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

7/17

(Catalog # K205-100; 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. Nitric oxide (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 a 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: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). nNOS accounts for the production of NO in central nervous system, where NO participates in cell communication and information storage. eNOS produces NO in blood vessels and is involved in regulation of vascular function. In contrast to other isoforms, iNOS is expressed *de novo* under oxidative stress conditions and produces large amounts of NO as a part of body's defense mechanism. BioVision's Nitric Oxide Synthase Activity Assay Kit provides an accurate and convenient method to assay NOS activity in a variety of samples. In this assay, nitric oxide generated by NOS undergoes a series of reactions and reacts with Griess Reagent 1 and 2 to generate a colored product with a strong absorbance at 540 nm. The assay is simple, sensitive and high-throughput adaptable and can detect as low as 5 µU of NOS activity.

II. Application:

- Detection of NOS activity

III. Sample Type:

- Purified recombinant protein
- Tissue or cell extracts

IV. Kit Contents:

Components	K205-100	Cap Code	Part Number
NOS Assay Buffer	25 ml	WM	K205-100-1
NOS Dilution Buffer	1.5 ml	Blue/White Dot	K205-100-2
NOS Substrate	0.5 ml	White	K205-100-3
NOS Cofactor 1	1 Vial	Blue	K205-100-4
NOS Cofactor 2 (25X)	0.1 ml	Amber	K205-100-5
Nitrate Reductase	1 Vial	Green	K205-100-6
NOS (Positive Control)	4 µl	Yellow	K205-100-7
Enhancer	1 Vial	Purple	K205-100-8
Nitrite Standard	1 Vial	Orange	K205-100-9
Griess Reagent 1	10 ml	NM	K205-100-10
Griess Reagent 2	10 ml	Amber	K205-100-11

V. User Supplied Reagents and Equipment:

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

VI. Storage Conditions and Reagent Preparation:

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

- **NOS Assay Buffer:** Bring to room temperature (RT) before use. Store at 4°C or -20°C.
- **NOS Dilution Buffer:** Ready to use. Store at 4°C or -20°C.
- **NOS Substrate:** Ready to use. Aliquot and store at -20°C. Avoid repeated freeze/thaw. Keep on ice while in use.
- **NOS Cofactor 1:** Reconstitute with 110 µl of dH_2O to make 10 mM stock solution. Aliquot and store at -20°C. Freeze/thaw should be limited to 1 time. Dilute 10 mM stock solution with dH2O to make 1 mM working solution just before use. Make as much as needed. Keep on ice while in use. Working solution can be stored at 4°C for 6-8 hrs.
- **NOS Cofactor 2:** Aliquot and store at -20°C. Avoid repeated freeze/thaw. Make 1X working solution with dH_2O just before use. Keep on ice while in use.
- **Nitrate Reductase:** Reconstitute with 1.1 ml Assay Buffer. Aliquot and store at –20°C. Avoid repeated freeze/thaw. Keep on ice while in use.
- **NOS (Positive Control)**: Aliquot and store at -80°C. Freeze/thaw should be limited to 1 time. During use, keep the solution on ice at all times since the enzyme loses activity at higher temperature.
- **Enhancer:** Reconstitute with 1.2 ml Assay buffer. Keep on ice during use. Store at -20°C.
- **Nitrite Standard**: Reconstitute with 1 ml Assay Buffer. Vortex and mix well to generate a 10 mM Standard. Store at 4°C when not in use (do not freeze). The reconstituted standard is stable for 4 months when stored at 4°C.
- **Griess Reagents R1 and R2**: Ready to use. Store at 4°C.

VII. Nitric Oxide Synthase Activity Assay Protocol:

1. **Sample Preparation:** Rinse tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80°C) to a prechilled tube. Add 200 µl cold NOS Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice. Transfer the tissue

homogenate to a cold microfuge tube. To prepare cell extract, add 100-200 µl cold NOS Assay Buffer containing protease inhibitor cocktail (not provided) to fresh or frozen cells (2-5 x 10^6) and homogenize to disrupt the cells. Centrifuge the tissue or cell homogenate at 10,000 X g, 4°C for 10 min. Transfer the clarified supernatant to a fresh pre-chilled tube & keep on ice. Measure the protein concentration. Use lysates immediately to assay NOS activity.

2. **Nitrite Standard:** Add 5 µl of the reconstituted 10 mM Nitrite Standard to 995 µl Assay Buffer to generate 50 µM working Standard solution. Mix well. Add 0, 5, 10, 15, 20, and 25 µl of the working Standard solution into a series of wells in 96-well plate to generate 0, 250, 500, 750, 1000, and 1250 pmol/well Nitrite Standard. Adjust the volume to 60 µl/well with NOS Assay Buffer.
3. **NOS Activity:** Add 30-60 µl (200-400 µg protein) of cell/tissue homogenate or purified protein into desired wells in a 96-well plate. For Positive Control, dilute NOS enzyme 1:20 in NOS Dilution Buffer just before use. Add 5-10 µl of diluted NOS enzyme into desired well(s). Make up the volume of samples and Positive Control wells to 60 µl/well with NOS Assay Buffer.

   Notes:

   a. We recommend using the tissue/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 pilot experiment and testing several amounts to ensure the readings are within the Standard Curve range.

   c. **Optional**: For samples having background, prepare parallel sample well(s) as sample background control(s). Use same amount of tissue/cell homogenate or purified enzyme as in the sample well. Adjust the final volume to 300 µl with Assay Buffer.
4. **Reaction Mix:** Prepare enough reaction mix for the number of wells (Standards, Positive Control and sample) to be analyzed. For each well, prepare 45 µl reaction mix:

Diluted NOS Cofactor 1	10 µl
NOS Cofactor 2 (1X)	20 µl
NOS Substrate	5 µl
Nitrate Reductase	5 µl

   Mix and add 40 µl of the Reaction Mix into Standard, Positive Control, and sample wells. Mix well and incubate at 37°C for 1 hr. After incubation, add 95 µl of NOS Assay Buffer to Standard, Positive Control, and sample wells and subsequently add 5 µl of the enhancer into each well. Mix and incubate at room temperature for 10 min.
5. **Measurement:** Add 50 µl of Griess Reagent 1 and 50 µl of Griess Reagent 2 to Standard, Positive Control and sample wells. Mix and incubate for 10 min. Read absorbance (540 nm) using a microplate reader.
6. **Calculation:** Subtract 0 Standard reading from all readings. Plot the Nitrite Standard Curve. If sample background control is significant, then subtract sample background control reading from sample reading. Apply ΔA_{540} to the Standard curve to get B pmoles of nitrite generated during the reaction.

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

Where, **B** is Nitrite amount in sample well from Standard Curve (pmol).
T is reaction time (min.) (60 min.)
C is amount of protein (µg)

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

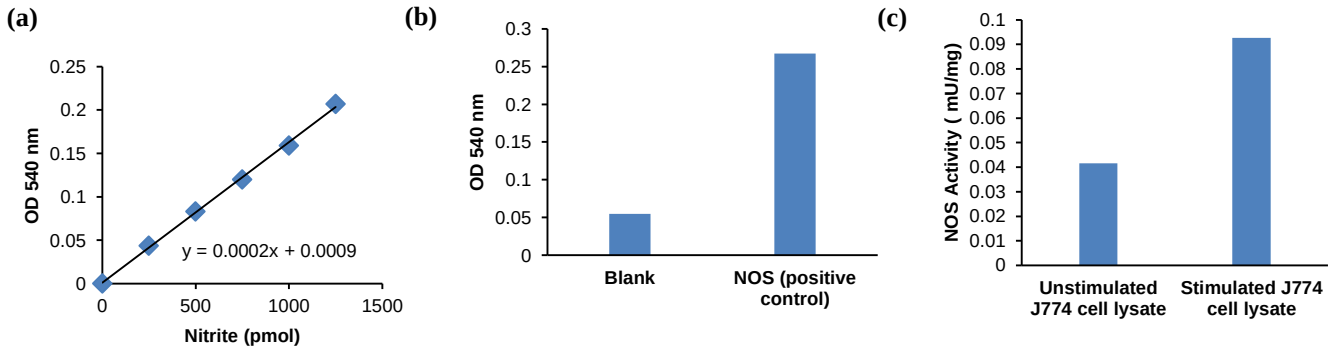

Figure: (a) Nitrite Standard Curve, (b) Measurement of NOS Positive Control activity (10 µl), and (c) Detection of endogenous NOS activity in J774.1A cell lysate (225 µg) stimulated with or without 200 ng/ml LPS and 100 ng/ml murine IFN-gamma Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Nitric Oxide Synthase Activity Assay Kit (Fluorometric) (K206)
Nitric Oxide Colorimetric Assay Kit (K262)
Diphenyleneiodonium chloride (2358)
NOC-18 (2492)
EZCell™ Intracellular Nitric Oxide Synthase Detection Kit (K207)
Nitric Oxide Fluorometric Assay Kit (K252)
L-NMMA acetate (2348)
eNOS Antibody (3426)

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