Na+K+-ATPase assay kit(red blood cell)

(CAT/NO.: BC056 Size: 100T/48S, 50T/24S)

1. Significance

ATPase exists in tissue cells or on the cell membranes. As a type of protease anchored on the cell membrane, it plays an important role in transmission of energy or information and transport of substance. Under the circumstance of hypoxia or suffering certain diseases, the activity of ATPase may vary from the ordinary activity. Also, genetic disorder may cause the activity out of ordinary.

2. Principle of Measurement

ATPase catalyzes the hydrolysis of ATP with the product of ADP and phosphate. The amount of phosphate can be determined and thus measure the activity of ATPase.

3.Composition and Preparation(The kit is valid for 6 months)

	State	50T/24 Samples	100T/ 48 Samples	Preservation
Reagent I	Liquid	1 Bottle×13ml	2 Bottles×13ml	4°C for 6 months
Reagent II	Liquid	1 Bottle×4ml	2 Bottles×4ml	4°C for 6 months
Reagent III	Powder	4 Bottles	8 Bottles	-20°C for 6 months
Preparation of Reagent III: Dissolve each bottle of powder with 1ml double distilled water (DDW) and the excess can be preserved at -20°C for a week.				
Reagent IV	Liquid	1 Bottle×5ml	2 Bottles×5ml	4°C for 6 months
Reagent V	Solution A	4 Bottles×7ml	8 Bottle×7ml	4°C for 6 months
	Solution B	4 Bottles×6ml	8 Bottles×6ml	4°C for 6 months (avoid light)
Note: Gel may generated at low temperature and under this circumstance, solution should be warmed at 60°C for 10 min to fully dissolve. Both Solution should be kept away from phosphate contamination.				
Reagent VI	Liquid	1 Bottle×50ml	2 Bottles×50ml	RT for 6 months
Reagent VII	Liquid	1 Bottle×5ml	1 Bottle×5ml	4°C for 6 months
Reagent X	Stock Solution	2 Bottles×0.1ml	4 Bottles×0.1ml	4°C for 6 months
	Diluent	2 Bottles×0.9ml	4 Bottles×0.9ml	4°C for 6 months
Preparation of Reagent X: Dilute 1 bottle of stock solution with 1 bottle of diluent and the excess can be preserved at 4°C				
Preparation of 0.1mM Phosphate Solution: Dilute 0.1ml reagent VII with DDW to a final volume of 10ml.				
Preparation of 0.02mM Standard Phosphate Solution: Dilute 0.1mM solution (1ml) with DDW to 5ml.				
Preparation of Substrate Solution: Mix reagent I, II and III at the ratio of 13:4:4 (v/v) prior to use.				
Preparation of Reagent V (Chromogenic Agent): 0.5 h prior to use, pre-warm solution B. Reagent V (A) is then added and fully mix the solution. The solution can be preserved at 2-8°C for 5 days and is capable of measurement of 13 tubes. Also, the solution A and B can be mixed with the ratio 7:6 (v/v) if small amount of reagent V needed.				

4. Sample Pre-Treatment

a. RBC Count (See Appendix) and Measurement of Hemoglobin (Assay Kit Available)

b. Hemolysate Preparation

i. Packed RBC Solution

Take blood and add heparin as anticoagulant. Centrifuge at 1,000 rpm for 10 min and discard the supernatant. Extract packed RBC and dissolve with DDW with the ratio of 1:49 (v/v) and mix thoroughly. The thoroughly mixed solution should be transparent. Lower the concentration of hemolysate if the results received are too high.

ii. Whole Blood Solution

Take blood and shake softly, and then add DDW with the ratio of 1:24 (v/v). Mix till the solution is transparent. Lower the concentration of hemolysate if the results received are too high.

Note: Loss of activity may occur and thus the hemolysate should be measured right after its preparation. Other preparations should be done before the preparation of hemolysate.

Note: The excess anticogulated whole blood can be preserved for 2-3 days under 4°C or more than a week under -20°C。

Note: Samples cannot be diluted with phosphate in presence.

Note: Pre-experiment should be done in order to let the absolute absorbance (Sample absorbance subtracting Reference absorbance) to be around 0.2.

5. Standard Procedures

I . Enzymatic Reaction

Compositions (ml)	Reference	Sample
DDW	0.16	0.1
Sample		0.1
Reagent X		0.06
Reagent I	0.26	0.26
Reagent II	0.08	0.08
Reagent III	0.08	0.08
Mix and Warm at 37°C for 10 min		
Reagent IV	0.1	0.1
Sample	0.1	

Mix and centrifuge at 3,500 rpm for 10 min and extract supernatant.

II . Phosphate Determination

Compositions (ml)	Blank	Standard	Reference	Sample
DDW	0.3			
0.02mM Standard Phosphate Solution		0.3		
Supernatant			0.3	0.3
Reagent V	1	1	1	1

Mix and Set Aside for 2 min at RT				
Reagent VI	1	1	1	1

Mix and set aside for 5 min at RT. Regulate the spectrophotometer at 636nm with DDW.

Record the absorbed optical density (OD) for each tube with 1cm light path.

6. Simple Method

I. Enzymatic Reaction

Composition (ml)	Reference	Sample
DDW	0.16	0.1
Sample		0.1
Reagent X		0.06
Substrate Solution	0.42	0.42
Mix and Warm at 37°C for 10 min		
Reagent IV	0.1	0.1
Sample	0.1	

Mix and centrifuge at 3k-4k rpm for 10 min and extract supernatant for phosphate determination.

Note: The preparation of substrate solution is mentioned at page 1.

II. Phosphate Determination

Exactly the same as the phosphate determination step mentioned above.

Note: The cuvettes should be washed with tap water 10 times and then washed with DDW for 4-5 times to avoid contamination by phosphate.

7. Calculation Formula and Example

I. According to RCBs

a. Definition

One ATPase activity unit is defined as 1μmol phosphate generated by the hydrolysis of ATP catalyzed by ATPase within 10^7 red blood cells within an hour.

b. Formula

$$\text{ATpase Activity U}/10^7\text{RCBs} = \frac{OD_{Sample} - OD_{Ref}}{OD_{Standard} - OD_{Blank}} \times \frac{C_{Standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{CoD}{7.8} \times \frac{CoD}{Pre} \div \left(\frac{No.RBCs}{ml} \div 10^7\right)$$

c. Example

Diluted the hemolysate with the ratio 1:24 and measured the OD values. OD values were 0.052, 0.255, 0.226, and 0.278 respectively. Also the number of RBCs per milliliter was 8.004×10^6/ml.

$$\underset{U/10^7RCBs}{\text{ATpase Activity}} = \frac{OD_{sample} - OD_{Ref}}{OD_{stan} - OD_{Blank}} \times \frac{C_{standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{COD}{7.8} \times 25 \div \left(\frac{No.RBCs}{ml} \div 10^7\right)$$

$$= \frac{0.278 - 0.226}{0.255 - 0.052} \times 0.02 \div \frac{1}{6} \times 7.8 \times 25 \div (8.004 \times 10^6 \div 10^7) = 7.49U/10^7RBCs$$

II. According to the volume of whole blood

a. Definition

One ATPase activity unit is defined as 1μmol phosphate generated by the hydrolysis of ATP catalyzed by ATPase within 1ml whole blood within an hour.

b. Formula

$$\underset{U/ml}{\text{ATpase Activity}} = \frac{OD_{sample} - OD_{Ref}}{OD_{standard} - OD_{Blank}} \times \frac{C_{standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{COD}{7.8} \times \frac{COD}{pre}$$

c. Example

Diluted the hemolysate with the ratio 1:24 and measured the OD values. OD values were0.052, 0.255, 0.226, and 0.278 respectively.

$$\underset{U/ml}{\text{ATpase Activity}} = \frac{OD_{sample} - OD_{Ref}}{OD_{standard} - OD_{Blank}} \times \frac{C_{standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{COD}{7.8} \times 25$$

$$= \frac{0.278 - 0.226}{0.255 - 0.052} \times 0.02 \div \frac{1}{6} \times 7.8 \times 25 = 5.99U/ml$$

III. According to the amount of Hemoglobin

a. Definition

One ATPase activity unit is defined as 1μmol phosphate generated by the hydrolysis of ATP catalyzed by ATPase within 1g Hemoglobin within an hour.

b. Formula

$$\underset{U/mg}{\text{ATpase Activity}} = \frac{OD_{sample} - OD_{Ref}}{OD_{standard} - OD_{Blank}} \times \frac{C_{standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{COD}{7.8} \times \frac{COD}{pre} \div \frac{C_{Hb}}{g/ml}$$

c. Example

Diluted the hemolysate with the ratio 1:24 and measured the OD values. OD values were 0.052, 0.255, 0.226, and 0.278 respectively. The Hemoglobin content is 144.14mg/ml

$$\underset{U/g}{\text{ATpase Activity}} = \frac{OD_{sample} - OD_{Ref}}{OD_{standard} - OD_{Blank}} \times \frac{C_{standard}}{0.02mM} \div \frac{Time}{\frac{1}{6}h} \times \frac{COD}{7.8} \times 25 \div \frac{C_{Hb}}{g/ml}$$

$$= \frac{0.278 - 0.226}{0.255 - 0.052} \times 0.02 \div \frac{1}{6} \times 7.8 \times 25 \div (144.14 \div 1000) = 41.58U/g$$

Note: CoD represents the coefficient of dilution and 25 is the CoD for pretreatment.

Appendix I Red Blood Cells Count

There are total 3 methods and choose one to count the number of RBCs.

I. Direct Count with Hemocytometer

a. Preparation of Diluent: 3.8 g trisodium citrate, 1 ml Formaldeyde (Methanol)?, and 100ml DDW. Mix the mixture thoroughly and preserve at 4°C.

b. Extract 10μl whole blood (anticoagulation) and dilute with 2ml diluent and mix thoroughly.

c. Take one drop on the hemocytometer.

d. Count the No. of RBCs on top-left, top-right, bottom-left, bottom right and central squares. Multiply the number counted with 10^9 to give No. RBCs in 1L whole blood.

II. Count with Photoelectric Turbidimetry

Dilute 20μl whole blood with 4ml diluent and mix thoroughly. Add the solution to the cuvette with 1cm light path. Regulate the meter with DDW at 540nm and record the absorbance.
Establish the standard curve with and find the number of RBCs based on the standard curve.

III. Count with the Amount of Hemoglobin

Dilute 10μl whole blood with 2.5ml hemoglobin assay solution and mix thoroughly. Set aside for 10 min and record the absorbance at 540nm. Multiply the absorbance with 367.7 to get the amount of hemoglobin and find the number of RBCs based on the standard curve.