# ab83369– Alkaline Phosphatase Assay Kit (Colorimetric)

For rapid, sensitive and accurate measurement of Alkaline Phosphatase (ALP) activity in various samples.
For research use only - not intended for diagnostic use.

**For overview, typical data and additional information please visit:**
**http://www.abcam.com/ab83369** (use http://www.abcam.cn/ab83369 for China, or http://www.abcam.co.jp/abab83369 for Japan)

**Background:**

Alkaline Phosphatase Assay Kit (Colorimetric) (ab83369) provides a simple, rapid, sensitive and direct method for accurate Alkaline Phosphatase (ALP) activity quantification in cell lysates, cell culture media, tissue extracts, serum, plasma, and other biological fluids.

Alkaline Phosphatase dephosphorylates the p-nitrophenyl phosphate (pNPP) substrate, which turns yellow (pNP, $\lambda_{max}$ = 405 nm). The kit detects 10-250 µU of ALP in samples.

**Assay Summary:**

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

Sample preparation
↓
Standard curve preparation
↓
Add enzyme mix and substrate to respective wells
↓
Incubate 60 min at 25°C then stop reactions
↓
Measure optical density (OD405 nm)

## QUICK ASSAY PROCEDURE

- Solubilize pNPP Substrate, thaw ALP Assay Buffer I, Enzyme Mix II and Stop Solution I, (aliquot if necessary); prepare equipment
- Prepare samples in duplicate
- Prepare standard curve
- Set up plate for standard (120 µL) and samples (80 µL).
- Add 10 µL of Enzyme Mix II to each Standard.
- Add 50 µL 5 mM pNPP Substrate to each sample well.
- Incubate plate at 25 °C for 60 mins.
- Stop reactions (20 µL Stop Solution I)
- Measure plate at OD 405 nm

**Precautions & Limitations:**

**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.
- 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.

**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. Reconstituted components are stable for 2 months. Do not use kit or components if they have exceeded the expiry date.

**Materials Supplied:**

| Item | Quantity | Storage Temperature (on receipt) | Storage temperature (reconstituted) |
|---|---|---|---|
| ALP Assay Buffer I | 100 mL | -20°C | -20°C |
| pNPP Substrate | 1 vial | -20°C | 4°C |
| Enzyme Mix II | 1 vial | -20°C | 4°C |
| Stop Solution I | 10 mL | -20°C | -20°C |

**Materials Required, Not Supplied:**

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

- Microplate reader capable of measuring absorbance (OD) at 405 nm (colorimetric)
- 96 well clear plate with clear flat bottom (colorimetric assay)
- Orbital shaker
- Microcentrifuge
- Dounce homogenizer (if using cells or tissue)
- 1 x PBS pH 7.4
- MilliQ water or other type of double distilled/deionized water (ddH2O)

**Reagent Preparation:**

- Briefly centrifuge small vials at low speed prior to opening.
- Equilibrate reagents to room temperature before use.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.

**ALP Assay Buffer I:** Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

**Enzyme Mix II:** Reconstitute Enzyme Mix II with 1 mL ALP Assay Buffer I. 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.

**pNPP Substrate:** Reconstitute 2 *p*NPP tablets in 5.4 mL ALP Assay Buffer I to make a 5 mM solution; this is enough for 100 assays. The *p*NPP solution is stable for 12 hours on ice. Store unused tablets at -20°C.
*__Note:__ never touch the tablets with bare hands.

**Stop Solution I:** Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

**Sample Preparation:**

1. We recommend performing several dilutions or doses 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-80 °C. Alternatively, 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. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Interferences:

2. Inhibitors of ALP such as EDTA, oxalate, fluoride and citrate should be avoided in sample preparation.

**Cells (adherent or suspension) samples:**
1. Harvest the number of cells necessary for each assay (initial recommendation = 1 x $10^5$ cells).
2. Wash cells in cold PBS.
3. Resuspend cells in 50 µL of ALP Assay Buffer I.
4. Homogenize tissue with a Dounce homogenizer sitting on ice.
5. Centrifuge sample for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube.
7. Keep on ice.

**Tissue Samples:**
1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
2. Wash tissue in cold PBS.
3. Homogenize tissue in 4-6 volumes of ALP Assay Buffer I with a Dounce homogenizer sitting on ice, with 10 - 50 passes.
4. Centrifuge samples for 15 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
5. Collect supernatant and transfer to a clean tube.
6. Keep on ice.

**Liquid Samples: (Plasma, Serum, and other biological fluids):**
Plasma and serum samples can be tested directly; they do not require additional sample preparation. Too find the optimal values and ensure your readings will fall within the standard curve, we recommend an initial 10-fold dilution in ALP Assay Buffer I.
*Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

**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. For cell culture medium phenol red-free medium is recommended.
*Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

**Standard Preparation:**

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.
- Each dilution has enough standard to set up duplicate readings (2 x 120 µL).
- If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.

Prepare 1 mM dilution of pNPP Standard as follows:

1. 1 mM dilution: dilute 40 µL pNPP Substrate 5 mM Standard in 160 µL of ALP Assay Buffer I. Gently pipette up and down a few times to ensure all standard is removed from tip. Mix well by inversion.

**For colorimetric assay:** Using 1 mM pNPP standard, add 0, 4, 8, 12, 16, and 20 µl of the 1 mM standard into a series of wells, generating 0, 4, 8, 12, 16, and 20 nmoles of pNPP/well. Adjust the volume to 120 µl/well with Assay Buffer, or, prepare standard curve dilution as described in the table below in a microplate or microcentrifuge tubes (sufficient for duplicate standard curves):

| Standard # | pNPP 1 mM Standard (µL) | ALP Assay Buffer I (µL) | Final volume standard in well (µL) | End amount pNP in well (nmol/well) |
|---|---|---|---|---|
| 1 | 0 | 300 | 120 | 0 |
| 2 | 10 | 290 | 120 | 4 |
| 3 | 20 | 280 | 120 | 8 |
| 4 | 30 | 270 | 120 | 12 |
| 5 | 40 | 260 | 120 | 16 |
| 6 | 50 | 250 | 120 | 20 |

**Assay Procedure:**

- Keep enzymes and heat labile components and samples on ice during the assay.
- Equilibrate all other materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls, and samples in duplicate.

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

**ALP Reaction:**

1. Add 20 µL Stop Solution I to Sample Background Control wells to terminate ALP activity in these samples. Mix well by pipetting up and down.
2. Add 10 µL of Enzyme Mix II to each pNPP Standard well. Mix by pipetting up and down.
3. Add 50 µL of 5 mM pNPP Solution to each well containing Sample and Background Sample Controls. Do not add solution to Standard wells.
4. Mix and incubate at 25 °C for 60 minutes, protected from light. The enzyme will convert pNPP substrate to an equal amount of colored p-Nitrophenol (pNP).
5. Stop reaction in Sample wells and Standard wells by adding 20 µL Stop Solution I.
   ***Note: Do not add Stop Solution I to Sample Background Control wells as the Stop Solution I has already been added to the control when prepared**
6. Gently shake the plate and measure the absorbance immediately on a microplate reader at OD 405 nm for Colorimetric assay.

**Calculations:**

1. Average the duplicate reading for each standard, sample and background control (if required).
2. Subtract the mean absorbance value of the blank (Standard #1) from all standard readings. This is the corrected absorbance.
3. Plot the corrected absorbance values for each standard as a function of the end amount of pNP in nmoles.
4. Calculate the linear equation of the standard curve using a linear regression and determine the slope.
5. If sample background controls were required, subtract the OD value of the sample background control from the corresponding sample OD. Otherwise, subtract the mean absorbance value of the blank (Standard #1) from the sample OD. This is the corrected sample absorbance.
6. Apply the corrected sample absorbance to the standard curve to determine the nmoles of pNP in the sample well.
7. ALP activity (µmoles/min/mL or U/mL) in the test samples is calculated as:

$$ALP\ activity = \frac{B}{\Delta T * V} \times D = mU/ml$$

Where:

$B$ = amount of pNP in sample well calculated from standard curve (nmoles).

$\Delta T$ = reaction time (minutes, ΔT = 60 for endpoint assay).

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

$D$ = sample dilution factor (before addition to the well).

**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 Substrate 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 Substrate 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.

**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 it. 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?**
A. This is an endpoint assay and measurements are taken after the reaction has stopped. If the corrected sample readings are outside the range of the standard curve, it is best to repeat the assay with either a smaller dose or an increased sample dilution. Modifying the length of the assay by too much could risk an incomplete standard curve.

**Q. During the sample preparation, once I have added ALP Assay Buffer I/Assay Buffer to my cell samples, can I then freeze these samples to -20°C?**
A. Samples homogenized in the ALP Assay Buffer I can be frozen in aliquots at – 80 °C until analysis.

**Q. Can the incubation steps for pNPP Substrate 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 ALP Assay Buffer I, take the supernatant from that and use different volumes of this supernatant for the subsequent assay. This is to ensure that one of the sample wells fall within the range of the standard curve.

**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.

**Q: What protein quantification method can be used with this kit?**
A: We recommend a BCA assay. Components in the assay buffer can interfere with some Bradford assays and other methods of protein quantification.

**Technical Hints**

For additional helpful hints and tips on using our assay kits please visit:
https://www.abcam.com/en-us/support/product-support

## Technical Support

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