

Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase

For Research Use Only

Store Nitrate Reductase Enzyme at -20°C

Store NADPH Part A and NADPH Part B at -20°C

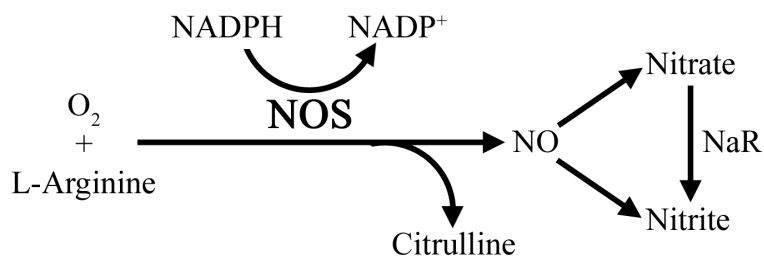
Store all other kit components at 4°C

INTRODUCTION

The traditional method for measuring nitric oxide synthase (NOS) activity is performed by radiochemical assay that measures the conversion of L-[^3H]arginine to L-[^3H]citrulline. This method is expensive and requires regulation of radioactive materials. The Ultrasensitive Colorimetric NOS Assay Kit is a low-cost novel assay that allows for the detection of NOS activity without the need for radioactivity. Our Ultrasensitive NOS Assay Kit employs a NADPH recycling system to allow NOS to operate linearly for hours as nitric oxide-derived nitrate and nitrite accumulate. NOS can be assayed spectrophotometrically by measuring the accumulation of its stable degradation products, nitrate and nitrite. The ratio of these two products in biological fluids, tissue culture media, etc. may vary substantially. Hence, for accurate assessment of the total nitric oxide generated, one must monitor both nitrate and nitrite. An excellent solution to this problem is the enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase (NaR), followed by quantization of nitrite using Griess Reagent. This kit allows for efficient high-throughput screening of NOS activity in resting cells or cell lysates as well as biological fluids and tissue homogenates. The kit is also ideal for in vitro NOS assays using recombinant purified NOS. All materials necessary to perform the entire assay in a 96-well microplate format are provided with the kit.

PRINCIPLES OF THE PROCEDURE

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.



This kit can be used to accurately measure as little as $1 \text{ pmol}/\mu\text{L}$ ($\sim 1 \mu\text{M}$) NO produced in aqueous solutions. Very little sample is required (5 to $100 \mu\text{L}$ depending on the $[\text{NO}]$ in the sample). The completed reaction is read at 540 nm.

MATERIALS PROVIDED

Component	Contents	Quantity	Storage	Cat. No.
Reaction Buffer	20 mM HEPES; 0.5 mM EDTA	50 mL	4°C	NB 78-1
NADPH Part A	NADP ⁺ , Glucose 6-Phosphate, L-Arginine	1.0 mL	-20°C	NB 78-2
NADPH Part B	Glucose 6-Phosphate dehydrogenase	1.0 mL	-20°C	NB 78-3
Nitrate Reductase	Nitrate Reductase	1 U	-20°C	NB 78-5
Nitrate Reductase Buffer	Proprietary buffer	1.2 mL	4°C	NB 78-6
Color Reagent #1	Sulfanilamide in 3N HCl	7.0 mL	4°C	NB 78-7
Color Reagent #2	N-(1-Naphthyl) ethylenediamine dihydrochloride	7.0 mL	4°C	NB 78-8
Nitrite Standard	500 pmol / μ L NaNO ₂	1.5 mL	4°C	NB 78-9
Microtiter Plates	96-well low binding, flat-bottom plate	1	RT	NB 78-10

MATERIALS NEEDED BUT NOT PROVIDED

1. Deionized water
 2. Microplate reader with 540 nm filter.
 3. Precision pipettes ranging from 5 μ L - 1.0 mL and disposable tips.
 4. Test tubes to dilute the standards and unknowns.
 5. Microcentrifuge tubes or microtiter plate for incubation of cell extracts or purified NOS.
 6. Centrifuge for microcentrifuge tubes or microtiter plate if incubating in microtiter plate.
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STORAGE CONDITIONS

1. Store this kit and its components according to the label.
 - The Nitrate Reductase, NADPH Part A and NADPH Part B should be stored at -20°C.
 - The reconstituted Nitrate Reductase may be stored at -20°C for up to six months.
 - The diluted standard curve may be stored at 4°C for up to 24 hours.
 - All other kit components should be stored at 4°C.
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WARNINGS AND PRECAUTIONS

1. Do not use components beyond the specified expiration date.
 2. This kit is designed to work properly as provided and instructed. Additions, deletions, or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
 3. Universal precautions should be employed with handling of the components provided in this kit.
 4. Use aseptic techniques when opening and removing reagents from vials and bottles.
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PROCEDURAL NOTES

1. This assay can be used with cell lysates or purified NOS for in vitro assays. If using purified NOS it is important the required cofactors are added to the reaction. OBR sells a NOS Cofactor Mix specifically designed for use with this kit under product number **NS70**.
2. If using cell lysates for the assay, it is recommended that they are suspended in phosphate buffered saline (PBS) and protein concentration determined.
3. It is possible for the entire assay to be run in a 96-well microplate if the researcher has a centrifuge equipped with a microplate rotor. The initial incubation should be carried out in the 96-well V-bottom plate while the Color Reaction should be done in the 96-well flat bottom plate.
4. For ease in setting up multiple samples, the Reaction Buffer can be combined with NADPH Part A and Part B and then aliquoted equally to each sample.
5. If the NO concentration in your sample is low, you can increase the sample volume in the Color Reaction to 100 μ L while decreasing the buffer volume.

- For best results, complete the reading of the plate within 20 minutes.

REAGENT PREPARATION

- Nitrate Reductase:** Reconstitute with 1 mL of Nitrate Reductase Buffer. Vortex briefly every five minutes for fifteen minutes total.
 - Nitrite Standard:** Dilute the 500 μM solution that is supplied with the kit to 100 μM by adding 240 μL of Nitrite Standard to 960 μL of Reaction Buffer.
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STANDARD PREPARATION

Follow **Table 1** below to construct a standard curve using the 100 μM Nitrite Standard diluted above.

Table 1: Preparation of the Standard Curve.

Standard	Final Concentration (μM)	ddH ₂ O (μL)	100 μM 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

PROCEDURE

In V-well microplate or microcentrifuge tube

- 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.
- Add 200 μL Reaction Buffer.
- Add 10 μL of NADPH Part A.
- Add 10 μL of NADPH Part B.
- If using purified NOS, add 10 μL of the NS70 – NOS Cofactor Mix.
- Mix and incubate for 1 - 6 hours at 37°C.
- Chill on ice for 5 minutes.
- 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.
- Centrifuge at 12,500 rpm for 5 minutes at 4°C.

In flat-bottom microtiter plate

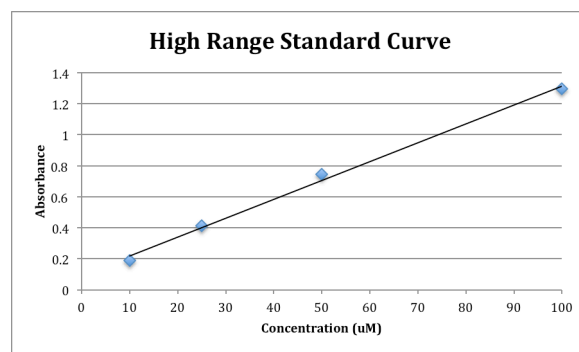
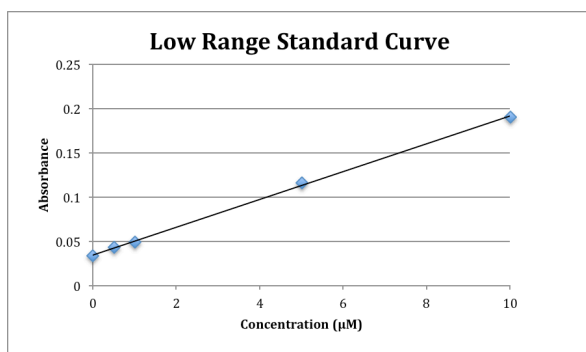
- Add 100 μL of Standards or Samples to the microplate in duplicate. Depending on [NO], the samples may need to be diluted in Reaction Buffer. See **Scheme 1** for a sample plate layout.
- Add 50 μL Color Reagent #1 and shake briefly.
- Add 50 μL Color Reagent #2. Shake for 5 minutes at room temperature.
- Read absorbance values at 540 nm in Microtiter plate reader.

Scheme I: Sample Microplate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	B0	B0	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

CALCULATIONS

1. Subtract the average O.D. value of the blank wells (S₀) from all other pairs of wells.
2. Average the O.D. values for each pair of duplicate wells.
3. Plot a standard curve using the average O.D. value for each standard value versus the concentration of standard. If samples are less than 10 μM , plot standards B0, S1, S2, S3, and S4. For samples over 10 μM , plot standards S4, S5, S6, and S7.
4. Determine the concentration of each unknown by interpolation from the standard curve.
5. Samples can be compared by determining $\mu\text{moles of NO produced} / \mu\text{g protein} / \text{unit time}$.

Figure 1: Typical Standard Curves**REFERENCES**

1. Ghigo, D., et al. (2006). *Nitric Oxide* **15**:148-153
2. Schmidt, H.H., et al. (1995). *Biochemica* **2**:22-23

DISCLAIMER

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