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Life Science Specialties, LLC

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NWLSS™
Nitric Oxide (Nitrate/Nitrite) Enzymatic Assay

Product NWK-EN001

For Research Use Only

Enzymatic assay system for measurement of nitric oxide in biological samples. This method employs a nitrate reductase to reduce nitrates to nitrate before measuring using Griess reagent.

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Introduction:

Nitric Oxide (NO) is a biologically relevant free radical and cell signaling molecule. In normal cellular physiology it was originally characterized as an endothelial relaxation factor (EDRF) and is most commonly associated with its role in regulation of vasodilation. From a pathogenic standpoint the free radical nitric oxide (NO•) is known to combine with superoxide (O_2•) to form peroxynitrite (ONOO-) which is a potent oxidant. Since ONOO– (NO_3-) is essentially an unstable isomer of nitrate, it can react with tyrosine residues in proteins to create nitrotyrosine, a biomarker of oxidative mediated modification of proteins.

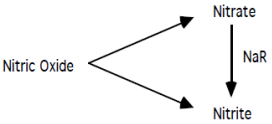
Nitric oxide degrades rapidly to nitrate (NO_3) and nitrite (NO_2) in aqueous biological systems. When NO_3 is reduced to NO_2 measurement of nitrite provides a simple, indirect means to quantify nitric oxide produced in a wide array of experimental model systems.

Intended Use:

This NWLSS™ Nitric Oxide (Nitrate/Nitrite) Enzymatic Assay kit is designed for use in quantifying nitric oxide (NO) in biological samples.

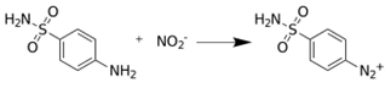
Test Principle:

The test method is based on measurement of total sample nitrites using Griess Reagent. Since Griess reagent does not detect nitrate NO_3, This kit employs nitrate reductase for reduction of nitrate to nitrite (Fig. 1).



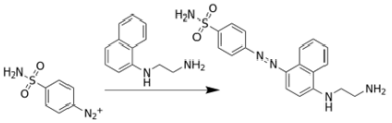
Using purified plant nitrate reductase with NADH helps prevent NADPH interference with the Griess reaction as has been previously reported.

Figure 1: Nitrate Reduction



After the appropriate sample reduction step nitrites are converted to nitrous acid (HNO_2) in Sulfanilamide, HCl solution. HNO2 subsequently reacts with sulfanilamide to form sulfanilamide diazonium salt (Fig 2).

Figure 2: Sulfanilamide reaction with HNO_2



Diazonium salt is then reacted with N-(1-Naphthyl)-ethylenediamine (NED) to produce a chromophore directly measureable at 540 nm (Fig. 3).

Figure 3: Diazonium Salt Reaction with NED

General Specifications:

Format:	96 wells
Number of tests:	Triplicate = 24 Duplicate= 40
Specificity:	Nitric Oxide as total Nitrite
Sensitivity:	1 pmol/mL or 1µM in the assay
Sample Volume:	10 - 50 µM
Effective Range:	1 µM - 100 µM

Kit Contents

Microplate (96 well clear, low binding, flatbottom)	1 X 96 wells
MOPS Buffer (Sample Dilution Buffer)	1 X 25 mL
Nitrate Standard (500 µM KNO_3)	1 X 1.5 mL
Nitrate Reductase (Lyophilized)	1 Unit
Nitrate Reductase Buffer	1.5 mL
NADH (Lyophilized)	2 mg
Reagent A: (Sulfanilamide (p-Aminobenzenesulfonamide) in 3N HCl)	1 X 7 mL
Reagent B: (N-(1-Naphthyl) ethylenediamine dihydrochloride in deionized H_2O)	1 X 7 mL

Required Materials Not Provided:

Adjustable pipettes with disposable tips (range of 10 µL to 1,000 µL)
Deionized water.

Microcentrifuge tubes.

Vortex Mixer.

Required Instrumentation:

Microtiter plate reader with 540 nm capability.

Warnings, Limitations, Precautions:

Individual components may be harmful if swallowed, inhaled or absorbed through the skin. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact with skin or eyes occurs, rinse the site immediately with water and consult a physician.

Storage Instructions:

Upon receipt, store Nitrate Reductase enzyme at –20 °C. Store NADH in the dark at room temperature. Store all other components at 4°C until immediately before use.

Assay Preparation

1. Determine the number of wells required to assay standards, samples and controls for the appropriate replicates.

2. Create an assay template showing positioning of standards, controls and samples. Include blank wells also.

Reagent Preparation:

Nitrate Reductase

Reconstitute with 1.0 mL *Nitrate Reductase Buffer* and incubate at room temperature for 20 minutes. Vortex gently at 0, 10, and 20 minutes.

NADH

Add 1.28 mL deionized water to the vial to create 2 mM NADH Reagent. Label as *Working NADH Reagent.*

All other reagents are supplied ready to use.

Standard Preparation:

1. Standard Supplied: 1.5 mL of 500 µM KNO_3

2. Label 8 tubes (S_7 to S_0) as 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0 µM.

3. In tube 8 (S_7): Add 100 µL of 500 µM $NaNO_2$ Standard as supplied to 900 µL deionized H_2O. Mix well. S_7 is now 50 µM.

4. Perform a serial dilution by transferring 400 µL of 50 µM S_7 to S_6 then 400 µL S_6 to S_5 and so on through to S_1 to create all standards down to 0.78 µM. Leave S_0 as deionized H_2O only zero or blank.

Sample Handling/Preparation:
Samples with low levels of nitrite:
If the nitrite in a sample is too low, sample volume may be increased while decreasing the volume of buffer added.

Samples with high levels of protein:
Excess protein in samples can produce a precipitate that may interfere with the measurement of NO. If there is visible precipitate after addition of Reagent A, if possible, samples can be diluted to reduce the amount of protein present and retested.

Note: If sample proteins cannot be diluted enough to allow for accurate testing using this assay, we recommend using our Non-Enzymatic Nitric Oxide Assay, product number NWK-NNO01.

Assay Procedure:
1. Add 85 µL of each **standard** and **sample** to replicate wells according to assay template.

2. Add 10 µL reconstituted *Nitrate Reductase* to each well.

3. Add 10 µL of *Working NADH Reagent* to each well and shake the plate for 20 minutes at room temperature.

4. Add 50 µL of **Reagent A** to each well and shake briefly.

3. Add 50 µL of **Reagent B** to each well and shake for 5 minutes at room temperature.

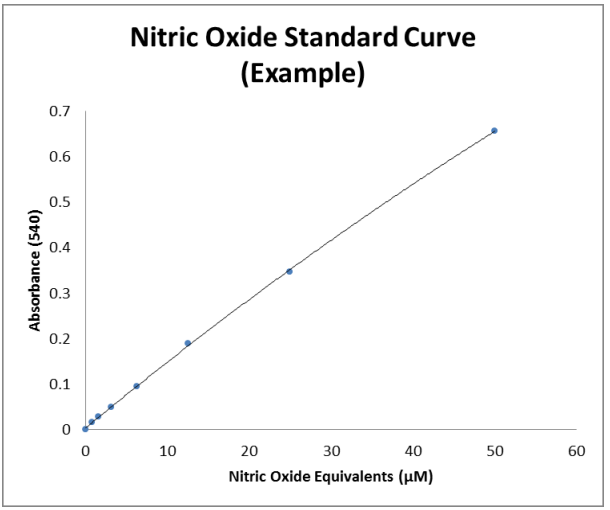
5. Read and record absorbance at 540 nm.

Data Analysis

1. Average the A_{540} values for each replicate of sample, standard and blank.

2. Subtract the average A_{540} value of the blank wells from the average A_{540} for each standard and sample replicate.

3. Plot a standard curve as concentration NO_2 (µM Nitric Oxide Equivalents) vs. Absorbance (A_{540}).



4. Calculate the concentration of nitrite in each sample using the equation as derived from the standard curve generated.

Notes: Each plate tested must have its own standard curve. The Example standard curve above is for illustration purposes only.

Values obtained must be multiplied by dilution factors incurred during sample precipitation and reduction steps and any other dilutions that may have been necessary.

Since 1 µM Nitric Oxide Equivalents = 1 pmol/mL NO_3, the standard curve can also be plotted as NO_3 concentration (pmol/mL) vs. Absorbance (A_{540}).

REFERENCES

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

2. Campbell, E. R., et. Al., (2000) *American Laboratory* February, 90-92.

Statement of Limited Warranty:

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User Notes:

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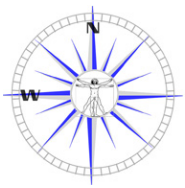

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