



OXFORD BIOMEDICAL RESEARCH  
P.O. Box 522, Oxford MI 48371 • USA  
USA: 800-692-4633 • Fax: 248-852-4466  
www.oxfordbiomed.com

---

## Colorimetric Assay for Nitric Oxide Product No. NB 98

For Research Use Only

---

Microtiter plate format for the quantitative measurement of NO in aqueous solutions by reduction of NO<sub>3</sub> to NO<sub>2</sub>

---

### INTRODUCTION

---

#### The Analyte

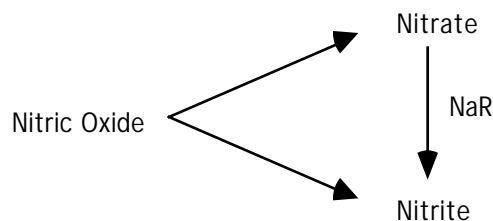
This kit employs immunoaffinity purified Nitrate Reductase (E.C. # 1.6.6.1) from *Zea Mays* to allow you to measure total nitric oxide (NO) produced in *in vitro* experimental systems following enzymatic conversion to nitrite.

Nitric oxide can be spectrophotometrically assayed 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 quantitation of nitrite using Griess Reagent.

In addition to providing all necessary components in a microtiter format, this kit employs affinity purified *Zea Mays* nitrate reductase and NADH, thereby circumventing some of the potential problems reported for nitric oxide measurement using NADPH dependent nitrate reductases.

#### Principles of the Procedure

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This kit employs the NADH-dependent enzyme nitrate reductase (NaR) for quantitative conversion of nitrate to nitrite prior to quantitation of nitrate using Griess reagent — thus providing for accurate determination of total NO production.



This kit can be used to accurately measure as little as 1 pmol/mL (~1μM) NO produced in aqueous solutions. Very little sample is required (5 to 85μL depending on the [NO] in the sample). The completed reaction is read at 540 nm.

---

## REAGENTS

---

### Materials Provided

1. **NITRATE REDUCTASE (NB98a):** The enzyme is provided in lyophilized form to improve stability. The enzyme must be reconstituted just prior to use, according to the following protocol: Reconstitute the enzyme (NB98a) with 500  $\mu$ L of buffer (NB98b) on ice for 20 minutes with minimal shaking & vortexing. Reconstituted enzyme should be used within 24 hours (keep on ice) or stored at  $-20^{\circ}\text{C}$ . Stability drops after reconstitution and dilution! After reconstitution the enzyme is at the appropriate concentration of 1 unit in 500  $\mu$ L.
2. **BUFFER (NB98b):** 50 mM MOPS/1 mM EDTA, pH 7.0. 25 mL provided.
3. **NADH (NB98c):** 2mg NADH (b-Nicotinamide adenine dinucleotide, reduced form). To obtain a 2 mM working solution, add 1.28 mL deionized water to the vial prior to use. Store in the dark at RT.
4. **COLOR REAGENT #1 (NB98d):** Sulfanilamide (p-Aminobenzenesulfonamide) dissolved in 3N HCl. 7 mL provided.
5. **COLOR REAGENT #2 (NB98e):** N-(1-Naphthyl) ethylenediamine dihydrochloride dissolved in deionized  $\text{H}_2\text{O}$ . 7 mL provided.
6. **NITRATE STANDARD (NB98f):** 500 pmol / mL  $\text{KNO}_3$  (equivalent to 500  $\mu$ M NO). 1.5 mL provided.
7. **MICROTITER PLATE (NB98g):** One (96 well) low protein binding plate with flat-bottom wells.
8. **MICROTITER PLATE TEMPLATE (NB98h):** One printed template for duplicate assays.
9. **REAGENT RESERVOIRS (NB98i):** Three plastic troughs for dispensing and pipetting reagents.

### Materials Needed but not Provided

1. **Reagent grade water:** Distilled or deionized.
2. **Microplate reader** with 540nm filter. (Note: The wavelength of the filter can be 530 to 560 nm, but 540 nm is the absorption maximum).
3. **Precision pipettes** ranging from 1.0  $\mu$ L -1000  $\mu$ L, 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.
4. **Clean test tubes** to dilute the standards and unknowns.
5. **Vortex mixer.**

---

## PROCEDURE

---

1. Prepare standards as detailed on the next page.
2. Determine the number of wells to be used and the organization of the samples on the Microplate (e.g. see Scheme II).
3. Add samples to wells in duplicate, 5  $\mu$ L to 85  $\mu$ L depending on the [NO] in the sample.
4. Add sufficient buffer to each sample to bring the volume to 85  $\mu$ L. (e.g. 80  $\mu$ L for 5  $\mu$ L of sample).
5. Add 5  $\mu$ L of the reconstituted Nitrate Reductase to each sample.
6. Add 10  $\mu$ L of 2 mM NADH to each well and shake the plate for 20 minutes at room temperature.
7. Add 50  $\mu$ L Color Reagent #1 and shake briefly.
8. Add 50  $\mu$ L Color Reagent #2. Shake for 5 minutes at room temperature.
9. Read absorbance values at 540 nm in Microtiter plate reader.
10. Plot the standard curve and estimate the concentrations of the samples from the curve.

### Standard Preparation

- A. 500  $\mu\text{M}$  Nitrate standard is provided.
- B. Take 1.0 mL of A, add 250  $\mu\text{L}$  deionized  $\text{H}_2\text{O}$  and mix = 400  $\mu\text{M}$
- C. Take 0.7 mL of B, add 0.7 mL deionized  $\text{H}_2\text{O}$  and mix = 200  $\mu\text{M}$
- D. Take 1.0 mL of C, add 1.0 mL deionized  $\text{H}_2\text{O}$  and mix = 100  $\mu\text{M}$
- E. Take 1.0 mL of D, add 1.0 mL deionized  $\text{H}_2\text{O}$  and mix = 50  $\mu\text{M}$
- F. Take 1.0 mL of E, add 1.5 mL deionized  $\text{H}_2\text{O}$  and mix = 20  $\mu\text{M}$
- G. Take 0.5 mL of F, add 0.5 mL deionized  $\text{H}_2\text{O}$  and mix = 10  $\mu\text{M}$

Standard	Final [Std.] $\mu\text{M}$	Buffer $\mu\text{L}$ /well	Std. Solution $\mu\text{L}$ /well	NaR $\mu\text{L}$ /well	NADH $\mu\text{L}$ /well
S <sub>0</sub>	0	85	0	5	10
S <sub>1</sub>	0.5	80	5 $\mu\text{L}$ of G	5	10
S <sub>2</sub>	1.0	80	5 $\mu\text{L}$ of F	5	10
S <sub>3</sub>	2.5	80	5 $\mu\text{L}$ of E	5	10
S <sub>4</sub>	5.0	80	5 $\mu\text{L}$ of D	5	10
S <sub>5</sub>	10	80	5 $\mu\text{L}$ of C	5	10
S <sub>6</sub>	20	80	5 $\mu\text{L}$ of B	5	10
S <sub>7</sub>	25	80	5 $\mu\text{L}$ of A	5	10

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

### Scheme II Sample Microtiter protocol

	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>

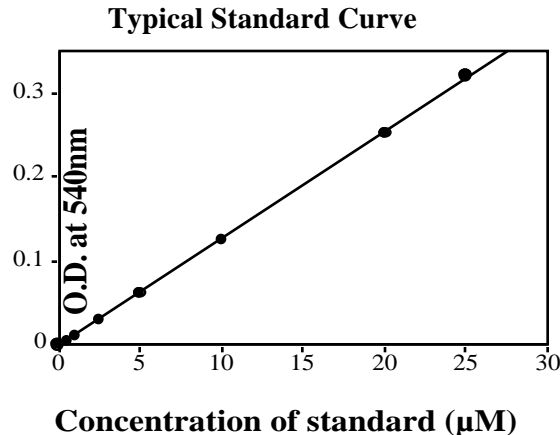
S<sub>0</sub> = blanks: Add buffer in place of standard or sample.

---

## 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.
4. Determine the concentration of each unknown by interpolation from the standard curve.  
A typical standard curve is shown here.



---

## NOTES

---

1. Reconstituted enzyme should be used within one week if stored at  $4^{\circ}\text{C}$ ; one year if stored at  $-20^{\circ}\text{C}$ .
2. It is better to complete reading of the plate within 20 minutes.
3. Color reagent #2 and the NADH solution should be kept in the dark.
4. If the NO concentration in your sample is low, you can increase the sample volume to  $100\ \mu\text{L}$  by decreasing the buffer volume to zero.
5. Samples containing very high levels of protein (resulting in  $\geq 1\ \text{mg/well}$  in this assay) may produce a precipitate that may interfere with the accurate measurement of NO. If a precipitate is visible after addition of color reagent #1, remove excess proteins (e.g. by boiling and centrifuging or diluting the samples) prior to performing the assay. Alternatively, the reaction may be performed in a conical microtiter plate. Then, prior to reading absorbances, centrifuge the plate and transfer  $100\ \mu\text{L}$  of supernatant from each well to the corresponding well of a plate with optically clear flat-bottomed wells.  
Note: If it is necessary to analyze samples with high protein concentrations (e.g.  $85\ \mu\text{L/well}$  of undiluted serum) our non-enzymatic NO kit (catalog number NB88) can be used.
6. If additional microtiter plates are used, they should be low protein binding plates. If used to read absorbances, they must have optically clear, flat-bottomed wells.

---

## REFERENCES

---

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

---

## **SAFETY**

---

### **PRECAUTIONS**

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. The use of gloves, lab coat and eye protection is recommended when working with any type of laboratory reagent.
2. Do not pipette solutions by mouth.
3. Do not eat, smoke or apply make-up in areas where sample specimens or kit reagents are handled.
4. Contents may be harmful if swallowed, inhaled or absorbed through the skin.

### **FIRST AID**

Call a physician. If swallowed, give water or milk to dilute and induce vomiting. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes, assure adequate flushing by separating eyelids with fingers. If inhaled, remove to an area with fresh air. In case of skin contact, wash with soap or mild detergent and large amounts of water.

---

## **DISCLAIMER**

---

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.

---

## **ORDERING INFORMATION**

---

For additional kits or a complete catalog please call 1-800-692-4633.

---

## **TECHNICAL SUPPORT**

---

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.

---

## **LIMITED WARRANTY**

---

Oxford Biomedical Research, Inc. makes no warranty of any kind, expressed or implied, except that this product will meet our specifications at the time of delivery. Buyer's exclusive remedy and Oxford Biomedical Research Inc.'s sole liability shall be limited to the refund of the purchase price or, at the discretion of Oxford Biomedical Research, Inc., the replacement of all materials that do not meet our specifications. Oxford Biomedical Research, Inc. shall not be otherwise liable for incidental or consequential damages including, but not limited to, the costs of handling and shipping.

Oxford Biomedical Research, Inc.  
P.O. Box 522  
Oxford, MI 48371 U.S.A.

Orders: 800-6924633  
Technical Service: 248-852-8815  
Fax: 248-852-4466  
E-mail: [info@oxfordbiomed.com](mailto:info@oxfordbiomed.com)

Made in the U.S.A.