EnzyChrom™ Nitric Oxide Synthase Assay Kit (ENOS-100)

Quantitative Colorimetric Determination of Nitric Oxide Synthase Activity at 540nm

DESCRIPTION

Nitric oxide (NO) is a reactive radical that plays an important role in many key physiological functions. NO, an oxidation product of arginine by nitric oxide synthase (NOS), is involved in host defense and development, activation of regulatory proteins and direct covalent interaction with functional biomolecules.

Simple, direct and non-radioactive procedures for measuring NOS are becoming popular in Research and Drug Discovery. BioAssay Systems' EnzyChrom™ Nitric Oxide Synthase Assay Kit involves two steps: a NOS reaction step during which NO is produced followed by an NO detection step. Since the NO generated by NOS is rapidly oxidized to nitrite and nitrate, the NO production is measured following reduction of nitrate to nitrite using an improved Griess method. The procedure is simple and the time required for sample pretreatment and assay is reduced to as short as 40 min.

KEY FEATURES

Sensitive and accurate. Detection range 0.25 - 25 U/L in 96-well plate.

Rapid and reliable. Can be completed in 40 min if reduction of NO_3^- to NO_2^- is performed at 60°C.

APPLICATIONS

Direct Assays: NOS activity in biological samples.

Drug Discovery/Pharmacology: effects of drugs on NOS activity.

KIT CONTENTS (100 tests in 96-well plates)

Assay Buffer:	10 mL	**Substrate**:	600 µL	**GDH**:	120 µL
Reagent A:	12 mL	**Reagent B**:	500 µL	**Reagent C**:	12 mL
Reagent D:	Dried (2 Tubes)	**Reagent E**:	1.5 mL	**$ZnSO_4$**:	1 mL
Standard:	1 mL	**NaOH**:	1 mL		

Storage conditions. The kit is shipped on ice. Store Assay Buffer, Substrate, Reagent D, Reagent E and GDH at -20°C. Store all other reagents at 4°C. Shelf life of six months after receipt. Use Reagent D within 1 week after reconstitution.

Precautions: reagents are for research use only. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Prior to assay, equilibrate all components to room temperature. Reconstitute one tube of Reagent D with 300 µL dH2O. (If assaying more than 60 wells, reconstitute both tubes of Reagent D) Store unused reconstituted Reagent D at -20˚C and use within 1 week. Prewarm Assay Buffer to 37°C. Keep GDH on ice. If precipitates are present in Reagent B, warm at 37˚C until redissolved (~10-15 min).

Sample treatment: tissue or cell samples are homogenized in 1 x PBS (pH 7.4). Centrifuge at 10,000*g* or higher at 4°C. Use supernatant for NOS assay.

Standard preparation: Prepare 200 µL 500 µM Premix by mixing 100 µL 1.0 mM Standard and 100 µL distilled water. Dilute standards in 1.5-mL centrifuge tubes as described in the Table.

No	Premix + H_2O	Nitrite (µM)
1	50 µL + 0 µL	500
2	30 µL + 20 µL	300
3	15 µL + 35 µL	150
4	0 µL + 50 µL	0

NOS Reaction: If samples will not require deproteination (i.e. purified NOS), add 20 µL of each sample and standard to separate labeled eppendorf tubes. Each sample requires at least two tubes: one reaction tube and one sample blank tube. Immediately prior to starting the reaction, prepare enough NOS Working Reagent (NOS WR) for all sample reaction tubes and standards by mixing per reaction tube: 65 µL Assay Buffer, 4 µL Substrate, 4 µL Reconstituted Reagent D, 10 µL Reagent E and 1 µL GDH. For the sample blanks, use 8 µL dH_2O instead of the Substrate and Reagent D. Add 80 µL of the appropriate NOS WR to each tube and incubate at 37°C for 20 min. After 20 min immediately add 200 µL of the NO Detection Reagent (NO DR) (see next section: NO Measurement) to each tube to kill the NOS reaction.

For samples requiring deproteination which include serum, plasma, whole blood, cell culture media containing FBS, tissue or cell lysates, add 25 µL of each sample and standard to separate labeled eppendorf tubes. Each sample requires at least two tubes: one reaction tube and one sample blank tube. Immediately prior to starting the reaction, prepare enough NOS WR for all sample reaction tubes and standards by mixing per reaction tube: 80 µL Assay Buffer, 5 µL Substrate, 5 µL Reconstituted Reagent D, 13 µL Reagent E and 1 µL GDH. For the sample blanks, use 10 µL dH_2O instead of the Substrate and Reagent D. Add 100 µL of the appropriate NOS WR to each tube and incubate at 37°C for 20 min. After 20 min immediately proceed to the deproteination step.

Deproteination. Add 7 µL $ZnSO_4$ to each sample and standard tube. Vortex and then add 7 µL NaOH. Vortex again and centrifuge 10 min at 14,000 rpm. Transfer 100 µL of the clear supernatant to a clean tube and proceed to the NO Measurement step.

NO Measurement: Immediately prior to starting the reaction, prepare enough NO Detection Reagent (NO DR) for all samples and standards by mixing per reaction tube: 100 µL Reagent A, 4 µL Reagent B and 100 µL Reagent C. Add 200 µL of the NO DR to each sample and standard tube and incubate for 5 min at 60°C. (Alternatively, the reaction can be run at 37°C for 60 min or RT for 150 min.)

Briefly centrifuge the reaction tubes to pellet any condensation and transfer 250 µL of each reaction to separate wells in a 96 well plate. Read OD at 500-570nm (peak 540 nm).

CALCULATION

Subtract blank OD (Std 4) from the standard OD values and plot the OD against standard concentrations. Determine the slope using linear regression fitting. The NOS activity of the Sample is then calculated as

$$\text{NOS Activity} = \frac{OD_{SAMPLE} - OD_{BLANK}}{\text{Slope}} \times \frac{1}{t} \quad (\text{U/L})$$

OD_{SAMPLE} and OD_{BLANK} are optical density values of the sample and sample blank, respectively. *t* is the reaction time (20 min).

Unit definition: one unit of NOS catalyzes the production of 1 µmole of nitric oxide per minute under the assay conditions (pH 7.5 and 37°C).

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipetting devices, eppendorf tubes, eppendorf centrifuge, clear, flat bottomed 96 well plates or cuvettes, plate reader or spectrophotometer and heat block or hot water bath (optional).

GENERAL CONSIDERATIONS

Antioxidants and nucleophiles (e.g. β-mercaptoethanol, glutathione, dithiothreitol and cysteine) may interfere with this assay. Avoid using these compounds during sample preparation. However, if β-mercaptoethanol or dithiothreitol must be used, an equal concentration needs to be added to the standards.

LITERATURE

1. Ghigo, D. (2006). Cycling of NADPH by glucose 6-phosphate dehydrogenase optimizes the spectrophotometric assay of nitric oxide synthase activity in cell lysates. *Nitric Oxide* 15: 148-53.
2. Knowles, R. G. and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.* 298: 249-58.
3. Förstermann, U. et al. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem Pharmacol.* 42:1849-57.