

## Ultrasensitive Colorimetric NOS Assay

Product Number: NB 78

Store components as labeled prior to use.

FOR RESEARCH USE ONLY.

## Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase

PLEASE READ ALL INSTRUCTIONS PRIOR TO BEGINNING THIS ASSAY

Store Nitrate Reductase Enzyme at  $-20^{\circ}\text{C}$

Store NADPH Part A and NADPH Part B at  $-20^{\circ}\text{C}$

Store all other kit components at  $4^{\circ}\text{C}$

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### INTRODUCTION

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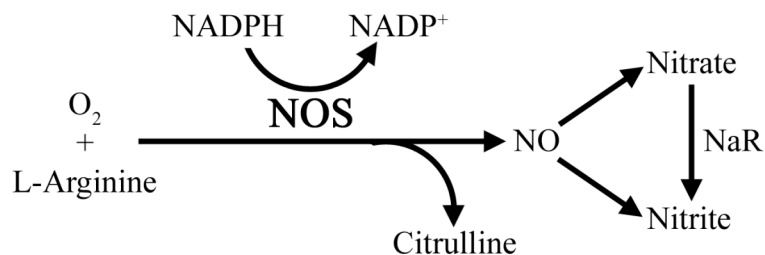
The traditional method for measuring nitric oxide synthase (NOS) activity is performed by radiochemical assay that measures the conversion of L-[ $^3\text{H}$ ]arginine to L-[ $^3\text{H}$ ]citrulline. This method is expensive and requires regulation of radioactive materials. The Ultrasensitive Colorimetric 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.

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### PRINCIPLES OF THE PROCEDURE

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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 [NO] in the sample). The completed reaction is read at  $540 \text{ nm}$ .

## MATERIALS PROVIDED

**Table 1: Materials Provided**

Component	Contents	Volume	Storage	Cat. No.
Reaction Buffer	50 mM HEPES 0.5 mM EDTA	50 mL	4°C	NB 78-1
NADPH Part A	NADP <sup>+</sup> , 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 / $\mu$ L NaNO <sub>2</sub>	1.5 mL	4°C	NB 78-9
Microtiter Plates	96-well low binding, flat-bottom and v-bottom plates	1 each	RT	NB 78-10
Reagent Reservoirs	Plastic troughs for dispensing reagents	3	RT	NB 78-4

## MATERIALS NEEDED BUT NOT PROVIDED

- Reagent grade water:** Distilled and deionized.
- Microplate reader** with 540nm filter. (Note: The wavelength of the filter can be 530 to 560 nm, but 540 nm is the absorbance maximum).
- Precision pipettes** ranging from 5  $\mu$ L - 1.0 mL and disposable tips. NOTE: If all 96 wells are to be used at one time it is suggested that a multi-channel pipettor be used.
- Test tubes** to dilute the standards and unknowns.
- Microcentrifuge tubes or microtiter plate** for incubation of cell extracts or purified NOS.
- Centrifuge** for microcentrifuge tubes or microtiter plate if incubating in microtiter plate.

## STORAGE CONDITIONS

The Ultrasensitive Colorimetric NOS Assay kit is shipped on wet ice. Upon arrival, the Nitrate Reductase, NADPH Part A and NADPH Part B should be stored at -20°C until the time of use. After initial use of the kit, store each component according to the Materials Provided located in Table 1 of these instructions.

## PROCEDURAL NOTES

- Reconstitute the Nitrate Reductase by adding 1 mL of Nitrate Reductase Buffer and vortexing briefly every 5 minutes for 15 minutes total. This should be stored at -20°C and used within 6 months.
- The assay can be used with cell lysates or purified NOS for in vitro assays. If using purified NOS it is important that the required cofactors are added to the reaction. OBR sells a NOS Cofactor Mix specifically designed for use with this kit under product number **NS 70**.
- If using cell lysates for the assay, it is recommended that they are suspended in phosphate buffered saline (PBS) and protein concentration determined.
- It is recommended that all samples and standards be assayed in duplicate.
- 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.
- 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.
- If the NO concentration in your sample is low, you can increase the sample volume in the Color Reaction to 100  $\mu$ L while decreasing the buffer volume.
- For best results, complete the reading of the plate within 20 minutes of stopping the color reaction.

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## PROCEDURE

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### **In V-well microplate or microcentrifuge tube**

1. Add 40–500  $\mu\text{g}$  of protein from lysates or 0.2 - 1.0 Unit of recombinant or purified NOS in a volume of 30  $\mu\text{l}$  to a tube or well.
2. Add 200  $\mu\text{L}$  of Reaction Buffer
3. Add 10  $\mu\text{L}$  of NADPH Part A
4. Add 10  $\mu\text{L}$  of NADPH Part B
5. Mix and incubate for 1 - 6 hours at 37°C.

**Note:** While samples are incubating it is recommended to determine the number of wells to be used and the organization of the samples and standards on the microplate (e.g. see Table 3).

6. Prepare standards as detailed below and store at 4°C.
7. Chill the samples on ice for 5 minutes.
8. Add 10 $\mu\text{L}$  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, in duplicate, 100  $\mu\text{L}$  of standards to the appropriate wells.
  11. Add, in duplicate, 5-100  $\mu\text{L}$  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 Assay Buffer to each sample to bring the volume to 100  $\mu\text{L}$ . (e.g. 80  $\mu\text{L}$  buffer for 20  $\mu\text{L}$  of sample).
  13. Add 50 $\mu\text{L}$  Color Reagent #1 and shake briefly.
  14. Add 50 $\mu\text{L}$  Color Reagent #2. Shake for 5 minutes at room temperature.
  15. Read the absorbance values at 540 nm in Microtiter plate reader.
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## STANDARD PREPARATION

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- A. 500  $\mu\text{M}$  NO equivalent standard is provided.
- B. Take 1.0 mL of A, add 4mL deionized H<sub>2</sub>O and mix = 100  $\mu\text{M}$  NO
- C. Take 1.0 mL of B, add 1mL deionized H<sub>2</sub>O and mix = 50  $\mu\text{M}$  NO
- D. Take 1.0 mL of C, add 1mL deionized H<sub>2</sub>O and mix = 5  $\mu\text{M}$  NO
- E. Take 1.0 mL of D, add 1.5 mL deionized H<sub>2</sub>O and mix = 10  $\mu\text{M}$  NO
- F. Take 1.0 mL of E, add 1.0 mL deionized H<sub>2</sub>O and mix = 5  $\mu\text{M}$  NO
- G. Take 1.0 mL of F, add 4mL deionized H<sub>2</sub>O and mix = 1  $\mu\text{M}$  NO
- H. Take 1.0 mL of G, add 1mL deionized H<sub>2</sub>O and mix = 0.5  $\mu\text{M}$  NO

**Table 2: Standard concentrations and well locations.**

Standard	Final Concentration ( $\mu\text{M}$ )	H <sub>2</sub> O ( $\mu\text{L}$ /well)	Std. Solution $\mu\text{L}$ /well
S <sub>0</sub>	0	100	0
S <sub>1</sub>	0.5	0	100 of H
S <sub>2</sub>	1.0	0	100 of G
S <sub>3</sub>	5.0	0	100 of F
S <sub>4</sub>	10.0	0	100 of E
S <sub>5</sub>	25.0	0	100 of D
S <sub>6</sub>	50.0	0	100 of C
S <sub>7</sub>	100.0	0	100 of B

**NOTE:** Standard solutions may be stored at 4°C for later use.

**Table 3: Sample microplate layout**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S <sub>0</sub>	S <sub>0</sub>	U <sub>1</sub>	U <sub>1</sub>	U <sub>9</sub>	U <sub>9</sub>	U <sub>17</sub>	U <sub>17</sub>	U <sub>25</sub>	U <sub>25</sub>	U <sub>33</sub>	U <sub>33</sub>
B	S <sub>1</sub>	S <sub>1</sub>	U <sub>2</sub>	U <sub>2</sub>	U <sub>10</sub>	U <sub>10</sub>	U <sub>18</sub>	U <sub>18</sub>	U <sub>26</sub>	U <sub>26</sub>	U <sub>34</sub>	U <sub>34</sub>
C	S <sub>2</sub>	S <sub>2</sub>	U <sub>3</sub>	U <sub>3</sub>	U <sub>11</sub>	U <sub>11</sub>	U <sub>19</sub>	U <sub>19</sub>	U <sub>27</sub>	U <sub>27</sub>	U <sub>35</sub>	U <sub>35</sub>
D	S <sub>3</sub>	S <sub>3</sub>	U <sub>4</sub>	U <sub>4</sub>	U <sub>12</sub>	U <sub>12</sub>	U <sub>20</sub>	U <sub>20</sub>	U <sub>28</sub>	U <sub>28</sub>	U <sub>36</sub>	U <sub>36</sub>
E	S <sub>4</sub>	S <sub>4</sub>	U <sub>5</sub>	U <sub>5</sub>	U <sub>13</sub>	U <sub>13</sub>	U <sub>21</sub>	U <sub>21</sub>	U <sub>29</sub>	U <sub>29</sub>	U <sub>37</sub>	U <sub>37</sub>
F	S <sub>5</sub>	S <sub>5</sub>	U <sub>6</sub>	U <sub>6</sub>	U <sub>14</sub>	U <sub>14</sub>	U <sub>22</sub>	U <sub>22</sub>	U <sub>30</sub>	U <sub>30</sub>	U <sub>38</sub>	U <sub>38</sub>
G	S <sub>6</sub>	S <sub>6</sub>	U <sub>7</sub>	U <sub>7</sub>	U <sub>15</sub>	U <sub>15</sub>	U <sub>23</sub>	U <sub>23</sub>	U <sub>31</sub>	U <sub>31</sub>	U <sub>39</sub>	U <sub>39</sub>
H	S <sub>7</sub>	S <sub>7</sub>	U <sub>8</sub>	U <sub>8</sub>	U <sub>16</sub>	U <sub>16</sub>	U <sub>24</sub>	U <sub>24</sub>	U <sub>32</sub>	U <sub>32</sub>	U <sub>40</sub>	U <sub>40</sub>

S<sub>0</sub> = Blanks: Add Assay Buffer in place of standard or sample.

U<sub>1-40</sub> = Unknown sample to be assayed.

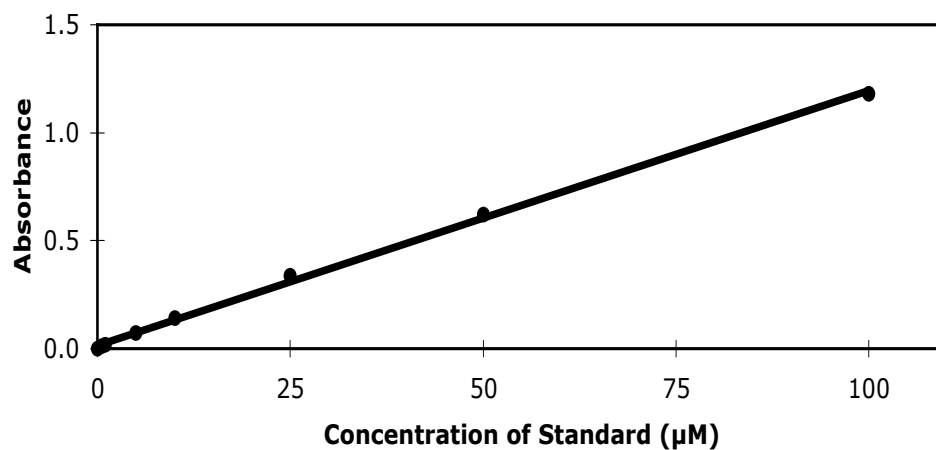
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## CALCULATIONS

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1. Subtract the average O.D. value of the blank wells (S<sub>0</sub>) 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.
4. Determine the concentration of each unknown by interpolation from the standard curve.
5. Samples can be compared by determining  $\mu$ moles of NO produced /  $\mu$ g protein / unit time.  
A typical standard curve is shown here.

**Figure 1: Typical Standard Curve**



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## WARNINGS AND PRECAUTIONS

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1. **DO NOT** use components beyond the specified expiration date.
2. Universal precautions should be employed with handling of the components provided in this kit.
3. Use aseptic techniques when opening and removing reagents from vials and bottles.

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## REFERENCES

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

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## SAFETY

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1. Normal precautions exercised in handling laboratory reagents should be followed at all times. The reagents supplied here are not considered hazardous according to 29 CFR 1910.1200. The chemical, physical, biological, and toxicological properties of these reagents may not, as yet, have been thoroughly investigated.
2. The use of gloves, lab coat and eye protection is recommended when working with any type of laboratory reagent.
3. Contents may be harmful if swallowed, inhaled or absorbed through the skin.

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## DISCLAIMER

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This information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. Oxford Biomedical Research, Inc. shall not be held liable for any damage resulting from handling or from contact with the above product. See Catalog for additional terms and conditions of sale.

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## TECHNICAL SUPPORT

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If you need technical information or assistance with assay procedures, please call our Technical Support Department at 1-800-692-4633 or 1-248-852-8815. Our staff will be happy to answer your questions about this or any other product in the Oxford Biomedical line.

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