Nitric Oxide (NO) Assay Kit

(Cat/No.:BC140 Size:50T/48S,25T/24S)

1. Assay principle (Nitrate Reductase Method)

NO has active chemical property, it is converted to nitrate (NO^{3-}) & nitrite (NO^{2-}), NO^{2-} can also convert to NO^{3-}. The method use nitrate reductase to reduce NO^{3-} to NO^{2-} selectively and determine the concentration by the depth of color.

2. Assay significance

Nitric oxide (NO) is chemically reactive and is metabolized in vivo to form nitrate (NO^{3-}) and nitrite (NO^{2-}). Only the sum of serum nitrate (NO^{3-}) and nitrite (NO^{2-}) concentrations (NO^{3-} + NO^{2-}) can accurately reflect the in vivo NO level. For the determination of serum (NO^{3-} + NO^{2-}) content, some domestic institutions adopt the metallic cadmium reduction method. However, this method has cumbersome operation (requiring serum deproteinization), difficult reaction control (metallic cadmium can further reduce NO^{2-}), and fails to completely reduce NO^{3-} to NO^{2-}, resulting in poor accuracy.

The proposed assay is a sensitive, simple, rapid, stable, and easy-to-popularize method.

3. Reagents composition & Preparation:(The validity period of the kit is 3 months)

Reagents composition	25T/24S	50T/48S	Stored
Reagent 1	Solution 6mL×1 bottle	Solution 6mL×2 bottle	Stored at -20℃
Reagent 2	Solution 6mL×1 bottle	Solution 6mL×2 bottle	Stored at -20℃
Preparation of the mixed reagent: Mix reagent 1 with reagent 2 in a ratio of 1:1. Mix thoroughly and set aside for use. Prepare as much as needed, and prepare immediately before use.			
Reagent 3	Solution 6mL×1 bottle	Solution 12mL×1 bottle	Stored at 4℃
Reagent 4	Solution 3mL×1 bottle	Solution 6mL×1 bottle	Stored at 4℃
Reagent 5	Powder ×1 vial	Powder ×1 vial	4℃ in the dark
	For each powder, add 10 mL of double-distilled water and heat it to around 95℃ to fully dissolve.	For each powder, add 20 mL of double-distilled water and heat it to around 95℃ to fully dissolve.	stored at room temperature
Reagent 6	Powder ×1 vial	Powder ×1 vial	4℃ in the dark

	Mix with 4 mL of double-distilled water at room temperature and let it dissolve. The color will turn dark coffee color and it should not be used anymore.	Mix with 8 mL of double-distilled water at room temperature and let it dissolve. The color will turn dark coffee color and it should not be used anymore.	4℃ in the dark
Reagent 7	Solution 4mL×1 bottle	Solution 8mL×1 bottle	stored at room temperature
Preparation of Chromogenic Reagent: Mix Reagent 5:Reagent 6:Reagent 7 at a ratio of 2.5:1:1. Prepare only the required amount. If to be used up within one month, it can be prepared in one batch; store the remaining chromogenic reagent at room temperature in the dark. Crystallization may occur in cold weather—dissolve in a 100℃ water bath before reuse.			
Standard (10mmol/L)	0.5mL×1 vial	0.5mL×1 vial	stored at below -20℃
	Preparation of **100 μmol/L Standard Working Solution**: Take 50 μL of 10 mmol/L standard stock solution, dilute to a final volume of 5 mL with double-distilled water (i.e., 100-fold dilution), mix thoroughly, and prepare fresh before use.		
Double-distilled water	40mL×1 bottle	40mL×2 bottle	Stored at 4℃

[Note]: If Reagent 2 requires multiple uses, aliquot and store it after the first thawing to avoid repeated freeze-thaw cycles.Since Reagent 5 is a supersaturated solution, it is recommended to heat while stirring with a glass rod during preparation to ensure complete dissolution.If not fully used in one experiment, crystallization may still occur when reused—reheat to dissolve it before use. (Water bath heating is recommended: Take a clean small beaker or reagent vial, add Reagent 5 powder and double-distilled water, then place the beaker/vial in a 90–100℃ water bath. Caution: Do not tip it over! Secure the container with metal objects or small stones around it to prevent tipping.)

4. Required instruments and reagents

Visible light spectrophotometer with 0.5 cm light path cuvettes (or microplate reader (550 nm) with 96-well plates), vortex mixer, laboratory electric stove, 37℃ water bath (or incubator), centrifuge, beakers, etc.

5. Operation steps

(1) Pretreatment of Specimens (Serum, Gastric Juice, Urine, Cell Culture Medium, etc.) Before Detection

1.Operation Table:

	Blank	Standard	Sample
Double-distilled water (mL)	0.1		
100 μmol/L standard solution (mL)		0.1	
Sample (mL)			0.1

Mixed reagent (mL)	0.4	0.4	0.4
Mix well and incubate at 37℃ for 60 minutes.			
Reagent 3 (mL)	0.2	0.2	0.2
Reagent 4 (mL)	0.1	0.1	0.1
Mix sufficiently by vortex for 30 seconds, place quescently for 40 minutes, centrifugate at 4000 rpm for 10 minutes, take supernatant for chromogenic reaction.			
Supernatant (ml)	0.5	0.5	0.5
Chromogenic agent (ml)	0.6	0.6	0.6
Mix well, let it stand at room temperature for 10 minutes. Set the wavelength to 550nm, the optical path to 0.5cm. Zero the instrument with double-distilled water. Measure the absorbance value A of each tube (or take 200μL of the reaction solution from each tube and add it to the 96-well plate, and read the value at 550nm with the microplate reader).			

6. Calculation Formula

$$\textbf{NO content(μmol/L)}=\frac{A_{sample}-A_{blank}}{A_{standard}-A_{blank}}\times C_{standard}\times N$$

$C_{standard}$:Standard solution concentration: 100 μmol/L;
N: Dilution factor of the sample before testing

7. Calculation Example

Example 1： 0.1 mL of chicken serum was taken for testing. The results are as follows: the absorbance of the blank tube was 0.020, that of the standard tube was 0.129, and that of the test tube was 0.069. The serum was not diluted; the dilution factor was 1. Then the calculation is as follows:

$$\textbf{NO content(μmol/L)}=\frac{0.060-0.020}{0.129-0.020}\times 100\times 1\times\frac{1}{0.05}=36.70\ \textbf{μmol/L}$$

Example 2： 0.1 mL of rat cerebrospinal fluid was taken for testing. The results are as follows: the absorbance of the blank tube was 0.019, that of the standard tube was 0.125, and that of the test tube was 0.041. The dilution factor was 1. The calculation result is:

$$\textbf{NO content(μmol/L)}=\frac{0.041-0.019}{0.125-0.019}\times 100\times 1=20.75\ \textbf{μmol/L}$$

8. Pre-treatment of the sample for testing

1.Operation Table:

	Blank	Standard	Sample
Double-distilled water (mL)	0.5	0.4	
100 μmol/L standard solution (mL)		0.1	
Sample (mL)			0.5
Mixed reagent (mL)	0.4	0.4	0.4
Mix well and incubate at 37℃ for 60 minutes.			

Reagent 3 (mL)	0.2	0.2	0.2
Reagent 4 (mL)	0.1	0.1	0.1
Mix sufficiently by vortex for 30 seconds, place quescently for 40 minutes, centrifugate at 4000 rpm for 10 minutes, take supernatant for chromogenic reaction.			
Supernatant (ml)	0.8	0.8	0.8
Chromogenic agent (ml)	0.6	0.6	0.6
Mix well, let it stand at room temperature for 10 minutes. Set the wavelength to 550nm, the optical path to 0.5cm. Zero the instrument with double-distilled water. Measure the absorbance value A of each tube (or take 200μL of the reaction solution from each tube and add it to the 96-well plate, and read the value at 550nm with the microplate reader).			

2.Calculation Example:

$$\textbf{Tissue NO content(μmol/gprot)}=\frac{A_{sample}-A_{blank}}{A_{standard}-A_{blank}}\times C_{standard}\div Cpr$$

Or

$$\textbf{Tissue NO content(μmol/gprot)}=\frac{A_{sample}-A_{blank}}{A_{standard}-A_{blank}}\times C_{standard}\div \frac{W}{V_{total\ sample}}$$

$C_{standard}$:Standard concentration: 20 mol/L (0.4 mL double-distilled water and 0.1 mL 100 mol/L standard solution are added to the standard tube, which is equivalent to a 5-fold dilution—thus the standard concentration here is 20 mol/L)

Cpr: Concentration of protein in the homogenate, gprot/L (prot refers to protein);

W: Sample quality, g;

$V_{total\ sample}$:Total volume of the homogenate during tissue sample homogenization (Unit: L)

3.Calculation Example:

Example 1： Take 0.5 mL of supernatant from 10% mouse brain tissue homogenate and perform detection according to the operation table. The results are as follows: absorbance of the blank tube (A_{blank}) = 0.071, absorbance of the standard tube ($A_{standard}$) = 0.156, absorbance of the assay tube (A_{assay}) = 0.096. Meanwhile, the protein concentration of the 10% homogenate supernatant is measured as 4.249 gprot/L. The calculation results are as follows:

$$\textbf{Mouse liver NO content(μmol/gprot)}=\frac{0.096-0.071}{0.156-0.071}\times 20\div 4.249=1.384\ \textbf{μmol/gprot}$$

Example 2： Take 0.5 mL of supernatant from 10% Chinese sturgeon brain tissue homogenate and perform detection according to the operation table. The results are as follows: absorbance of the blank tube (A_{blank}) = 0.070, absorbance of the standard tube ($A_{standard}$) = 0.153, absorbance of the assay tube (A_{assay}) = 0.132. Meanwhile, the protein concentration of the 10% homogenate supernatant is measured as 4.533 gprot/L. The calculation results are as follows:

Sturgeon brain NO content(μmol/gprot)=$\frac{0.132-0.070}{0.153-0.070} \times 20 \div 4.533 = 3.296$ **μmol/gprot**

9. Optimal Reference Sampling Volume

1. Take 300 μL of bovine and ovine serum; take 100 μL of rabbit and mouse serum.
2. Generally take 500 μL of 10% tissue homogenate.
3. Generally take 500 μL of cell suspension; generally take 100 μL of cell culture medium.

10. Normal reference range

Sample	Referenced sampling volume	Referenced NO content
Rat blood serum	100μL	38.0±23.4μmol/L
Mouse blood serum	100μL	49.96±23.76μmol/L
Rabbit blood serum	100μL	99.16±39.5μmol/L
Dog blood serum	100μL	89.51±32.13μmol/L

11. Notes

1. Since the NO content in tissues is relatively low, the tissue homogenate is generally prepared at 10% or 20%. Centrifuge the prepared tissue homogenate at 2500 rpm for 10 minutes—do not use excessively high speed or prolonged centrifugation. Use a relatively large sample volume, preferably 0.3–0.5 mL.
2. Avoid repeated freeze-thaw cycles for Reagent 1, Reagent 2, and the standard. If experiments need to be performed in batches (more than 3 times), aliquot Reagent 1, Reagent 2, and the standard for storage during the first experiment, and take out the required amount according to the reagent dosage for each experiment. Store the prepared chromogenic reagent in the dark.
3. If specimens such as serum (plasma) or tissues are not tested immediately, store them below -70℃—valid for 6 months.
4. Both the double-distilled water used for reagent preparation and that added to the blank tube must be double-distilled water or triple-distilled water free of NO^{2-}. (Double-distilled water is provided in the kit.)
5. After the reaction between the sample and the substrate is completed, centrifuge and collect the supernatant. Never aspirate the precipitate—otherwise, the absorbance will increase significantly, which will obviously affect the authenticity of the results.
6. Selection of test tubes:
   ① Use disposable plastic test tubes or centrifuge tubes;
   ② If glass tubes are used, clean them as follows: First, soak in detergent for more than 30 minutes, boil for 0.5–1 hour, scrub thoroughly, then rinse with tap water 15–20 times.