ab83367
Acid Phosphatase Assay Kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Acid Phosphatase activity in various samples.

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

Acid Phosphatase Assay Kit (colorimetric) (ab83367) is a high sensitivity, simple, direct and HTS-ready colorimetric assay designed to measure AP activity in serum and other samples. It is suitable for research and drug discovery. The kit uses $p$-nitrophenyl phosphate ($p$NPP) as a phosphatase substrate which turns yellow ($\lambda_{\text{max}} = 405$ nm) when dephosphorylated by AP. The kit can detect μU acid phosphatase activity in samples.

Acid phosphatases (AP) dephosphorylate phosphate groups from phosphate esters under acid conditions. Different acid phosphatase isozymes are found in different organs, and their serum levels are used as a diagnostic for disease in the corresponding organs. Elevated prostatic acid phosphatase levels may indicate the presence of prostate cancer and elevated tartrate-resistant acid phosphatase levels may indicate bone disease.
INTRODUCTION

2. ASSAY SUMMARY

- Sample preparation
- Add $\text{pNPP}$ to samples
- Standard curve preparation
- Incubate with AP enzyme solution
- Add stop solution
- Measure optical density (OD 405 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. Modifications to the kit components or procedures may result in loss of performance.

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 section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. 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>AP Assay Buffer</td>
<td>100 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>pNPP (10 tablets)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>On ice</td>
</tr>
<tr>
<td>AP 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>

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Colorimetric microplate reader – equipped with filter for OD 570 nm
- 96 well plate: black plates (clear bottoms) for fluorometric assay; clear plates for colorimetric assay
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)
7. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.
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.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- 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 complete removal of all solutions and buffers from tubes or plates during wash steps.

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

- Make sure the heat block/water bath and microplate reader are switched on.
9. **REAGENT PREPARATION**

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **AP Assay Buffer:**

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

9.2 **pNPP Substrate / Standard Solution:**

Dissolve 2 tablets pNPP into 5.4 mL Assay Buffer to make 5 mM pNPP working solution. Two tablets are sufficient for 100 assays. **NEVER TOUCH THE TABLETS WITH BARE HANDS.** The pNPP solution is stable for 12 hours on ice.

9.3 **AP Enzyme:**

Reconstitute AP Enzyme with 1 mL Assay Buffer. **NEVER FREEZE!** The enzymes are stable for up to 2 months at 4°C after reconstitution.

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.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1.1 Prepare a 1 mM $\text{pNPP}$ standard (substrate) by adding 40 $\mu$L of 5 mM $\text{pNPP}$ solution with 160 $\mu$L Assay Buffer.

10.1.2 Using 1 mM $\text{pNPP}$ 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 Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End $[\text{pNPP}]$ in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>360</td>
<td>120</td>
<td>0 nmol/well</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>348</td>
<td>120</td>
<td>4 nmol/well</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>336</td>
<td>120</td>
<td>8 nmol/well</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>324</td>
<td>120</td>
<td>12 nmol/well</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>312</td>
<td>120</td>
<td>16 nmol/well</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>300</td>
<td>120</td>
<td>20 nmol/well</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 complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue 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.

*NOTE:* Use phenol red free media when using cell culture supernatant as this interferes with the colorimetric reading.

11.1 **Cell (adherent or suspension) samples:**

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = $1 \times 10^5$ cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of Assay Buffer.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge 3 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.2 **Tissue samples:**

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

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 100 µL of Assay Buffer.
11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge samples for 2 – 5 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 **Plasma, Serum and Urine and other biological fluids:**

Plasma, serum and urine samples can be tested directly by adding sample to the microplate wells.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (1/2 – 1/5 – 1/10).

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.
12. **ASSAY PROCEDURE and DETECTION**

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 **Set up Reaction wells:**
- Standard wells = 120 µL standard dilutions.
- Sample wells = 1 – 80 µL samples (adjust volume to 80 µL/well with Assay Buffer).
- (Optional) Sample background = 1 – 80 µL samples (adjust volume to 80 µL/well with Assay Buffer).
- Background control wells = 120 µL Assay Buffer.

12.2 Add 20 µL of Stop Solution to the sample background wells.

12.3 Add 50 µL of 5 mM pNPP to samples and background control wells. Mix well.

12.4 Add 10 µL of AP enzyme solution to each well containing the pNPP standards. Mix well.

12.5 Incubate at 25ºC for 60 minutes protected from light.

*NOTE:* The AP enzyme will convert pNPP substrate to an equal amount of colored p-Nitrophenol (pNP).

12.6 Stop reactions by adding 20 µL Stop Solution to the sample and standard wells.

12.7 Gently shake the plate.

12.8 Measure output on a microplate reader.
- Colorimetric assay: measure OD 405 nm.
13. **CALCULATIONS**

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the sample background from all standard and sample readings if applicable.

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

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of pNP.

13.5 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).

13.6 Extrapolate sample readings from the standard curve plotted using the following equation:

\[ A = \frac{(Corrected\ absorbance - (y-\ intercept))}{Slope} \]

13.7 Concentration of AP in the test samples is calculated as:

\[ AP\ Activity = \left(\frac{A}{\sqrt{BT}}\right) \times D \]

Where:
A = Amount of pNP in the sample well (in µmol).
B = Sample volume added into the reaction well (mL).
T = reaction time (in min)
D = Sample dilution factor.

**Unit Definition:**
One unit of AP is the amount of enzyme causing the hydrolysis of one micromole of $p$NPP to $p$NP per minute at pH 5.0 and 25°C.

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 $p$NP standard calibration curve obtained using colorimetric reading.
Figure 2: Acid phosphatase enzyme samples 40 minute assay
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 pNPP Solution, AP enzyme mix and stop solution; (aliquot if necessary); get equipment ready
- Prepare appropriate standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Add 20 µL of Stop Solution to the sample background wells.
- Add 50 µL of 5 mM pNPP to samples and background control wells.
- Add 10 µL of AP enzyme solution to each well containing the pNPP standards.
- Incubate 25°C 60 mins protected from light.
- Stop reactions by adding 20 µL Stop Solution to sample and standard wells.
- Gently shake the plate.
- Measure output on a microplate reader (OD405 nm).
## 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>Colorimetric: 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 PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></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>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 linear pattern</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 on protocol</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 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>
18. **INTERFERENCES**

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure.

- Any samples that contain color e.g. phenol red in cell culture medium.
- Tartrate.
- Fluoride.
- EDTA.
- Oxalate.
- Citrate.
19. **NOTES**
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