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

Catalog No：E-BC-K817-M

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

Measuring instrument：Microplate reader (530-550 nm)

Detection range：0.46-100 μmol/L

Elabscience® Nitric Oxide (NO) 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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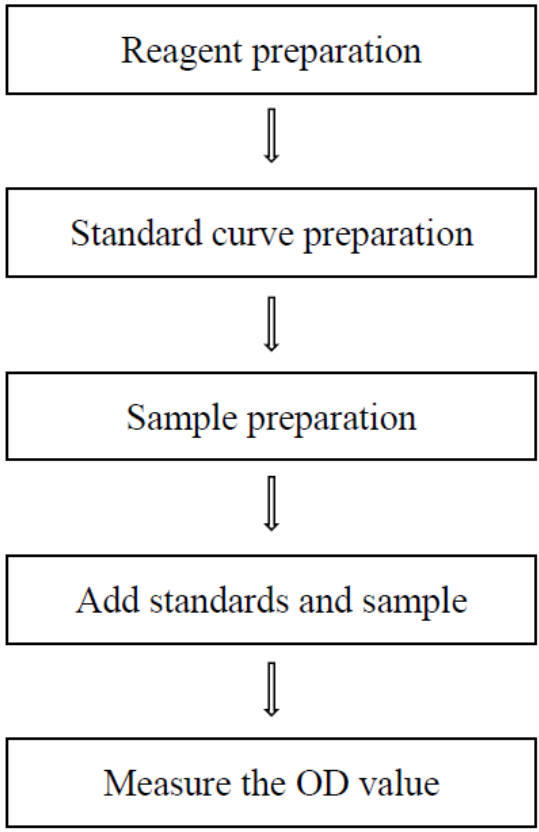
Assay summary



Intended use

The kit is suitable for detecting the content of Nitric Oxide (NO) in serum, plasma, urine, animal tissues and cells.

Detection principle

Nitric oxide (NO) is chemically reactive and is rapidly metabolized in the body to nitrite (NO_2^-) and nitrate (NO_3^-), with NO_2^- further converting to NO_3^-. This method utilizes a reducing agent to convert NO_3^- to NO_2^-, and the color development with a chromogenic reagent can be detected at 540 nm to determine the content of NO.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Precipitant Agent	8 mL × 1 vial	16 mL × 1 vial	2-8°C, 6 months, shading light
Reagent 2	Reductant Agent	powder × 2 vials	powder × 4 vials	2-8°C, 6 months, shading light
Reagent 3	Acid Solution	7 mL × 1 vial	14 mL × 1 vial	2-8°C, 6 months
Reagent 4	Chromogenic Agent A	4 mL × 1 vial	8 mL × 1 vial	2-8°C, 6 months, shading light
Reagent 5	Chromogenic Agent B	4 mL × 1 vial	8 mL × 1 vial	2-8°C, 6 months, shading light
Reagent 6	1 mmol/L Standard	1 mL × 1 vial	1 mL × 1 vial	2-8°C, 6 months, shading light
	Microplate	48 wells	96 wells	No requirement
	Plate Sealer	2 pieces		
	Sample Layout Sheet	1 piece		

Note: All the reagents should be stored according to the table. The reagents from different kits can not be mixed or used interchangeably. For liquid reagents with small volumes or powders, centrifuge them before use to prevent loss.

Instruments

Microplate reader (530-550 nm, optimum wavelength: 540 nm), Incubator (37°C)

Materials required but not provided

Double distilled water, 1×PBS (0.01 M, pH 7.4), 2 M NaOH

Reagent preparation

① Equilibrate all the reagents to 25°C before use.

② Preparation of Working Solution of Reductant Agent ：

Take one bottle of reductant agent and add 3 mL of Acid Solution to dissolve it completely. The unused working solution of reductant agent can be stable for 2 days when stored at 2-8°C protected from light.

③ 100 μmol/L Standard Solution preparation：

Before testing, prepare a sufficient amount of 100 μmol/L standard solution according to the test wells. For example, prepare 1000 μL of a 100 μmol/L standard solution (mix 900 μL of double distilled water and 100 μL of a 1 mmol/L standard solution thoroughly). The 100 μmol/L standard solution should be stable for 3 days when stored at 2-8°C protected from light.

④ Standard curve preparation：

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

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

Serum, plasma, urine and cell supernatant:

① Mix well 100 μL of the sample and 20 μL of precipitant agent for more than 2 minutes.

② According to the volume ratio of sample solution: 2 M NaOH = 20:1 (mix well 120 μL of the sample and 6 μL of 2 M NaOH), mix for more than 2 minutes.

③ Centrifuge at 10000×g for 10 min at 4°C. Collect supernatant and keep it on ice for detection. The prepared sample supernatant store at -20°C and use within 2 days.

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 180 μL PBS (0.01 M, pH 7.4) with a dounce homogenizer at 4°C.

④ Centrifuge at 10000×g for 10 min at 4°C. Collect supernatant and for each well,mix well 100 μL of the supernatant and 20 μL of precipitant agent for more than 2 minutes to obtain the sample solution.

⑤ According to the volume ratio of sample solution: 2 M NaOH = 20:1 (mix well 120 μL of the sample and 6 μL of 2 M NaOH), mix for more than 2 minutes.

⑥ Centrifuge at 10000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and keep it on ice for detection. The prepared sample supernatant store at -20°C and use within 2 days.

Cell sample

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 PBS (0.01 M, pH 7.4) with a ultrasonic cell disruptor at 4°C.

④ Centrifuge at 10000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and for each well, mix well 100 μL of the supernatant and 20 μL of precipitant agent for more than 2 minutes to obtain the sample solution.

⑤ According to the volume ratio of sample solution: 2 mol/L NaOH = 20:1 (mix well 120 μL of the sample and 6 μL of 2 mol/L NaOH), mix for more than 2 minutes.

⑥ Centrifuge at 10000×g for 10 min at 4°C. Collect supernatant and keep it on ice for detection. The prepared sample supernatant store at -20°C and use within 2 days.

Dilution of sample

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

Sample type	Dilution factor
10% Mouse liver tissue homogenization	1
10% Mouse kidney tissue homogenization	20-30
10% Mouse heart tissue homogenization	1
10% Mouse spleen tissue homogenization	1
Rats serum	1
Human plasma	1
Human urine	1
1×10^6 HL-60 cells	1
1×10^6 H9c2 cells	1
1×10^6 Hela cells	1
1×10^6 Jurkat cells	1

Note: The diluent is PBS (0.01 M, pH 7.4). 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 solution with different concentrations to the corresponding wells.

Sample well: Add 100 μL of sample to the corresponding wells.

② Add 100 μL of working solution of reductant agent to each well.

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

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

⑤ Mix fully with microplate reader for 5 s. Incubate at 37°C for 60 min. Measure the OD value of each well.

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 Corrected OD value.

3. Plot the standard curve by using Corrected 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:

Serum, plasma, urine and cell supernatant:

$$\text{NO content (μmol/L)} = (\Delta A - b) \div a \times f$$

Tissue sample:

$$\text{NO content (μmol/kg wet weight)} = (\Delta A - b) \div a \div m \times v \times f$$

Cell sample:

$$\text{NO content (nmol/10^6)} = (\Delta A - b) \div a \div n \times v \times f$$

[Note]

ΔA: Corrected OD, $OD_{Sample} - OD_{Blank}$.

m: The wet of sample, g.

V: The volume of homogenate solution added to the reaction system, mL

f: Dilution factor of sample before test.

n: The number of cell sample/10^6

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

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

Three human serum samples were assayed in replicates of 20 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	15	30	60
%CV	4.7	1.6	1.0

Inter-assay Precision

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

Three human serum samples were assayed 17 times in duplicate by three operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	15	30	60
%CV	4.8	3.8	3.4

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 105%.

	Sample 1	Sample 2	Sample 3
Expected Conc. (μmol/L)	15	30	60
Observed Conc. (μmol/L)	15.9	31.5	62.4
Recovery rate (%)	106	105	104

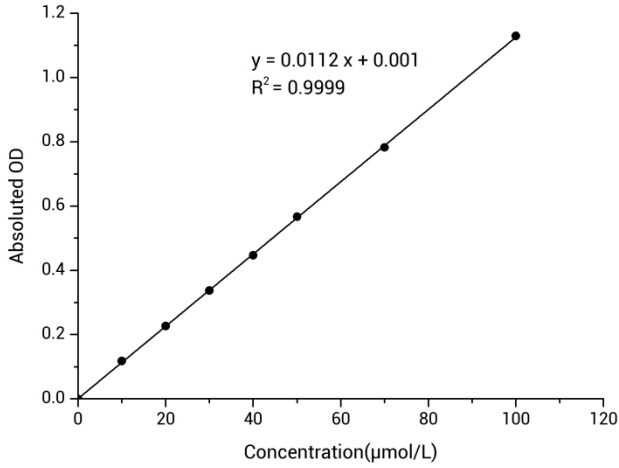
Sensitivity

The analytical sensitivity of the assay is 0.46 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 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	10	20	30	40	50	70	100
OD	0.101	0.218	0.331	0.439	0.548	0.668	0.871	1.219
	0.100	0.218	0.324	0.436	0.547	0.667	0.896	1.241
Average OD	0.101	0.218	0.328	0.438	0.548	0.668	0.884	1.230
Corrected OD	0	0.118	0.227	0.337	0.447	0.567	0.783	1.130



Appendix II Example Analysis

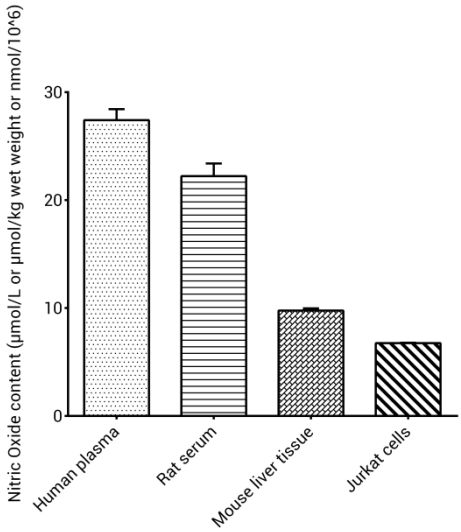
Example analysis：

Take 100 μL of the treated human plasma to the well of microplate. Proceed according to the operating steps. The results are as follows:

standard curve: y = 0.0112 x + 0.001. The OD of sample is 0.409, the OD of blank is 0.101, ΔA = 0.409 - 0.101 = 0.308, and the calculation result is:

$$\text{NO content (μmol/L)} = (0.308 - 0.001) \div 0.0112 = 27.41\ \text{μmol/L}$$

Detect Human plasma,Rat serum, 10% mouse liver tissue homogenization, 1×10^6 Jurkat 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.