ Note: Reconstituted components are stable for 2 months.
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>Quantity</th>
<th>Storage temperature (before prep)</th>
<th>Storage temperature (after prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP Assay Buffer</td>
<td>100 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>pNPP</td>
<td>10 tablets</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>ALP Enzyme</td>
<td>1 vial</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>10 mL</td>
<td>-20°C</td>
<td>-20°C</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 405 nm
- Double distilled water (ddH$_2$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
- Dounce homogenizer (if using tissue)
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 ALP Assay Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 ALP Enzyme:
Reconstitute ALP Enzyme with 1mL Assay Buffer. Keep on ice during the assay. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store aliquots at 4°C (do not freeze enzyme once reconstituted). Use within 2 months.

9.3 pNPP Solution:
△ Note: never touch the tablets with bare hands.
Reconstitute 2 pNPP tablets in 5.4 mL Assay buffer to make a 5 mM solution; this is enough for 100 assays. The pNPP solution is stable for 12 hours on ice. Store unused tablets at -20°C.

9.4 Stop Solution:
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.
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 1mM pNPP standard by diluting 40µL pNPP 5mM Standard in 160 µL of Assay Buffer.

10.2 Using 1mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>1 mM Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End amount pNPP in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>300</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>290</td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>280</td>
<td>120</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>270</td>
<td>120</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>260</td>
<td>120</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>250</td>
<td>120</td>
<td>20</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 120 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.

11.1 Cell lysates:
11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1 x 10^5 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend cells in 100 – 200 µL of Assay Buffer.
11.1.4 Centrifuge samples at 4°C at top speed for 15 minutes in a cold microcentrifuge to remove any insoluble material.
11.1.5 Collect supernatant and transfer to a new tube.
11.1.6 Keep sample on ice.

11.2 Tissue lysates:
11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg tissue).
11.2.2 Homogenize in 4 – 6 volumes of Assay Buffer using a Dounce homogenizer (10 – 50 passes) on ice.
11.2.3 Centrifuge samples at 4°C at top speed for 15 minutes in a cold microcentrifuge to remove any insoluble material.
11.2.4 Collect supernatant and transfer to a new tube.
11.2.5 Keep sample on ice.

11.3 Plasma, Serum and Urine (and other biological fluids):
Plasma and serum samples can be tested directly; they do not require additional sample preparation.
Dilute sample 10 times in Assay Buffer.
However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.

11.4 **Cell culture medium:**
Cell culture medium can be analyzed directly; it does not require additional sample preparation. However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.

⚠️ **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.

⚠️ Note: Set up Sample Background Controls for colored samples, as the color may interfere with the reading.

12.1 Reaction wells set up:
- Standard wells = 120 µL standard dilutions.
- Sample wells = 2-80 µL samples (adjust volume to 80 µL/well with Assay Buffer).
- Sample Background Control wells (for colored samples only) = 2-80 µL samples (adjust volume to 80 µL/well with Assay Buffer).

12.2 ALP Reaction:
12.2.1 Add 20 µL Stop Solution to Sample Background Control wells to terminate ALP activity in these samples. Mix well by pipetting up and down.
12.2.2 Add 50 µL of 5 mM \( p \)NPP Solution (Step 9.3) to each well containing Sample and Background Sample Controls. Do not add solution to Standard wells.
12.2.3 Add 10 µL of ALP enzyme solution to each \( p \)NPP Standard well. Mix by pipetting up and down.
12.2.4 Incubate plate at 25°C for 60 minutes protected from light. The enzyme will convert \( p \)NPP substrate to an equal amount of colored p-Nitrophenol (\( p \)NP).
12.2.5 Stop reaction in Sample wells and Standard wells by adding 20 µL Stop Solution.

⚠️ Note: do not add Stop Solution to Sample Background Control wells as the Stop Solution has already been added to the control when prepared in Step 12.2.1.

12.3 Measurement:
12.3.1 Gently shake the plate.
12.3.2 Measure output at OD 405 nm on a microplate reader.
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 Standard curve calculation:

13.1.1 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.1.2 Average the duplicate reading for each standard.

13.1.3 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Measurement of ALP activity in the sample:

13.2.1 Average the duplicate reading for each sample.

13.2.2 If significant and applicable, determine the background corrected change in absorbance intensity for each well of sample by subtracting the OD value of the background control.

13.3 ALP activity (µmol/min/mL or U/mL) in the test samples is calculated as:

\[
ALP \text{ Activity} = \left( \frac{B}{\Delta T \times V} \right) \times D
\]

Where:

B = amount of ρNP in sample well calculated from standard curve (µmol).

ΔT = reaction time (minutes).

V = original sample volume added into the reaction well (mL).

D = sample dilution factor.
Unit Definition:
All the Units mentioned in this protocol are Glycine Units.
Glycine Units: The amount of enzyme causing the hydrolysis of one micromole of pNPP per minute at pH 9.6 and 25°C (glycine buffer).
DEA Units: The amount of enzyme causing the hydrolysis of one micromole of pNPP per minute at pH 9.8 and 37°C (diethanolamine buffer).
Unit Conversion: One Glycine unit as described above is equivalent to approximately three DEA units. This reaction system is in Glycine buffer.
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 pNP (p-Nitrophenol) standard calibration curve.

ALP levels in cell culture supernatants

Figure 2. ALP measured in cell culture supernatants showing activity (U) per liter (L) of tested sample. Samples were diluted 1-16 fold.
**Figure 3.** ALP measured in biological fluids showing activity (U) per liter (L) of tested sample. Samples were diluted 4-64 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.

- Solubilize ρNPP tablets, prepare ALP enzyme (aliquot if necessary); get equipment ready.
- Prepare samples.
- Prepare standard curve.
- Prepare background control samples = 80 µL Assay Buffer + 20 µL Stop Solution.
- Add 50 µL of 5mM ρNPP solution to test and background samples (do not add to standard wells).
- Add 10 µL of ALP enzyme solution to each ρNPP Standard wells.
- Incubate at 25°C for 60 minutes in the dark.
- Add 20 µL Stop solution to sample and standard wells.
- Measure immediately at OD 405 nm.
### 16. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay not working</strong></td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at assay 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 microplate</td>
<td>Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates</td>
</tr>
<tr>
<td><strong>Sample with erratic readings</strong></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</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><strong>Lower/higher readings in samples and standards</strong></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>Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td><strong>Standard readings do not follow a linear pattern</strong></td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever 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 concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td><strong>Unanticipated results</strong></td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:
- Inhibitors of ALP such as EDTA, oxalate, fluoride and citrate should be avoided in sample preparation.

18. FAQs

Q. Can this kit be used with isolated protein samples that contain protease inhibitor? Are there any other components that could affect the performance of this kit when one uses protease inhibitors?
A. The only chemicals you need to be wary of are EDTA, oxalate, fluoride, and citrate.

Q. Once the pNPP solution has been prepared, can I freeze the pNPP solution (e.g. at -20°C) so that I can use it later? If so, what is the maximum time I can store it at -20°C?
A. The pNPP solution should be used within 12 hours of making. Once you collect all samples, you can make the fresh pNPP solution and use it right away.

Q. Can samples be measured at different time points? During the sample preparation, once I have added ALP assay buffer to my cell samples, can I then freeze these samples to -20°C?
A. Samples homogenized in the assay buffer can be frozen in aliquots at -80°C until analysis.

Q. Can the incubation steps for pNPP and the ALP enzyme be combined into a single one hour incubation step? Or is it necessary to let the two incubations take place separately?
A. Yes, you can do the incubation simultaneously.
Q. I was thinking to keep the volume (and number) of cells stable and change the dose concentration of the drug I am testing with this assay. Why do you recommend different cell volumes?
A. We are not taking different volume of cells, but different volume of the cell homogenate. Plate equal number of cells in each well, treat them with the drugs, then trypsinize out the cells, get the cell pellet, wash with ice cold PBS, homogenize the resultant pellet in the assay buffer, take the supernatant from that and use different volumes of this supernatant for the subsequent assay.

Q. How do we normalize our final readings?
A. If you are beginning with variable number of cells, you can normalize against the total cell number or protein quantity used.

Q. We are looking for a kit to detect secreted alkaline phosphatase (SEAL) reporter gene in serum samples. Can we use this kit?
A. If you want to assay for the gene, you need to do DNA isolation from the serum and use a PCR based reaction to detect the specific gene. This assay is to detect the enzyme’s activity. A DNA isolation kit from serum samples (for example, DNA Isolation Kit – Plasma/Serum (ab156893)) and some SEAL specific primers would be ideal for you.
19. Notes
Technical Support

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Austria
wissenschaftlicherdienst@abcam.com | 019-288-259

France
supportscientifique@abcam.com | 01.46.94.62.96

Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154

Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com

UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
can.technical@abcam.com | 877-749-8807

US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823

China
cn.technical@abcam.com | +86-21-5110-5938 | 400-628-6880

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

Singapore
sg.technical@abcam.com | 800 188-5244

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

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