Northwest
Life Science Specialties, LLC

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NWLSS™ Nitric Oxide Synthase (NOS) Assay

Product NWK-NOS01

For Research Use Only

This kit is ideally suited for testing NOS activity of cells in culture after washing. Cell lysates and tissue homogenates can also be tested but requires that baseline nitric oxide be tested and subtracted from timed results.

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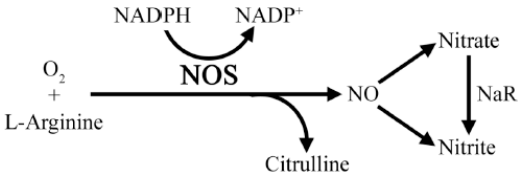
Introduction:
Nitric oxide synthase (NOS) is involved in the synthesis of nitric oxide from L-arginine. Mammalian NOS include three distinct isoforms; inducible, endothelial and neuronal. The traditional method for measuring nitric oxide synthase (NOS) activity is performed by radiochemical assay that measures the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. This method is expensive and requires regulation of radioactive materials

In contrast, we offer a non-radioactive method that utilizes a simple NADPH recycling system to allow NOS to operate linearly for hours as nitric oxide derived degradation products nitrate and nitrite accumulate. NOS activity can therefore be assayed spectrophotometrically by measuring total nitrite accumulation over time.

Intended Use:
This Nitric Oxide Synthase assay kit is designed for use in quantifying nitric oxide (NO) in biological samples.

Test Principle:
NADPH and L-arginine are required for the continual operation of NOS and production of nitric oxide (NO). In aqueous solution, NO rapidly degrades to nitrate and nitrite. Spectrophotometric quantization of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This kit employs recombinant nitrate reductase (NaR) for conversion of nitrate to nitrite prior to quantization of nitrite using Griess reagent, thus providing for accurate determination of total NOS activity.



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

Microplates (96-well low binding, flat-bottom and v-bottom plates)	1 X 96 wells
Reaction Buffer (50 mM HEPES 0.5 mM EDTA	1 X 25 mL
Nitrite Standard (500 µM $NaNO_2$)	1 X 1.5 mL
Nitrate Reductase (Lyophilized)	1 Unit
Nitrate Reductase Buffer	1.2 mL
NADPH Part A ($NADP_+$, Glucose 6-Phosphate, L-Arginine)	1 mL
NADPH Part B (Glucose 6-Phosphate dehydrogenase)	1 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

Required Materials Not Provided:

Adjustable pipettes with disposeable 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, NADPH Part A and NADPH Part B at –20 °C. 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.

3. Note that it is necessary to set up a baseline nitrate/nitrite reading for each sample to be assayed for NOS activity.

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.

All other reagents are supplied ready to use.

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

2. Label 8 tubes (S_7 to S_0) as 100, 50, 25, 10, 5, 2.5, 1.25, 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. Add 240 µL of 500 µM Nitrite Standard to 960 µL Reaction Buffer. Label as 100 µM Diluted Standard Standard and store on ice until used. The standard curve is then created by further diluting the Diluted Standard according to Table 2 below.

Standard	Final Concentration (μM)	ddH_2O (μL)	Diluted Standard (μL)
B_0	0	1000	0
S_1	0.5	995	5
S_2	1	990	10
S_3	5	950	50
S_4	10	900	100
S_5	25	750	250
S_6	50	500	500
S_7	100	0	250

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.

Assay Procedure:

In the V-well microplate or microcentrifuge tube

1. Add 40–500 µg of protein from lysates or 0.2 - 1.0 Unit of recombinant or purified NOS in a volume of 30 µl to a tube or well.

2. Add 200 µL Reaction Buffer

3. Add 10 µL of NADPH Part A

4. Add 10 µL of NADPH Part B

5. Mix and incubate for 1 - 6 hours at 37°C.

6. Prepare standards as detailed below and store at 4°C.

7. Chill on ice for 5 minutes.

8. Add 10 µL of the reconstituted Nitrate Reductase to each sample, vortex tube or tap plate to mix and incubate for 20 minutes at room temperature.

9. Centrifuge at 12,500 rpm for 5 minutes at 4°C.

In the flat-bottom microtiter plate

10. Add100 µL of standards to the appropriate wells.

11. Add 5-100 µL of sample to the determined wells. The amount of sample per well is dependent upon the amount of NO in the sample.

12. Add sufficient buffer to each sample to bring the volume to 100 µL. (e.g. 80 µL buffer for 20 µL of sample).

13. Add 50 µL Reagent A and shake briefly.

14. Add 50 µL Reagent B. Shake for 5 minutes at room temperature.

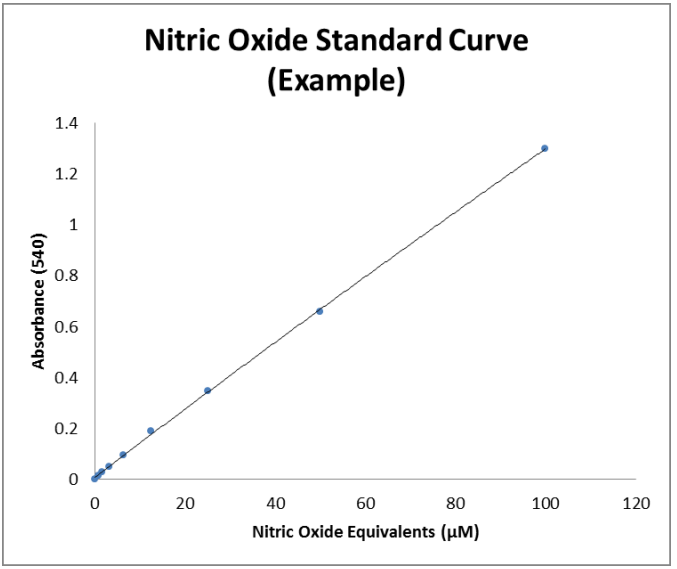
15. Read and record absorbance values at 540 nm

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.

5. For cell lysates and tissue homogenates subtract baseline NO values from final NO value obtained after timed incubation period. Values after subtraction represent NO produced due to NOS activity

6. Values for NOS activity of samples can be compared by determining µM NO/unit time normalized to total protein in the sample.

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:

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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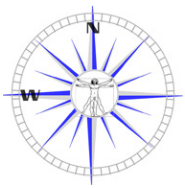

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