



BIOXYTECH[®] Nitric Oxide Synthase Assay Kit
Colorimetric Assay for the Determination of NOS Activity
For Research Use Only.
Not For Use In Diagnostic Procedures.
Catalog No. 22113

INTENDED USE

The BIOXYTECH[®] Nitric Oxide Synthase Assay Kit is intended for the quantitative determination of total nitrite as an indicator of nitric oxide synthase (NOS) activity in biological samples. It may be used for the assay of total nitrite in urine, plasma, serum and tissue culture medium.

INTRODUCTION

Three distinct isoforms of NOS have been identified: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS or bNOS). Nitric Oxide Synthase is a remarkably complex enzyme which acts on molecular oxygen and arginine in neurons, endothelial cells, platelets, neutrophils and other cells to produce nitric oxide (NO). Nitric oxide is a unique second messenger molecule that readily diffuses through cell membranes to exert a variety of biological actions in mammalian cells. Excess generation of NO leads to the formation of peroxynitrite, destruction of iron-sulfur clusters, thiol nitrosylation and nitration of protein tyrosine residues. The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-).

NADPH is an essential cofactor for the function of the NOS enzyme. Unfortunately, NADPH interferes with the Griess reagents, the most commonly used reagents for nitrite detection. There are two common methods to reduce the NADPH interference. Very small concentrations of NADPH can be used, in conjunction with a catalytic system for recycling of the spent NADP⁺ back to NADPH; or the excess NADPH is removed or destroyed. This assay uses lactate dehydrogenase (LDH) to destroy the excess NADPH.

PRINCIPLES OF THE PROCEDURE

NO exists primarily as NO_2 and NO_3 in biological systems. Its measurement can be a useful tool in estimating NOS activity. The principle of this assay is based on the measurement of NO_2 produced in the sample during a timed reaction compared with a heat-inactivated control sample. Nitrate reductase is utilized for the enzymatic reduction of nitrate to nitrite. Spectrophotometric quantitation of nitrite using Griess Reagents is straightforward and sensitive. In acidic solution, nitrite is converted to nitrous acid (HNO_2), which diazotizes

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sulfanilamide. This sulfanilamide-diazonium salt is then reacted with N-(1-Naphthyl)-ethylenediamine to produce a chromophore which is measured at 540 nm.

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Prepare a 1 mM solution of NADPH in Assay Buffer.
Make a fresh solution each day.

REAGENTS

Materials Provided

- Assay Buffer
- Nitrate Reductase
- Co-Factor Preparation
- Nitrate Standard
- Lactate Dehydrogenase
- Griess Reagent 1 (R1)
- Griess Reagent 2 (R2)
- NADPH, Tetrasodium Salt
- Microtiter Plate with Plate Cover

Other Items Required

- Plate reader capable of absorbance (OD) measurements at 540 nm.
- Adjustable pipettor
- Deionized water or HPLC grade water (preferred)

Warnings and Precautions

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For In Vitro Use Only.

Wear appropriate laboratory attire, including eye protection and disposable gloves. In case of accidental exposure to skin, eyes or mucous membranes, thoroughly rinse affected area with water.

Kit Storage

Store the reagents at -20°C . The unopened reagents are stable frozen until the expiration date on the label.

Reagent Preparation

- **Assay Buffer**
Dilute the contents of the Assay Buffer vial to 100 mL with HPLC-Grade water. This Assay Buffer should be used for dilution of samples as needed prior to assay.
- **Nitrate Reductase**
Reconstitute the contents of the vial with 1.2 mL of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Do not freeze-thaw this solution more than once.
- **Co-Factor Preparation**
Reconstitute the contents of the vial with 1.2 mL of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Do not freeze-thaw this solution more than once.
- **Nitrate Standard**
Reconstitute the contents of the vial with 1.0 mL of Assay Buffer. Mix well. Store at $2-8^{\circ}\text{C}$, do not freeze. The reconstituted standard is stable approximately four months when refrigerated.
- **Lactate Dehydrogenase**
Reconstitute the contents of the vial with 1.2 mL of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Do not freeze-thaw this solution more than once.
- **Griess Reagent R1 and R2**
These reagents are ready to use. Do not dilute with water or assay buffer.
- **NADPH**

SAMPLE COLLECTION AND PREPARATION

Collection

Nitric oxide synthase is relatively unstable; therefore, tissue samples should be harvested quickly after animal euthanasia. Harvest the culture cells in Assay Buffer, and transfer them to microcentrifuge tubes. Collect plasma samples in citrate or EDTA tubes; as heparin may form a precipitate with R1.

Storage and Handling of Sample

If assays are performed at a later time, it is best to freeze intact tissues or harvested culture cells prior to homogenization. Wrap the tissues in aluminum foil, flash freeze in liquid nitrogen and then store at -70°C. Keep plasma or serum on ice during processing, or freeze at -70°C if stored for later assay.

Sample Processing

- **Tissue Homogenates**

Homogenize the sample in PBS, pH 7.4, and centrifuge at 10,000 x g for 20 minutes. Ultracentrifuge the supernatant solution at 100,000 x g for 15 minutes. Ultrafilter using a 10 or 30 kDa molecular weight cut-off filter by centrifugation. Filtration of the 100,000 x g supernatant solution through a 0.45 micron filter prior to ultrafiltration can increase the ultrafiltration rate. Assay the sample for total nitrite using a maximum of 40 µL of the filtrate.

- **Culture Medium**

Some types of tissue culture media contain very high nitrate levels, e.g., RPMI 1640. These types of media should not be used for cell culture if the goal of an experiment is to measure small changes in nitrite levels. Cellular nitrate/nitrite production can be quantitated by subtracting the level of nitrite present in the media (in the absence of cells) from the total nitrite level present during cell growth. The effect of media components on color development can be assessed by making a nitrate standard curve in the presence of a fixed volume of the culture media (30 µL works well) and comparing it to a nitrate standard curve made in buffer alone. Cloudy samples may be centrifuged prior to use.

- **Plasma and Serum Samples**

Ultrafilter plasma or serum samples through a 10 to 30 kDa molecular weight cut-off filter using a centrifuge or microcentrifuge device. The filters should be pre-rinsed with HPLC grade water prior to ultrafiltration of serum or plasma. Drain all water from filter prior to use to prevent unintended dilution of sample. This procedure will reduce background absorbance due to the presence of hemoglobin and improve color formation using the Griess reagents. Assay for nitrite using a maximum of 40 µL of the filtrate. Heparinized plasma may form a precipitate upon addition of Griess Reagent R1, thus making the sample unusable for analysis. Citrate or EDTA are recommended as anticoagulants for plasma preparation.

- **Urine Samples**

Urine can be used directly after dilution to the proper concentration in Assay Buffer. Urine contains relatively high levels of nitrate (200 – 2000 mM), so dilution of approximately 1:10 to 1:50 may be necessary.

Sample Notes

The subcellular distribution of NOS is tightly regulated in tissues. Endothelial NOS is largely membrane associated as a result of N-terminal myristoylation. nNOS is found primarily in the cytoplasmic fractions in adult rat brain, yet in skeletal muscle, it is predominately associated with membrane fractions. Nitric oxide synthase in soluble and membrane-associated fractions can be separated by centrifuging the homogenized tissues at 100,000 x g for 60 minutes. The supernatant contains soluble NOS, while the pellet, which is resuspended in homogenization buffer, contains membrane-associated NOS.

PROCEDURE

Assay Notes

The conditions for the NOS reactions can be set up as required by your experimental design, but the following parameters should be observed.

- **Sample volume.** The amount of sample utilized in the assay can vary from <10 μL to 60 μL , depending on the activity of the enzyme. A convenient amount of sample for each assay will be 60 μL . Allow for duplicate or triplicate analysis of the samples.
- **Stopping the NOS reaction.** The NOS reaction must be stopped by heat inactivation. The use of acid to stop the NOS reaction will lead to erroneous results for two reasons: 1.) NO will be released from nitrite under acidic conditions; and 2.) Nitrate reductase is inhibited by a variety of acids even when the pH has been adjusted to neutral. Following heat inactivation, centrifuge the samples to pellet the denatured protein.
- **Negative Control.** A heat-inactivated, zero time point control should be included for each NOS preparation. This serves as a measure of endogenous nitrate, nitrite, and background interference. A new negative control should be made for a sample whenever the sample volume used in the assay changes.
- **Determining linearity of the assay.** The use of a single time point assay to measure the activity of an enzyme will only be valid when the steady-state reaction is in a linear phase. Typically, this is done by stopping the reaction when <20% of the substrate(s) has been utilized. Another concern is the stability of the enzyme for extended periods of time. NOS is not a highly stable enzyme and activity often decreases during the assay, particularly at 37°C. The linearity of the assay can be assessed by measuring several time points for a single reaction condition (example, every minute for 5 – 10 minutes) and plotting the absorbance as a function of time. Once linearity has been established, single time-point assays for NOS can be performed.
- **Plate configuration.** There is no specific pattern for using the wells on the plate. However, it is necessary to have some wells (at least 2) designated as absorbance blanks (containing 200 μL of Assay Buffer or water). The absorbance of these wells must then be subtracted from the absorbance measured in all of the other wells. The standard curve can be placed in any wells you choose. The remaining wells on the plate are available for the assay of samples.

- **Interferences.** Antioxidants will interfere with the color development reaction. Azide, ascorbic acid, dithiothreitol and β -mercaptoethanol will interfere with color development when present at concentrations as low as 100 μM . Alkyl amines, most sugars, lipids or amino acids (except those containing thiol groups) do not interfere. Phosphate concentrations greater than 50 mM will interfere with the conversion of nitrate to nitrite.

Preparation of the Nitrate Standard Curve

Dilute the reconstituted Nitrate Standard 1/10 in Assay Buffer (e.g., 0.1 mL + 0.9 mL). Pipet the following volumes of diluted Nitrate Standard and Assay Buffer into the microtiter plate wells to create a calibration curve. Duplicate assay of standards is recommended.

Well	μL Nitrate Standard	μL Assay Buffer	Concentration (nmoles/mL)
1	0	60	0
2	5	55	5
3	10	50	10
4	15	45	15
5	20	40	20
6	25	35	25
7	30	30	30
8	35	25	35

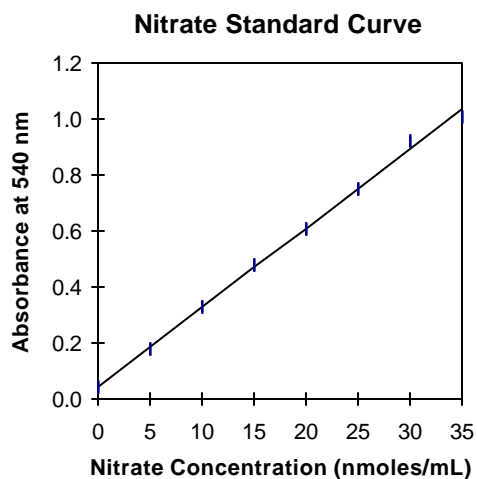
Assay Method

1. Add 200 μL of water or Assay Buffer to two wells to serve as blank for the microtiter plate. Do not perform any of the following steps on these wells.
2. Add up to 60 μL of sample to each sample well (40 μL for plasma, serum or tissue homogenates). The final volume must be adjusted to 60 μL using the Assay Buffer solution.
3. Add 10 μL of freshly prepared NADPH solution (1 mM) to each well.
4. Add 10 μL of Nitrate Reductase solution to each well.
5. Incubate at room temperature for 40 minutes (60 minutes for plasma, serum and tissue homogenates).
6. Add 10 μL of the Co-Factor Preparation solution and 10 μL for the Lactate Dehydrogenase solution to each well.
7. Incubate at room temperature for 20 minutes.
8. Add 50 μL of Griess Reagent R1 to each well.
9. Add 50 μL of Griess Reagent R2 to each well.
10. Allow the color to develop for 10 minutes at room temperature.
11. Use the plate blank wells (step 1 above) to zero the plate reader.
12. Read the absorbance at 540 nm.

RESULTS

Plotting the Standard Curve

Make a plot of absorbance at 540 nm as a function of nitrate concentration and determine the equation of the line by linear regression. The following curve is only an example, your results may vary.



Calculating Sample Activity

Utilizing the slope and y-intercept from the equation of the standard curve, and the sample absorbance at 540 nm, the equation for sample activity is:

$$\text{NOS Activity} = \{(A_{540} - b) / m\}(200 / VS) / \text{TRXN}$$

$$= \text{nmoles/mL/min}$$

Where:

A₅₄₀ is the sample absorbance at 540 nm.

b is the y-intercept (linear regression of the standard curve).

m is the slope (linear regression of the standard curve).

200 μL is the final volume of the reaction in the assay.

VS is the volume of sample added to the well (μL).

TRXN is the NOS reaction incubation time in minutes.

Example:

The equation for the above nitrate standard curve is $y = 0.028x + 0.045$. A plasma sample assayed at 40 μL and incubated for 60 minutes has an average absorbance of 0.432. The activity for this sample is:

$$\text{NOS Activity} = \{(A_{540} - b) / m\}(200 / VS) / \text{TRXN}$$

$$= \{(0.432 - 0.045) / 0.028\}(200 / 40) / 60$$

$$= 1.15 \text{ nmoles/mL/min}$$

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