***Oxis*Research™**
A Division of OXIS Health Products, Inc.

BIOXYTECH® Nitric Oxide Assay

Colorimetric Assay for Determination of Total Nitrite
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 22110

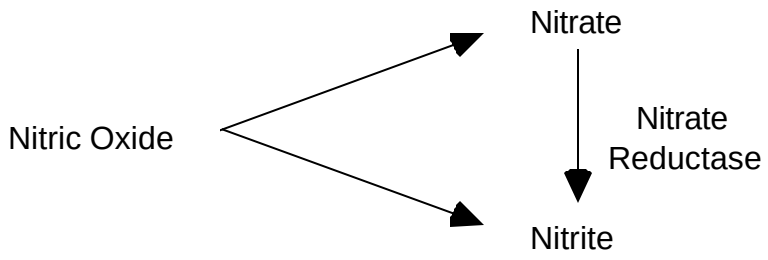
INTRODUCTION

This assay provides reagents for use in the determination of nitrite as an indicator of nitric oxide production in biological samples. Nitric oxide is rapidly converted to nitrite and nitrate in typical oxygenated aqueous solutions. Because an excellent colorimetric reagent (the Greiss reagent) exists for the determination of nitrite, it is common practice to use enzymatic or chemical reduction to convert all nitrate to nitrite in a sample and measure total nitrite as an indicator of nitric oxide production. This assay provides for enzymatic reduction of nitrate by nitrate reductase, followed by spectrophotometric analysis of total nitrite using Greiss reagent.

In addition to providing all necessary components in a microtiter format, this kit employs affinity purified Zea Mays nitrate reductase and NADH, thereby circumventing some of the potential problems reported for nitrite measurement using NADPH dependent nitrate reductases.

Principles of the Procedure

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite (Figure 1). Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward and sensitive, but does not measure nitrate, causing a possible underestimation of nitric oxide. This kit employs the NADH-dependent enzyme nitrate reductase for enzymatic reduction of nitrate to nitrite (Figure 1) prior to quantitation of nitrite using Griess reagent. In acid solution, nitrite is converted to nitrous acid (HNO_2) which diazotizes sulfanilamide. This sulfanilamide-diazonium salt is then reacted with N-(1-Naphthyl)-ethylenediamine (NED) to produce a chromophore which is measured at 540 nm. This kit can be used to accurately measure as little as 1 µM of nitrite (final concentration in the assay). Very little sample is required (5 µL to 85 µL for most samples).



REAGENTS

Materials Provided

- **NITRATE REDUCTASE:** Lyophilized enzyme. 1 unit
- **NITRATE REDUCTASE BUFFER:** Glycerol buffer. 1.2 mL
- **BUFFER:** 50 mM MOPS buffer with 1 mM EDTA, pH 7.0 25 mL
- **NADH:** β-Nicotinamide adenine dinucleotide, reduced form. 2 mg
- **COLOR REAGENT #1:** Sulfanilamide (p-Aminobenzenesulfonamide) in 3N HCl 7 mL
- **COLOR REAGENT #2:** N-(1-Naphthyl) ethylenediamine dihydrochloride in deionized H_2O 7 mL
- **NITRATE STANDARD:** 500 µM KNO_3 1.5 mL
- **MICROTITER PLATE:** One 96 well, low protein binding plate, with flat-bottom wells. 1 each
- **MICROTITER PLATE TEMPLATE:** One printed template for duplicate assays. 1 each
- **REAGENT RESERVOIRS:** Plastic troughs for duplicate assays. 3 each

Materials Required But Not Provided

- Microtiter plate reader with 540 nm filter (it is useful to have a plate mixing/shaking option).
- Note: The wavelength of the filter can be 530 to 560 nm, but 540 nm is the absorbance maximum.
- Precision pipettes ranging from 5 µL to 5 mL and appropriate disposable tips. NOTE: If all 96 wells are to be used at one time, it is suggested that a multichannel pipettor be used.
- Clean test tubes to dilute the standards and unknowns.
- Vortex mixer.
- Deionized H_2O.

Warnings and Precautions

- Do not smoke, eat or drink in areas where samples and reagents are handled.
- Wear disposable gloves when handling samples and reagents.
- Do not pipette reagents or samples by mouth.
- In case of accidental exposure of skin, mucous membranes or eyes to the components of this kit, thoroughly wash the exposed area with water.
- Reagents may contain sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents, flush with large volumes of water to prevent azide accumulation.
- For *in vitro* use only. For research purposes only. Not for use in diagnostic procedures.

Reagent Storage and Handling

Store Nitrate Reductase enzyme at –20 ^{o}C. Store NADH in the dark at room temperature. Store all other components at 4°C until immediately before use.

PROCEDURE

Reagent Preparation

1. Nitrate Reductase: Reconstitute just prior to use with 1.0 mL Nitrate Reductase Buffer at room temperature for 20 minutes with gentle vortexing at 0, 10 and 20 minutes. Reconstituted enzyme can be stored at –20 ^{o}C for up to 6 months. The concentration of this solution is 1 Unit/mL.

2. NADH: Add 1.28 mL deionized water to the 2 mg vial provided. The concentration of this solution is 2 mM. Store in the dark at room temperature upon arrival. Reconstituted NADH should be stored at –20 ^{o}C; avoid freeze-thaw cycles.

Standard Preparation

Prepare a series of standards by diluting the 500 µM standard to the following concentrations: 100, 50, 25, 10, 5, 1, 0.1 and 0.5 µM

S_8 500 µM nitrate standard is provided.

S_7 Take 1.0 mL of S_8, add 4 mL deionized H_2O and mix = 100 µM

S_6 Take 1.0 mL of S_7, add 1.0 mL deionized H_2O and mix = 50 µM

S_5 Take 1.0 mL of S_6, add 1.0 mL deionized H_2O and mix = 25 µM

S_4 Take 1.0 mL of S_5, add 1.5 mL deionized H_2O and mix = 10 µM

S_3 Take 1.0 mL of S_4, add 1.0 mL deionized H_2O and mix = 5 µM

S_2 Take 1.0 mL of S_3, add 4 mL deionized H_2O and mix = 1 µM

S_1 Take 1.0 mL of S_2, add 1.0 mL deionized H_2O and mix = 0.5 µM

NOTE: Standards can be stored in closed vials at 2-8° C for later use.

Assay Protocol

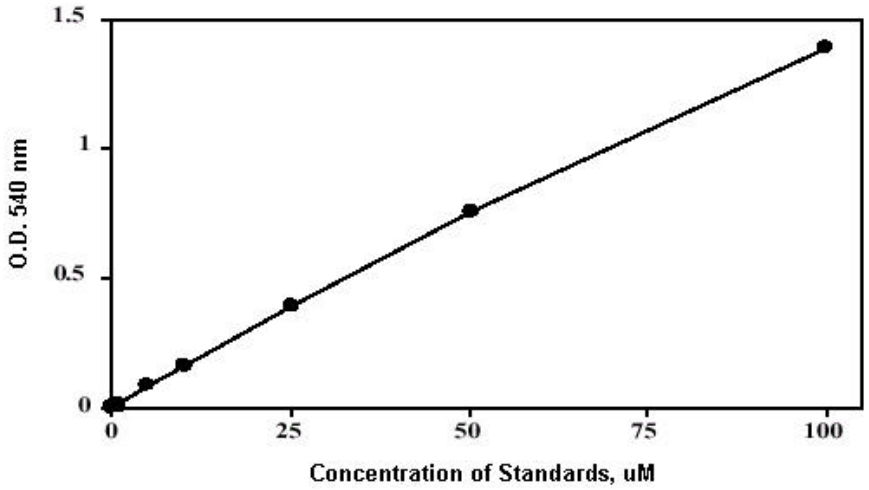
1. Determine the number of wells to be used and the organization of standards and samples on the microplate. See Microtiter Plate template.
2. Prepare reagents and standards as above.
3. Add 85 µL of each standard to be used, in duplicate, to respective wells.
4. Depending on the nitrite concentration in the sample, add between 5 µL to 85 µL of each sample to wells in duplicate. If the expected nitrite concentration of a sample is unknown, it may be helpful to perform more than one dilution.
5. Add sufficient buffer to each sample to bring the total volume to 85 µL (e.g., 80 µL for 5 µL of sample).
6. Add 10 µL of reconstituted Nitrate Reductase to each sample.
7. Add 10 µL of 2 mM NADH to each well and shake the plate for 20 minutes at room temperature.
8. Add 50 µL of Color Reagent #1 and shake briefly.
9. Add 50 µL of Color Reagent #2 and shake for 5 minutes at room temperature.
10. Measure absorbance values at 540 nm in the microtiter plate reader.
11. Plot the standard curve and estimate the concentrations of the samples from the curve.

Add standards and water to wells as follows to give an 85 µL sample volume:

Standard	Standard Preparation (mM)	Final Concentration in Assay (mM)	Standard to Pipet	Water to Pipet
S_0	**0**	**0**	**0 mL**	**85 mL**
S_1	**0.5**	**0.21**	**85 mL S_1**	**0 mL**
S_2	**1.0**	**0.42**	**85 mL S_2**	**0 mL**
S_3	**5.0**	**2.1**	**85 mL S_3**	**0 mL**
S_4	**10.0**	**4.2**	**85 mL S_4**	**0 mL**
S_5	**25.0**	**10.4**	**85 mL S_5**	**0 mL**
S_6	**50.0**	**20.7**	**85 mL S_6**	**0 mL**
S_7	**100**	**41.5**	**85 mL S_7**	**0 mL**

Calculations

1. Average the absorbance values for each pair of duplicate wells.
2. Subtract the average absorbance value of the blank wells (S_0) from all other pairs of wells.
3. Plot a standard curve using the net average absorbance value for each standard value versus the concentration of standard. The plot does not need to be linear.
4. Determine the concentration of each unknown by interpolation from the standard curve. A typical standard curve is shown below.



NOTES

1. Reconstituted enzyme should be used within 6 months if stored at –20 °C; one year if stored at –80 °C.
2. It is best to complete reading of the plate within 20 minutes.
3. Color reagents and the NADH solution should be kept in the dark.
4. Low sample NO concentrations may be improved by increasing sample volume to 85 µL (decreasing buffer volume).
5. Samples containing significant levels of protein (e.g., serum or tissue culture media) may produce a precipitate that may interfere with accurate measurement of nitrite. If a precipitate is visible after addition of Color Reagent #1, remove excess proteins (e.g., by diluting samples 1/2 with buffer or by boiling and centrifuging the samples) prior to performing the assay. Alternatively, the reaction may be performed in a conical bottomed microtiter plate. Then, prior to reading absorbances, centrifuge the plate and transfer 85 µL of supernatant from each well to the corresponding well of a plate with optically clear flat-bottomed wells.
5. If additional microtiter plates are used, they should be low protein binding plates and have optically clear, flat-bottomed wells.

REFERENCES

1. Schmit, H.H., et. al. Biochemica **2**:22-23 (1995).

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