BioTek
Get a Better Reaction.

Application Note

# Nitric Oxide Determination using the PowerWave 200 Scanning Microplate Spectrophotometer and KC4 Data Reduction Software

*Nitric oxide is synthesized by the enzyme nitric oxide synthase in a variety of different cell types at levels that vary according to the intended function. Because of its role in normal and pathophysiological processes, the determination of nitric oxide is of tremendous importance. Most importantly, in all instances nitric oxide is converted in vivo to either nitrite or nitrate. Here we describe the determination of nitrite and nitrate using the PowerWave™ 200 scanning microplate spectrophotometer in conjunction with KC4 data reduction software.*

## Introduction

The free radical nitric oxide (NO) is produced by a number of different cell types for a variety of biological functions. Nitric oxide is a product of the oxidation of L-arginine to L-citrulline in a two step process catalyzed by the enzyme nitric oxide synthase (NOS). Two major isoforms of nitric oxide synthase have been identified. The constitutive isoform found in neurons and endothelial cells, produces very low amounts of nitric oxide in a calcium and calmodulin dependent fashion. NO activates soluble guanlyate cyclase in target cells, resulting in increased levels of cGMP, which in turn facilitates neuronal transmission and vascular relaxation, and inhibits platelet aggregation (3). The inducible isoform, found in macrophages, fibroblasts, and hepatocytes, produces NO in relatively large amounts in response to inflammatory or mitogenic stimuli and acts in a host defensive role through its oxidative toxicity (1). Regardless of the source or role, the free radical NO has a very short half life ($T_{1/2}$= 4 seconds), reacting with several different molecules normally present to form either nitrate ($NO_3^-$) or nitrite ($NO_2^-$). Because the ratio of nitrite to nitrate is variable and unpredictable, the best index of NO production is the total of both nitrate and nitrite.

The determination of NO is in actuality the determination of the total of nitrate and nitrite. The reduction of nitrate to nitrite is catalyzed by nitrate reductase in the presence of the cofactor NADPH (Figure 1A). Subsequent determination of nitrite by a two step process (Figure 1B) provides information on the “total” of nitrate and nitrite. Nitrite only determinations can then be made in a parallel assay where the samples were not reduced prior to the colorimetric assay. Actual nitrate levels are then calculated by the subtraction of nitrite levels from the total.

BioTek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, Vermont 05404-0998 USA
 TEL: 888-451-5171 FAX: 802-655-7941 Outside the USA: 802-655-4740 E-mail: customercare@biotek.com
**www.biotek.com**

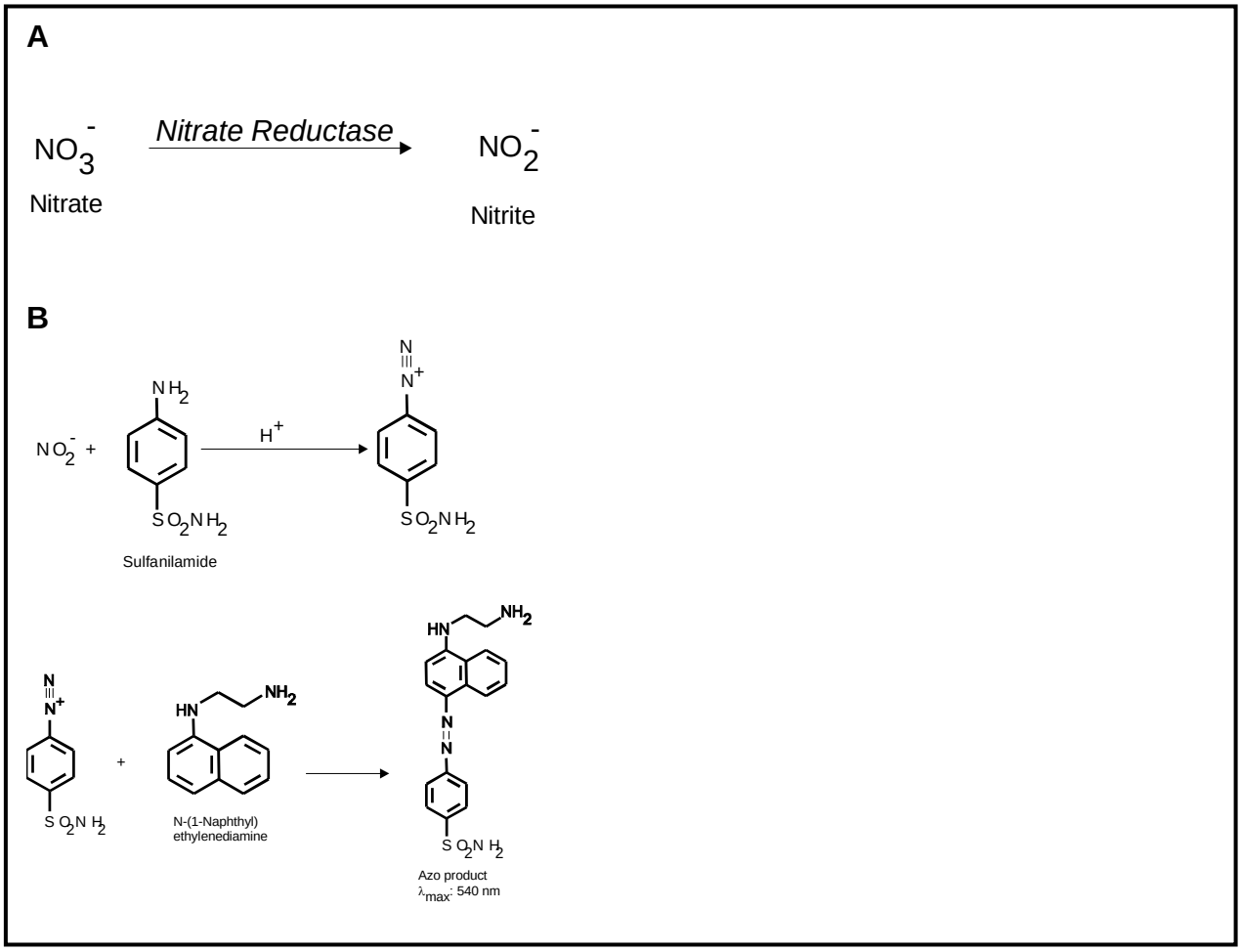


**Figure 1. Enzymatic and chemical reactions involved in the formation of colored azo product.** (A) The enzymatic reduction of nitrate to nitrite by nitrate reductase in the presence of the cofactor NADPH. (B) Two step conversion of nitrite to a colored azo-product by chemically reacting with sulfanilamide and N-(1-naphthyl) ethylenediamine.

## Materials and Methods

The assay performed in microplates is essentially that described by Green et al. (2) except that nitrate is reduced enzymatically (3). The reagents can be purchased in a kit (Catalogue No. 780001: Cayman Chemical, Ann Arbor, MI) or obtained as individual components from Sigma Chemical, St. Louis MO. Because the assay determines only nitrite (NO2-), nitrate (NO3-) must be reduced prior to the determination.

In order to perform a nitrate plus nitrite or “total” determination a nitrate standard curve was prepared by diluting a 200 mM stock solution. Dilutions were made such that the final nitrate concentration (200 ml final volume) ranged from 0 to 35 mM in 80 ml total volume. Standards were run in triplicate, while samples were run in duplicate wells. After the standards and samples were dispensed into the microplate wells 10 ml of enzyme co-factor mixture (40 mM NADPH Tris pH 7.6) was added to each well followed by the addition of 10 ml of nitrate reductase enzyme mixture (14 mU) for a final volume of 100 ml. The microplate was covered with an adhesive microplate cover and allowed to incubate at room temperature for 60 minutes. Following incubation, a standard curve for nitrite was made as described for nitrate except that a 200 mM stock of nitrite (NO2-) was used and the final volume for dilution was 100 ml rather than 80 ml. Standards and samples were dispensed in a volume of 100 ml into the empty wells of the same microplate used for the “total” determination.

After pipeting the standard curve and samples for nitrite only determination into the microplate, 100 ml of Griess reagent [1% sulfanilamide, 0.1% N-(1-Naphthyl) ethylene-diamine, in 5% H3PO4] was added to all wells (total and nitrite only assays). The color was allowed to develop for 10 minutes at room temperature and the absorbance at 540 nm was then determined using a PowerWave 200 scanning microplate spectrophotometer with KC4 data reduction software on a separate PC controlling reader function and data capture (Bio-Tek Instruments, Winooski, VT).

## Results

The absorbance was determined for nitrate and nitrite concentrations ranging from 0 to 35 mM. Over this range the absorbance increased in a linear fashion. Using KC4, a least
When samples containing known mixtures of both nitrate and nitrite were measured, a good correlation between the predicted and experimentally determined values was observed (Table 1).

| | Predicted | | | Experimental | | |
|---|---|---|---|---|---|---|
| sample | total [&] | nitrite | nitrate | total | nitrite | nitrate[@] |
| 1 | 40 | 5 | 35 | 42.06 | 8.07 | 33.99 |
| 2 | 35 | 10 | 25 | 37.35 | 13.25 | 24.10 |
| 3 | 35 | 25 | 10 | 38.29 | 27.96 | 10.33 |
| 4 | 40 | 35 | 5 | 42.47 | 36.88 | 5.59 |

@ Note that nitrate determinations were calculated by subtracting nitrite values from the total.
& Note that all concentration values indicated are expressed in mM.

**Table 1. Comparison of predicted vs. experimentally determined nitrate and nitrite concentrations.** Four samples containing mixtures of nitrate and nitrite were run in parallel for “total” (nitrate + nitrate) and nitrite only determination. Concentrations were determined from the appropriate standard curve and compared to predict values. This means squared linear regression analysis can be generated with a coefficient of determination (r2) value for nitrate and nitrite of 0.999 and 0.996 respectively. The close agreement of absorbance values for the nitrate and nitrite curves indicates that the reduction of nitrate to nitrite was complete.

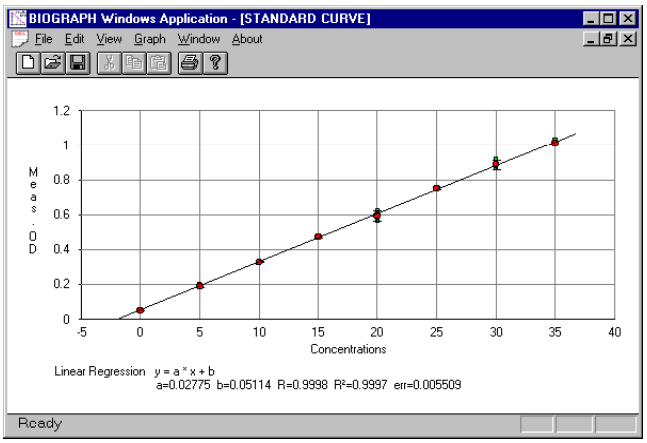


**Figure 2. Linearity of the Nitrate Assay.** Concentration curve from 0 to 35 mM nitrate with linear regression analysis. Image depicts the screen output from KC4 of a typical standard curve of a nitrate assay. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

When the linear range of the assay was examined, the absorbance was found to be linear for nitrite (NO2-) concentrations as high as 100 mM (Figure 4). Under the same conditions, unreduced nitrate (NO3-) up to 100 mM was found not to react to any extent (Figure 5). Therefore, it is absolutely necessary to reduce nitrate to nitrite in order to determine “total”

nitrate/nitrite. These data also indicate that nitrate does not interfere with nitrite only determination.

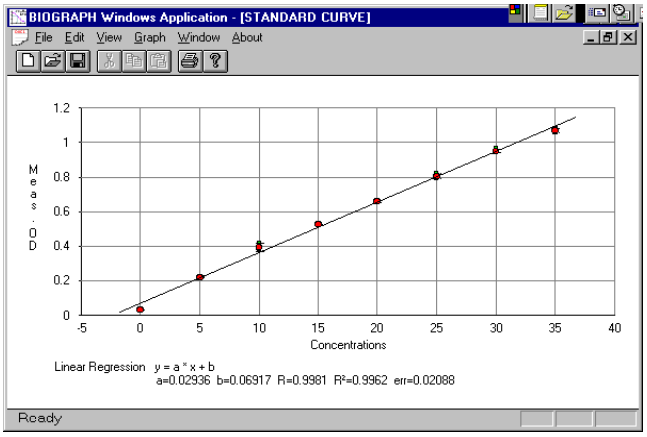


**Figure 3. Linearity of the Nitrite Assay.** Concentration curve from 0 to 35 mM nitrite with linear regression analysis. Image depicts the screen output from KC4 of a typical standard curve of an nitrite assay. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

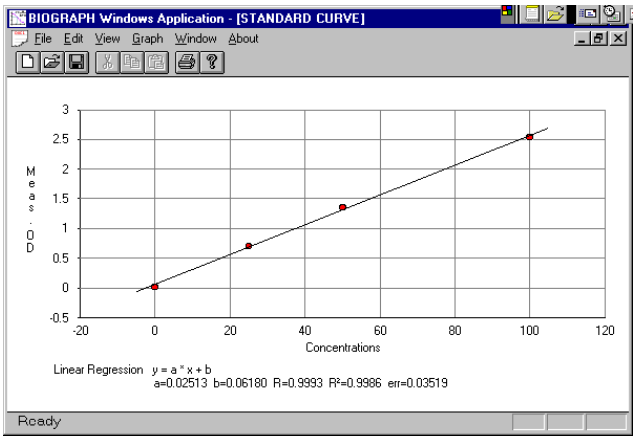


**Figure 4. Range of Linearity of the Nitrite Assay.** Concentration curve from 0 to 100 mM nitrite with linear regression analysis. Image depicts the screen output from KC4 of a nitrite assay. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

As demonstrated in Figure 6, only in the nitrite sample is there a symmetrical peak in absorbance with a lmax of 536 nm present. This is in close agreement with the wavelength of 540 nm, which we choose to perform this assay. The nitrate sample, as well as the reagent blank exhibits a broad band of absorbance in the UV range (200-350 nm) as seen in the nitrite sample, but virtually no absorbance at 540 nm.

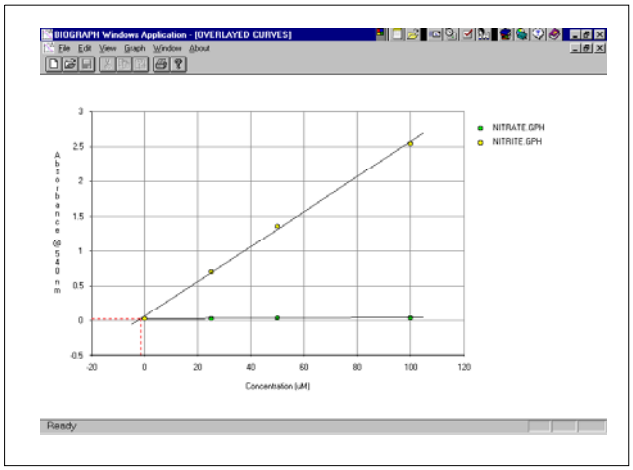


**Figure 5. Comparison of nitrate and nitrite concentration curves.** Concentration curve from 0 to 100 mM nitrite or nitrate with linear regression analysis. Image depicts the screen output from KC4 of a curve comparison between nitrite and nitrate without enzymatic reduction with nitrate reductase enzyme.

## Discussion

Many different cell types produce NO under both normal and abnormal physiological conditions. NO reacts rapidly to form either nitrate and/or nitrite, thus its production can be monitored through the formation of these end products. Here we have demonstrated that the PowerWaveÔ 200 scanning microplate spectrophotometer in conjunction with KC4 data reduction software can perform a colorimetric assay for nitrate and nitrite, thus NO production.

There are several agents that can interfere with the color formation of the assay including antioxidants, azide, ascorbic acid, dithiotheitol, and, b-mercaptoethanol. In addition, phosphate concentrations greater than 50 mM have also been shown to interfere with the conversion of nitrate to nitrite. However, alkyl amines, most sugars, lipids or amino acids (except those containing thiol groups) do not interfere with the assay (4).

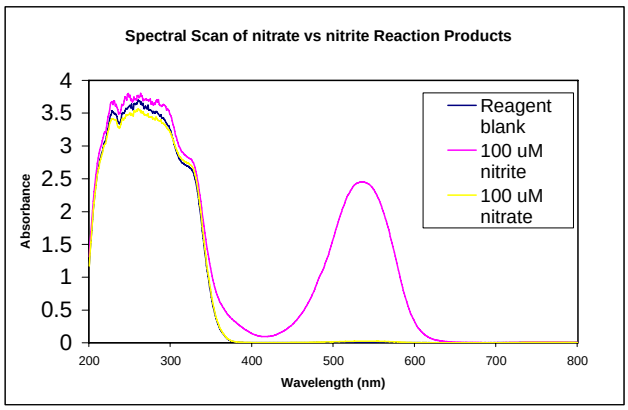


**Figure 6. Spectral scans of assay reaction products.** Final products from a nitrite assay were scanned from 200 nm to 800 nm in 1 nm increments using a PowerWave 200 microplate scanning spectrophotometer. Data was collected using KC4, exported to Excel (Microsoft Redmond, WA), and the background absorbance of the microplate subtracted for each wavelength.

In reality, the analysis of nitrate is a quantitation of the total of both nitrate and nitrite. While the colorimetric reaction does not take place with NO3-, the reduction of nitrate to nitrite prior to the colorimetric reaction allows for the quantitation of both. The determination of nitrate can then be made by subtracting the nitrite value from total. Theoretically, the nitrate and nitrite standard curves should be identical, as nitrate is converted to nitrite, but in reality slight differences almost always exist due to incomplete reduction of nitrate.

While we choose to use 540 nm for absorbance measurements rather than the maximal wavelength (lmax) of 536 nm, the PowerWave™ 200 is capable of measuring absorbance at any wavelength between 200 nm and 800 nm in 1 nm increments. Using 540 nm does not affect the result and more closely mimics the availability of wavelength specific filters which are required on many other absorbance readers.

The use of KC4 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software allows the user to define any configuration of plate map necessary. With several different curve fit algorithms to choose from, regression analysis of the standards and the subsequent concentration determinations of samples can be accomplished with a high degree of confidence. Likewise, the software is capable of performing statistical analysis on sample groups, as well as any mathematical calculation required by the user. In the case of the experiments demonstrated, the two standard curves (nitrate and nitrite) were defined on the same microplate using different plate maps.

## References


1. Nathan, C. (1992) Nitric oxide as a secretory roduct of mammalian cells, FASEB Journal 6:3051-3064.
2. Green, L.C., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, and S.R. Tannenbaum (1982) Analysis of Nitrate, Nitrite, and [15N] Nitrate in Biological Fluids, Analytical Biochemistry 126:131-138.
3. Misko, T.P., R.J. Schilling, D. Salvemini, W.M. Moore, and M.G. Currie (1993) A Fluorometric Assay for the Measurement of Nitrite in Biological Samples, Analytical Biochemistry, 214:11-16.
4. Nitrate/Nitrite Assay Kit Instructions (1995) Cayman Chemical Company, Ann Arbor MI.



**Paul Held, Ph. D.**
**Senior Scientist & Applications Lab Manager**



**Rev. Date: 2/7/01**