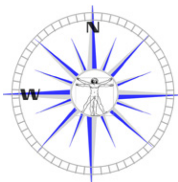

Northwest

Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™
Nitric Oxide (Nitrate/Nitrite) Non-Enzymatic Assay

Product NWK-NNO01

For Research Use Only

Non-enzymatic assay system for measurement of nitric oxide in biological samples. This method employs a robust cadmium reduction system and does not rely on the enzyme nitrate reductase.

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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.

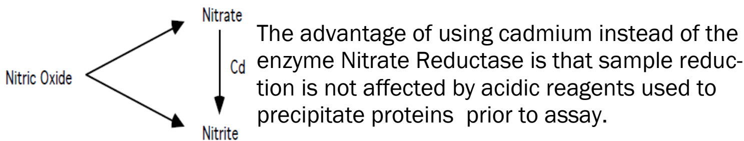
Nitric oxide degrades rapidly to nitrate (NO_3) and nitrite (NO_2) in aqueous biological systems. When NO_3 is reduced to NO_2 measurement of nitrite provides a simple, indirect means to quantify nitric oxide produced in a wide array of experimental model systems.

Intended Use:

This Nitric Oxide (Total Nitrite) assay kit is designed for use in quantifying nitric oxide (NO) in biological samples with high protein content.

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 metallic cadmium for reduction of nitrate to nitrite (Fig. 1).



The advantage of using cadmium instead of the enzyme Nitrate Reductase is that sample reduction is not affected by acidic reagents used to precipitate proteins prior to assay.

Figure 1: Nitrate Reduction

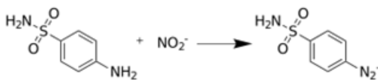


Figure 2: Sulfanilamide reaction with HNO_2

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

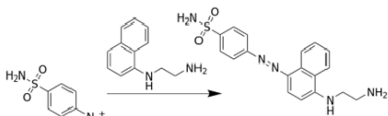


Figure 3: Diazonium Salt Reaction with NED

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

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
Nitrite Standard (500 µM $NaNO_2$)	1 X 1.5 mL
ZnSO4 (30% wt./vol.)	1 X 2 mL
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
Granulated Cadmium (cadmium (Cd++) beads)	25g
Acid Wash Solution (0.1 M HCl)	125 mL
Basic Wash Solution (0.1M NH_4OH)	125 mL

Required Materials Not Provided:

Adjustable pipettes with a range of 10 µL to 1,000 µL with disposable tips.

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:

Store all components at 4°C until immediately before use. Do not freeze.

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:

Cadmium Beads:

Cadmium beads must be washed according to the procedure below prior to use. The beads may be prepared ahead of time as long as they can be stored in an airtight container under inert gas such as argon or nitrogen.

1. Each sample to be tested will require 0.5 g cadmium (generally 6 or 7 beads).

2. Each 0.5 g of cadmium must be washed twice (2 times each) with 1 mL of each of the following in order: deionized H_2O, Acid Wash (0.1 M HCl and Basic Wash (0.1M NH_4OH).

3. Add the necessary cadmium to an appropriate sized sealable container. For example 10 samples would require 5 g cadmium beads.

4. Add the required volume of deionized H_2O for the first washing. For example wash 5 g cadmium beads with 10 mL H_2O. Seal container and mix well by inversion. Decant solution and repeat this step once more. Decant second wash solution and remove excess liquid using a pipette or by dabbing with a lint free paper towel.

5. In the same manner as step 4 wash the beads twice (2X) more times with Acid Wash Solution followed by two (2) more washes using Basic Wash Solution followed by removal of excess liquid and dabbing dry with lint free paper towel.

Washed and dried cadmium is now ready for use at 0.5 g cadmium for each sample to be reduced.

Standard Preparation:

1. Standard Supplied: 1.5 mL of 500 µM $NaNO_2$

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.

<u>Sample Protein Precipitation:</u>

Samples with high protein content, such as plasma, cell lysates, tissue homogenates or other biological samples must be treated with $ZnSO_4$ to precipitate proteins prior to sample reduction and assay.

1. Adjust sample volumes (10 - 50 µL) to 190 µL with deionized water.

2. Add 10 µL of 30% (wt/vol) $ZnSO_4$ solution to each sample.

3. Vortex to mix well then incubate at room temperature for 15 minutes.

4. Centrifuge at 3500 RPM for 5 minutes.

5. Harvest the clarified supernatant directly into reducing protocol below or store at -20 °C for future assay.

Note: Samples may need to be additionally diluted if nitrite levels test beyond the highest standard (50 µM).

<u>Sample Reduction Protocol:</u>

1. Label 1.5 mL microcentrifuge tubes according to each sample to be assayed. Add approximately 0.5 g of washed and dried Cadmium Beads to each tube.

2. Add the deproteinated sample from above to the appropriately labeled centrifuge tube containing 0.5 g cadmium beads. Incubate overnight at room temperature overnight while mixing.

3. Transfer the sample to a new (clean) microcentrifuge tube. Centrifuge for 5 minutes at 3,000 x g. Supernatant is now ready for assay.

Note: For best results, samples should be assayed within 1 hour. Used Cadmium Beads should be collected and can be re-used after washing as shown in the earlier Reagent Preparation section.

<u>Assay Procedure:</u>

1. Add 100 µL of each **standard** to replicate wells according to assay template.

2. Add 100 100 µL of each **sample** to replicate wells according to assay template.

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

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

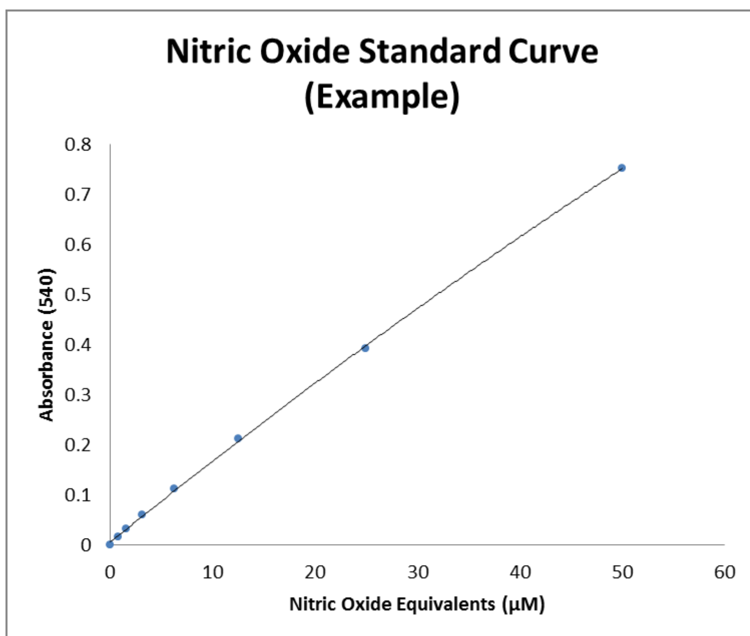
5. Read and record absorbance at 540 nm.

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 $NaNO_2$, the standard curve can also be plotted as NO_2 concentration (pmol/mL) vs. Absorbance (A_{540}).

REFERENCES

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

<u>Statement of Limited Warranty:</u>

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

User Notes:

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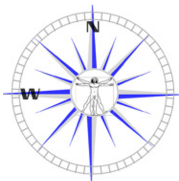

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