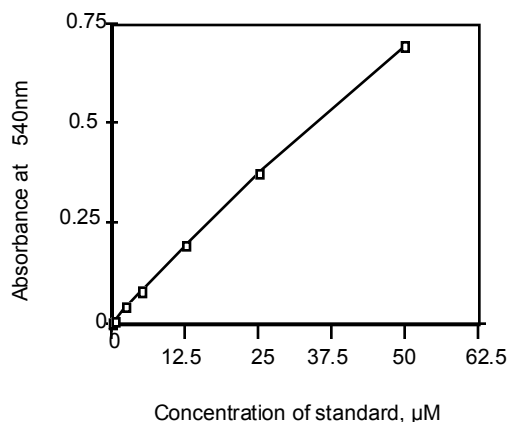


HELPFUL HINTS

1. Although the color is stable for several hours after it has developed, it is better to complete the reading of the plate within 20 minutes.
2. Color reagent #2 should be kept in the dark.
3. If the nitrite concentration in your sample is low, you can increase the sample volume by decreasing the water volume at procedure 5 and 7.
4. If additional microtiter plates are used, they should be low protein binding plates and have optically clear, flat-bottomed wells.

TYPICAL STANDARD CURVE



REFERENCES

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

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Revision 1096
Part No. 7594



BIOXYTECH® Nitric Oxide Non-Enzymatic Assay

Colorimetric, Non-Enzymatic Assay for Determination of Total Nitrite

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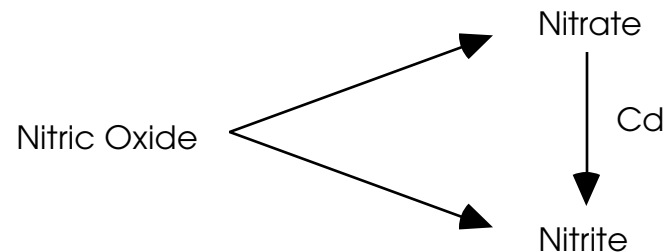
INTRODUCTION

This assay provides reagents for use in the determination of nitrite as an indicator of nitric oxide production in biological samples. Nitric oxide is rapidly converted to nitrite and nitrate in typical oxygenated aqueous solutions. Because an excellent colorimetric reagent (the Greiss reagent) exists for the determination of nitrite, it is common practice to use enzymatic or chemical reduction to convert all nitrate to nitrite in a sample and measure total nitrite as an indicator of nitric oxide production. This assay provides for chemical reduction of nitrate by granulated cadmium, followed by spectrophotometric analysis of total nitrite using Greiss reagent.

In addition to providing all necessary components in a microtiter format, this kit provides for elimination of possible interference by proteins in concentrated biological samples by $ZnSO_4$ precipitation. The granulated cadmium beads provide for a quantitative and inexpensive assay.

PRINCIPLE

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite (Figure 1). Spectrophotometric determination of nitrite using Greiss reagent is straightforward and sensitive, but does not measure nitrate, causing a possible underestimation of nitric oxide. This kit employs granular cadmium metal for chemical reduction of nitrate to nitrite (Figure 1) prior to quantitation of nitrite using Greiss reagent. In acid solution, nitrite is converted to nitrous acid (HNO_2) which diazotizes sulfanilamide. This sulfanilamide-diazonium salt is then reacted with N-(1-Naphthyl)-ethylenediamine (NED) to produce a chromophore which is measured at 540 nm. This kit can be used to accurately measure as little as 1 µM of nitrite (final concentration in the assay). Very little sample is required (10 µL to 100 µL for most samples).



CONTENTS OF THE REAGENT BOX

1. **GRANULATED CADMIUM:** 25 g cadmium beads.
2. **ZnSO₄ SOLUTION:** 2 mL of 30% (wt/vol) ZnSO₄.
3. **MICROCENTRIFUGE TUBES:** 50 x 1.5 mL microcentrifuge tubes.
4. **COLOR REAGENT #1:** 7 mL Sulfanilamide (p-Aminobenzenesulfonamide) dissolved in 3N HCl.
5. **COLOR REAGENT #2:** 7 mL N-(1-Naphthyl) ethylenediamine dihydrochloride dissolved in deionized H₂O.
6. **NITRITE STANDARD:** 1.5 mL 500 µM NaNO₂.
7. **MICROTITER PLATE:** One 96 well low protein binding plate with flat-bottom wells.
8. **MICROTITER PLATE TEMPLATE:** One printed template for duplicate assays.
9. **REAGENT RESERVOIRS:** Three plastic troughs for dispensing and pipetting reagents.
10. **CADMIUM BEAD WASHING SOLUTIONS:** 125 mL 0.1M HCl, 125 mL 0.1M NH₄OH.

ITEMS REQUIRED BUT NOT PROVIDED

1. Microplate reader with 540nm filter.
(Note: The wavelength of the filter can be 530 to 560 nm, but 540 nm is the absorbance maximum).
2. Microplate mixer if microplate reader does not have a mixing/shaking option.
3. Precision pipettes 10 µL - 100 µL; 1 mL to 5 mL, and disposable tips. NOTE: If all 96 wells are to be used at one time it is suggested that a multichannel pipettor be used.
4. Clean test tubes to dilute the standards and unknowns.
5. Vortex mixer.
6. Mixing device to agitate microfuge tubes overnight.

REAGENT STORAGE

- Store kit at 2° - 8° C.

SAFETY PRECAUTIONS

- Do not pipette solutions by mouth.
- Do not eat or smoke in areas where specimens or kit reagents are handled.
- Safety glasses and gloves should be worn to prevent skin and eye contact.
- Wear protective clothing such as lab coats to prevent contact.

EXPERIMENTAL PROCEDURE

1. Add 0.5 g cadmium beads (approximately 6-7 beads) to a microcentrifuge tube for each sample and for each standard.
2. Wash the cadmium beads twice with 1 mL of each of the following in order: H₂O, 0.1M HCl, and 0.1M NH₄OH (pH 9.6).
3. Prepare standards as detailed on the next page.
4. Determine the number of wells to be used and the organization of the samples on the Microtiter plate (e.g., see Scheme I).
5. For samples with high protein content, such as serum, culture medium or other biological samples, ZnSO₄ is added for protein precipitation. Each sample (10 - 50 µL) is adjusted to 190 µL with water, and then 10 µL of 30% (wt/vol) ZnSO₄ solution is added. The total volume of sample will be 200 µL. The samples are then mixed by vortexing, incubated at room temperature for 15 min and centrifuged (3,000-4,000 RPM) for 5 minutes.
6. The resulting supernatants are then transferred to the microcentrifuge tubes containing the granulated Cd and incubated at room temperature overnight with agitation. Recentrifuge and use the supernatants for assay.
7. Add samples to wells in duplicate, 20 - 100 µL.
8. Add sufficient water to each sample to bring the volume to 100 µL (e.g., 80 µL for 20 µL of sample).
9. Add 50 µL Color Reagent #1 and shake briefly.
10. Add 50 µL Color Reagent #2. Shake for 5 minutes at room temperature.
11. Read absorbance values at 540 nm in microtiter plate reader.
12. Plot the standard curve and estimate the concentrations of the samples from the curve.

STANDARD PREPARATION

- A. 500 µM Nitrite standard is provided.
- B. Take 1.0 mL of A, add 4 mL deionized H₂O and mix = 100 µM
- C. Take 1.0 mL of B, add 1 mL deionized H₂O and mix = 50 µM
- D. Take 1.0 mL of C, add 1 mL deionized H₂O and mix = 25 µM
- E. Take 1.0 mL of D, add 1.5 mL deionized H₂O and mix = 10 µM
- F. Take 1.0 mL of E, add 1 mL deionized H₂O and mix = 5 µM
- G. Take 1.0 mL of F, add 4 mL deionized H₂O and mix = 1 µM
- H. Take 1.0 mL of G, add 1 mL deionized H₂O and mix = 0.5 µM

Add standards to wells as follows:

Standard	Standard Preparation (µM)	Final Concentration in Assay (µM)	Standard to Pipet (µL)
S ₀	0	0	100 of H ₂ O
S ₁	0.5	0.25	100 of H
S ₂	1.0	0.5	100 of G
S ₃	5.0	2.5	100 of F
S ₄	10.0	5.0	100 of E
S ₅	25.0	12.5	100 of D
S ₆	50.0	25.0	100 of C
S ₇	100.0	50.0	100 of B

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

Scheme I

	1	2	3	4	5	6	7	8	9	10	11	12
A	S ₀	S 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

U = Unknown (sample)

CALCULATIONS

1. Subtract the average absorbance value of the blank wells (S₀) from all other pairs of wells.
2. Average the absorbance values for each pair of duplicate wells.
3. Plot a standard curve using the average absorbance 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 on the next page.
5. Multiply the concentration of the unknown found in the assay by its dilution factors to obtain the concentration in the original sample.