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

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; multichannel or repeating pipettor recommended
3. A source of nitrate- and nitrite-free pure water; glass-distilled or pure water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 11)

INTRODUCTION

Background

Nitric oxide (NO) is a highly membrane-permeable signaling molecule with roles in various physiological functions, including neurotransmission, vasodilation, the immune response, and muscle relaxation.[1-3] It is produced by the three isoforms of nitric oxide synthase (NOS) enzymes, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), which are expressed in a cell type- and tissue-specific manner. All NOS isoforms oxidize arginine to generate citrulline and NO using molecular oxygen and NADPH as co-substrates (see Figure 1).[1] This process requires additional cofactors: FAD, FMN, and tetrahydrobiopterin, as well as calcium and heme iron. In the canonical NO signaling pathway, NO activates soluble guanylate cyclase (sGC) in smooth muscle cells, resulting in increased synthesis of cGMP and vasodilation.[1,2,4,5] NO is also produced at high concentrations in response to inflammatory or mitogenic stimuli and reacts with superoxide anions to form peroxynitrite, a highly reactive molecule that induces oxidative damage, such as peroxidation of lipids and nitration of protein tyrosine residues.[2,5] NO radicals can also covalently attach to thiol groups in cysteine residues to form S-nitrosothiol (SNO), a process known as protein S-nitrosylation. Due to the radical nature of NO, it has a half-life of only 3-5 seconds in biological tissues, making it difficult to directly measure NO *in vivo*.[6] NO is readily oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) following reaction with various molecules, and measurement of NO_2^- and NO_3^-, therefore, is commonly used as an indirect measure of NO production.

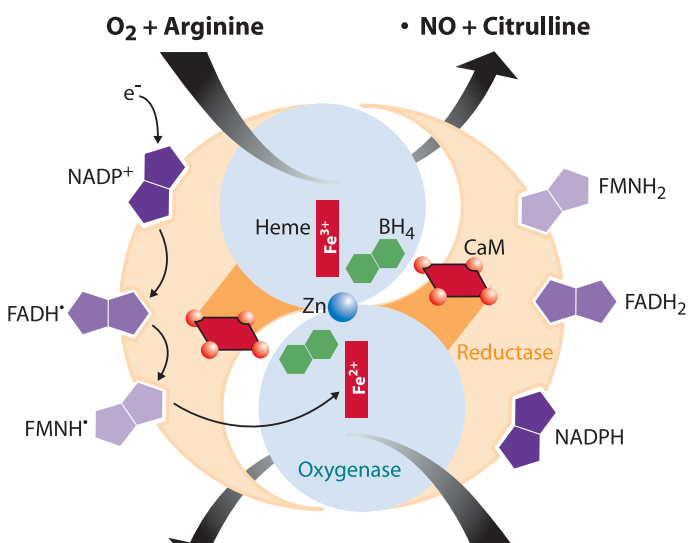


Figure 1. Nitric oxide synthesis
NO undergoes a series of reactions with several molecules present in biological fluids. These include:

$$NO + O_2^- \longrightarrow ONO_2^- + H^+ \longrightarrow NO_3^- + H^+$$
$$2NO + O_2 \longrightarrow N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^-$$
$$NO + NO_2 \longrightarrow N_2O_3 + H_2O \longrightarrow 2NO_2^-$$

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 and individual nitrate and nitrite concentrations in a simple two-step process utilizing nitrate and nitrite standards. 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 2).[7] Photometric measurement of the absorbance due to this azo chromophore accurately determines NO_2^- concentration.

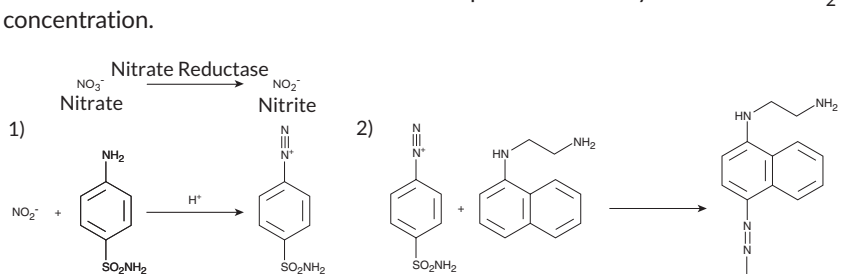


Figure 2. Chemistry of the Griess Reagents

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)**

   This vial contains 2 ml of concentrated Nitrate/Nitrite Assay Buffer. Thaw at room temperature and dilute the contents with 98 ml nitrate- and nitrite-free, deionized ultrapure water or equivalent. Be certain to rinse the vial to remove any salts that may have precipitated. The Nitrate/Nitrite Assay Buffer (1X) will be stable for at least two months when stored at 4°C.

   NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.

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

   This vial contains a lyophilized powder of nitrate reductase. Reconstitute the contents of the vial with 1.2 ml of Nitrate/Nitrite Assay Buffer (1X) and place on ice. One vial provides a sufficient volume to assay 100 wells. If additional wells will be utilized, reconstitute the second vial. Store this solution at -20°C. Freezing and thawing of it should be limited to one time.

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

   This vial contains a lyophilized powder of nitrate reductase cofators. Reconstitute the contents of the vial with 1.2 ml of Nitrate/Nitrite Assay Buffer (1X) and place on ice. One vial provides a sufficient volume to assay 100 wells. Store this solution at -20°C. Freezing and thawing of it should be limited to one time.

4. **Nitrate Standard - (Item No. 780014)**

   This vial contains a lyophilized powder of nitrate. Remove the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitute the contents of the vial with 1 ml of Nitrate/Nitrite Assay Buffer (1X) to obtain a solution of 2 mM nitrate. The reconstituted standard will be stable for at least four months when stored at 4°C. *Do not freeze!* Prepare the nitrate standard dilutions following procedures on page 15 and in Table 1.

5. **Nitrite Standard - (Item No. 780016)**

   This vial contains a lyophilized powder of nitrite. Remove the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitute the contents of the vial with 1 ml of Nitrate/Nitrite Assay Buffer (1X) to obtain a solution of 2 mM nitrite. The reconstituted standard will be stable for at least four months when stored at 4°C. *Do not freeze!* Prepare the nitrite standard dilutions following procedures on page 17 and in Table 2.

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

   These reagents are ready to use as supplied. Store at 4°C. Do not re-freeze.

Sample Preparation

The kit has been validated in plasma, serum, urine, saliva, and culture media. 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. **Plasma**

   Collect blood in vacutainers containing EDTA, or sodium citrate for plasma samples. Do not use heparin for coagulation. To obtain plasma, centrifuge blood at 1,000 x g for 15 minutes at 4°C. Pipette off the top plasma layer without disturbing the white buffy layer. It is recommended to deproteinate plasma samples using ultrafiltration with 10 or 30 kDa spin filters, following the manufacturer's protocol. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. Typically, plasma samples require dilutions of at least 1:2 or greater. Dilute the samples using Nitrate/Nitrite Assay Buffer (1X) prior to the assay.

2. **Serum**

   Collect blood in vacutainers without a coagulant for serum samples. Allow samples to clot undisturbed for 30-60 minutes at room temperature. To obtain serum, centrifuge at 1,000-2,000 x g for 15-30 minutes at 4°C. Pipette off the serum layer. It is recommended to deproteinate serum samples using ultrafiltration with 10 or 30 kDa spin filters, following the manufacturer's protocol. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. Typically, serum samples require dilutions of at least 1:2 or greater. Dilute the samples using Nitrate/Nitrite Assay Buffer (1X) prior to the assay.

3. **Urine**

   Urine samples should be assayed immediately after collection or stored at -20°C. Urine contains relatively high levels of nitrate (200-2,000 µM).[8] Interference in urine is infrequent. Dilute urine samples with Nitrate/Nitrite Assay Buffer (1X) at least 1:10 to 1:60 to fall within the range of the standard curve.

4. **Saliva**

   Centrifuge saliva at 1,500 x g for 15 minutes if visible precipitate is present. Transfer supernatant into a clean test tube. Saliva can be assayed directly after dilution with Nitrate/Nitrite Assay Buffer (1X). Saliva contains relatively high levels of nitrate (200-660 µM in adults), dilutions of approximately 1:10-1:20 may be necessary.[7,8]

5. **Culture Media**

   Some types of cell 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 the assay can be assessed by making a Nitrate Standard curve in the presence of 40% or less of the culture media in buffer (depending on the final sample dilution in the reaction) and comparing it to a Nitrate standard curve made in buffer alone.

5. **Tissue homogenates**

   Mince tissue into small pieces, homogenize in PBS, pH 7.4 containing protease inhibitors of choice, then centrifuge at 10,000 x g for 20 minutes at 4°C. Transfer the supernatant to another tube and store on ice or at -80°C for long-term storage. *Optional:* centrifuge supernatant at 100,000 x g for 30 minutes at 4°C. It is recommended to deproteinate the supernatant using ultrafiltration with 10 or 30 kDa spin filters, following the manufacturer's protocol. Dilute homogenates at least 1:2 with the Nitrate/Nitrite Assay buffer (1X) prior to the assay.

ASSAY PROTOCOL

Plate Set Up

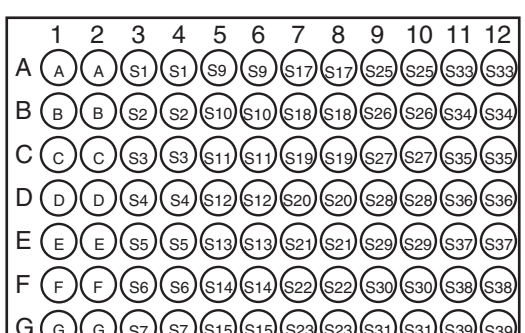
There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 3, on page 14. It is suggested that the contents of each well are recorded on the template sheet provided (see page 30).

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.

General Information

- The final volume in the assay is 200 µl in all of the wells.
- All reagents should be prepared as described above. The reconstituted Nitrate Reductase Enzyme and the Nitrate Reductase Cofactors preparations should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- Twenty-four samples can be assayed in triplicate or forty in duplicate.
- The assay is performed at room temperature.
- Monitor the absorbance at 540-550 nm.



A-H = Standard wells
S1-S40 = Sample wells

Figure 3. Sample plate

Determination of Total Nitrate + Nitrite Concentration

NOTE: To measure total nitrate + nitrite levels, use the nitrate standard curve. To measure nitrite levels alone, use the nitrite standard curve. To measure nitrate levels alone, prepare both standard curves.

Nitrate Standard Curve Preparation

A nitrate standard curve must be prepared in order to quantify sample total nitrate + nitrite concentrations. Dilute 100 µl of the 2 mM Nitrate Standard with 900 µl of Nitrate/Nitrite Assay Buffer (1X) to yield a 200 µM bulk standard. Prepare standard curve dilutions directly into the designated wells according to Table 1, below.

Well	Bulk Nitrate Standard (µl)	Assay Buffer (µl)	Nitrate Standard 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. Preparation of the nitrate standards for the colorimetric assay format
*Concentration is calculated for the final 200 µl assay volume after addition of the Griess Reagents.

Measurement of Total Nitrate + Nitrite

1. **Standard Wells:** 80 µl of standard is directly prepared in designated wells on the plate (see **Sample plate**, Figure 3, on page 14 and Table 1, on page 15.
2. **Sample Wells:** Add 80 µl of sample to at least two wells. To obtain reproducible results, the amount of target added to the wells should fall within the range of the assay. When necessary, samples should be diluted with Nitrate/Nitrite Assay Buffer (1X).
3. Add 10 µl of the reconstituted Nitrate Reductase Cofactors Preparation to all standard and sample wells.
4. Add 10 µl of the reconstituted Nitrate Reductase Enzyme Preparation to all standard and sample wells.
5. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate at room temperature as follows:

Sample Type	Incubation Time
Urine, Saliva	1 hour
Tissue Culture Medium	2 hours
Plasma, Serum, Homogenized Tissue	3 hours

6. Add 50 µl of Griess Reagent 1 to the sample and standard wells.
7. Immediately add 50 µl of Griess Reagent 2 to the sample and standard wells.
8. Incubate for 10 minutes at room temperature. It is not necessary to cover the plate.
9. Read absorbance at 540-550 nm.

Determination of Nitrite Concentration

Nitrite Standard Curve Preparation

Dilute 100 µl of the 2 mM Nitrite Standard with 900 µl of Nitrate/Nitrite Assay Buffer (1X) to yield a 200 µM bulk standard. Prepare standard curve dilutions directly into the designated wells according to Table 2, below.

Well	Bulk Nitrite Standard (µl)	Assay Buffer (µl)	Nitrite Standard 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. Preparation of the nitrite standards for the colorimetric assay format
*Concentration is calculated for the final 200 µl assay volume after addition of the Griess Reagents.

Measurement of Nitrite

1. **Standard Wells:** 100 µl of standard is directly prepared in designated wells on the plate (see **Sample plate**, Figure 3, on page 14 and Table 2, on page 17.
2. **Sample Wells:** Add 100 µl of sample to at least two wells. To obtain reproducible results, the amount of nitrite added to the wells should fall within the range of the assay. When necessary, samples should be diluted with Nitrate/Nitrite Assay Buffer (1X).
3. Add 50 µl of Griess Reagent 1 to the sample and standard wells.
4. Immediately add 50 µl of Griess Reagent 2 to the sample and standard wells.
5. Incubate for 10 minutes at room temperature. It is not necessary to cover the plate.
6. Read absorbance at 540-550 nm.

ANALYSIS

Calculations

Total (Nitrate + Nitrite) Concentration Determination:

1. Determine the average absorbance of each of the standards and samples.
2. Subtract the average absorbance value of standard A from all other standards and samples to obtain corrected standard or sample measurements (CSM) for each standard and sample.
3. Plot the CSM values of each standard as a function of the final concentration of nitrate from Table 1, on page 15. See Figure 4, on page 21, for a typical standard curve.
4. Calculate the total [nitrate + nitrite] of the samples using the equation obtained from the linear regression of the standard curve substituting the CSM for each sample.

$$\text{Total [Nitrate + Nitrite] (µM)} = \left[\frac{\text{CSM - (y-intercept)}}{\text{Slope}}\right] \text{x 2.5 x Sample dilution}$$

Nitrite Concentration Determination:

1. Determine the average absorbance of each of the standards and samples.
2. Subtract the average absorbance value of standard A from all other standards and samples to obtain corrected standard or sample measurements (CSM) for each standard and sample.
3. Plot the CSM values of each standard as a function of the final concentration of nitrite from Table 2, on page 17. See Figure 4, on page 21, for a typical standard curve.
4. Calculate the nitrite of the samples using the equation obtained from the linear regression of the standard curve substituting the CSM for each sample.

$$[\text{Nitrite}]\ (\mu M) = \left[\frac{\text{CSM - (y-intercept)}}{\text{Slope}}\right] \times 2 \times \text{Sample dilution}$$

Nitrate Concentration Determination:

Use the equation below to calculate [nitrate] in your samples:

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

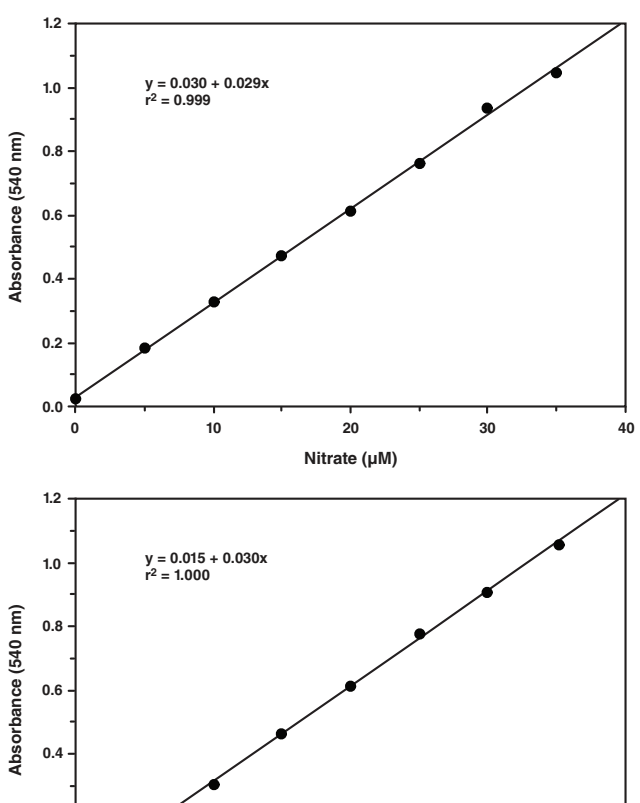


Figure 4. Typical standard curves

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:

The Lower Limit of Detection (LLOD) of the total nitrate + nitrite assay is 0.70 µM (0.14 nmol/well).

The LLOD of the nitrite assay is 0.62 µM (0.12 nmol/well).

The Lower Limit of Quantification (LLOQ) for the total nitrate + nitrite assay is 1.25 µM (0.25 nmol/well).

The LLOQ for the nitrite assay is 0.63 µM (0.13 nmol/well).

Linearity

Plasma, serum, urine, and saliva samples were serially diluted with Nitrate/Nitrite Assay Buffer (1X) and evaluated for linearity using the Nitrate/Nitrite Colorimetric Assay Kit. The results are shown in the table below.

Dilution	Nitrate + Nitrite Concentration (µM)	Dilution Linearity
Urine		
20	1,079	100%
40	1,099	102%
80	1,085	101%
EDTA Plasma		
2	74.0	100%
4	68.2	92%
8	76.9	104%
Serum		
2	25.2	100%
4	25.7	102%
Saliva		
8	499.0	100%
16	544.1	109%
32	610.6	122%

Table 3. Linearity in plasma, serum, urine and saliva

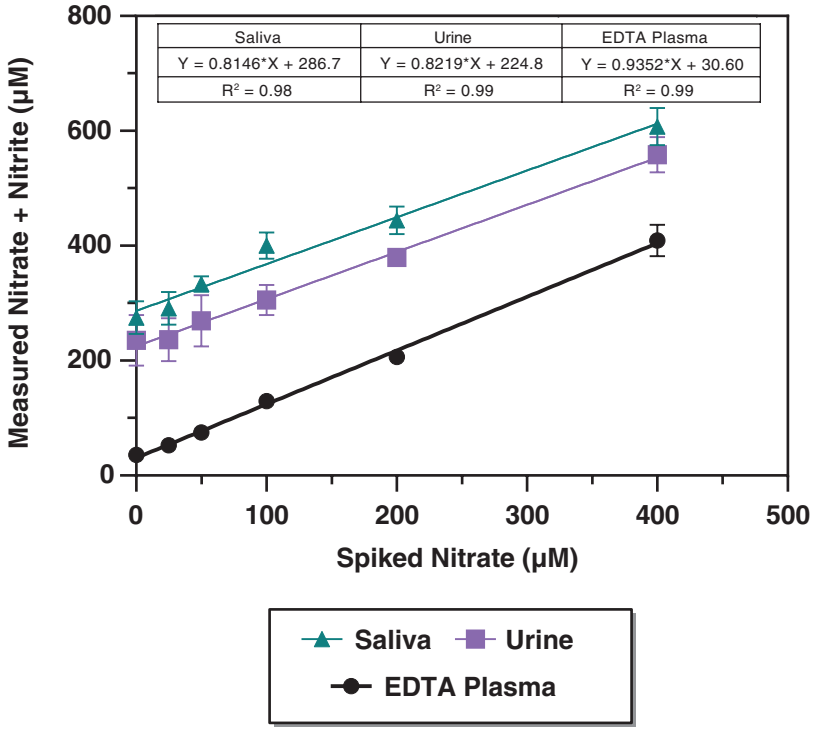


Figure 5. Spike and recovery of Nitrate in human plasma (EDTA), saliva, and urine. Human plasma, urine, and saliva were spiked with different amounts of nitrate, processed as described in the Sample Preparation section, serially diluted with Nitrate/Nitrite Assay Buffer (1X), and evaluated using the Nitrate/Nitrite Colorimetric Assay Kit. The error bars represent standard deviations obtained from multiple dilutions of each sample.

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 µM. Alkyl amines, most sugars, lipids, or amino acids (except those containing thiol groups) do not interfere.[5] 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	Prepare a new standard curve and repeat the experiment

References

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Nitrate/Nitrite Assay Buffer	Dilute 1:50 with nitrate- and nitrite-free pure or ultrapure water to make a 1X Assay Buffer solution.
Nitrate Reductase Enzyme Preparation	Reconstitute with 1.2 ml of Assay Buffer (1X).
Nitrate Reductase Cofactors Preparation	Reconstitute with 1.2 ml of Assay Buffer (1X).
Nitrate Standard	1. Reconstitute with 1.0 ml of Assay Buffer (1X) to make a 2 mM solution. 2. Dilute 1:10 to make a 200 µM bulk standard. 3. Further dilute the 200 µM bulk standard according to Table 1, page 15, to make solutions for a standard curve.
Nitrite Standard	1. Reconstitute with 1.0 ml of Assay Buffer (1X) to make a 2 mM solution. 2. Dilute 1:10 to make a 200 µM bulk standard. 3. Further dilute the 200 µM bulk standard according to Table 2, page 17, to make solutions for a standard curve.
Griess Reagents 1 and 2	These are ready to use as supplied.

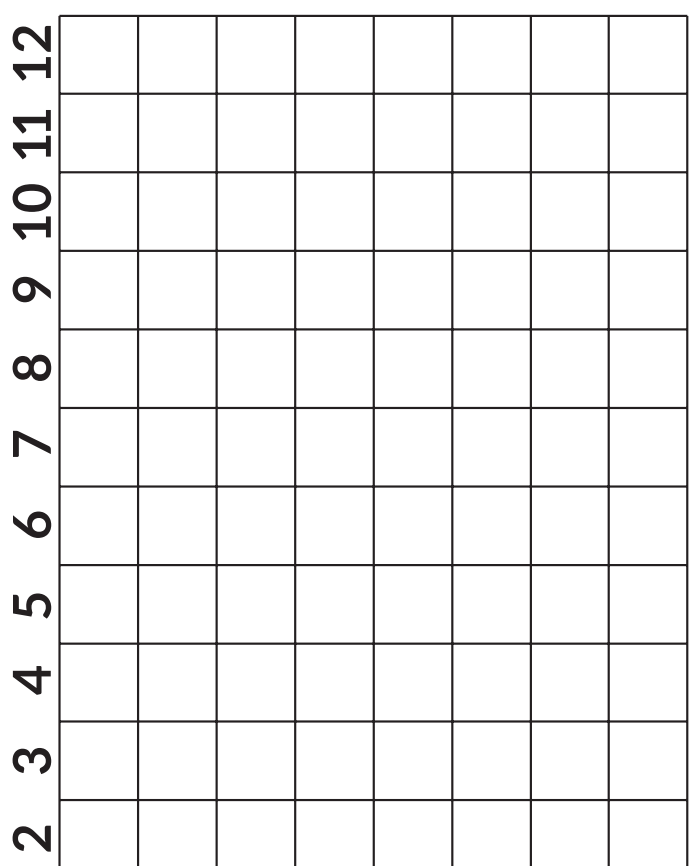
Table 4. Preparation Summary of Assay Reagents.

	Standard Wells (µl)	Sample Wells (µl)
Standard	80	--
Sample	--	80
Nitrate Reductase Cofactors	10	10
Nitrate Reductase Enzyme	10	10
Nitrate/Nitrite Assay Buffer (1X)	--	--
Cover plate; incubate at room temperature for 1-3 hours depending on sample type		
Griess Reagent 1	50	50
Griess Reagent 2	50	50
Incubate at room temperature for 10 minutes		
Read Absorbance at 540-550 nm		

Table 5. Pipetting summary for Nitrate + Nitrite Reaction

	Standard Wells (µl)	Sample Wells (µl)
Standard	100	--
Sample	--	100
Nitrate/Nitrite Assay Buffer (1X)	--	--
Griess Reagent 1	50	50
Griess Reagent 2	50	50
Incubate at room temperature for 10 minutes		
Read Absorbance at 540-550 nm		

Table 6. Pipetting summary for Nitrite Reaction



NOTES

Warranty and Limitation of Remedy

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