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

**Catalog No：E-BC-K135-M**

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

**Measuring instrument：Microplate reader（520-550 nm）**

**Detection range：1.38-40 μmol/L**

# **Elabscience®** Nitric Oxide (NO) Colorimetric Assay Kit (Nitrate Reductase 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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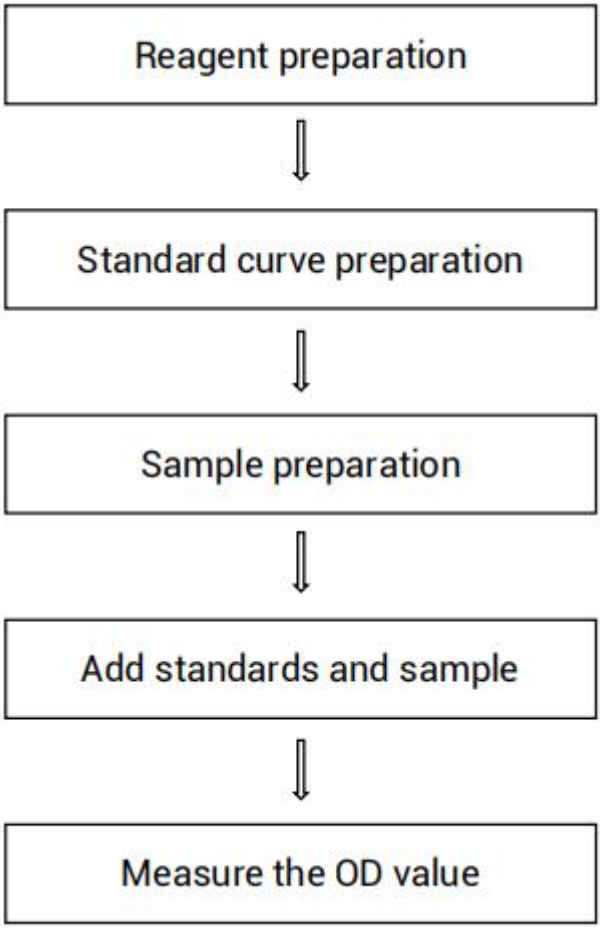
**Assay summary**



## Intended use

The kit can detect the concentration of NO in serum, plasma, urine, tissue,cell and supernatant of cell samples.

## Detection principle

Nitric oxide (NO) has active chemical properties and is quickly metabolized into NO2- and NO3- in the body, while NO2- is further transformed into NO3-. This method uses nitrate reductase specifically to reduce NO3- to NO2-, and the concentration of NO is measured by the color depth.

## Kit components & storage

| Item | Component | Size 1(48 T) | Size 2(96 T) | Storage |
|---|---|---|---|---|
| Reagent 1 | Enzyme Reagent | Powder × 2 vials | Powder × 4 vials | -20℃, 12 months, shading light |
| Reagent 2 | Substrate | Powder × 1 vial | Powder × 1 vial | -20℃, 12 months, shading light |
| Reagent 3 | Sulfate Solution | 1.5 mL × 1 vial | 3 mL × 1 vial | -20℃, 12 months |
| Reagent 4 | Alkaline Reagent | 0.75 mL × 1 vial | 1.5 mL × 1 vial | -20℃, 12 months |
| Reagent 5 | Chromogenic Agent A | Powder × 1 vial | Powder × 1 vial | -20℃, 12 months, shading light |
| Reagent 6 | Chromogenic Agent B | Powder × 1 vial | Powder × 1 vial | -20℃, 12 months, shading light |
| Reagent 7 | Acid Reagent | 1.5 mL × 1 vial | 3 mL × 1 vial | -20℃, 12 months |
| Reagent 8 | 1 mmol/L Standard | 0.75 mL × 1 vial | 1.5 mL × 2 vials | -20℃, 12 months |
| | Microplate | 48 wells | 96 wells | No requirement |
| | Plate Sealer | 2 pieces | | |

| | | | |
|---|---|---|---|
| | Sample Layout Sheet | 1 piece | |

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:**

Vortex mixer, 37℃ Incubator, Centrifuge, Microplate reader (520-550 nm, optimum wavelength: 530 nm)

## Reagent preparation

① Keep enzyme reagent and substrate on ice during use. Equilibrate all the reagents to room temperature before use.

② The preparation of enzyme stock solution：
Dissolve one vial of enzyme reagent with 1 mL of ultrapure water, mix well. Store at 2-8°C for 6 hours protected from light.

③ The preparation of substrate working solution：
Dissolve one vial of substrate with 5 mL of ultrapure water, mix well. Store at -20°C for 3 days protected from light.

④ The preparation of enzyme working solution：
For each well, prepare 60 μL of enzyme working solution (mix well 30 μL of enzyme stock solution and 30 μL of substrate working solution). The enzyme working solution should be prepared on spot. Store at 2-8°C for 6 hours protected from light.

⑤ The preparation of chromogenic A working solution：
Dissolve one vial of chromogenic A with 6 mL of ultrapure water, heat

and dissolve in 90°C water bath. The working solution can be stored at 2-8°C with shading light for 3 days and be heated to dissolved in 90°C water bath when it is used again.

⑥ The preparation of chromogenic B working solution：
Dissolve one vial of chromogenic B with 4 mL of ultrapure water, mix well to dissolve. Store at 2-8°C for 3 days protected from light.

⑦ The preparation of chromogenic working solution：
Before testing, please prepare sufficient chromogenic working solution according to the test wells. For example, prepare 90 μL of chromogenic working solution (mix well 50 μL of chromogenic A working solution, 20 μL of chromogenic B working solution and 20 μL of acid reagent). The chromogenic working solution should be prepared on spot and protect from light.

⑧ The preparation of 40 μmol/L standard solution：
Dilute 168 μL of 1 mmol/L standard with 4032 μL of ultrapure water, mix well. The 40 μmol/L standard solution should be prepared on spot and protect from light.

⑨ The preparation of standard curve：
Always prepare a fresh set of standards. Discard working standard dilutions after use.
Dilute 40 μmol/L standard solution with ultrapure water to a serial concentration. The recommended dilution gradient is as follows: 0, 8, 16, 20, 24, 28, 32, 40 μmol/L. Reference is as follows:

| Item | ① | ② | ③ | ④ | ⑤ | ⑥ | ⑦ | ⑧ |
|---|---|---|---|---|---|---|---|---|
| **Concentration (μmol/L)** | **0** | **8** | **16** | **20** | **24** | **28** | **32** | **40** |
| **40 μmol/L standard (μL)** | 0 | 200 | 400 | 500 | 600 | 700 | 800 | 1000 |
| **ultrapure water (μL)** | 1000 | 800 | 600 | 500 | 400 | 300 | 200 | 0 |

## 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°C for a month.

**Tissue sample:**

① Harvest the amount of tissue needed for each assay (initial recommendation 40 mg).

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

③ Homogenize 40 mg tissue in 360 μL normal saline (0.9% NaCl) with a dounce homogenizer at 4℃.

④ Centrifuge at 10000×g for 10 min 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).

**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 300 μL normal saline (0.9% NaCl) with a ultrasonic cell disruptor at 4℃.

④ Centrifuge at 10000×g for 10 min 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 urine | 20-30 |
| Mouse serum | 1-3 |
| Chicken plasma | 1 |
| Rat urine | 20-30 |
| 10% Rat brain tissue homogenate | 1 |
| 10% Rat kidney tissue homogenate | 1 |
| 10% Vegetable leaf tissue homogenate | 1 |
| 1×10^6 Jurkat cell | 1 |

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 The prepared chromogenic working solution should be stored with shading light.

② After adding the enzyme working solution and sample or standard to the EP tube, it should be mixed fully.

③ Avoid the precipitation at the bottom of EP tube when take the supernatant of the incubation reaction.

## Operating steps

### 1. Incubation reaction

① Standard tube: Take a* μL of standard solution with different concentrations to 1.5 mL EP tubes.
Sample tube: Take a* μL of sample to 1.5 mL EP tubes.
[Note]: a* = Sample volume= Standard volume. For tissue and cell samples, a* is 100-200 μL. For serum or plasma, a* is 80-100 μL.

② Add 60 μL of enzyme working solution to each tube.

③ Mix fully and incubate at 37°C with shading light for 60 min.

④ Add 20 μL of sulfate solution to each tube.

⑤ Add 10 μL of alkaline reagent to each tube.

⑥ Mix fully and stand at room temperature for 5 min, centrifuge at 10000×g for 10 min, then take the supernatant for detection.

### 2. Chromogenic reaction

① Standard well: Take 50 μL of chromogenic working solution to the corresponding wells.
Sample well:Take 50 μL of chromogenic working solution to the corresponding wells.

② Standard well: Take 120 μL of supernatant from standard tube to the corresponding wells.
Sample well:Take 120 μL of the supernatant from sample tube to the corresponding wells.

③ Mix fully with microplate reader for 5 s and stand at room temperature for 5 min. Measure the OD value of each well at 530 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) and other liquid sample:**

$$\text{NO content (μmol/L)} = (\Delta A_{530} - b) \div a \times f$$

**2. Tissue and cell sample:**

$$\text{NO content (μmol/gprot)} = (\Delta A_{530} - b) \div a \times f \div C_{pr}$$

**[Note]**

$\Delta A_{530}$: the absolute OD value of sample, $\Delta A_{530} = OD_{sample} - OD_{blank}$

f: dilution factor of the sample before tested.

$C_{pr}$: the protein content of sample (gprot/L).

# 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) | 5.50 | 18.50 | 32.00 |
| %CV | 6.5 | 5.8 | 5.7 |

### 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) | 5.50 | 18.50 | 32.00 |
| %CV | 8.3 | 7.9 | 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 95%.

| | Standard 1 | Standard 2 | Standard 3 |
|---|---|---|---|
| Expected Conc. (μmol/L) | 12 | 22.5 | 31 |
| Observed Conc. (μmol/L) | 11.3 | 21.6 | 29.5 |
| Recovery rate (%) | 94 | 96 | 95 |

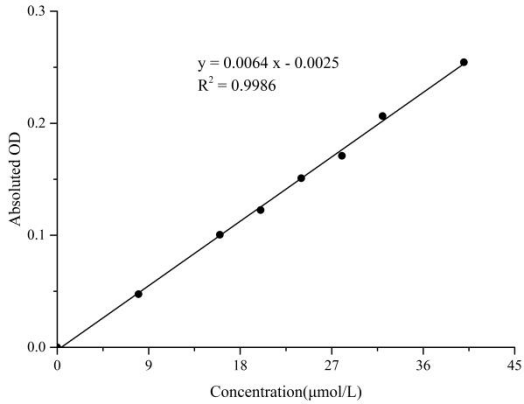
### Sensitivity

The analytical sensitivity of the assay is 1.38 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 | 8 | 16 | 20 | 24 | 28 | 32 | 40 |
|---|---|---|---|---|---|---|---|---|
| OD value | 0.043 | 0.092 | 0.144 | 0.166 | 0.194 | 0.214 | 0.249 | 0.295 |
| | 0.043 | 0.089 | 0.143 | 0.165 | 0.194 | 0.214 | 0.250 | 0.300 |
| Average OD | 0.043 | 0.091 | 0.144 | 0.166 | 0.194 | 0.214 | 0.250 | 0.298 |
| Absoluted OD | 0 | 0.048 | 0.101 | 0.123 | 0.151 | 0.171 | 0.207 | 0.255 |



## Appendix Π Example Analysis

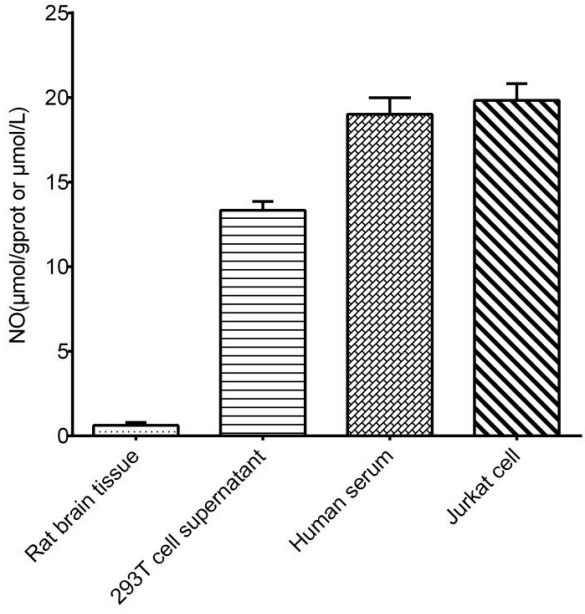
**Example analysis：**

For rat brain tissue, take 100 μL of 10% rat brain tissue homogenate, and carry the assay according to the operation steps. The results are as follows:

standard curve: y = 0.0064x + 0.0025, the average OD value of the sample is 0.089, the average OD value of the blank is 0.060, the concentration of protein in sample is 4.74 gprot/L, and the calculation result is:

$$\text{NO content (μmol/gprot)} = (0.089 - 0.060 - 0.0025) \div 0.0064 \div 4.74 = 0.87 \ \text{μmol/gprot}$$

Detect 10% rat brain tissue homogenate (the concentration of protein is 4.74 gprot/L), 293T cell supernatant, human serum and Jurkat cell (the concentration of protein is 1.2 gprot/L) 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.