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

Catalog No：E-BC-K035-M

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

Measuring instrument：Microplate reader (540-550 nm)

Detection range：0.16-100 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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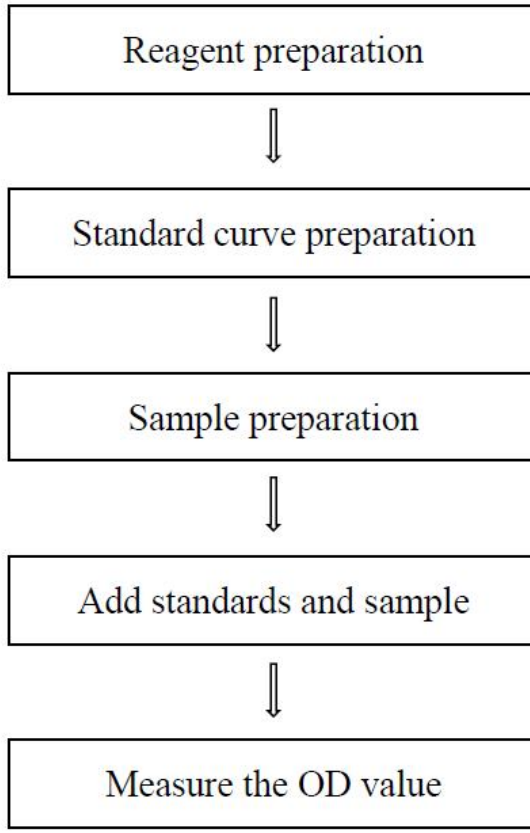
Assay summary

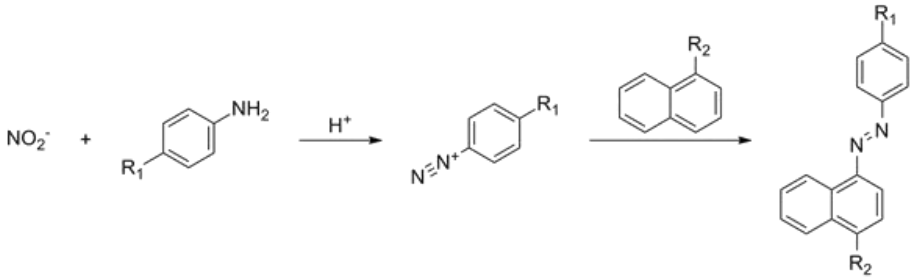


Intended use

This kit can be used to measure nitric oxide (NO) in serum, plasma, saliva, animal and plant tissue samples.

Detection principle

NO is easily oxidized to form NO2- in vivo or in aqueous solution, and a reddish azo compound is formed with the color developing agent, and the concentration of the azo compound is linearly related to the concentration of NO. The concentration of NO can be calculated indirectly by measuring the OD value at 550 nm.



Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Sulphate Solution	12 mL × 1 vial	24 mL × 1 vial	2-8℃, 12 months
Reagent 2	Alkali Reagent	12 mL × 1 vial	12 mL × 1 vial	2-8℃, 12 months
Reagent 3	Chromogenic Agent A	1.9 mL × 1 vial	1.9 mL × 2 vials	2-8℃, 12 months shading light
Reagent 4	Chromogenic Agent B	Powder × 1 vial	Powder × 1 vial	2-8℃, 12 months shading light
Reagent 5	Acid Solution	1.3 mL × 1 vial	1.3 mL × 2 vials	2-8℃, 12 months
Reagent 6	Sodium Nitrite Standard	Powder × 1 vial	Powder × 2 vials	-20℃, 12 months
	Microplate	48 wells	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(540-550 nm), Micropipettor, Vortex mixer, Centrifuge

Reagents:

Double distilled water, Normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4)

Reagent preparation

① Equilibrate other reagents to room temperature before use.

② If there is any crystal precipitation in chromogenic agent A, please dissolve it fully with water bath at above 60℃ before use.

③ The preparation of chromogenic agent B working solution：
Dissolve one vial of chromogenic agent B with 3.8 mL of double distilled water, mix well to dissolve. Store at 4°C for 2 month protected from light.

④ The preparation of chromogenic reagent：
For each well, prepare 80 uL of chromogenic reagent (mix well 30 uL of chromogenic agent A, 30 uL of chromogenic agent B working solution and 20 uL of acid solution). The chromogenic reagent should be prepared on spot and can't be used when its color gets darker.

⑤ The preparation of 2 mmol/L sodium nitrite standard：
Dissolve one vial of sodium nitrite standard with 2 mL of double-distilled water. The 2 mmol/L sodium nitrite standard should be prepared on spot.

⑥ The preparation of standard curve：

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

Dilute 2 mmol/L sodium nitrite standard with double distilled water diluent to a serial concentration. The recommended dilution gradient is as follows: 0, 10, 20, 30, 40, 60, 80, 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**	**60**	**100**
2 mmol/L sodium nitrite (μL)	0	10	20	30	40	60	80	100
Double distilled water (μL)	2000	1990	1980	1970	1960	1940	1920	1900

Sample preparation

① Sample preparation

Serum and plasma: detect directly. If not detected on the same day, the serum or plasma can be stored at -80℃ for a month.

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℃.

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

⑤ Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M).

② Dilution of sample

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

Sample type	Dilution factor
Human serum	1
Human plasma	1
Rat serum	1
Rat plasma	1
10% Mouse liver tissue homogenization	1
10% Epipremnum aureum tissue homogenization	1

Note: The diluent is normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4). For the dilution of other sample types, please do pretest to confirm the dilution factor

The key points of the assay

① Use disposable EP tubes or clean glass tubes with stopper for centrifugation.

② The supernatant for assay should not contain sediment, otherwise it will affect the results.

③ All reagents should be prepared the day before the assay, let it fully dissolved. Please add reagents to the bottom of well vertically and slowly, avoid to add on the wall of well and generate bubble.

④ Serum samples can be stored for 3 days at 4℃ and for a month at -20℃..

Operating steps

① Standard tubes: add a* μL of sodium nitrite standard solution with different concentrations to 1.5 mL EP tubes.

Sample tubes: add a* μL of sample to 1.5 mL EP tubes.

[Note]: a* = Sample volume= Standard volume. For serum or plasma samples, a* is 200-300 μL. For tissue samples, a* is 100-300 μL.

② Add 200 μL of sulphate solution and mix fully with a vortex mixer.

③ Add 100 μL of alkali reagent and mix fully with a vortex mixer.

④ Stand for 15 min at room temperature, centrifuge at 3100 g for 10 min. (If there is precipitate in the supernatant, please transfer the supernatant to a new EP tube and centrifuge again.).

⑤ Take 160 μL of supernatant to the corresponding wells of microplate for chromogenic reaction.

⑥ Add 80 μL of chromogenic reagent to each well, oscillate for 2 min and stand at room temperature for 15 min.

⑦ Measure the OD value at 550 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:

$$\text{NO content (μmol/L)} = (\Delta A_{550}\text{-b}) \div a \times f$$

2. Tissue:

$$\text{NO content (μmol/gprot)} = (\Delta A_{550}\text{-b}) \div a \times f \div C_{pr}$$

[Note]

ΔA_{550}: Absolute OD, $OD_{Sample} - OD_{Blank}$.

f: Dilution factor of sample before test.

C_{pr}: Concentration of protein in sample, mgprot/mL

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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)	1.30	36.50	74.20
%CV	2.8	2.1	2.3

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)	1.30	36.50	74.20
%CV	3.5	3.8	3.8

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 102%.

	Standard 1	Standard 2	Standard 3
Expected Conc. (μmol/L)	15	34	78
Observed Conc. (μmol/L)	15.2	35.4	78.8
Recovery rate (%)	101	104	101

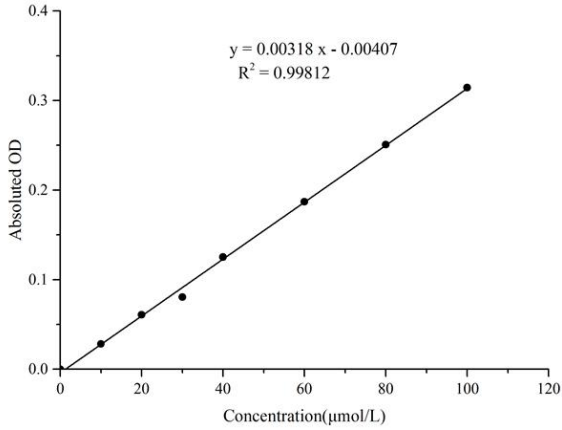
Sensitivity

The analytical sensitivity of the assay is 0.16 μmol/L NO. 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	60	80	100
Average OD	0.036	0.065	0.097	0.117	0.162	0.223	0.287	0.351
Absoluted OD	0.000	0.028	0.061	0.081	0.125	0.187	0.251	0.314



Appendix II Example Analysis

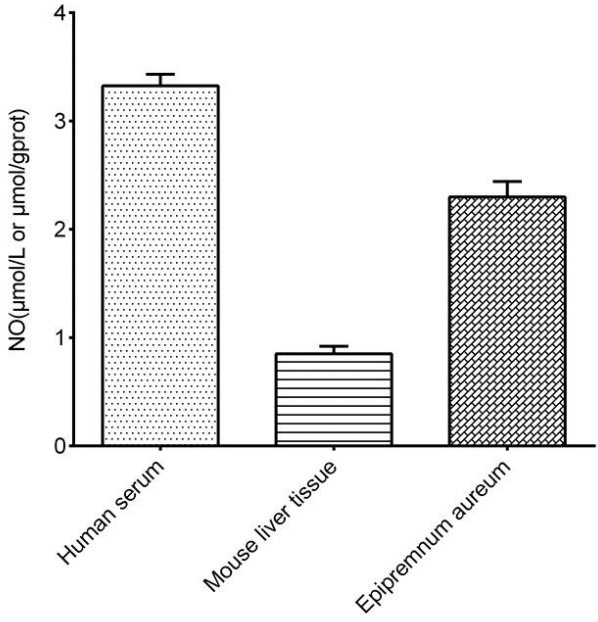
Example analysis：

Dilute human serum for 2 times, carry the assay according to the operation steps. The results are as follows:

standard curve: y = 0.00215 x + 0.00514, the average OD value of the sample is 0.056, the average OD value of the blank is 0.035, the concentration of protein in sample is 9.23 gprot/L, and the calculation result is:

$$\text{NO content (μmol/L)} = (0.056 - 0.035 - 0.00514) \div 0.00215 = 3.20\ (\mu mol/L)$$

Detect human serum (dilute for 2 times, a*=100 μL), 10% mouse liver tissue homogenate (the concentration of protein is 1.82 gprot/L, a*=300 μL), 5% Epipremnum aureum leaves tissue homogenate (the concentration of protein in sample is 0.99 gprot/L, a*=300 μL), according to the protocol, the result is as follows：



Appendix III Publications

1. Xia Y, Zeng Y, Jiang R. Effect of chronic periodontitis on the endothelial glycocalyx of rat penile corpus cavernosum[J]. Andrology, 2024.
2. Li J, Hu H, Xu X, et al. Mechanisms of action of ethyl acetate fractions of Liparis nervosa (Thunb.) Lindl. as potential central anti-nociceptive agents[J]. Inflammopharmacology, 2024: 1-17.
3. Chen J, Wu L, Xie X, et al. Carthamus tinctorius L. protects cerebral ischemia/reperfusion injury via arachidonic acid/p53-mediated apoptosis axis[J]. Frontiers in Pharmacology, 2024, 15: 1504109.

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.