Technical Manual

Nitric Oxide (NO) Colorimetric Assay Kit

- Catalogue Code: MAES0050
- Size: 96T
- Research Use Only

1. Key features and Sample Types

Detection method:

Colorimetric method

Specification:

96T

Range:

0.16-100 µmol/L

Sensitivity:

0.16 µmol/L

Storage:

2-8°C and -20°C for 6 months

Expiry:

See Kit Label

Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 6 months.

Do not use components from different batches of kit.

2. 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.

3. Intended Use

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

4. 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.

5. Kit components & storage

Item	Specification	Storage
Sulphate Solution	24 mL × 1 vial	2-8°C, 6 months
Alkali Reagent	12 mL × 1 vial	2-8°C, 6 months
Chromogenic Agent A	1.9 mL × 2 vials	2-8°C, 6 months, avoid direct sunlight
Chromogenic Agent B	Lyophilized × 1 vial	2-8°C, 6 months, avoid direct sunlight
Acid Solution	1.3 mL × 2 vials	2-8°C, 6 months
Sodium Nitrite Standard	Lyophilized × 2 vials	-20°C, 6 months
Microplate	96 wells	No requirement
Plate Sealer	2 pieces	

Materials required but not supplied

- Micropipettor
- Incubator
- Centrifuge
- Microplate Reader (540-550 nm)
- Tips (10 μL, 200 μL, 1000 μL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water
- Normal Saline (0.9% NaCl)
- PBS (0.01 M, pH 7.4)

6. Assay Notes:

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

2. Prevent the formulation of bubbles when the supernatant is transferred into the microplate.

7. Reagent preparation:

1. Bring all reagent to room temperature before use.

2. Preparation of chromogenic agent B working solution: Dissolve a vial of chromogenic agent B with 3.8 mL of double distilled water fully. The prepared solution can be stored at 4°C for 2 months with avoid direct sunlight.

3. Preparation of chromogenic reagent: Mix the chromogenic agent A, chromogenic agent B working solution and acid solution 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 sodium nitrite standard (2 mmol/L): Dissolve standard lyophilized with 2 mL of double distilled water. Prepare the needed amount before use.

8. Sample Preparation

1. Serum sample:
Fresh blood should be incubated at 25°C for 30 min to clot the blood. Centrifuge the sample at 2000 g for 15 min at 4°C. Take the serum (which is the upper light yellow clarified liquid layer) to preserve it on ice for detection. If not detected on the same day, the serum can be stored at -80°C for a month.

2. Plasma sample:
Place the fresh blood sample into a tube of anticoagulant and centrifuge at 700-1000g for 10 min at 4°C. 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. If not detected on the same day, the plasma can be stored at -80°C for a month.

3. Tissue sample:
Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8°C. Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of PBS (0.01 M, pH 7.4) (2-8°C) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 10000 g at 4°C. Take the supernatant to preserve it on ice for detection. If not detected on the same day, the tissue sample (without homogenization) can be stored at -80°C for a month.

Sample Notes:

The concentration should be determined before preforming the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

Dilution of Samples:

Large variances in results may be seen when performing pre-experiments. 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 normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4);

9. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: 550 nm

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.

10. Operation Steps

The preparation of standard curve

Dilute sodium nitrite standard (2 mmol/L) 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.

The measurement of samples

1. **Standard tube:** Take a* μL of sodium nitrite 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 serum or plasma samples, a* is 200-300 μL. For tissue, 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.)Take 160 μL of supernatant to the corresponding wells of microplate for chromogenic reaction.

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

6. Measure the OD value at 550 nm with microplate reader.

Operation Table

Pre-treatment

	Standard tube	Sample tube
Standards with different concentrations (μL)	a*	
Sample (μL)		a*
Sulphate solution (μL)	200	200
Alkali reagent (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.		

Chromogenic reaction

	Standard well	Sample well
Supernatant (μL)		20
Chromogenic reagent (μL)	200	200
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.		

11. Calculations

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. The standard curve is: y= ax + b.

1. Serum (plasma) sample:

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

2. Tissue sample:

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

y: $OD_{Standard} - OD_{Blank}$ (OD_{Blank} is the OD value when the standard concentration is 0).
x: The concentration of standard.
a: The slope of standard curve .
b: The intercept of standard curve.
ΔA_{550}: $OD_{Sample} - OD_{Bllank}$
f: Dilution factor of sample before test.
C_{pr}: Concentration of protein in sample, gprot/L.

12. Performance Characteristics

Detection Range	0.16-100 μmol/L
Sensitivity	0.16 μmol/L
Average recovery rate (%)	102
Average inter-assay CV (%)	3.7
Average intra-assay CV (%)	2.4

Analysis

Dilute human serum, 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 , and the calculation result is:

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

= 3.20 (μmol/L)

Safety Notes

Some of the reagents in the kit contain dangerous substances. Prevent touching 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.

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

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