Nitric Oxide Synthase Subtypes (TNOS, iNOS, cNOS) Activity Assay Kit

Note: It is necessary to predict 2-3 large difference samples before the formal determination.

Operation Equipment: Spectrophotometer

Cat No: BC5690

Size:50T/24S

Components:

Extract solution I: Liquid 40mL×1. Store at -20℃.

Extract solution II: Liquid 0.6mL×1. Store at -20℃. It is volatile and sealed immediately after use.

Buffer solution: Liquid 30mL×1. Store at 2-8℃.

Reagent I: Liquid 5.2mL×1. Store at 2-8℃.

Reagent II: Powder×2. Store at -20℃. It is in the glass bottle. Add 6mL Buffer solution into one Reagent II. It can be stored at -20℃ for 4 weeks after dispensing to avoid repeated freezing and thawing.

Reagent III: Liquid 50μL×1. Store at 2-8℃.

Reagent III working solution: Reagent III and Buffer solution are mixed by the ratio of 8μL: 792μL (0.8mL, 10T) to make Reagent III working solution according to sample number before use.

Reagent IV: Powder×2. Store at -20℃. Add 0.6mL Buffer solution into one Reagent IV before use. It can be stored at -20℃ for 4 weeks after dispensing to avoid repeated freezing and thawing.

Reagent V: Powder×2. Store at -20℃. It is in the glass bottle. Add 1.2mL Buffer solution into one Reagent V. It can be stored at -20℃ for 4 weeks after dispensing to avoid repeated freezing and thawing.

Working solution: Reagent I, Reagent II, Reagent III working solution and Reagent IV are mixed by the ratio of 0.8mL: 2.0mL: 0.8mL: 0.2mL (3.8mL, 10T) to make Working solution according to sample number before use.

Reagent VI: Liquid 1.3mL×1. Store at 2-8℃.

Reagent VII: Liquid 2.5mL×1. Store at 2-8℃.

Reagent VIII: Liquid 50μL×1. Store at 2-8℃.

Reagent VIII working solution: Reagent VIII and Buffer solution are mixed by the ratio of 10μL: 450μL (0.46mL, about 11T) to make Reagent VIII working solution according to sample number before use.

Chromogenic solution A: Liquid 15mL×1. Store at 2-8℃.

Chromogenic solution B: Liquid 15mL×1. Store at 2-8℃.

Chromogenic solution: Chromogenic solution A and Chromogenic solution B are mixed by the ratio of 1: 1 to make Chromogenic solution according to sample number before use.

Standard: Liquid 1mL×1, 10μmol/mL sodium nitrite solution. Store at 2-8℃. Mix 5μL 10μmol/mL sodium nitrite solution and 995μL distilled water to prepare a 0.05μmol/mL standard solution before use.

Note: Reagent II, Reagent IV and Reagent V are lyophilized powder. The differences in the amount of these powders may seem to be large, but the actual qualities are the same. It does not affect the detection.

Product Description:

Nitric oxide synthase (NOS, EC 1.14.13.39, written as total NOS (TNOS) after) is a type of enzyme that catalyzes the synthesis of NO from L-arginine in living organisms. It mainly exists in various cells such as vascular smooth muscle, macrophages, endothelial cells, neurons, liver cells, and glomerular mesangial cells. According to the different dependence of its enzyme activity on calcium ions, it can be divided into structural NOS (constructive NOS, cNOS) and injury induced NOS (inducible NOS, iNOS). The former requires a certain concentration of calcium ions to activate, while the latter does not rely on exogenous calcium ions.

NOS catalyzes L-arginine, molecular oxygen and NADPH to form NO and $NADP^+$. NO is easily oxidized to form NO_2^- and NO_3^- in aqueous solution. Under acidic conditions, NO_2^- and diazonium sulfonamide produce diazo compounds. The compounds could further couple with naphthyl vinyl diamine. The product has a characteristic absorption peak at 550 nm and its absorbance value can be measured to calculate NOS activity.

Reagents and Equipment Required but Not Provided:

Spectrophotometer, centrifuge, balance, transferpettor, mortar/homogenizer/cell ultrasonic crusher, 1mL glass cuvette, ice and distilled water.

Procedure:

I. Sample preparation

1. **Tissue:** It is suggested that 0.2 g of tissue with 0.98 mL of Extract solution I and 0.02 mL of Extract solution II and fully homogenized on ice bath. Centrifuge at 12000g for 15 minutes at 4℃ to remove insoluble materials and take the supernatant on ice for testing.
2. **Bacteria/Cells:** Collect bacteria/cells into the centrifuge tube, after centrifugation discard supernatant. It is suggested that add 0.98 mL of Extract solution I and 0.02 mL of Extract solution II to 10 million of bacteria/ cells. Use ultrasonication to split bacteria/cells (place on ice, ultrasonic power 200W, working time 3 seconds, interval 7 seconds, repeat for 5 minutes). Centrifuge at 12000g for 15 minutes at 4℃ to remove insoluble materials and take the supernatant on ice for testing.
3. **Liquid samples:** Detect directly. Centrifuge before detecting if there are precipitation in the samples.

Note: Extract solution I and Extract solution II are mixed by the ratio of 0.98mL: 0.02mL to prepare according to sample number before use.

II. Determination

1. Preheat spectrophotometer for 30 min, adjust the wavelength to 550 nm and set counter to zero with distilled water.
2. Add reagents in 2ml EP tube as the following:

Reagent (μL)	Total NOS test tube(A_{T1})	iNOS test tube(A_{T2})	Standard tube(A_S)	Blank tube(A_B)
Sample	240	240	-	-
Working solution	380	380	-	-
Reagent V	80	-	-	-
Reagent VI	40	-	-	-
Buffer solution	-	120	-	-
Mix and react at 37℃ for 60min, bath at 100℃ for 5min and cool to room temperature. Centrifuge at 11000g for 10 minutes at 4℃ and take all supernatant.			-	-
Supernatant	all supernatant	all supernatant	-	-
Reagent VII	40	40	-	-
Reagent VIII working solution	40	40	-	-
Mix and react at 37℃ for 30min.			-	-
Standard solution	-	-	240	-
Distilled water	-	-	580	820
Chromogenic solution	400	400	400	400
Mix and react for 10min at room temperature. Detect the absorbance value at 550 nm and record as A_{T1}, A_{T2}, A_S and A_B. $\Delta A_{T1}=A_{T1}-A_B$. $\Delta A_{T2}=A_{T2}-A_B$. $\Delta A_S=A_S-A_B$. Blank tube and standard tube need to test once or twice.				

III. NOS activity calculation:

1. Protein concentration:

Unit definition: One unit of enzyme activity is defined as the amount of enzymes catalyzes production of 1 nmol NO in the reaction system per minute every milligram protein.

(1) TNOS activity (U/mg prot) $=(\Delta A_{T1}\div\Delta A_S\times C_S)\times Vs\div(Vs\times Cpr)\times10^3\div T\times F=0.83\times\Delta A_{T1}\div\Delta A_S\div Cpr\times F$

(2) iNOS activity (U/mg prot) $=(\Delta A_{T2}\div\Delta A_S\times C_S)\times Vs\div(Vs\times Cpr)\times10^3\div T\times F=0.83\times\Delta A_{T2}\div\Delta A_S\div Cpr\times F$

(3) cNOS activity (U/mg prot) = TNOS activity- iNOS activity

2. Sample mass:

Unit definition: One unit of enzyme activity is defined as the amount of enzymes catalyzes production of 1 nmol NO in the reaction system per minute every gram sample.

(1)TNOS activity (U/g mass) $=(\Delta A_{T1}\div\Delta A_S\times C_S)\times Vs\div(W\times Vs\div V_E)\times10^3\div T\times F=0.83\times\Delta A_{T1}\div\Delta A_S\div W\times F$

(2) iNOS activity (U/g mass) $=(\Delta A_{T2}\div\Delta A_S\times C_S)\times Vs\div(W\times Vs\div V_E)\times10^3\div T\times F=0.83\times\Delta A_{T2}\div\Delta A_S\div W\times F$

(3) cNOS activity (U/g mass) = TNOS activity- iNOS activity

3. Bacteria/Cells number:

Unit definition: One unit of enzyme activity is defined as the amount of enzymes catalyzes production of 1 nmol NO in the reaction system per minute every 10^6 bacteria/cells.

(1) TNOS activity (U/10^6 cell) =($\Delta A_{T1}\div\Delta A_S\times C_S$) ×Vs÷(N×Vs÷$V_E$)×$10^3$÷T×F =0.83×$\Delta A_{T1}\div\Delta A_S$÷N×F

(2) iNOS activity (U/10^6 cell) =($\Delta A_{T2}\div\Delta A_S\times C_S$) ×Vs÷(N×Vs÷$V_E$)×$10^3$÷T×F =0.83×$\Delta A_{T2}\div\Delta A_S$÷N×F

(3) cNOS activity (U/10^6 cell) = TNOS activity- iNOS activity

4. Liqiud volume:

Unit definition: One unit of enzyme activity is defined as the amount of enzymes catalyzes production of 1 nmol NO in the reaction system per minute every milliliter liqiud sample.

(1) TNOS activity (U/mL) =($\Delta A_{T1}\div\Delta A_S\times C_S$) ×Vs÷Vs×$10^3$÷T×F =0.83×$\Delta A_{T1}\div\Delta A_S$×F

(2) iNOS activity (U/ mL) =($\Delta A_{T2}\div\Delta A_S\times C_S$) ×Vs÷Vs×$10^3$÷T×F =0.83×$\Delta A_{T2}\div\Delta A_S$×F

(3) cNOS activity (U/ mL) = TNOS activity- iNOS activity

C_S: sodium nitrite concentration of standard solution, 0.05μmol/mL;

Vs: Add sample supernatant volume, 0.24 mL;

V_E: Add volume of Extract solution I and Extract solution II, 1mL;

Cpr: Sample protein concentration, mg/mL;

W: Sample mass, g;

N: Cell amount, 10^6 for one unit;

10^3: Unit conversion factor, 1μmol=10^3 nmol;

T: Reaction time, 60 min;

F: Dilution factor

Note:

1. NOS is not stable and easy to inactivate. It is recommended to use fresh samples for experiments and stored at -20℃ for fresh samples if not tested immediately.
2. The prepared Reagent II need to be stored at -20℃ after taking out the required volume according to the sample number.
3. If ΔA_T is less than 0.005, or A_T is closed to A_B, it is recommended to increase added sample supernatant volume or prolong time of the first reaction at 37℃ before determination. If ΔA_T is more than 0.4， it is recommended to dilute the sample with Buffer solution before determination. And modify the calculation formula.

Experimental example:

1. Take 0.2047g fresh mouse liver samples, add 0.98 mL of Extract solution I and 0.02 mL of Extract solution II, grind the homogenate with ice bath. Then operate according to the determination steps, using 1mL glass cuvette and calculate $\Delta A_{T1}=A_{T1}-A_B$=0.088-0.000=0.088, $\Delta A_{T2}=A_{T2}-A_B$ =0.045-0.000=0.045, $\Delta A_S=A_S-A_B$=0.380-0.000=0.380. The result is calculated according to the

sample mass:

TNOS activity (U/g mass) =0.83×ΔA_{T1}÷ΔA_S÷W×F = 0.939 U/g
iNOS activity (U/g mass) =0.83×ΔA_{T2}÷ΔA_S÷W×F = 0.480 U/g
cNOS activity (U/g mass) = TNOS activity- iNOS activity= 0.459 U/g

2. Take 240μL horse serum and operate according to the determination steps, using 1mL glass cuvette and calculate $\Delta A_{T1}=A_{T1}-A_B$=0.036-0.000 =0.036, $\Delta A_{T2}=A_{T2}-A_B$ =0.021-0.000 =0.021, $\Delta A_S=A_S-A_B$=0.380-0.000=0.380. The result is calculated according to the sample mass:

TNOS activity (U/mL) =0.83×ΔA_{T1}÷ΔA_S÷W×F = 0.079 U/mL
iNOS activity (U/mL) =0.83×ΔA_{T2}÷ΔA_S÷W×F = 0.046 U/mL
cNOS activity (U/mL) = TNOS activity- iNOS activity= 0.033 U/mL

References:

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[2] Dawson J, Knowles RG. A microtiter-plate assay of nitric oxide synthase activity[J]. Molecular Biotechnology, 1999, 12(3): 275-279.

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[4] Kazakov A, Hall R, Jagoda P. et al. Inhibition of endothelial nitric oxide synthase induces and enhances myocardial fibrosis [J]. Cardiovascular Research, 2013, 100(2):211-221.

Related Products:

BC1470/BC1475	Nitric Oxide (NO) Content Assay Kit
BC5480/BC5485	Nitric Oxide (NO) Content Assay Kit
BC5680/BC5685	Nitric Oxide Synthase (NOS) Activity Assay Kit