ELK Biotechnology

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Alkaline Phosphatase (ALP) Assay Kit

InstructionCAT/NO.: BC046 Plate Reader Method

1. Principle of Measurement

ALP catalyzes the hydrolysis of disodium phenyl phosphate and one of the product, phenol can be reacted with 4-amino-antipyrene while the product of this reaction can be further oxidized by potassium ferricyanide to give red quinone product. The amount of quinone product can be measured by plate reader and thus the activity of ALP can be calculated based on it.

2. Reagent Composition

Reagent	Functions	Format/96T	Format/48T	Preservation Condition
Reagent I	Buffer Solution	1 Bottle×6ml	1 Bottle×3ml	4°C for 6 months
Reagent II	Substrate	1 Bottle×6ml	1 Bottle×3ml	4°C for 3 months (avoid light)
Reagent III	Chromogenic Agent	1 Bottle×18ml	1 Bottle×9ml	4°C for 6 months (avoid light)
Reagent IV	Phenol Stock Solution (1.1mg/ml)	1 Bottle×0.5ml	1 Bottle×0.5ml	4°C for 6 months (avoid light)
0.1mg/ml Phenol Standard Solution Preparation: Dilute the reagent IV stock solution with distilled water with the ratio of 1:10 (v/v) right before the measurement.				

3. Sample Collection and Preservation

1. Collect samples including serum, plasma (heparin for coagulation purpose), and supernatant from cultured cells should be collected with standardized methods. Tissue or cultured cells should be collected and pretreated based on methodology prior to the measurements.
2. Excess samples can be preserved at temperatures below -20°C. The lower the surrounding temperature, the longer the preservation period.

4. Procedures of Measurement

I. Pretreatment

a. Serum/Plasma Samples
Samples can be measured directly. Were the OD results too high, dilute the samples with physiological saline in order to results within the desirable range.

b. Tissue Samples
Weigh the tissues precisely and add physiological saline with the ratio 1 g tissues to 9 ml saline. Homogenize in an ice water ice bath and centrifuge the homogenate at 2,500 rpm for 10 min and extract the supernatant for further measurement.

c. Cultured Cells or Culture Medium
For Culture Medium: Extract the medium and centrifuge at 1,000-1,500 rpm for 10 min and extract the supernatant for further measurement.

For Cultured Cells:

i. Collection of Cultured Cells:
For suspended cells, the samples can be centrifuged at 1,000 rpm for 10 min. Discard the supernatant and keep the pellets.
For cells anchored on the walls, discard the culture medium and the cells can be scraped down directly. Or can be digested with 0.25% pancreatin solution for 2-3 min and then add culture medium to terminate the digestion. After the scrape or the digestion, transfer the solution into an Eppendorf (EP) tube and centrifuge it at 1,000 rpm for 10 min. Discard the supernatant and add 1ml phosphate buffer solution (pH7.4). Shake the tube softly and centrifuge it again on the same condition. Discard the supernatant and keep the precipitates for further treatment.

ii. Disruption of Cells:
Disruption via Homogenization: Add phosphate buffer solution to the pellets with the ratio of 10^5 cells to 0.3-0.5 ml buffer solution and homogenize the mixture in an ice water bath with a homogenizer.

Disruption via Sonication: Add phosphate buffer solution to the pellets with the ratio of 10^5 cells to 0.3-0.5 ml buffer solution and sonicate the mixture with the power of 300W in an ice water bath. The sonication should be done 5 times with 3-5 second-period and the interval of 30 seconds.

Disruption by Repeated Freezing and Thawing: Transfer the cells into an EP tube and add some DDW into the tube. Place the tube into liquid nitrogen for 3-5 seconds and transfer the tube into a refrigerator with the surrounding temperature of -20°C. After 20-30 seconds, place the tube at RT to thaw the sample. Repeat the procedures three more times to complete the disruption. Note that the EP tubes cannot be transferred from liquid nitrogen directly to RT.

Disruption with Chemicals: For cells anchored on the walls, remove the supernatant and add lysate for disruption purpose. The disruption process lasts for 30-40 min and after the process, extract the mixture and dilute it with saline or phosphate buffer solution if necessary.

II. Procedures

Composition (μL)	Blank	Standard	Sample
Double Distilled Water (DDW)	5 (30 for cells and culture medium)		
0.1mg/ml Phenol Solution		5 (30μl 0.02mg/ml phenol solution for culture medium or cell samples)	
Sample			5 (30 for cells and culture medium)
Reagent I	50	50	50
Reagent II	50	50	50
Mix thoroughly and warm the mixture in a water bath at 37°C for 15 minutes.			
Reagent III	150	150	150

Shake the microplate softly for homogenization purpose and measure the optical density (OD) values at 520 nm with a plate reader.

5. Technical Parameters

No.	Parameter	Requirement
1	OD value for Blank Tube	≤0.100
2	Coefficient of Variation (CV) within a Batch of Assay Kits	≤3%
3	CV among Batches of Assay Kits	≤5%
4	Assay Kit Recovery Rate	98%
5	Regression Coefficient in its Detection Range	R^2=0.9999
6	Accepted Wavelength Range	490-530 nm

6. Calculation Formula and Examples

I. Serum or Plasma Samples

a. Definition

One king unit (KU) is defined as one milligram phenol generated in 15 minutes catalyzed by enzymes in 100 ml serum or plasma at 37°C.

b. Formula

$$\frac{Enzyme\ Activity}{KU/100ml} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \times \frac{V}{100ml} \times CoD$$

CoD represents the coefficient of dilution in the pre-treatment procedure and can be 1 if dilution is unnecessary.

c. Examples

i. 5μl human serum was prepared and measured and the OD values were 0.0233, 0.2603 and 0.1585 respectively.

$$\frac{Enzyme\ Activity}{KU/100ml} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \times \frac{V}{100ml} \times CoD$$

$$= \frac{0.1585 - 0.0233}{0.2603 - 0.0233} \times 0.1mg/ml \times 100ml \times 1 = 5.7046\ KU/100ml$$

ii. Rat serum was diluted with the same volume of physiological saline and then the mixture was measured with the OD values equal to 0.0233, 0.2603 and 0.2694 respectively.

$$\frac{Enzyme\ Activity}{KU/100ml} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \times \frac{V}{100ml} \times CoD$$

$$= \frac{0.2694 - 0.0233}{0.2603 - 0.0233} \times 0.1mg/ml \times 100ml \times 2 = 20.768\ KU/100ml$$

II. Tissue Samples

a. Definition

One king unit is defined as one milligram phenol generated in 15 minutes catalyzed by enzymes in 1g tissue proteins at 37°C.

b. Formula

$$\frac{Enzyme\ Activity}{KU/g} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \div \frac{C_{Protein}}{g/ml}$$

c. Examples

i. 2% rat hepatic tissue homogenate was prepared and 5μl was measured and the OD values were 0.0233, 0.2603 and 0.0629 respectively. Also, the protein concentration for the homogenate was 3.528 mg/ml.

$$\underset{KU/g}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \div \frac{C_{Protein}}{g/ml}$$

$$= \frac{0.0629 - 0.0233}{0.2603 - 0.0233} \times 0.1mg/ml \div (3.528 \div 1000)g/ml = 4.736\ KU/g$$

ii. 10% fish hepatic tissue homogenate was prepared and measured. The OD values were 0.0233, 0.2603 and 0.1765 respectively. Also, the protein concentration was 4.1386mg/ml.

$$\underset{KU/g}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.1mg/ml} \div \frac{C_{Protein}}{g/ml}$$

$$= \frac{0.1765 - 0.0233}{0.2603 - 0.0233} \times 0.1mg/ml \div (4.1386 \div 1000)g/ml = 15.619\ KU/g$$

III. Culture Medium Samples

a. Definition

One king unit is defined as one milligram phenol generated in 15 minutes catalyzed by enzymes in 100ml medium at 37°C.

b. Formula

$$\underset{KU/100ml}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.02mg/ml} \times \frac{V}{100ml} \times CoD$$

c. Example

30μl culture medium was extracted and measured with the OD values equal to 0.0263, 0.3172 and 0.2698 respectively.

$$\underset{KU/100ml}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.02mg/ml} \times \frac{V}{100ml} \times CoD$$

$$= \frac{0.2698 - 0.0263}{0.3172 - 0.0263} \times 0.02mg/ml \times 100ml \times 1 = 1.6741\ KU/100ml$$

IV. Cultured Cell Samples

a. Definition

One king unit is defined as one milligram phenol generated in 15 minutes catalyzed by enzymes in 1g tissue proteins at 37°C.

b. Formula

$$\underset{KU/g}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.02mg/ml} \div \frac{C_{Protein}}{g/ml}$$

c. Example

Osteoblasts cultured were treated with 0.2ml lysate (0.1% TritonX-100 Solution) and the then measured with procedures mentioned above. The OD values were 0.0263, 0.3172 and 0.1465 respectively. The protein concentration was 0.8546mg/ml.

$$\underset{KU/g}{Enzyme\ Activity} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.02mg/ml} \div \frac{C_{Protein}}{g/ml}$$

$$= \frac{0.1465 - 0.0263}{0.3172 - 0.0263} \times 0.02mg/ml \div (0.8546 \div 1000)g/ml = 9.6700\ KU/g$$

7. Notes

I. In case of lack of optical filter with required wavelength, choose the optical filter with similar wavelength like 510 or 530 nm instead.

II. Please be considerate while adding the reagents to ensure the minimum amount reagents left on the tips and to avoid splitting of the reagents.

III. Tips of the pipettes should be placed as low as possible while adding the reagents to avoid incompleteness of the reaction caused by reagents anchored on the walls.

IV. Shake the microplate softly to avoid splitting of the reagents. It is recommended to shake vertically followed by shaking horizontally.

V. As the absorbance values may differ among different microplates, it is recommended to measure the absorbance values of microplates to minimize the error cause by different microphates.

VI. This assay kit is designed for scientific research only.

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Appendix I: Standard Curve Establishment

1. Pre-Treatment

The phenol stock solution was diluted with DDW to 50, 20, 10, 5, 5/2, 5/3 and 5/4 times the initial solution volume respectively. The resulted phenol solutions with the concentrations 0.022, 0.055, 0.11, 0.22, 0.44, 0.66, 0.88 mg/ml along with the stock solution were measured as the procedures mentioned below.

2. Procedures

Compositions (μ l)	Blank	Standard
DDW	5	
Phenol Solutions with Different Concentrations		5
Reagent I (Buffer Solution)	50	50
Reagent II (Substrate Solution)	50	50
Mix thoroughly and Warm the mixture in a water bath at 37° C for 15 min.		
Reagent III (Chromogenic Agent)	150	150

Mix immediately and record the absorbance values at 520 nm.

3. Standard Curve