Nitric Oxide (NO) Colorimetric Assay Kit

Catalog No: E-BC-K035-M

Method: Colorimetric method

Specification: 96T (Can detect 80 samples without duplication)

Instrument: Microplate reader

Sensitivity: 0.16 μmol/L

Detection range: 0.16-100 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.

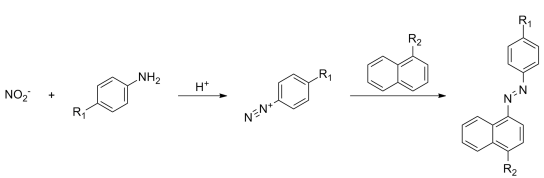
▲Background

The half-life of NO is extremely short, it exists in form of nitrate or nitrite produced by vascular endothelial cell, vascular smooth muscle cell, platelet, and macrophage and so on. The concentration of NO can be indirectly measured by detecting that of nitrate or nitrite.

NO react with oxygen and water to generate nitrate or nitrite which can form a kind of pale red azo compound when meet with nitrate chromogenic reagent, the absorbance of the compound can be measured to calculate the concentration of NO indirectly.

▲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	24 mL × 1 vial	2-8℃ , 6 months
Reagent 2	Alkali Reagent	12 mL × 1 vial	2-8℃ , 6 months
Reagent 3	Chromogenic Agent A	1.9 mL × 2 vials	2-8℃ , 6 months, shading light
Reagent 4	Chromogenic Agent B	Powder × 1 vial	2-8℃ , 6 months, shading light
Reagent 5	Acid Solution	1.3 mL × 2 vials	2-8℃ , 6 months
Reagent 6	Sodium Nitrite Standard	Powder × 2 vials	-20℃ , 6 months
	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.

▲Materials prepared by users

Instruments

Microplate reader(540-550 nm), Micropipettor, Vortex mixer, Centrifuge

Consumptive material

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

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 3.8 mL of double distilled water fully. The prepared solution can be stored at 4℃ for 2 months with shading light.
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 Sodium Nitrite Standard

   Dissolve reagent 6 with 2 mL of double distilled water. Prepare the needed amount 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 (0.16-100 μmol/L).

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

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

Note: The diluent is double distilled water, normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4).

Assay protocol	
Ambient temperature	25-30℃
Optimum detection wavelength	0

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

▲Plate set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73
B	B	B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74
C	C	C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75
D	D	D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76
E	E	E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77
F	F	F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78
G	G	G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79
H	H	H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80

Note: A, blank well; B-H, standard wells; S1-S80, sample wells.

▲Operating steps

1. The preparation of standard curve

Dilute 2 mmol/L sodium nitrite standard with double distilled water to a serial concentration. The recommended dilution gradient is as follows: 0, 10, 20, 30, 40, 60, 80, 100 μmol/L.

2. The measurement of samples

(1) Standard tubes: Take a* μL of sodium nitrite standard solution with different concentrations to 1.5 mL EP tubes.
Sample tubes: Take 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 , a* is 100-300 μL.

(2) Add 200 μL of reagent 1 and mix fully with a vortex mixer.

(3) Add 100 μL 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 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.

▲Operation table

1. Pre-treatment

	Standard	Sample
Sodium nitrite standard solution with different concentrations (μL)	a*	
Sample (μL)		a*
Reagent 1 (μL)	200	200
Reagent 2 (μL)	100	100
Mix fully with a vortex mixer and stand for 15 min at room temperature, centrifuge at 3100 g for 10 min, take 160 μL of the supernatant for chromogenic reaction.		

2. Chromogenic reaction

	Standard	Sample
Supernatant (μL)	160	160
Chromogenic reagent (μL)	80	80
Mix thoroughly for 2 min, stand for 15 min at room temperature, measure the OD of each well with microplate reader immediately at 550 nm wavelength.		

▲Calculation

Plot the standard curve by using OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve with graph software (or EXCEL). The concentration of the sample can be calculated according to the formula based on the OD value of sample. If the sample have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. The actual concentration is the calculated concentration multiplied by dilution factor. The standard curve is: y= ax + b.

1. Serum (plasma):

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

2. Tissue:

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

Note:

y: The absolute OD value of standard;

x: The concentration of standard;

a: The slope of standard curve;

b: The intercept of standard curve.

ΔA_{550}: Absolute OD ($OD_{Sample} - OD_{Blank}$)

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

Appendix I Performance characteristics			
Detection range	0.16-100 μmol/L	Average intra-assay CV (%)	2.4
Sensitivity	0.16 μmol/L	Average inter-assay CV (%)	3.7
Average recovery rate (%)	102		

▲Example analysis

Dilute human serum for 2 times, carry the assay according to the operation table.

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:

NO content(μmol/L)= (0.056-0.035-0.00514)÷0.00215=3.20 (μmol/L)

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).

▲ 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.)

▲ Note 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.