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Nitric Oxide Synthase (NOS) Activity Assay Kit II (Colorimetric)

06/21

(Catalog # K2094-100; 100 assays; Store at -80 °C)

I. Introduction:

Nitric oxide synthases (EC 1.14.13.39, NOS) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. NO plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. In presence of NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, calmodulin and heme, NOS catalyzes the five-electron oxidation of the guanidino nitrogen of L-arginine with molecular oxygen to generate NO and L-citrulline. There are three isoforms of NOS including endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). nNOS accounts for the production of NO in the central nervous system and participates in cell communication and information storage. eNOS produces NO in the blood vessels and is involved in the regulation of vascular function. In contrast to other isoforms, iNOS is expressed *de novo* during oxidative stress and large amounts of NO are produced as part of body's defense mechanism. **BioVision's Nitric Oxide Synthase Activity Assay Kit II** provides an accurate and convenient method to assay NOS activity in a variety of sample types. In this assay, NO generated by NOS undergoes a series of reactions and then reacts with Griess Reagent 1 and 2 to generate a colored product with a strong absorbance at 540 nm. The assay is simple, fast, sensitive and is high-throughput adaptable.

II. Application:

- Detection of NOS activity in various sample types

III. Sample Types:

- Purified recombinant protein
- Tissue or cell extracts

IV. Kit Contents:

Components	K2094-100	Cap Code	Part Number
NOS Assay Buffer	25 ml	WM	K2094-100-1
NOS Lysis Buffer	25 ml	Red	K2094-100-2
NOS Substrate	0.5 ml	White	K2094-100-3
NOS Cofactor 1	1 vial	Blue	K2094-100-4
NOS Cofactor 2 (25X)	0.1 ml	Amber	K2094-100-5
Nitrate Reductase	1 vial	Green	K2094-100-6
NOS (Positive Control)	50 µl	Yellow	K2094-100-7
Enhancer	1 vial	Purple	K2094-100-8
Nitrite Standard	1 vial	Orange	K2094-100-9
Griess Reagent 1	10 ml	NM	K2094-100-10
Griess Reagent 2	10 ml	Amber	K2094-100-11

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer
- Protease Inhibitor Cocktail (BioVision Cat. # K271 or equivalent)

VI. Storage Conditions and Reagent Preparation:

Store the kit at -80 °C, protected from light. Once opened, store the kit components as recommended. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **NOS Assay Buffer:** Bring to room temperature (RT) before use. Store at 4 °C or -20 °C.
- **NOS Lysis Buffer:** Ready to use. Store at 4 °C or -20 °C.
- **NOS Substrate:** Ready to use. Divide into aliquots and store at -20 °C. Avoid repeated freeze/thaw. Keep on ice while in use.
- **NOS Cofactor 1:** Reconstitute the vial in 300 µl of dH_2O to prepare 10 mM NOS Cofactor 1 working solution. Divide into aliquots and store at -20 °C. Avoid repeated freeze-thaw cycles. Keep on ice while in use.
- **NOS Cofactor 2 (25X):** Divide into aliquots and store at -20 °C. Avoid repeated freeze/thaw cycles. Prepare 1X NOS Cofactor 2 working solution with dH_2O just before use. Keep on ice while in use.
- **Nitrate Reductase:** Reconstitute the vial in 1.1 ml NOS Assay Buffer. Divide into aliquots and store at -20 °C. Avoid repeated freeze-thaw cycles. Keep on ice while in use.
- **NOS (Positive Control):** Divide into aliquots and store at -80 °C. Avoid repeated freeze-thaw cycles. During use, keep the solution on ice at all times since the enzyme loses activity at higher temperatures.
- **Enhancer:** Reconstitute the vial in 1.2 ml NOS Assay buffer. Keep on ice during use. Store at -20 °C.
- **Nitrite Standard:** Reconstitute the vial in 100 µl NOS Assay Buffer. Vortex & mix well to generate a 100 mM Nitrite Standard solution. Store at 4 °C when not in use. **Do not freeze.** The reconstituted Nitrite Standard solution is stable for 4 months when stored at 4 °C.
- **Griess Reagents 1** and **Griess Reagent 2:** Ready to use. Store at 4 °C.

VII. Nitric Oxide Synthase Activity Assay Protocol:

1. **Sample Preparation:** Rinse the tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80 °C) to a pre-chilled tube. Add 200-300 µl cold NOS Lysis Buffer containing protease inhibitor cocktail (not provided, **BioVision Cat# K271**) and thoroughly homogenize the tissue on ice. To prepare the cell extract, add 200-300 µl cold NOS Lysis Buffer containing protease inhibitor cocktail (not provided)

to fresh or frozen cells (2-5 x 10^6 cells) and homogenize to disrupt the cells. Centrifuge the tissue or the cell homogenate at 10,000 x *g*, 4 °C for 10 min. Transfer the clear supernatant to a fresh pre-chilled tube and keep on ice. Measure the protein concentration using BCA or any preferred protein assay. Use the lysates immediately for the NOS activity assay. Add 30-60 µl (200-400 µg protein) of cell or tissue homogenate or purified protein into the desired wells of a 96-well plate. For **Positive Control**, Add 5 µl of NOS (Positive Control) into the desired well(s). Adjust the volume of samples and Positive Control wells to 57.5 µl/well with NOS Assay Buffer.

Notes:

a. We recommend using the tissue or cell homogenate immediately to measure the NOS activity. If desired, snap freeze the lysate and store at -80 °C.

b. For Unknown Samples, we suggest doing a pilot experiment and testing several amounts to ensure that the readings are within the Nitrite Standard Curve range.

c. Optional: For samples with high background, prepare a parallel sample well(s) containing same amount of sample labeled as **Sample Background Control**(s).

2. **Nitrite Standard Preparation:** Add 5 µl of reconstituted 100 mM Nitrite Standard to 495 µl NOS Assay Buffer to generate 1 mM Nitrite Standard solution. Add 0, 2, 4, 6, 8, and 10 µl of 1 mM Nitrite Standard solution into a series of wells in a 96-well plate to generate 0, 2, 4, 6, 8 and 10 nmol/well Nitrite Standard. Adjust the volume to 57.5 µl/well with NOS Assay Buffer.

3. **Reaction Mix Preparation:** Prepare enough Reaction Mix for the number of wells (Standards, Positive Control and sample) to be analyzed. For each well, prepare 40 µl Mix:

	Reaction Mix	Background Mix
NOS Assay Buffer	10 µl	15 µl
NOS Cofactor 2 (1x)	20 µl	20 µl
NOS Substrate	5 µl	--
Nitrate Reductase	5 µl	5 µl

Mix and add 40 µl of the Reaction Mix to Standard, Positive Control, and Sample wells. Add 40 µl of Background Mix to the Sample Background Control wells. Add 2.5 µl of 10 mM NOS Cofactor 1 working solution to all the wells including Sample, Sample Background Control, Standards and Positive Control and mix well. Incubate the plate at 37 °C for 20- 30 min. After incubation, add 95 µl of NOS Assay Buffer to all wells followed by the addition of 5 µl of Enhancer into each well. Mix and incubate the plate at RT for 10 min.

4. **Measurement:** Add 50 µl of Griess Reagent 1 and 50 µl of Griess Reagent 2 to all the wells including Sample, Sample Background Control, Standard, and Positive Control. Mix and incubate for 10 min. Measure the absorbance at 540 nm in a microplate reader in endpoint mode.

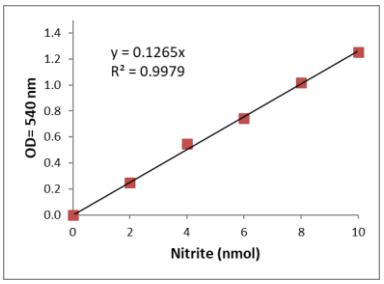
5. **Calculation:** Subtract 0 Standard reading from all readings. Plot the Nitrite Standard Curve. If the Sample Background Control reading is significant, subtract the Sample Background Control reading from the Sample readings to get the corrected Sample reading. Apply the corrected Sample reading to the Standard Curve to get B nmoles of Nitrite generated during the reaction.

$$\textbf{Sample Nitric Oxide Synthase Specific Activity} = \frac{\mathbf{B}}{\mathbf{T \times C}} = \textbf{nmol/min/mg = mU/mg}$$

Where, **B** is the Nitrite amount in the sample well from the Standard Curve (nmol).
T is the reaction time (min.)
C is the amount of protein (mg)

Unit Definition: One unit of NOS activity is the amount of enzyme required to yield 1.0 µmol of nitric oxide/min. at 37 °C.

(a)



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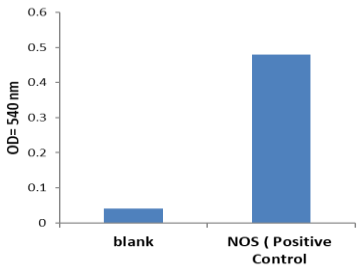

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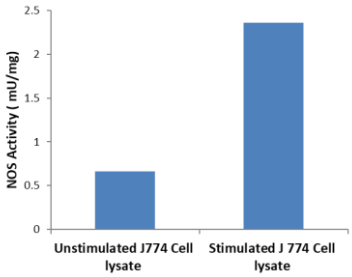

Figures: (a) Nitrite Standard Curve. **(b)** Measurement of NOS Positive Control activity (10 µl). **(c)** Detection of endogenous NOS activity in J774.1A cell lysate (150 µg) stimulated with or without 200 ng/ml LPS and 100 ng/ml murine IFN-gamma. Assays were performed following the kit protocol.

VIII. Related Products:

Nitric Oxide Synthase Activity Assay Kit (Fluorometric) (K206)
Nitric Oxide Colorimetric Assay Kit (K262)
Nitric Oxide Synthase (NOS) Inhibitor Screening Kit (K208)
EZCell™ Intracellular Nitric Oxide Synthase Detection Kit (K207)
Nitric Oxide Fluorometric Assay Kit (K252)
DDAH Activity Assay Kit (Colorimetric) (K2089)

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