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

Catalog No：E-BC-K895-M

Specification：96T (39 samples)

Measuring instrument：Microplate reader(530-550 nm)

Detection range：0.68-100.00 μmol/L

Elabscience® Nitrate Colorimetric Assay Kit

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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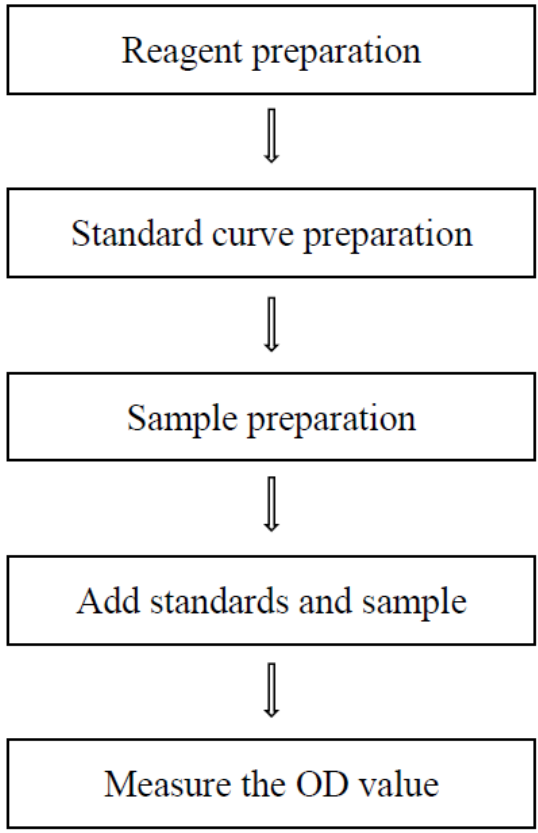
Assay summary



Intended use

The kit can be used to measure the nitrate (NO_3^-) content in serum (plasma), urine, tissue, cell and cell culture supernatant samples.

Detection principle

Nitric oxide (NO) is chemically active and is rapidly metabolized in the body to nitrite (NO^{2-}) and nitrate (NO^{3-}), and NO^{2-} is further converted into NO^{3-}. In this kit, NO^{3-} was reduced to NO^{2-} by reductant agent, and reacts with chromogenic agent to produce color substance. The nitrate content can be determined at 540 nm.

Kit components & storage

Item	Component	Size (96 T)	Storage
Reagent 1	Precipitant Agent	4 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 2	Reductant Agent	Powder × 3 vials	2-8°C, 12 months, shading light
Reagent 3	Acid Solution	11 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 4	Chromogenic Agent A	8 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 5	Chromogenic Agent B	8 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 6	1 mmol/L Standard	1 mL × 1 vial	2-8°C, 12 months, shading light
	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:

Microplate reader (530-550 nm, optimum wavelength: 540 nm), Vortex mixer

Reagent:

PBS (0.01 M, pH 7.40), 2 mol/L NaOH

Reagent preparation

① Equilibrate all the reagents to 25℃ before use.

② The preparation of reductant working solution:

Dissolve one vial of reductant reagent with 3 mL of acid solution, mix well. Store at 2-8 ℃ for 2 days protected from light.

③ The preparation of 100 μmol/L standard solution:

Before testing, please prepare sufficient 100 μmol/L standard solution. For example, prepare 2000 μL of 100 μmol/L standard solution (mix well 200 μL of 1 mmol/L standard and 1800 μL of double distilled water). Store at 2-8 ℃ for 3 days protected from light.

④ The preparation of standard curve:

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

Dilute 100 μmol/L standard with double distilled water to a serial concentration, the recommended dilution gradient is 0, 10, 20, 30, 40, 50, 70, 100 μmol/L. Reference is as follows:

Item	①	②	③	④	⑤	⑥	⑦	⑧
Concentration (μmol/L)	**0**	**10**	**20**	**30**	**40**	**50**	**70**	**100**
100 μmol/L standard (μL)	0	40	80	120	160	200	280	400
Double distilled water (μL)	400	360	320	280	240	200	120	0

Sample preparation

① Sample preparation

Serum (plasma), urine and supernatant of cell samples:

① Take 300 μL of serum (plasma), urine or supernatant of cell, add 60 μL of precipitant agent, vortex for at least 2 min, mix fully as sample solution.

② Mix well 300 μL of sample solution and 15 μL of 2 mol/L NaOH for more than 2 min.

③ Centrifuge at 10000×g for 10 min at 4℃, collect supernatant and keep it on ice for detection. The prepared solution should be used up within 2 days, and store at -20℃.

Tissue sample:

① Harvest the amount of tissue needed for each assay (initial recommendation 0.1 g).

② Homogenize 0.1 g tissue in 0.9 mL of PBS (0.01 M, pH 7.40) with a dounce homogenizer at 4℃.

③ Centrifuge at 10000×g for 10 min to remove insoluble material. Collect supernatant and keep it for detection.

④ Vortex 300 μL of supernatant and 60 μL of precipitant agent for at least 2 min, mix fully as sample solution. Mix well 300 μL of sample solution and 15 μL of 2 mol/L NaOH for more than 2 min.

④ Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection. The prepared solution should be used up within 2 days, and store at -20℃.

⑤ **Cell samples:**

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

② Homogenize 1×10^6 cells in 0.2 mL PBS (0.01 M, pH 7.40) with a ultrasonic cell disruptor at 4℃.

③ Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it for detection.

④ Vortex 300 μL of supernatant and 60 μL of precipitant agent for at least 2 min, mix fully as sample solution. Mix well 300 μL of sample solution and 15 μL of 2 mol/L NaOH for more than 2 min.

⑤ Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection. The prepared solution should be used up within 2 days at -20℃.

② Dilution of sample

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

Sample type	Dilution factor
Human serum	1
Rat serum	1
Mouse serum	1
Mouse plasma	1
Mouse urine	10-20
10% Mouse kidney tissue homogenate	1-2
10% Mouse liver tissue homogenate	1-2
10% Young garlic shoot tissue homogenate	1
1×10^6 Jurkat cells	1
1×10^6 HL-60 cells	1
1×10^6 293T cells	1
Cell culture supernatant	1

Note: The diluent is PBS (0.01 M, pH 7.40). For the dilution of other sample types, please do pretest to confirm the dilution factor.

Operating Steps

① Standard well: add 100 μL of standard with different concentrations into the corresponding wells.

Sample well: add 100 μL of sample into the corresponding wells.

Control well: add 100 μL of sample into the corresponding wells.

Blank control well: add 100 μL of double distilled water into the corresponding wells.

② Add 100 μL of reductant working solution into standard wells and sample wells.

Add 100 μL of acid solution into control wells and blank control wells.

③ Add 50 μL of chromogenic agent A into each well.

④ Add 50 μL of chromogenic agent B into each well.

⑤ Mix fully with microplate reader for 5 s, incubate at 37℃ for 60 min. Measure the OD value of each well at 540 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), urine and cell culture supernatant:

$$\text{Nitrate content } (\mu\text{mol/L}) = (\Delta A_{540} - b) \div a \times f$$

2. Tissue sample:

$$\text{Nitrate content } (\mu\text{mol/kg wet weight}) = (\Delta A_{540} - b) \div a \div m \times v \times f$$

3. Cell samples:

$$\text{Nitrate content } (\text{nmol/10\^6}) = (\Delta A_{540} - b) \div a \div n \times v \times f$$

[Note]

$\Delta A_1 = A_{sample} - A_{blank}$ (The blank is the OD value when the standard concentration is 0).

$\Delta A_2 = A_{control} - A_{blank\ control}$.

$\Delta A_{540} = \Delta A_1 - \Delta A_2$.

m: The weight of the sample, g.

V: The volume of PBS in the preparation of step, mL.

n: The number of the cell samples, 10^6.

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 (μmol/L)	15.00	35.00	60.00
%CV	1.2	1.9	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 (μmol/L)	15.00	35.00	60.00
%CV	7.8	8.3	9.3

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 95.7%.

	Standard 1	Standard 2	Standard 3
Expected Conc. (μmol/L)	15	35	60
Observed Conc. (μmol/L)	14.1	33.3	58.8
recovery rate(%)	94	95	98

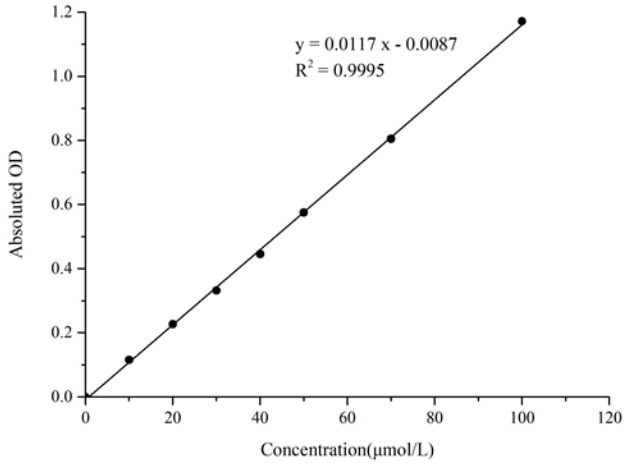
Sensitivity

The analytical sensitivity of the assay is 0.68 μmol/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 (μmol/L)	0	10	20	30	40	50	70	100
OD	0.109	0.231	0.345	0.447	0.558	0.684	0.931	1.299
	0.117	0.226	0.335	0.443	0.560	0.692	0.905	1.270
Average OD	0.113	0.229	0.340	0.445	0.559	0.688	0.918	1.285
Absoluted OD	0	0.116	0.227	0.332	0.446	0.575	0.805	1.172



Appendix Ⅱ Example Analysis

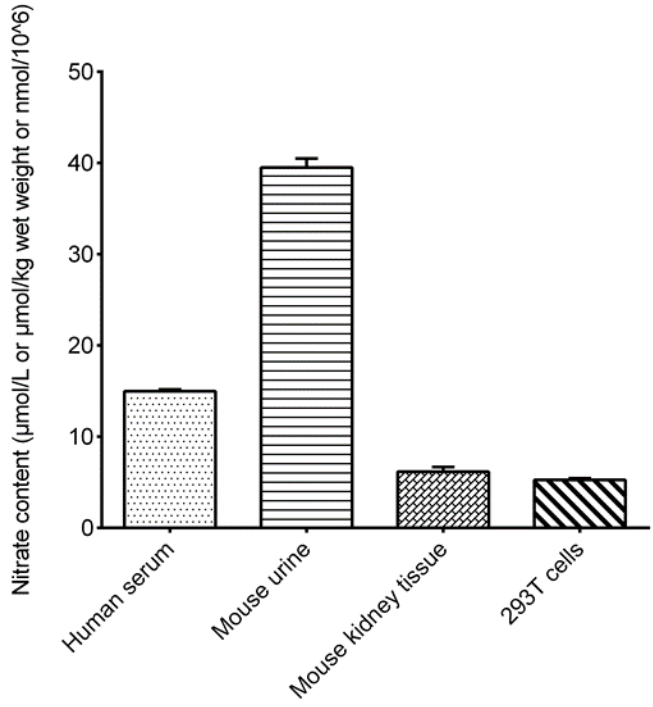
Example analysis:

Take 100 μL of human serum sample and carry the assay according to the operation steps. The results are as follows:

Standard curve: y = 0.0117 x - 0.0087, the average OD value of the sample well is 0.305, the average OD value of the blank well is 0.113, the average OD value of the control well is 0.070, the average OD value of the blank control well is 0.044, A_2 = 0.070 - 0.044 = 0.026, ΔA_{540} = 0.192 - 0.026 = 0.166,and the calculation result is:

$$\text{Nitrate content } (\mu\text{mol/L}) = (0.166 + 0.0087) \div 0.0117 = 14.932\ \mu\text{mol/L}$$

Detect human serum, mouse urine (diluent for 10 times), 10% mouse kidney tissue homogenate (diluent for 2 times) and 1×10^6 293T cells, 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.