（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-K832-M

Specification：48T(32 samples)/96T(80 samples)

Measuring instrument：Microplate reader（630-650 nm）

Detection range：0.001 -0.20 mmol/L

Elabscience® Phosphate Colorimetric Assay Kit

(Malachite Green Method)

This manual must be read attentively and completely before using this product.
If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623
Tell: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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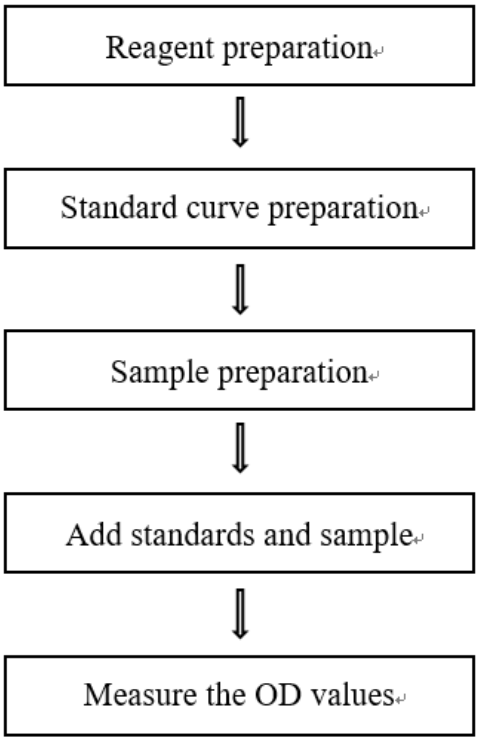
Assay summary



Intended use

This kit can be used to measure phosphate content in serum, plasma, tissue and cell samples.

Detection principle

Phosphorus is an important mineral, one of the essential nutrients for all animals, and is essential for maintaining phosphate homeostasis.

Malachite green method is a sensitive method for phosphate analysis. The principle is that the compounds formed by phosphoric acid and molybdate under acidic conditions will form color substances with malachite green. The depth of color is proportional to the content of phosphate. The phosphorus content can be calculated indirectly be measuring the OD value at 636 nm.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Chromogenic Agent A	6 mL×1 vial	12 mL×1 vial	2-8℃, 12 months, shading light
Reagent 2	Chromogenic Agent B	2 mL×1 vial	4 mL×1 vial	2-8℃, 12 months
Reagent 3	Protein Precipitator	30 mL×1 vial	60 mL×1 vial	2-8℃, 12 months
Reagent 4	10 mmol/L Standard Solution	0.5 mL ×1 vial	1 mL ×1 vial	2-8℃, 12 months
	Microplate	96 wells		No requirement
	Plate Sealer	2 pieces		

Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

Materials prepared by users

Instruments:

Micropipettor, Incubator, Vortex mixer, Centrifuge, Microplate reader (630-650 nm, optimum wavelength: 636 nm)

Reagents:

Normal saline（0.9% NaCl）

Reagent preparation

① Equilibrate all reagents to room temperature before use.

② Preparation of chromogenic working solution:

For each well, prepare 200 μL of chromogenic working solution (mix well 75μL of chromogenic agent A, 25μL of chromogenic agent B and 100μL of double distilled water). Incubate the prepared working solution at 37℃ for 1 h. The chromogenic working solution should be prepared on spot and used up within the same day.

③ Preparation of 0.2 mmol/L standard:

Dilute 20 μL of 10 mmol/L standard solution with 980 μL of double distilled water, mix well. The prepared solution should be prepared on spot. Store at 2-8℃ for 7 days.

④ The preparation of standard curve：

Always prepare a fresh set of standards. Discard working standard dilutions after use.

Dilute 0.2 mmol/L standard with double distilled water to a serial concentration. The recommended dilution gradient is as follows: 0.20, 0.15, 0.12, 0.10, 0.05, 0.02, 0.01, 0 mmol/L. Reference is as follows:

Item	①	②	③	④	⑤	⑥	⑦	⑧
Concentration (mmol/L)	**0**	**0.01**	**0.02**	**0.05**	**0.10**	**0.12**	**0.15**	**0.20**
0.2 mmol/L standard (μL)	0	10	20	50	100	120	150	200
Double distilled water (μL)	200	190	180	150	100	80	50	0

Sample preparation

① Sample preparation

Serum and plasma: For each well，add 10 μL of sample and 10 μL of protein precipitator, mix well. Centrifuge at 12000×g for 10 minutes, collect supernatant and keep it on ice for detection.

Tissue sample:

① Harvest the amount of tissue needed for each assay (initial recommendation 20 mg).

② Wash tissue in cold PBS (0.01 M, pH 7.4).

③ Homogenize 20 mg tissue in normal saline (0.9% NaCl) with a dounce homogenizer at 4℃.

④ Centrifuge at 12000×g for 10 minutes to remove insoluble material. Collect supernatant and keep it on ice for detection.

⑤ For each well，add 10 μL of supernatant and 10 μL of protein precipitator, mix well. Centrifuge at 12000×g for 10 minutes, collect supernatant and keep it on ice for detection.

Cell (adherent or suspension) samples:

① Harvest the number of cells needed for each assay (initial recommendation 1×10^6 cells).

② Wash cells with PBS (0.01 M, pH 7.4).

③ Homogenize 1×10^6 cells in 200 μL normal saline (0.9% NaCl) with a ultrasonic cell disruptor at 4℃.

④ Centrifuge at 12000×g for 10 min at 4℃ to remove insoluble material. Collect

supernatant and keep it on ice for detection.

⑤ For each well，add 10 μL of supernatant and 10 μL of protein precipitator, mix well. Centrifuge at 12000×g for 10 minutes, collect supernatant and keep it on ice for detection.

② Dilution of sample

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

Sample type	Dilution factor
10% Rat liver tissue homogenate	15-25
10% Rat heart tissue homogenate	15-25
10% Rat spleen tissue homogenate	15-25
10% Rat brain tissue homogenate	15-25
10% Rat kidney tissue homogenate	15-25
10% Mouse heart tissue homogenate	15-25
10% Mouse lung tissue homogenate	15-25
10% Mouse ovarian tissue homogenate	50-70
Rat serum	8-12
Rat plasma	8-12
Human plasma	8-12
10^6 HL-40 cell	2-8

Note: The diluent is normal saline (0.9% NaCl). For the dilution of other sample types, please do pretest to confirm the dilution factor

The key points of the assay

① When the absoluted OD value is more than 1.2, it is necessary to increase the dilution ratio and detect again.

② Incubate chromogenic agent A at 37℃ until no gelatinous substance before use.

③ Avoid the contamination of phosphorus, it is recommended to wash the experimental equipment for several times (about 10 times).

Operating steps

① Standard well: Take 20 μL of standard solution with different concentration to the well.

Sample well: Take 20 μL of sample to the well.

② Add 200 μL of chromogenic working solution to each well.

③ Mix fully with microplate reader for 5 s and incubate at 37℃ for 20 min with shading light.

④ Measure the OD value of each well at 636 nm with microplate reader.

Calculation

The standard curve:

1. Average the duplicate reading for each standard.

2. Subtract the mean OD value of the blank (Standard #①) from all standard readings. This is the absoluted OD value.

3. Plot the standard curve by using absoluted OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (**y = ax + b**) with graph software (or EXCEL).

The sample:

1. Serum (plasma) sample:

$$\text{Phosphate (mmol/L)} = \frac{\Delta A - b}{a} \times f \times 2^*$$

2. Tissue sample:

$$\text{Phosphate (mmol/kg wet weight)} = \frac{\Delta A - b}{a} \div \frac{m}{V} \times f \times 2^*$$

3. Cell sample:

$$\text{Phosphate (mmol/10^6)} = \frac{\Delta A - b}{a} \div \frac{n}{V} \times f \times 2^*$$

[Note]

ΔA: $OD_{Sample} - OD_{Blank}$.

m: The weight of tissue sample, g.

V: The volume of normal saline in the preparation step of sample, mL.

n: The number of cell sample/10^6.

2*: Dilution factor of sample in preparation of supernatant.

f: Dilution factor of sample before test.

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

Three human serum samples were assayed in replicates of 20 to determine precision within an assay (CV = Coefficient of Variation).

Parameters	Sample 1	Sample 2	Sample 3
Mean (mmol/L)	0.03	0.08	0.15
%CV	4.2	4.0	3.8

Inter-assay Precision

Three human serum samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (mmol/L)	0.03	0.08	0.15
%CV	7.9	8.3	7.8

Recovery

Take three samples of high concentration, middle concentration and low concentration to test the samples of each concentration for 6 times parallelly to get the average recovery rate of 98%.

	Standard 1	Standard 2	Standard 3
Expected Conc. (mmol/L)	0.016	0.077	0.13
Observed Conc. (mmol/L)	0.0	0.1	0.1
Recovery rate(%)	96	99	99

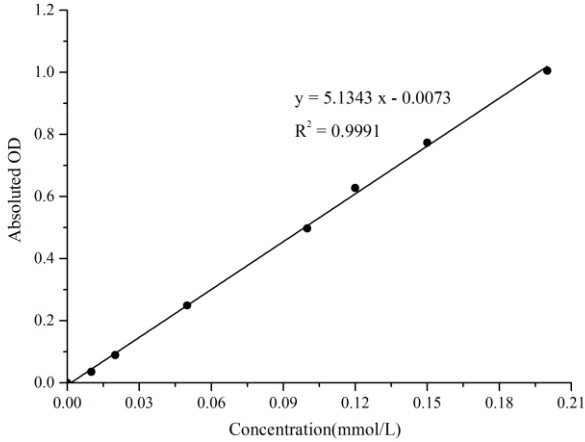
Sensitivity

The analytical sensitivity of the assay is 0.001 mmol/L. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

2. Standard curve:

As the OD value of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique or temperature effects), so the standard curve and data are provided as below for reference only:

Concentration (mmol/L)	**0**	**0.01**	**0.02**	**0.05**	**0.10**	**0.12**	**0.15**	**0.20**
Average OD	0.142	0.177	0.231	0.391	0.639	0.769	0.915	1.147
Absoluted OD	0.000	0.036	0.090	0.250	0.498	0.628	0.774	1.006



Appendix II Example Analysis

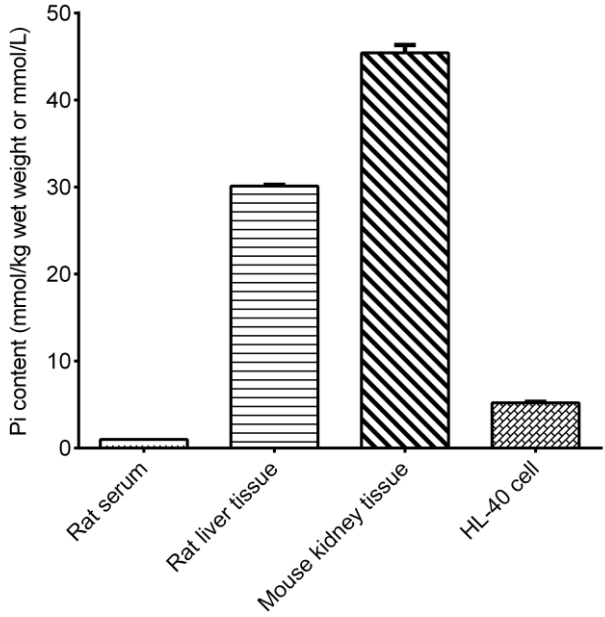
Example analysis:

For rat liver tissue, take 10% rat liver tissue homogenate, dilute for 20 times and carry the assay according to the operation steps. The results are as follows:

Standard curve: y = 5.1343 x - 0.0073, the average OD value of the blank is 0.142, the average OD value of the sample is 0.564, and the calculation result is:

Phosphate content (mmol/kg wet weight) = (0.564 – 0.142 + 0.0073) ÷5.1342 ÷(0.1 ÷0.9) ×20 ×2 = 30.06 mmol/kg wet weight

Detect rat serum (dilute for 10 times), 10% rat liver tissue homogenate (dilute for 20 times), 10% mouse kidney tissue homogenate (dilute for 20 times) and HL-40 cell (dilute for 3 times) according to the protocol, the result is as follows:



Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.