ELK Biotechnology
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Classified Nitric Oxide Synthase (NOS) Assay Kit

(Colorimetric Method)

Cat. No:BC099 Size:100T/48S

1.Assay principle:

Nitric oxide synthase can catalyze L-Arg and dioxygen to produce NO, NO reacts with nucleophile to produce colored compound. It is able to calculate NOS activity by measuring OD values at 530nm.

NOS is generally classified to 2 types: constitutive type (cNOS) & inducible type (iNOS). cNOS mainly exists in neurons & endotheliocytes, requires calcium; iNOS mainly exists in macrophages, doesn't require calcium. They can be classfied by calcium requirement.

2.Reagent composition&Preparation

(1) 100T/48S Assay Kit:

Reagent 1: Substrate buffer, 6ml×4 bottles, can be stored at -20℃ (or even colder) away from light for 3 months, when use, please thaw and shake it sufficiently. Unused reagent can be stored -20℃ (or even colder) away from light for next time.

Reagent 2: Promoter, light yellow or white powder×6 vials, diluent 0.6ml×6 vials. Can be stored at -20℃ (or even colder) for 6 months. When use, add 1 vial diluent (0.6ml) in 1 vial powder, mix sufficiently (turn 1.5ml small centrifuge tube upside down repeatedly, and let liquid in tube tip drops down by flipping). Unused reagent can be stored at -20℃ (or even colder) for less than 1 week. If powder becomes tan or brown, please you can not use it.

Reagent 3: Yellow chromogenic agent 6ml×2 bottles, can be stored at 4℃ for 3 months.

Reagent 4: Clearing reagent 6ml×2 bottles, can be stored at room temperature for 6 months. It will freeze in cold days, so please place it in 37℃ water bath until it becomes limpid, then you can use this reagent.

Reagent 5: Terminator 60ml×4 bottles, can be stored at 4℃ for 6 months. It will be turbid at low temperature, so please place it in 37℃ water bath until it becomes limpid, then you can use this reagent.

Reagent 6: Solution, 10ml×1 bottle, can be stored at 4℃ for 6 months. Please check the bottle carefully before use, if there are crystals at bottom or attach to inner surface, then place the bottle in 90～100℃ hot water, shake repeatedly until it becomes limpid, then you can use this reagent.

(2) 50T/24S Assay Kit:

Reagent 1: Substrate buffer, 6ml×2 bottles, can be stored at -20℃ (or even colder) away from light for 3 months, when use, please thaw and shake it sufficiently. Unused reagent can be stored -20℃ (or even colder) away from light for next time.

Reagent 2: Promoter, light yellow or white powder×3 vials, diluent 0.6ml×3 vials. Can be stored at -20℃ (or even colder) for 6 months. When use, add 1 vial diluent (0.6ml) in 1 vial powder, mix sufficiently (turn 1.5ml small centrifuge tube upside down repeatedly, and let liquid in tube tip drops down by flipping). Unused reagent can be stored at -20℃ (or even colder) for less than 1 week. If powder becomes tan or brown, please you can not use it.

Reagent 3: Yellow chromogenic agent 6ml×1 bottle, can be stored at 4℃ for 3 months.

Reagent 4: Clearing reagent 6ml×1 bottle, can be stored at room temperature for 6 months. It will freeze in cold days, so please place it in 37℃ water bath until it becomes limpid, then you can use this reagent.

Reagent 5: Terminator 60ml×2 bottles, can be stored at 4℃ for 6 months. It will be turbid at low temperature, so please place it in 37℃ water bath until it becomes limpid, then you can use this reagent.

Reagent 6: Inhibitor solution, 10ml×1 bottle, can be stored at 4℃ for 6 months. Please check the bottle carefully before use, if there are crystals at bottom or attach to inner surface, then place the bottle in 90～100℃ hot water, shake repeatedly until it becomes limpid, then you can use this reagent.

3.Operation Procedures

Note: Take samples and reagents out of fridge, place them in 37℃ water bath to

dissolve completely, Reagent 4 & Reagent 5 must be limpid, then you can start operations (imcomplete dissolving and turbidity will disturb results).

Operation table (TNOS means total NOS):

	TNOS blank tube	**TNOS sample tube**	**iNOS blank tube**	**iNOS sample tube**
Double distilled water (μl)	a*+100	100	a*	
Sample(μl)		a*		a*
Reagent 6 Inhibitor (μl)			100	100
Shake test tube shelf				
Reagent 1 substrate buffer (μl)	200	200	200	200
Reagent 2 Promoter (μl)	10	10	10	10
Reagent 3 Chromogenic agent (μl)	100	100	100	100
Mix sufficiently, react at 37℃ water bath for 15 minutes accurately				
Reagent 4 Clearing reagent (μl)	100	100	100	100
Reagent 5 Terminator (μl)	2000	2000	2000	2000
Mix sufficiently, transfer in cuvettes of 1cm light path, measure OD values of all tubes at 530nm (adjust zero by distilled water).				

Note: 1. a* is volume of sample & distilled water. Different samples have different a* values.

- 10% rat liver tissue homogenate: 50～100μl
- Rat/mouse blood serum: 30μl
- 10% rat kidney tissue homogenate: 50μl

- Dog blood serum: 30μl
- Cardiac muscle culture fluid: 100μl

2. Please check test tubes carefully before measurement, if there are crystals or turbidity, then place tubes in 37℃ water bath and shake them repeatedly until all solutions become limpid. Then you can start measurement.

4. Calculations

(1) Blood serum NOS assay:

① Unit definition: 1nmol NO producing per ml blood serum per minute is considered as 1 enzyme activity unit (U).

② Formulas:

$$\text{TNOS activity (U/ml)} = \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div 1000$$

$$= \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{38.3 \times 10^{-6}} \times \frac{2.51 + a^*}{a^*} \times \frac{1}{1 \times 15} \div 1000$$

$$\text{iNOS activity (U/ml)} = \frac{OD_{\text{iNOS-Sample}} - OD_{\text{iNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div 1000$$

$$= \frac{OD_{\text{iNOS-Sample}} - OD_{\text{iNOS-Blank}}}{38.3 \times 10^{-6}} \times \frac{2.51 + a^*}{a^*} \times \frac{1}{1 \times 15} \div 1000$$

③ Example:

Take 30μl blood serum to measure NOS activity according to operation table, in results, $OD_{\text{TNOS-Blank}}$ is 0.024, $OD_{\text{TNOS-Sample}}$ is 0.273, $OD_{\text{iNOS-Blank}}$ is 0.010, $OD_{\text{iNOS-Sample}}$ is 0.149, calculate as follows:

$$\text{TNOS activity (U/ml)} = \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div 1000$$

$$= \frac{0.273 - 0.024}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.03}{0.03} \times \frac{1}{1 \times 15} \div 1000 = 36.696 \ \ U/ml$$

$$\text{iNOS activity (U/ml)} = \frac{OD_{\text{iNOS-Sample}} - OD_{\text{iNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div 1000$$

$$= \frac{0.149 - 0.010}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.03}{0.03} \times \frac{1}{1 \times 15} \div 1000 = 20.485 \ \ U/ml$$

(2) Tissue NOS assay:

① Unit definition: 1nmol NO producing per mg tissue protein per minute is considered as 1 enzyme activity unit (U).

② Formulas:

$$\text{TNOS activity (U/mgprot)} = \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (mgprot/L)}$$

$$= \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{38.3 \times 10^{-6}} \times \frac{2.51 + a^*}{a^*} \times \frac{1}{1 \times 15} \div \text{Protein concentration (mgprot/L)}$$

$$\text{iNOS activity (U/mgprot)} = \frac{OD_{\text{iNOS-Sample}} - OD_{\text{iNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \frac{\text{Protein concentration}}{\text{(mgprot/L)}}$$

$$= \frac{OD_{\text{iNOS-Sample}} - OD_{\text{iNOS-Blank}}}{38.3 \times 10^{-6}} \times \frac{2.51 + a^*}{a^*} \times \frac{1}{1 \times 15} \div \frac{\text{Protein concentration}}{\text{(mgprot/L)}}$$

Note: mgprot means milligram protein.

③ Example:

a. Take 0.15g mouse liver tissue, add physiological saline of 9 times volume (1.35ml) to make 10% tissue homogenate, centrifugate at 3000rpm for 10 minutes (according to Experimental Methodology from our Institute), take 50μl supernatant to measure NOS activity, in results, $OD_{TNOS\text{-}Blank}$ is 0.021, $OD_{TNOS\text{-}Sample}$ is 0.159, $OD_{iNOS\text{-}Blank}$ is 0.008, $OD_{iNOS\text{-}Sample}$ is 0.054, protein concentration in 10% mouse liver homogenate is 14.012mgprot/ml, calculate as follows:

$$\text{TNOS activity (U/mgprot)} = \frac{OD_{\text{TNOS-Sample}} - OD_{\text{TNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \frac{\text{Protein concentration}}{\text{(mgprot/L)}}$$

$$= \frac{0.159 - 0.021}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.05}{0.05} \times \frac{1}{1 \times 15} \div (14.012 \times 10^3)\ mgprot/L$$

$$= 0.8777\ U/mgprot$$

$$\text{iNOS activity (U/mgprot)} = \frac{OD_{iNOS\text{-}Sample} - OD_{iNOS\text{-}Blank}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (mgprot/L)}$$

$$= \frac{0.054 - 0.008}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.05}{0.05} \times \frac{1}{1 \times 15} \div (14.012 \times 10^{3})\ mgprot/L$$

$$= 0.2926\ U/mgprot$$

b. Take 0.1g *Acipenser sinensis* brain tissue, add physiological saline of 9 times volume (0.9ml) to make 10% tissue homogenate, centrifugate at 3000rpm for 10 minutes (according to Experimental Methodology from our Institute), take 50μl supernatant to measure NOS activity, in results, $OD_{TNOS\text{-}Blank}$ is 0.023, $OD_{TNOS\text{-}Sample}$ is 0.122, $OD_{iNOS\text{-}Blank}$ is 0.006, $OD_{iNOS\text{-}Sample}$ is 0.058, protein concentration in 10% *Acipenser sinensis* brain homogenate is 4.5332mgprot/ml, calculate as follows:

$$\text{TNOS activity (U/mgprot)} = \frac{OD_{TNOS\text{-}Sample} - OD_{TNOS\text{-}Blank}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (mgprot/L)}$$

$$= \frac{0.122 - 0.023}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.05}{0.05} \times \frac{1}{1 \times 15} \div (4.5332 \times 10^{3})\ mgprot/L$$

$$= 1.9463\ U/mgprot$$

$$\text{iNOS activity (U/mgprot)} = \frac{OD_{iNOS\text{-}Sample} - OD_{iNOS\text{-}Blank}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (mgprot/L)}$$

$$= \frac{0.058 - 0.006}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.05}{0.05} \times \frac{1}{1 \times 15} \div (4.5332 \times 10^{3})\ mgprot/L$$

$$= 1.0223\ U/mgprot$$

6. Normal Reference Values

- Rat blood serum (TNOS): 18.69±3.97 U/ml (n=45)
- 10% rat kidney homogenate (TNOS): 0.536±0.134 U/mgprot (n=19)
- Dog blood serum (TNOS): 20.57±3.39 U/ml (n=18)
- Cardiac muscle culture solution (TNOS): 0.698±0.110U/ml (n=12)

7.Announcements

(1) Promoter (Reagent 2) should be used soon after preparation, it is better to use all promoter in 1 day, unused promoter can be stored at -20℃ or even colder for less than 1 week. Unprepared Reagent 2 promoter and diluent should be stored at -20℃ or even colder, if light yellow or white powder becomes brown or tan granules, then please discard it.

(2) Reagent 6 is supersaturated solution, some crystals may seed out when you use residuary reagent again, so please place this reagent in boiling water bath, stir by glass rod until dissolving completely.

(3) NOS activity may be low and unstable, if you don't want measure samples or homogenate supernatants immediately, then please store them at -20℃ or even colder.

(4) Avoid freeze-thawing Reagent 1 and prepared Reagent 2 repeatedly.

(5) NOS Reagent 5 becomes turbid at low temperature. If you have already added

Reagent 5 in sample tube, then please check whether there are crystals or turbidity towars light. If there are, then please place each sample tube in 37℃ water bath, shake for 5 minutes. You can start measurements when they become limpid.