MANUAL

RayBio® Alkaline Phosphatase (ALP) Activity Assay Kit (Colorimetric)

Catalog #: MA-ALP

ISO 13485:2016

Introduction

Alkaline phosphatase (ALP) is an enzyme that catalyzes the hydrolysis of phosphate esters in an alkaline environment, leading to the release of inorganic phosphate. This inorganic phosphate is crucial for various biological processes, including bone mineralization, glycolysis, and oxidative phosphorylation. Alterations in serum ALP levels are indicative of specific diseases and have been utilized as a biomarker for liver and bone disease, such as Paget's disease and Hyperphosphatasemia. Additionally, a significant increase in serum ALP levels has been associated with conditions like malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis.

RayBio® Alkaline Phosphatase (ALP) Activity Assay Kit provides a simple, reproducible, and sensitive tool of measuring Alkaline Phosphatase (ALP) Activity in plasma, serum, cell lysates, urine and other biological liquid samples. The assay involves monitoring the dephosphorylation of the chromogenic substrate *p*-nitrophenyl phosphate (*p*NPP) by ALP. First, ALP dephosphorylates *p*NPP, leading to the formation of *p*-nitrophenol. Then the phenolic hydroxyl group undergoes deprotonation under alkaline conditions, resulting in the generation of *p*-nitrophenolate. This reaction yields a yellow-colored product that can be quantified at 405nm. The rate of the reaction is directly proportional to the enzyme activity.

Storage

The entire kit may be stored at < -20 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate (Item A)	A 96-well (12 strips x 8 wells) plate	RT*
ALP Assay Buffer	5 ml	2–8 °C, a week, protect from light
ALP Substrate	1 amber bottle	Do not store and reuse
Alkaline Phosphatase Standard	1 vial (10 µl of 1U/µL)	Do not store and reuse
10X ALP Stop Solution	1 vial (2 mL)	RT, 3 months

RT = room temperature

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 405 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Tubes to prepare sample dilutions
4. Incubator at 37°C
5. 50 ml conical tubes
6. DiH2O

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes.
It is recommended that sample protein concentrations should be determined using a total protein assay. Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

2. Plasma samples:

Collect blood with an anticoagulant such as citrate or oxalate and mix by inversion (*Note:* Chelating anticoagulants cannot be used). Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Sample should be tested immediately or frozen at -80°C for storage. Typically, normal human plasma has Alkaline Phosphatase concentrations in the range of 7-127 U/L. Plasma does not need to be diluted prior to use in the assay.

3. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80°C for storage. Typically, normal human serum has Alkaline Phosphatase concentrations in the range of 7-127 U/L. Serum does not need to be diluted prior to use in the assay.

4. Urine samples:

To remove insoluble particles, spin at 10,000 x g for 5 min. The supernatant can be assayed directly.

Note: If the calculated Alkaline Phosphatase activity of the sample is higher than 100 U/L, dilute the sample in ALP assay buffer and repeat the assay.

Reagent Preparation

REAGENT	PREPARATION
Microplate (Item A)	No preparation
ALP Assay Buffer	
Alkaline Phosphatase Standard	See "Standard Preparation" section
ALP Substrate	Add 2.7 mL ALP Assay Buffer into the ALP Substrate bottle. Mix until it is completely dissolved. The reconstituted solution is stable for 12 hours on ice.
10X ALP Stop Solution	To make 1X ALP Stop Solution: Add 1.5 mL of 10X ALP Stop Solution into 13.5 mL DiH2O, mix well.

Standard Preparation

To prepare a dilution series of standards in the concentration range of 0 U/L – 100 U/L (see Table below), Keep the standards on ice during the assay.

1. Label 8 microtubes #1 through #8 for the following concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0 U/L.
2. Pipette 594 µL ALP Assay Buffer into labeled tube #1. Pipette 200 µL ALP Assay Buffer into labeled tubes #2 – tube #8.
3. Pipette 3 µL Alkaline Phosphatase Standard (provided) into 297 µL ALP Assay Buffer and mix thoroughly to make 10,000 U/L Alkaline Phosphatase Standard stock.
4. Pipette 6 µL 10,000 U/L Alkaline Phosphatase Standard stock into labeled tube #1, mix thoroughly and quick spin, to make 100 U/L Alkaline Phosphatase Standard.
5. To make the 50 U/L standard, pipette 200 µL of tube #1 into the tube labeled #2. Mix thoroughly and quick spin.
6. Repeat this step with each successive concentration, preparing a dilution series as shown in the Table below. Each time, use 200 µl of the prior concentration until the 1.56 U/L is reached. Mix each tube thoroughly before the next transfer. Standards should be prepared fresh, mix thoroughly and used immediately.

Labeled Tubes	Alkaline Phosphatase Standard (µL)	ALP Assay Buffer (µL)	Standard Activity (U/L)
1	6 µL of 10,000 U/L stock	594 µL	100
2	200 µL of Tube #1	200 µL	50
3	200 µL of Tube #2	200 µL	25
4	200 µL of Tube #3	200 µL	12.5
5	200 µL of Tube #4	200 µL	6.25
6	200 µL of Tube #5	200 µL	3.125
7	200 µL of Tube #6	200 µL	1.56
8	0µL	200 µL	0

Assay Procedure

Each Alkaline Phosphatase Standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add solutions to each well:
    a. For Blank well(s) (Standard 0 U/L): add 30 µL ALP Assay Buffer.
    b. For Samples and standards: add 10 µL ALP Assay Buffer and 20 µL sample/standard.

Well	ALP assay buffer	Standard	Sample
Blank (0 U/L)	30 µL	-	-
Standard	10 µL	20 µL	-
Sample	10 µL	-	20 µL

2. Initiate the reaction by adding 20 µL reconstituted ALP Substrate (See "Reagent Preparation") to each well.
3. Cover with the plate cover. Carefully shake the plate for a few seconds to mix.
4. Incubate the plate for 20 minutes at 37ºC in the dark.
5. Pipette 100 µL of 1X ALP Stop Solution to each well to stop the reactions.
6. Measure the absorbance at 405nm immediately using a plate reader.

Calculation of Results

Subtract the blanks

Average the absorbance value of the blank wells (Standard 0 U/L) and subtract this from the absorbance values of all the other wells. These are the corrected absorbance.

Plotting the standard curves

Make a plot of corrected absorbance at 405nm as a function of Alkaline Phosphatase activity.

Determination of sample Alkaline Phosphatase activity

Alkaline Phosphatase (U/L) = $\frac{OD_{Sample} - OD_{Blank}}{Slope} \times DF$

OD_{Sample} = Optical density (OD) reading of the Sample

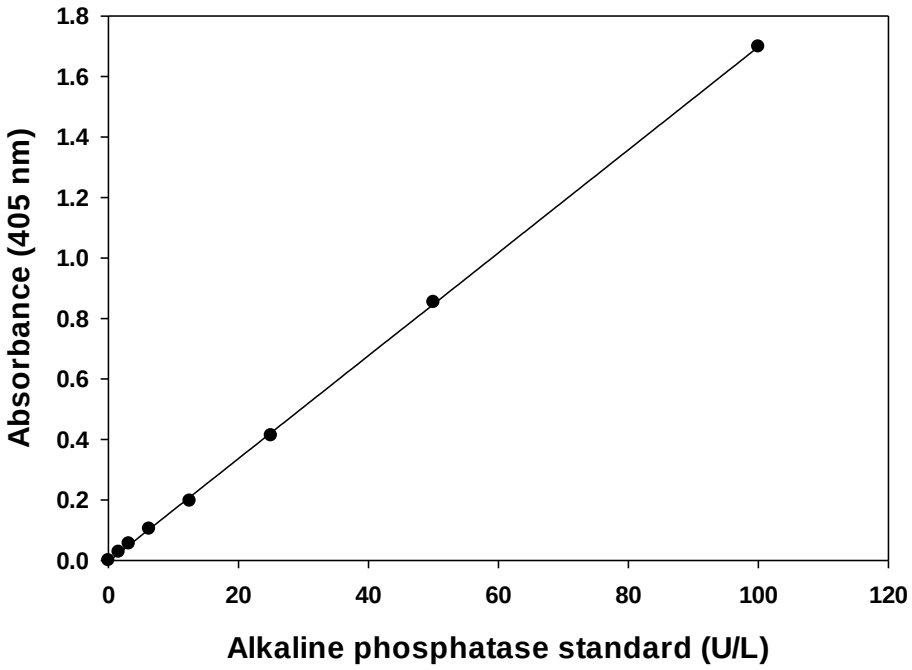
OD_{Blank} = Optical density (OD) reading of the Blank (Standard 0 U/L)

Slope is from the plot of Alkaline Phosphatase activity vs. Absorbance shown in Typical data below

DF = Sample Dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated Alkaline Phosphatase activity of the sample is higher than 100 U/L, dilute the sample in ALP assay buffer and repeat the assay.

A. Typical Data



These standard curves are for demonstration only. A standard curve must be run with each assay.

B. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 3.8%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 3.3%.

This product is for research use only.