Nitric Oxide (NO) Colorimetric Assay Kit

Catalog No: E-BC-K035-S

Method: Colorimetric method

Specification: 100 Assays (Can detect 96 samples without duplication)

Instrument: Spectrophotometer

Sensitivity: 0.97 μmol/L

Detection range: 0.97-700 μmol/L

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

General information

▲Intended use

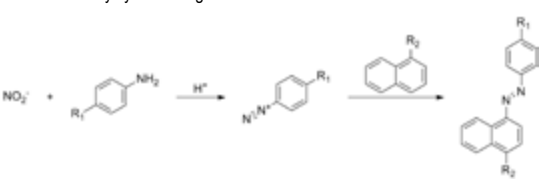
This kit can be used for detection of nitric oxide (NO) in serum, plasma,saliva,animal and plant tissue samples.

▲Background

NO is a kind of highly reactive free radical, which has the function of the second messenger and neurotransmitter, and it is also a kind of effector molecule, which has a wide range of physiological functions in vivo, such as relax vascular smooth muscle, regulate cerebral blood flow, mediate cytotoxic effect and immune regulation, participate in learning and memory, etc. Half life of NO is very short. NO in blood is mainly produced by vascular endothelial cells, vascular smooth muscle cells, platelets, macrophages and so on. It exists in the form of nitrate and nitrite, and the concentration of NO can calculate indirectly by the concentration of nitrate and nitrite.

▲Detection principle

NO is easily oxidized to form NO_2^- 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	Specification	Storage
Reagent 1	Sulphate Solution	50 mL × 4 vials	2-8°C , 6 months
Reagent 2	Alkali Reagent	50 mL × 2 vials	2-8°C , 6 months
Reagent 3	Chromogenic Agent A	38 mL × 1 vial	2-8°C , 6 months, shading light
Reagent 4	Chromogenic Agent B	Powder × 1 vial	2-8°C , 6 months, shading light
Reagent 5	Acid Solution	25 mL × 1 vial	2-8°C , 6 months
Reagent 6	Sodium Nitrite Standard	Powder × 2 vials	2-8°C , 6 months
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.			

▲Materials prepared by users

Instruments

Spectrophotometer (550 nm), Vortex mixer, Centrifuge, Analytical Balance, Micropipettor

Consumptive material

Tips (10 μL, 200 μL, 1000 μL), EP tubes (1.5 mL, 2 mL, 5 mL)

Reagents:

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

▲Safety data

Some of the reagents in the kit contain dangerous substances. It should be avoided to touch the skin and clothing. Wash immediately with plenty of water if touching it carelessly. All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

▲Precautions

Before the experiment, please read the instructions carefully, and wear gloves and work clothes.

▲The key points of the assay

1. It is recommended to use a disposable plastic tube or glass tube must be washed clean.
2. Hemolysis and turbid serum have an effect on the results of the experiment.
3. Serum samples can be stored for 3 days at 4℃ and for a month at -20℃ .
4. The supernatant for chromogenic reaction should not contain sediment, otherwise it will affect the results.

Pre-assay preparation

▲ Reagent preparation

1. If there is any crystal precipitation in reagent 3, please dissolve it fully with water bath at above 60℃ before use.
2. Preparation of reagent 4 working solution

   Dissolve a vial of reagent 4 with 37.5 mL of double distilled water fully. The prepared solution can be stored at 4℃ for 2 months with shading light. If the reagents appear darkened color, it should be abandon. It is recommended to prepare the needed amount and the concentration is 1.5 mg/mL.
3. Preparation of chromogenic reagent

   Mix the reagent 3, reagent 4 working solution and reagent 5 at a ratio of 3:3:2 fully. Prepare the fresh solution before use and it can't be used when its color gets darker.
4. Preparation of 2 mmol/L standard solution

   Dissolve the reagent 6 with 2 mL of distilled water. Prepare the fresh solution before use.
5. Preparation of 40 μmol/L sodium nitrite standard solution

   Dilute the 2 mmol/L standard solution with distilled water at a ratio of 1:49 and mix fully. Prepare the fresh solution before use.

▲ Sample preparation

The samples should be prepared as conventional methods. Also please refer to appendix II.

▲Dilution of sample

It is recommended to take 2~3 samples with expected large difference to do pre-experiment before formal experiment and dilute the sample according to the result of the pre-experiment and the detection range (0.97-700 μmol/L).

Assay protocol	
Ambient temperature	25-30°C
Optimum detection wavelength	550 nm

Instructions for the use of transferpettor:

(1) Equilibrate the pipette tip in that reagent before pipetting each reagent.

(2) Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Assay protocol

▲Operating steps

(1) Blank tube: Take a* mL of double distilled water to 1.5 mL EP tubes.
Standard tubes: Take a* mL of 40 μmol/L sodium nitrite standard solution to 1.5 mL EP tubes.
Sample tubes: Take a* mL of sample to 1.5 mL EP tubes.

Note: a* = Sample volume= Standard volume.

For serum or plasma samples, a* is 0.2-0.3 mL.

For tissue, a* is 0.1-0.3 mL.

(2) Add 1.6 mL of reagent 1 and mix fully with a vortex mixer.

(3) Add 0.8 mL of reagent 2 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 1.6 mL of supernatant to the corresponding tubes for chromogenic reaction.

(6) Add 0.8 mL of chromogenic reagent to each tube, mix fully and stand at room temperature for 20 min.

(7) Set the spectrophotometer to zero with double distilled water and measure the OD values of each tube at 550 nm with 1 cm optical path cuvette.

▲Operation table

1. Pre-treatment

	Blank tube	Standard tube	Sample tube
Double distilled water (mL)	a*		
40 μmol/L sodium nitrite standard solution (mL)		a*	
Sample (mL)			a*
Reagent 1 (mL)	1.6	1.6	1.6
Reagent 2 (mL)	0.8	0.8	0.8
Mix fully and stand for 15 min, centrifuge at 3100 g for 10 min, take the supernatant for chromogenic reaction.			

2. Chromogenic reaction

	Blank tube	Standard tube	Sample tube
Supernatant (mL)	1.6	1.6	1.6
Chromogenic reagent (mL)	0.8	0.8	0.8
Mix fully and stand at room temperature for 20 min. Set the spectrophotometer to zero with double distilled water and measure the OD values of each tube at 550 nm with 1 cm optical path cuvette.			

▲Calculation

1. Serum (plasma):

$$\text{NO content (μmol/L)} = \frac{\Delta A_1}{\Delta A_2} \times c \times f$$

2. Tissue:

$$\text{NO content (μmol/gprot)} = \frac{\Delta A_1}{\Delta A_2} \times c \times f \div C_{pr}$$

Note:

ΔA_1: $OD_{Sample} - OD_{Blank}$

ΔA_1: $OD_{Standard} - O_{DBlank}$

c: Concentration of sodium nitrite, 40 μmol/L.

f: Dilution factor of sample before test.

C_{pr}: Concentration of protein in sample, gprot/L.

▲Notes

1. This kit is for research use only.
2. Instructions should be followed strictly, changes of operation may result in unreliable results.
3. The validity of kit is 6 months.
4. Do not use components from different batches of kit.

Appendix I Performance characteristics

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Detection range	0.97-700 μmol/L	Average intra-assay CV (%)	3.4
Sensitivity	0.97 μmol/L	Average inter-assay CV (%)	5.2
Average recovery rate (%)	99		

▲Inter-assay CV

Take three kits of different batches to detect the samples of high concentration, middle concentration and low concentration respectively. The concentrations should be within the detection range. The samples of each concentration were tested 3 times (n=3) parallelly with each kit. Then calculate the corresponding values according to the following formula. The average Inter-assay CV is 5.2%.

$$\bar{x}_T = \frac{\bar{x}_1 + \bar{x}_2 + \bar{x}_3}{3}$$

$$R = \frac{\bar{x}_{max} - \bar{x}_{min}}{\bar{x}_T} \times 100\%$$

$$\bar{R} = \frac{R_1 + R_2 + R_3}{3} \quad (n=3)$$

$\bar{x}_{max}$--- The max values of $\bar{x}_i$

$\bar{x}_{min}$--- The min values of $\bar{x}_i$

$\bar{x}_T$---The average values of $\bar{x}_i$

R_i---The value of each batch number kit

▲Intra-assay CV

Take one kit to detect the samples of high concentration, middle concentration and low concentration respectively. The concentrations should be within the detection range. The samples of each concentration were tested 6 times (n=6) parallelly, and the average Intra-assay CV is 3.4%, which was calculated according to the following formula.

$$CV = \frac{S}{\bar{x}} \times 100\%$$

S--- Standard deviation

▲Sensitivity

OD values of standard curve and 20 blank samples were measured according to the operation table. Plot the standard curve and calculate the standard deviation of blank, Three standard deviations divided by the slope is the sensitivity (0.97 μmol/L) according to the formula of IUPAC.

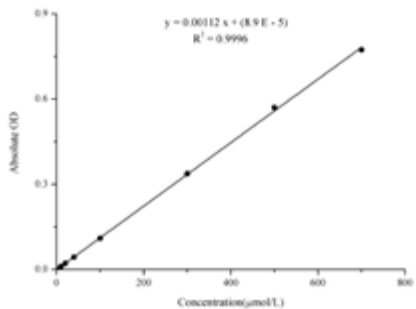
▲Recovery rate

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 99%.

▲Standard curve (It doesn't need to prepare the standard curve for this kit and the provided standard curve is for reference only)

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	40	100	300	500	700
Average OD	0.010	0.021	0.032	0.054	0.120	0.348	0.580	0.784
Absoluted OD	0	0.011	0.022	0.044	0.110	0.338	0.570	0.774



▲Example analysis

Take 0.3 mL of 10% mouse liver tissue homogenate, carry the assay according to the operation table.

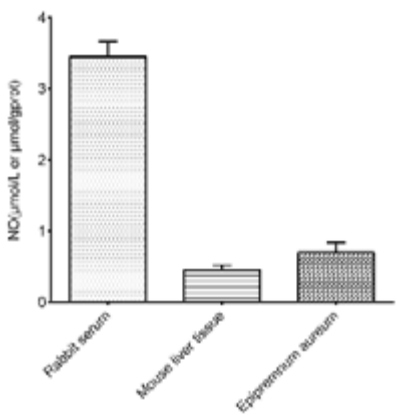
The results are as follows:

The average OD value of the sample is 0.010, the average OD value of the blank is 0.004, the average OD value of the standard is 0.065, the concentration of protein in sample is 8.65 gprot/L, and the calculation result is:

NO content(μmol/gprot)=(0.010-0.004)÷(0.065-0.004) ×40÷8.65

= 0.45 μmol/gprot

Detect rabbit serum (a*=0.1 mL), 10% mouse liver tissue homogenate (the concentration of protein is 8.65 gprot/L, a*=0.3 mL), 10% Epipremnum aureum tissue homogenate (the concentration of protein in is 1.62 gprot/L, a*=0.4 mL) , according to the protocol, the result is as follows:



Appendix II Sample preparation

The following sample pretreatment methods are for reference only.

▲Serum

Collect fresh blood and stand at 25℃ for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4℃ . Take the serum (which is the upper light yellow clarified liquid layer) to preserve it on ice for detection.

▲ Plasma

Take fresh blood into the tube which has anticoagulant, centrifuge at 1000-2000 g for 10 min at 4℃ . Take the plasma (which is the upper light yellow clarified liquid layer, don't take white blood cells and platelets in the middle layer) to preserve it on ice for detection.

▲ Tissue sample

Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8℃ . Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of homogenized medium (2-8℃) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 10000 g at 4℃ . Take the supernatant to preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M, E-BC-K168-S, E-BC-K165-S).

▲ Saliva

Gargle with clear water, collect the saliva 30 min later, centrifuge at 10000 g for 10 min at 4℃ . Take the supernatant and preserve it on ice for detection.

Note:

1. Homogenized medium: PBS (0.01 M, pH 7.4).

2. Homogenized method:

(1) Hand-operated: Weigh the tissue and mince to small pieces (1 mm^3), then put the tissues pieces to glass homogenized tube. Add homogenized medium into homogenized tube, place the tube into the ice bath with left hand, and insert the glass tamping rod vertically into the homogenized tube with the right hand to grind up and down for 6-8 min.

Or put the tissue into the mortar, and add liquid nitrogen to grind fully. Then add the homogenized medium to homogenize.

(2) Mechanical homogenate: Weigh the tissue to EP tube, add the homogenized medium to homogenize the tissue with homogenizer instrument (60 Hz, 90s) in the ice bath. (For samples of skin, muscle and plant tissue, the time of homogenization can be properly prolonged.)

▲ Notes for sample

1. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
2. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
3. If a lysis buffer is used to prepare tissue homogenates, there is a possibility of causing a deviation due to the introduced chemical substance.

Appendix III References

1. Davis K L, Martin E, Turko I V, Murad F. Novel effects of nitric oxide. Annu Rev Pharmacol Toxicol, 2001, 41(1): 203-236.

2. Sun J, Zhang X, Broderick M, Fein H. Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. Sensor, 2003, 3(8): 276-284.