Ultrasensitive Colorimetric Assay for
Nitric Oxide Synthase

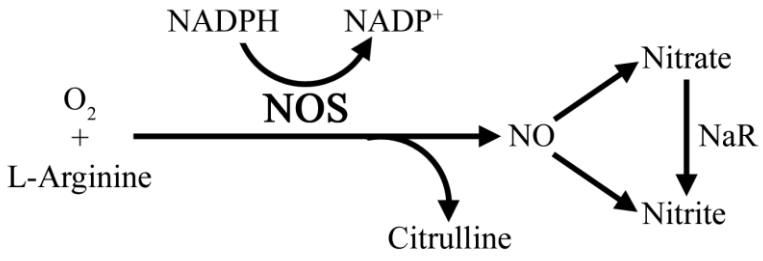
For Research Use Only
Store Nitrate Reductase Enzyme at –20°C
Store NADPH Part A and NADPH Part B at –20°C
Store all other kit components at 4°C

INTRODUCTION

The traditional method for measuring nitric oxide synthase (NOS) activity is performed by radiochemical assay that measures the conversion of L-[^{3}H]arginine to L-[^{3}H]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 pmol/µL (~1µM) NO produced in aqueous solutions. Very little sample is required (5 to 100 µL depending on the [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 78a
NADPH Part A	$NADP^+$, Glucose 6-Phosphate, L-Arginine	1.0 mL	-20°C	NB 78b
NADPH Part B	Glucose 6-Phosphate dehydrogenase	1.0 mL	-20°C	NB 78c
Nitrate Reductase	Nitrate Reductase	1 U	-20°C	NB 78d
Nitrate Reductase Buffer	Proprietary buffer	1.2 mL	4°C	NB 78e
Color Reagent #1	Sulfanilamide in 3N HCl	7.0 mL	4°C	NB 78f
Color Reagent #2	N-(1-Naphthyl) ethylenediamine dihydrochloride	7.0 mL	4°C	NB 78g
Nitrite Standard	500 pmol / µL NaNO2	1.5 mL	4°C	NB 78h
Microtiter Plate	96-well low binding, flat-bottom plate	1	RT	NB 78i

MATERIALS NEEDED BUT NOT PROVIDED

1. Deionized water
2. Microplate reader with 540 nm filter.
3. Precision pipettes ranging from 5 µL - 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.

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.

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.

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 µL while decreasing the buffer volume.
6. For best results, complete the reading of the plate within 20 minutes.

REAGENT PREPARATION

1. **Nitrate Reductase:** Reconstitute with 1 mL of Nitrate Reductase Buffer. Vortex briefly every five minutes for fifteen minutes total.
2. **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.

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_2O (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

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. If using purified NOS, add 10 µL of the NS70 – NOS Cofactor Mix.
6. Mix and incubate for 1 - 6 hours at 37°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 flat-bottom microtiter plate

10. 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.
11. Add 50 µL Color Reagent #1 and shake briefly.
12. Add 50 µL Color Reagent #2. Shake for 5 minutes at room temperature.
13. Read absorbance values at 540 nm in Microtiter plate reader.

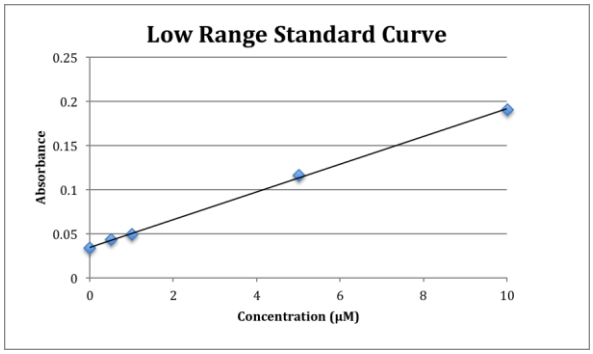
Scheme I: Sample Microplate Layout

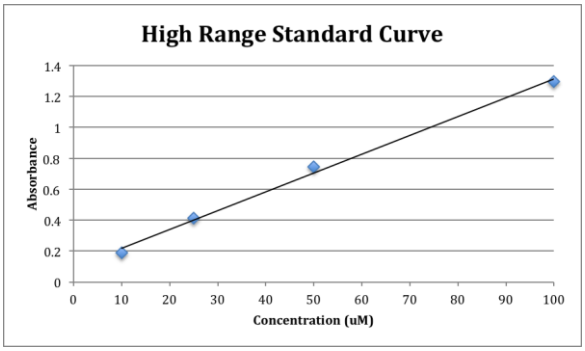
	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}

CALCULATIONS

1. Subtract the average O.D. value of the blank wells (S_0) 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 µmoles of NO produced / µg protein / 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

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