Technical Manual

H+K+-ATPase Activity Assay Kit

- **Catalogue Code: MAES0100**
- **Size: 100 Assays**
- **Research Use Only**

1. Key features and Sample Types

Detection method:

Colorimetric method

Specification:

100Assays

Storage:

2-8°C and -20°C for 3 months

Expiry:

See Kit Label

Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 3 months.

Do not use components from different batches of kit.

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2. Background

H^+K^+-ATPase is a member of the P-type ATPase family and mediates the exchange and transport of intracellular hydrogen ions and extracellular potassium ions. Gastric H^+K^+-ATPase (HKAg) mainly exists in gastric mucosal wall cells and a small amount in renal medulla. As a membrane-bound protein, HKAg has the function of acidifying gastric contents and activating pepsin, and can be used as a therapeutic target for peptic ulcer disease. However, colonic HKA (HKAc) exists in the colon or other tissues and mediates the reabsorption of active K.

3. Intended Use

The kit is used for the determination of H^+K^+-ATPase activity in animal tissue and cells samples.

4. Detection Principle

ATPase can decompose ATP to produce ADP and inorganic phosphorus. The activity of ATPase can be expressed by measuring the production amount of inorganic phosphorus in unit time. The inorganic phosphorus reacts with ammonium molybdate in acidic solution to form ammonium molybdate compound, which is reduced with reducing agent to form molybdenum blue, and has absorption peak at 660 nm. Determine the concentration of molybdenum blue to calculate the amount of inorganic phosphorus.

5. Kit components & storage

Item	Specification	Storage
Buffer Solution	20 mL × 1 vial	2-8°C, 3 months
Accelerator	8 mL × 1 vial	2-8°C, 3 months
Acid Solution	8 mL × 1 vial	2-8°C, 3 months
Substrate	Lyophilized × 1 vial	-20°C, 3 months
Inhibitor	Lyophilized × 1 vial	2-8°C, 3 months
Complexing Agent	6 mL × 1 vial	2-8°C, 3 months
Stop Solution	10 mL × 1 vial	2-8°C, 3 months
Reducing Agent	Lyophilized × 2 vials	2-8°C, 3 months, avoid direct sunlight
Chromogenic Agent	Lyophilized × 1 vial	2-8°C, 3 months
Sulphuric Acid (2.5 mol/L)	60 mL × 1 vial	2-8°C, 3 months
Standard Stock Solution	10 mL × 1 vial	2-8°C, 3 months

Materials required but not supplied

- Micropipettor
- Incubator
- Centrifuge
- Spectrophotometer (660 nm)
- Tips (10 µL, 200 µL, 1000 µL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water
- Normal Saline (0.9% NaCl)

6. Assay Notes:

1. The tubes used in assay must be disposed strictly without a trace of phosphorus. It is better to use disposable tubes or new tubes to avoid pollution of phosphorus which is the key for success.

2. All the containers of reagents should be dedicated, including the pipette of drawing sulfuric acid and distilled water containers.

3. The protein concentration of the sample to be tested should be less than 3 mg/mL.

7. Reagent preparation:

1. Bring all reagents to room temperature before use.

2. **Preparation of substrate application solution:** Dissolve a vial of substrate lyophilized with 5 mL of double distilled water. The prepared solution can be stored at -20°C for a week.

3. **Preparation of inhibitor application solution:** Dissolve a vial of inhibitor lyophilized with 5 mL of double distilled water and heat appropriately. The prepared solution can be stored at 2-8°C for a week.

4. **Preparation of stop application solution:** Dilute stop solution with double distilled water to the final volume of 15 mL before use. The prepared solution can be stored at 2-8°C for 3 months.

5. **Preparation of reducing agent application solution:** Dissolve 1 vial of reducing agent lyophilized with 30 mL of double distilled water before use. The prepared solution can be stored at 2-8°C with avoid direct sunlight for a week.

6. **Preparation of chromogenic agent application solution:** Dissolve 1 vial of chromogenic agent lyophilized with 60 mL of double distilled water before use. The prepared solution can be stored at 2-8°C for 3 months. If there is a small amount of insoluble lyophilized, take supernatant directly, it will not affect the results.

7. **Preparation of phosphorus assay reagent:** Mix double distilled water, sulphuric acid (2.5 mol/L), reducing agent application solution, chromogenic agent application solution at a ratio of 2:1:1:1. Prepared solution should be pale yellow. If it is colorless or blue, it should be invalid or phosphorus pollution. Prepare the fresh phosphorus assay reagent before use.

8. **Preparation of standard (0.5 μmol/mL):** Dilute the standard stock solution with double distilled water for 20 times. The prepared solution can be stored at 2-8℃ with for a week.

8. Sample Preparation

Sample requirements: The samples should be detect within 24 hours after collecting. Do not treat the samples with phosphorus-containing reagents and detergents such as SDS, Tween20, NP-40, Triton X-100.

1. Cell sample:
Collect the cells with cell scraper (Don't use trypsin or EDTA). Add normal saline (0.9% NaCl) at a ratio of cell number (10^6): normal saline (μL) =1: 300-500, then treat the sample with mechanical homogenate or sonication on ice. Centrifuge at 4ºC at 10000 g for 10 min and collect the supernatant for measurement. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M).

2. Tissue sample:
Take 0.02-1 g tissue sample, wash with normal saline (0.9% NaCl) at 2-8°C. Absorb the water with filter paper and weigh. Then add 9 times the volume of normal saline according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M).

Sample Notes:

The concentration should be determined before preforming the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

Dilution of Samples:

Dilute the 10% tissue homogenate to different concentrations of 2%, 1%, and 0.5%, then take 100 µL for pre-experiment according to the operation steps. The OD value of sample tube should be less than 1.0.

The recommended dilution factor for different samples is as follows (for reference only).

Sample Type:	Dilution Factor
10% Animal tissue homogenate	5
GES-1 cells (2.52mg/mL)	2

Note: The diluent of is normal saline (0.9% NaCl).

9. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: 660 nm

10. Operation Steps

Preparation of working solution A and B:

Prepare needed amount of the fresh **working solution A** (for control tube) and **working solution B** (for sample tube) according to the following table.

	Working solution A	Working solution B
Buffer solution (µL)	130 × (n+2*)	130 × (n+2*)
Accelerator (µL)		80 × (n+2*)
Acid Solution (µL)	120 × (n+2*)	
Substrate application solution (µL)	40 × (n+2*)	40 × (n+2*)
Inhibitor application solution (µL)	40 × (n+2*)	40 × (n+2*)
Complexing agent (µL)		40 × (n+2*)
Total amount of mixture reagent (µL)	330 × (n+2*)	330 × (n+2*)

Note: n refers to the number of sample. 2*: Prepare 2 more tubes of working solution A and working solution B, respectively.

Enzymatic reaction

1. **Control tube:** take 330 μL of working solution A to 1.5 mL EP tube.
   Sample tube: take 330 μL of working solution B to 1.5 mL EP tube.

2. Add 100 μL of sample to **sample tube**.

3. Mix fully and incubate at 37°C for 10 min.

4. Add 50 μL of stop application solution to each tube.

5. Add 100 μL of sample to **control tube**.

6. Mix fully and centrifuge at 2000 g for 10 min, take 400 μL supernatant of each tube for phosphorus assay.

Phosphorus assay

1. **Standard tube:** take 400 μL of 0.5 μmol/mL standard to 5 mL EP tube
   Control tube: take 400 μL of supernatant from corresponding control tube to 5 mL EP tube.
   Sample tube: take 400 μL of supernatant from corresponding sample tube to 5 mL EP tube.

2. Add 2000 μL of phosphorus assay reagent to each tube.

3. Mix fully, incubate at 45°C for 10 min and cool to room temperature.

4. Set the spectrophotometer to zero with distilled water and measure the OD of each tube at 660 nm with 1 cm optical path quartz cuvette.

Operation Table

Enzymatic reaction

	Control tube	Sample tube
Working solution A (μL)	330	
Working solution B (μL)		330
Sample (μL)		100
Mix fully and incubate at 37°C for 10 min.		
Stop application solution (μL)	50	50
Sample (μL)	100	
Mix fully and centrifuge at 2000 g for 10 min, take 400 μL supernatant of each tube for phosphorus assay.		

Phosphorus assay

	Standard tube	Control tube	Sample tube
0.5 μmol/mL standard (μL)	400		
Supernatant of control tube (μL)		400	
Supernatant of sample tube (μL)			400
Phosphorus assay reagent (μL)	2000	2000	2000
Mix fully, incubate at 45°C for 10 min and cool to room temperature. Set the spectrophotometer to zero with distilled water and measure the OD of each tube at 660 nm with 1 cm optical path quartz cuvette.			

11. Calculations

Unit definition:
1 μmol of inorganic phosphorus produced by the decomposition of ATP by ATPase of 1 mg of tissue protein per hour is defined as 1 ATPase activity unit.

$$H^+K^+\text{-ATPase activity (μmol Pi/mgprot/hour)} = \frac{A_2-A_1}{A_3} \times C \times 4.8^* \times 6^{**} \div C_{pr} \times f$$

A_1: the OD value of control
A_2: the OD value of sample
A_3: the OD value of standard
C: the concentration of standard, 0.5 μmol/mL
4.8*: the dilution factor of the sample in the reaction system

$$4.8 = \frac{\text{The total volume of reaction}}{\text{The volume of sample}}$$

$$= \frac{130+80+40+40+40+50+100}{100}$$

6:** the reaction time is 10 min, but the time in unit definition is an hour.
C_{pr}: Concentration of protein in sample, mgprot/mL
f: Dilution factor of sample before tested.

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12. Performance Characteristics

Average recovery rate (%)	109
Average inter-assay CV (%)	9.8
Average intra-assay CV (%)	4.4

Analysis

Dilute 10% rat kidney tissue homogenate with normal saline for 5 times, then take 100 μL of 2% rat kidney tissue homogenate, carry the assay according to the operation table.

The results are as follows:

The average OD value of the control is 0.189, the average OD value of the sample is 0.455, the average OD value of the standard is 0.736, the concentration of protein in sample is 6.95 mgprot/mL, and the calculation result is:

$$\text{H}^+\text{K}^+\text{-ATPase activity (μmol Pi/mgprot/hour)} = \frac{0.455-0.189}{0.736} \times 0.5 \times 4.8 \times 6 \div 6.95 \times 5 = 3.74 \text{ μmol Pi/mgprot/hour}$$

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Safety Notes

Some of the reagents in the kit contain dangerous substances. Prevent touching skin and clothing.

Wash immediately with plenty of water if touching it carelessly.

All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

Before the experiment, read the instructions carefully, and wear gloves and work clothes.

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Notes:

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