



Nitrate/Nitrite Colorimetric Assay Kit

Item No. 780001

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity	Storage
780022	Nitrate/Nitrite Assay Buffer	1 vial	4°C
780010	Nitrate Reductase Enzyme Preparation	2 vials	-20°C
780012	Nitrate Reductase Cofactor Preparation	2 vials	-20°C
780014	Nitrate Standard	1 vial	4°C
780016	Nitrite Standard	1 vial	4°C
780018	Griess Reagent R1	2 vials	4°C
780020	Griess Reagent R2	2 vials	4°C
400014	96-Well Solid Plate (Colorimetric Assay)	3 plates	RT
400012	96-Well Cover Sheet	3 covers	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 540-550 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water (preferably Milli-Q or equivalent)

INTRODUCTION

Nitric Oxide Background

Nitric Oxide (NO) is synthesized in biological systems by the enzyme Nitric Oxide Synthase (NOS). NOS is a remarkably complex enzyme which acts on molecular oxygen, arginine, and NADPH to produce NO, citrulline, and NADP⁺. This process requires five additional cofactors (FMN, FAD, Heme, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron; see Figure 1). Three distinct isoforms of NOS have been identified, as detailed in Figure 2 on page 6.

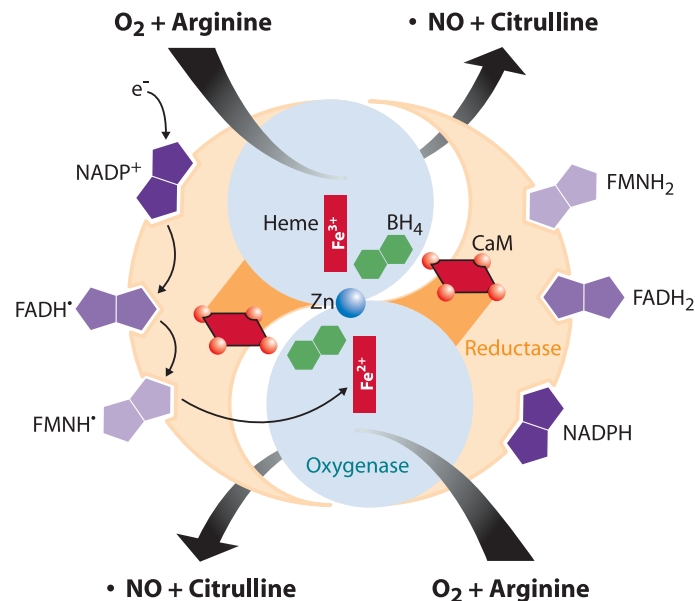


Figure 1. Nitric oxide synthesis

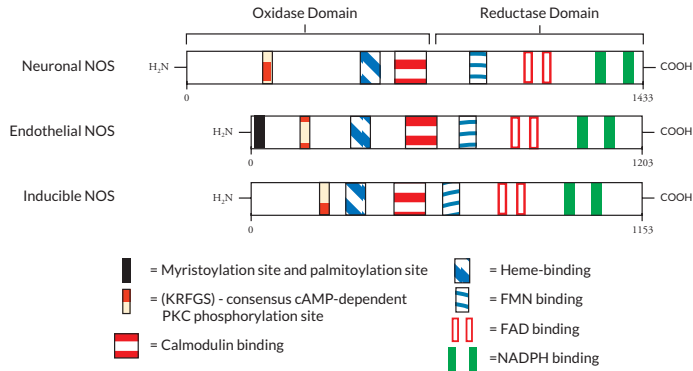
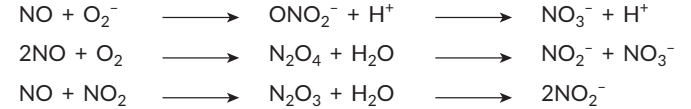


Figure 2. Nitric oxide synthase isoforms

NO is produced in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli.^{1,2} This NO is scavenged rapidly ($t_{1/2} = 4$ seconds) and acts in a paracrine fashion to transduce cellular signals. NO interacts with the heme prosthetic group of guanylate cyclase, activating the enzyme and leading to increased cGMP levels. NO is also produced by other cells (macrophages, fibroblasts, hepatocytes) in micromolar concentrations in response to inflammatory or mitogenic stimuli. In this case, the biological role is defense against non-self pathogens through oxidative toxicity. These very high NO levels lead to the formation of peroxynitrite, destruction of iron-sulfur clusters, thiol nitrosation, and nitration of protein tyrosine residues. Thus, the amount of NO produced in different biological systems can vary over several orders of magnitude and its subsequent chemical reactivity is diverse.

NO undergoes a series of reactions with several molecules present in biological fluids. These include:



The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of NO_2^- and NO_3^- is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both NO_2^- and NO_3^- .

About this Assay

Cayman's Nitrate/Nitrite Colorimetric Assay Kit provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents which convert nitrite into a deep purple azo compound (See Figure 3).³ Photometric measurement of the absorbance due to this azo chromophore accurately determines NO_2^- concentration.

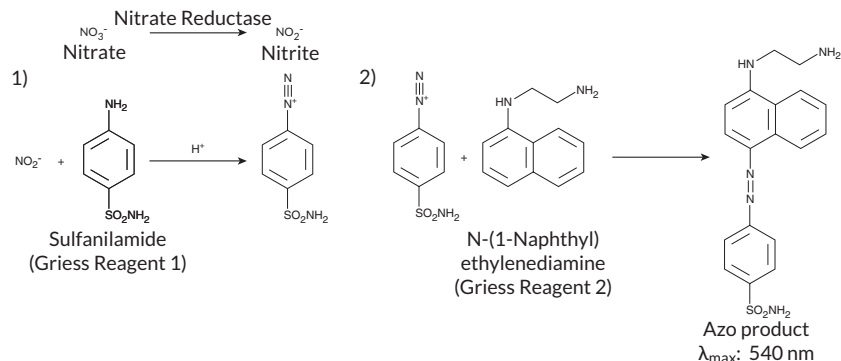


Figure 3. Chemistry of the Griess Reagents

NADPH is an essential cofactor for the function of the NOS enzyme. Unfortunately, NADPH interferes with the chemistry of the Griess reagents, which are the most commonly used reagents for nitrite detection. One way to prevent this interference is to use small amounts of NADPH in conjunction with a catalytic system for recycling spent NADP^+ back to NADPH. This is the system used in this Nitrate/Nitrite Colorimetric Assay Kit. It works well for the analysis of nitrate and nitrite in fluids such as plasma and urine, and is also available in a highly sensitive fluorometric version (Item No. 780051) for the detection of low levels of nitrite. However, it cannot be used to analyze nitrate and nitrite from an *in vitro* assay of NOS in which excess NADPH has been added. For these assays a second method (LDH method) is utilized and is available from Cayman in a 96-well plate format (Item No. 760871).

PRE-ASSAY PREPARATION

Reagent Preparation

1. Nitrate/Nitrite Assay Buffer - (Item No. 780022)

Dilute the contents of the Assay Buffer vial to 100 ml with UltraPure water (Milli-Q or equivalent). This Assay Buffer should be used for dilution of samples as needed prior to assay. The buffer will be stable for approximately two months at 4°C.

2. Nitrate Reductase Enzyme Preparation - (Item No. 780010)

Reconstitute the contents of the vial with 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of this solution should be limited to one time.

3. Nitrate Reductase Cofactors Preparation - (Item No. 780012)

Reconstitute the contents of the vial with 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of this solution should be limited to one time.

4. Nitrate Standard - (Item No. 780014)

Remove the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitute the contents of the vial with 1.0 ml of Assay Buffer. Vortex and mix sufficiently to ensure all powder in the vial, including any on the stopper, is in solution. Store at 4°C when not in use (*do not freeze!*). The reconstituted standard will be stable for about four months when stored at 4°C.*

5. Nitrite Standard - (Item No. 780016)

Remove the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitute the contents of the vial with 1.0 ml of Assay Buffer. Vortex and mix sufficiently to ensure all powder in the vial, including any on the stopper, is in solution. Store at 4°C when not in use (*do not freeze!*). The reconstituted standard will be stable for about four months when stored at 4°C.*

6. Griess Reagents R1 and R2 - (Item Nos. 780018 and 780020)

Do not add any water or Assay Buffer to these reagents, as they are ready for use. These reagents should be stored at 4°C (do not refreeze).

**NOTE: After reconstitution the standards must be further diluted prior to performing the assay (see pages 15, 18 for details).*

Sample Preparation

The kit has been validated in urine, culture media, and plasma. No sample purification from these sources is necessary other than some special instructions as described below. Store samples at -20°C or -80°C after collection.

1. Urine samples

Urine can be used directly after dilution to the proper concentration in Assay Buffer. Urine contains relatively high levels of nitrate (200-2,000 μM), so dilutions of approximately 1:10-1:50 may be necessary.

2. Culture Media

Some types of tissue culture media contain very high nitrate levels (*i.e.*, RPMI 1640). These types of media should not be used for cell culture if the goal of an experiment is to measure small changes in nitrate levels. Cellular nitrate/nitrite production can be quantitated by subtracting the level of nitrate/nitrite present in the media (in the absence of cells) from the total nitrate/nitrite level present during cell growth. The effect of media components on color development can be assessed by making a Nitrite Standard curve in the presence of a fixed volume of the culture media (40 μl works well) and comparing it to a Nitrite Standard curve made in buffer alone.

3. Plasma and serum samples

Ultrafilter plasma or serum samples through a 10 or 30 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. The filters, supplied through Amicon or Millipore, should be pre-rinsed with UltraPure water prior to ultrafiltration of serum or plasma. Ultrafiltration will reduce background absorbance due to the presence of hemoglobin and improve color formation using the Griess Reagents. Assay for nitrate and/or nitrite using a maximum of 40 μl of the filtrate. The conversion of nitrate to nitrite requires three hours for completion.

Heparinized plasma may form a precipitate upon addition of Griess Reagent R1, thus making the sample unusable for analysis. Citrate or EDTA are recommended as anticoagulants for plasma preparation.

4. Tissue homogenates

Homogenize the sample in PBS, pH 7.4, and centrifuge at 10,000 x g for 20 minutes. Ultracentrifuge the supernatant solution at 100,000 x g for 30 minutes (Centrifugation at 100,000 x g is optional, but will increase filtration rates). Ultrafilter using a 10 or 30 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. The filters, supplied through Amicon or Millipore, should be pre-rinsed with UltraPure water prior to ultrafiltration. Assay the sample for nitrate and/or nitrite using a maximum of 40 μl of the filtrate. The conversion of nitrate to nitrite requires three hours for completion.

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have some wells (at least two) designated as absorbance blanks (containing 200 μl of Assay Buffer or water). The absorbance of these wells must then be subtracted from the absorbance measured in all the other wells. Standard curves for nitrate and nitrite must also be included. If you plan to measure only total NO products (nitrate + nitrite), only the nitrate standard curve is required. If only nitrite is being measured, then only the nitrite standard curve is needed. The wells for the standard curves have been designated (as in A1-H2) in the instructions below. However, these standard curves can be placed in any wells you choose. The remaining wells on the plate can then be used for the assay of your samples. We suggest you record the contents of each well on the template sheet provided (see page 26).

This kit provides sufficient cofactors and reagents to run two 96-well plates measuring total NO ($\text{NO}_2^- + \text{NO}_3^-$) in all the wells. If you wish to test some samples for NO_2^- only (where reductase and cofactors are not required), there is sufficient Griess Reagent R1 and R2 to run a third 96-well plate of nitrite determinations. All three plates are supplied with this kit.

	1	2	3	4	5	6	7	8	9	10	11	12
A	(A)	(A)	(S1)	(S1)	(S9)	(S9)	(S17)	(S17)	(S25)	(S25)	(S33)	(S33)
B	(B)	(B)	(S2)	(S2)	(S10)	(S10)	(S18)	(S18)	(S26)	(S26)	(S34)	(S34)
C	(C)	(C)	(S3)	(S3)	(S11)	(S11)	(S19)	(S19)	(S27)	(S27)	(S35)	(S35)
D	(D)	(D)	(S4)	(S4)	(S12)	(S12)	(S20)	(S20)	(S28)	(S28)	(S36)	(S36)
E	(E)	(E)	(S5)	(S5)	(S13)	(S13)	(S21)	(S21)	(S29)	(S29)	(S37)	(S37)
F	(F)	(F)	(S6)	(S6)	(S14)	(S14)	(S22)	(S22)	(S30)	(S30)	(S38)	(S38)
G	(G)	(G)	(S7)	(S7)	(S15)	(S15)	(S23)	(S23)	(S31)	(S31)	(S39)	(S39)
H	(H)	(H)	(S8)	(S8)	(S16)	(S16)	(S24)	(S24)	(S32)	(S32)	(S40)	(S40)

A-H = Standards
S1-S40 = Sample wells

Figure 4. Sample plate format

Measurement of Nitrate + Nitrite

Preparation of nitrate standard curve

A nitrate standard curve must be performed in order to quantitate sample nitrate + nitrite concentrations. In a clean test tube place 0.9 ml of Assay Buffer. To this, add 0.1 ml of reconstituted Nitrate Standard and vortex. The concentration of this stock standard is 200 μM . Use this standard (200 μM) for the preparation of the nitrate standard curve as described below. The standard curve for nitrate is prepared by addition of reagents to the plate wells in the following way:

Well	Nitrate Standard (μl)	Assay Buffer (μl)	Final Nitrate Concentration* (μM)
A1, A2	0	80	0
B1, B2	5	75	5
C1, C2	10	70	10
D1, D2	15	65	15
E1, E2	20	60	20
F1, F2	25	55	25
G1, G2	30	50	30
H1, H2	35	45	35

Table 1. Nitrate standard concentrations

*The concentration is calculated for the final 200 μl assay volume after addition of the Griess Reagents.

Preparation of Samples for Total Nitrate + Nitrite Measurement

Samples containing nitrate (with or without nitrite) can be assayed by addition of up to 80 μl (40 μl with plasma or serum) of sample per well and should be done in triplicate. When using less than 80 μl of sample, the volume must be adjusted to 80 μl by addition of the appropriate amount of Assay Buffer. When necessary, dilution of samples should be done using the Assay Buffer solution. In the event that the approximate concentration of nitrate or nitrite is completely unknown, we recommend that several different dilutions of the sample be made.

The absorbance of the samples should be between 0.05 and 1.2 absorbance units, since the plate reader will give the most accurate values when the absorbance is in this range. In addition, high absorbance values imply high nitrate levels. Under these conditions, there may be incomplete conversion of nitrate to nitrite. The detection limit of the assay is approximately 1 μM nitrite. When using 80 μl of sample, this translates into 2.5 μM nitrate in the original sample.

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Performing the Assay

1. Add 200 μl of water or Assay Buffer to the blank wells. Do not add any other reagents to these wells.
2. Add up to 80 μl of sample or sample dilutions to the wells in a pattern you choose. The final volume must be adjusted to 80 μl using the Assay Buffer solution. *NOTE: Plasma samples should be assayed with no more than 40 μl when undiluted samples are used (Samples which have been diluted 1:2 or greater can use up to 80 μl in the assay). Caution should be taken when pipetting plasma samples to ensure no bubbles enter to the well as this will lead to erroneous results.*
3. Add 10 μl of the Enzyme Cofactor Mixture (Item No. 780012) to each of the wells (standards and unknowns).
4. Add 10 μl of the Nitrate Reductase Mixture (Item No. 780010) to each of the wells (standards and unknowns).
5. Cover the plate with the plate cover and incubate at room temperature for one hour. *NOTE: This incubation time should be increased to two hours when assaying tissue culture medium, and increased to three hours when assaying plasma or tissue nitrate + nitrite concentrations. It is not necessary to shake the plate during incubation.*
6. After the required incubation time, add 50 μl of Griess Reagent R1 (Item No. 780018) to each of the wells (standards and unknowns).
7. Immediately add 50 μl of Griess Reagent R2 (Item No. 780020) to each of the wells (standards and unknowns).
8. Allow the color to develop for 10 minutes at room temperature. It is not necessary to cover the plate. *NOTE: The 10 minute incubation is optimal for color development. However, if the plate has been left to develop for longer time periods the data is still valid, provided the Griess Reagents have been added to the standard curve and unknowns at the same time. Developing the standard curve at the same time as the unknowns ensures the presence of an accurate control.*
9. Read the absorbance at 540 nm or 550 nm using a plate reader.

Measurement of Nitrite

Preparation of Nitrite standard curve

Nitrite concentrations can be measured directly by performing the assay in the absence of substrate or enzymes. In a clean test tube place 0.9 ml of Assay Buffer. To this, add 0.1 ml of reconstituted Nitrite Standard and vortex. Use this diluted standard (200 μM) for the preparation of the nitrite standard curve as described below. The nitrite standard curve is prepared as follows:

Well	Nitrite Standard (μl)	Assay Buffer* (μl)	Final Nitrite Concentration** (μM)
A1,A2	0	100	0
B1,B2	5	95	5
C1,C2	10	90	10
D1,D2	15	85	15
E1,E2	20	80	20
F1,F2	25	75	25
G1,G2	30	70	30
H1,H2	35	65	35

Table 2. Nitrite standard concentrations

*UltraPure water can also be used

**The concentration is calculated for the final 200 μl assay volume after addition of the Griess reagents.

Measurement of sample nitrite

Measurement of samples with unknown nitrite concentrations can be done using up to 100 μl of sample. When using less than 100 μl of sample for nitrite determination, the volume must be adjusted to 100 μl using Assay Buffer or water. Samples can be diluted in water or Assay Buffer. Once again it is best to keep the absorbance of the sample at approximately 0.05-1.2. When using 100 μl of sample the detection limit for nitrite is approximately 2 μM in the original sample.

Performing the Assay

1. Add 200 μl of water or Assay Buffer to the blank wells. Do not add any other reagents to these wells.
2. Add up to 100 μl of sample to the chosen wells. When using less than 100 μl be sure to adjust the volume to 100 μl using Assay Buffer or water.
3. Add 50 μl of Griess Reagent R1 (Item No. 780018) followed by addition of 50 μl Griess Reagent R2 (Item No. 780020) to each of the wells (standards and unknowns).
4. Allow the color to develop for 10 minutes.
5. Measure the absorbance at 540 or 550 nm.

Calculations

Subtract the blanks

Average the absorbance value of the blank wells and subtract this from the absorbance values of all the other wells.

Plotting the standard curves

Make a plot of absorbance at 540-550 nm as a function of nitrate **OR** nitrite concentration. The nitrate standard curve is used for determination of total nitrate + nitrite concentration, whereas the nitrite standard curve is used for the determination of nitrite alone. In theory these two standard curves should be identical however, in practice a small discrepancy often occurs. Examples of typical standard curves are shown on page 21.

Determination of sample nitrate or nitrite concentrations

$$[\text{Nitrate} + \text{Nitrite}] (\mu\text{M}) = \left(\frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left(\frac{200 \mu\text{l}}{\text{volume of sample used } (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrite}] (\mu\text{M}) = \left(\frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left(\frac{200 \mu\text{l}}{\text{volume of sample used } (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrate}] (\mu\text{M}) = (\text{Nitrate} + \text{Nitrite}) - (\text{Nitrite})$$

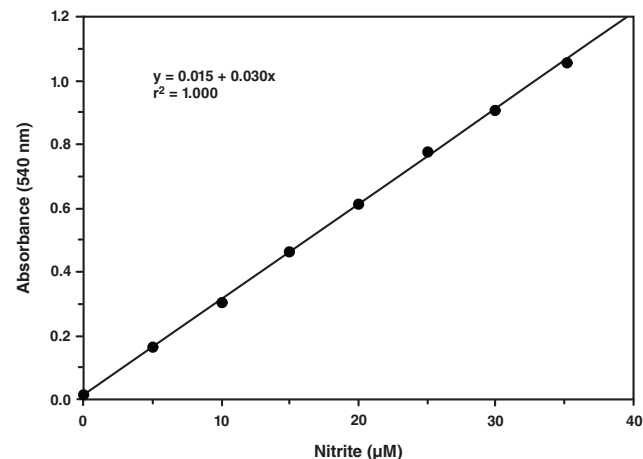
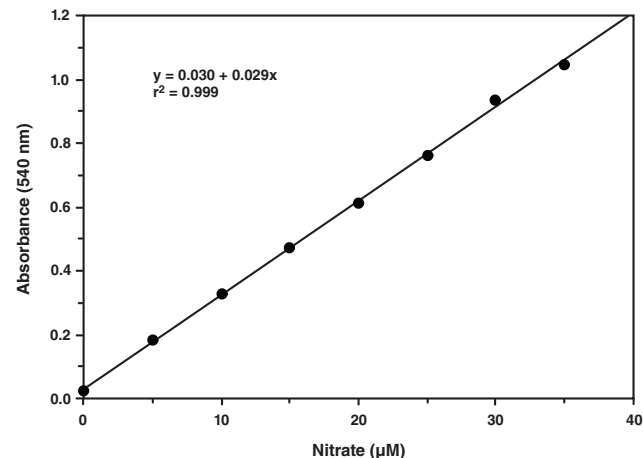


Figure 5. Typical standard curves

Performance Characteristics

Precision:

The inter-assay coefficient of variation is 3.4% (n=5).

The intra-assay coefficient of variation is 2.7% (n=84).

Sensitivity

When using the maximum amount of sample for the nitrate/nitrite assay (80 μ l), the detection limit is 2.5 μ M. The detection limit for plasma is higher since only 40 μ l of sample can be used. For the nitrite assay a maximum volume of 100 μ l can be used. In this case the detection limit is approximately 2.0 μ M.

RESOURCES

Interferences

Antioxidants will interfere with the color development reaction. Azide, ascorbic acid, dithiothreitol, and mercaptoethanol will interfere with color development when present at concentrations as low as 100 μ M. Alkyl amines, most sugars, lipids, or amino acids (except those containing thiol groups) do not interfere.⁴ Phosphate concentrations greater than approximately 50 mM will interfere with the conversion of nitrate to nitrite.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No color development in nitrate standard curve	Cofactors or enzymes (or both) not added	You will need to do a new standard curve; if you have not added one of these reagents to the sample wells, you will need to repeat the experiment

References

1. Moncada, S. The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.* **145**, 201-227 (1992).
2. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051-3064 (1992).
3. Green, L.C., Wagner, D.A., Glogowski, J., et al. Analysis of nitrate, nitrite, and [¹⁵N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138 (1982).
4. Nims, R.W., Darbyshire, J.F., Saavedra, J.E., et al. Colorimetric methods for the determination of nitric oxide concentration in neutral aqueous solutions. *Methods* **7**, 48-54 (1995).

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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website. This document is copyrighted. All rights are reserved.

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