Nitric Oxide (NO_2^-/NO_3^-) Assay

Catalog Number DE1500B

For the quantitative determination of Nitrate (NO_3^-) and Nitrite (NO_2^-) concentrations in cell culture supernates, serum, plasma, urine, saliva, and other biological fluids.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

TELEPHONE: +49 (0)6122 90980
FAX: +49 (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

R&D Systems Europe
77 boulevard Vauban
59041 LILLE CEDEX
France

FREEPHONE: +0800 90 72 49
FAX: +0800 77 16 68
E-MAIL: info@RnDSystems.co.uk

INTRODUCTION

Nitric oxide (NO) is a major secretory product of mammalian cells that initiates host defense, homeostatic and development functions by either direct effect or intercellular signaling. NO is the product of a five-electron oxidation of the amino acid L-arginine mediated by one of three isoforms of nitric oxide synthase (1). As a direct effector, NO is thought to activate regulatory proteins, kinases, and proteases that are directed by reactive oxygen intermediates (2). As a messenger molecule, NO covalently interacts with target molecules based on redox potential (3). Activation of the immune system is associated with an increase in macrophage NO production (4). NO exerts a variety of homeostatic influences as an activator of soluble guanylyl cyclase (5), a neuronal potentiator (6), a peripheral nervous system neurotransmitter (7), and a contraction regulator of both smooth muscle and vascular tissue (8). In addition, NO has been linked to the formation of olfactory (9) and synaptic memories and remodeling (10). The transient and volatile nature of NO makes it unsuitable for most convenient detection methods. However, since most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions have been used as a quantitative measure of NO production. After the conversion of NO_3^- to NO_2^-, the spectrophotometric measurement of NO_2^- is accomplished by using the Griess Reaction.

$$NO + O_2^- \longrightarrow ONO_2^- \xrightarrow{H^+} NO_3^- + H^+$$

$$2NO + O_2 \longrightarrow N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+$$

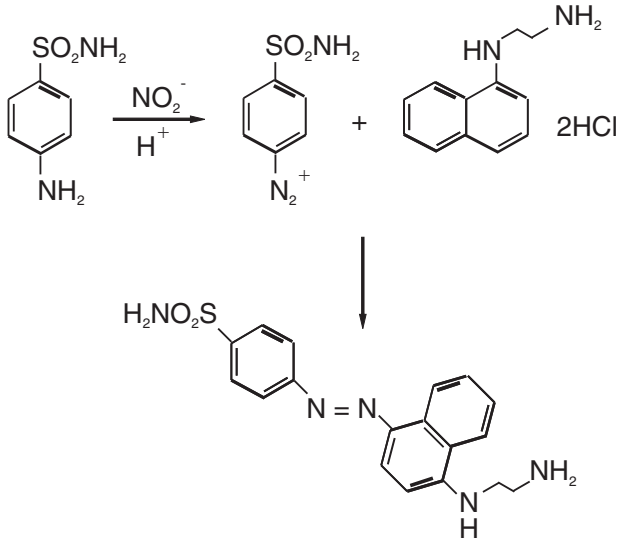
$$NO + NO_2^- \longrightarrow N_2O_3 \xrightarrow{H_2O} 2NO_2^- + 2H^+$$

The conversion of NO into nitrate and nitrite by these reactions varies in each system so both nitrate and nitrite concentrations should be measured.

R&D Systems' Nitric Oxide Assay involves the conversion of nitrate to nitrite by the enzyme nitrate reductase. The detection of nitrite is then determined as a colored azo-dye product of the Griess reaction that absorbs visible light at 540 nm. The concentration of NO is indirectly measured by determining both nitrate and nitrite levels in the sample. The relative levels of nitrate and nitrite can vary substantially depending on the ambient conditions and the redox state of the given biological fluid. Therefore, the most accurate determination of total nitric oxide production requires quantitation of both nitrate and nitrite.

PRINCIPLE OF THE ASSAY

This assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (11).



TECHNICAL HINTS

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix reagents from different lots.
- The reconstituted NADH and Nitrate Reductase must be kept on ice during the assay.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Pre-rinse the pipette tips when pipetting standards.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pipette standards and samples to the bottom of the wells.
- Add all other reagents to the side of the wells to avoid contamination.
- If samples generate values higher than the highest standard, further dilute the samples and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in optical density.
- Precautions should be taken to avoid the contamination of samples or buffers with outside sources of nitrates or nitrites. Possible sources of contamination are skin, saliva, food, drink, and water.
- Deionized or distilled water should be used to avoid the contamination of reagents with nitrate or nitrite.
- Care should be taken in the selection of gloves and disposable pipette tips as these products may be a source of nitrite or nitrate contamination. For more information, see reference 12.

REAGENTS

Microplates (Part R80-0144) - Two 96 well microplates with removable strips.

Nitrate Reductase (Part R80-1347) - 1 vial of lyophilized Nitrate Reductase, desiccated. **Store at ≤ -20° C.**

Nitrate Reductase Storage Buffer (Part R80-0255) - 4 mL of a buffer containing preservatives.

NADH (Part R80256 - inner vial; Part R80-0258 - bottle) - 2 vials of lyophilized reduced β-Nicotinamide adenine dinucleotide, desiccated. **Store in the dark.**

Nitrite Standard (Part R80-0224) - 0.5 mL of a Sodium Nitrite solution (2000 μmol/L) with preservative.

Nitrate Standard (Part R80-0223) - 0.5 mL of a Sodium Nitrate solution (1000 μmol/L) with preservative.

Reaction Buffer Concentrate (10X) (Part R80-0257) - 30 mL of a 10-fold concentrated buffer containing detergent and preservative.

Griess Reagent I (Part R80-0227) - 12 mL of Sulfanilamide in 2 N HCl.

Griess Reagent II (Part R80-0228) - 12 mL of *N*-(1-Naphthyl) ethylenediamine in 2 N HCl.

Plate Cover - 2 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at ≤ -20° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/
Reconstituted
Reagents</td><td>Nitrate Reductase</td><td rowspan="2">Aliquot and store tightly capped at ≤ -20° C in a manual defrost freezer for up to 45 days*. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Diluted NADH</td></tr>
<tr><td>Nitrate Reductase Storage Buffer</td><td rowspan="6">Store at 2 - 8° C for up to 3 months.*</td></tr>
<tr><td>Nitrite Standard</td></tr>
<tr><td>Nitrate Standard</td></tr>
<tr><td>Reaction Buffer (1X)</td></tr>
<tr><td>Griess Reagent I</td></tr>
<tr><td>Griess Reagent II</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 540 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Ice bath
- 37° C incubator
- Vortex mixer
- 500 mL graduated cylinder
- **10,000 Molecular Weight cutoff filter for preparation of samples**

PRECAUTION

Griess Reagents I and II supplied with this kit are caustic and contain organic chemicals. Wear eye, hand, face, and clothing protection when using this material. Refer to the Material Safety Data Sheets for disposal and handling information.

INTERFERENCE

The Griess reaction involves an oxidation and a nucleophilic reaction. Buffer or sample components that interfere with the oxidation and nucleophilic reaction may interfere with color formation. Examples of nucleophiles and antioxidants are azide, ascorbic acid, compounds containing sulphydryl groups such as cysteine, glutathione, DTT, and β-mercaptoethanol. If concentrations of these materials are in excess of 10 μM in the sample, the recovery of nitrite and nitrate should be determined using the nitrite and nitrate provided with the kit. To determine the recovery, nitrite and nitrate at concentrations similar to those used for the standard curve should be added to the buffer containing the suspected interfering compound, and to a similar buffer without the suspected interfering compound. If there are significant changes in the nitrate and nitrite concentrations found in the buffer containing the interfering compound, the effect should be determined and the suitable corrections made.

Nitrate Reductase converts nitrate to nitrite. Any sample or buffer component that may interfere with this enzyme will lower the conversion of sample nitrate to nitrite and therefore result in lower estimates of NO.

The nitrate salt content should be considered when choosing a tissue culture media. Some media may contain relatively high levels of nitrate, which may interfere with sensitive detections. Media that contain phenol red as a pH indicator do not interfere with the Griess reaction as the indicator is typically yellow colored under the conditions of the Griess reaction.

For samples containing high concentrations of NADPH:

Certain systems involve NO synthetase enzymes that utilize high concentrations (0.5 - 1.0 mM) of NADPH, which may inhibit the Griess Reaction slightly. Care should be taken to ensure that these samples are diluted sufficiently (10-fold or greater) in Reaction Buffer (1X) to minimize any affects of NADPH.

If samples contain excessive amounts of NADPH, the NADPH can be oxidized using Lactate Dehydrogenase (LDH) and pyruvic acid prior to color formation (13). To oxidize samples, follow the steps below:

a. Perform steps 1 - 8 as directed in the Nitrate Reduction Assay Procedure on page 10.

b. Add 10 μL of 1500 U/mL LDH (Sigma) in 30 mM sodium pyruvate (Sigma) to each well.

c. Incubate for 10 minutes at 37° C.

d. Proceed to step 9 (addition of Griess Reagent) of the Nitrate Reduction Assay Procedure.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernate - Remove particulates by centrifugation. Prepare samples as directed in Sample Preparation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Prepare samples as directed in Sample Preparation and assay immediately or aliquot and store at ≤ -80° C. **For storage, add 2-propanol at 6.5% (v/v) or antibiotic (*i.e.,* streptomycin or penicillin at 100 U/mL). Aliquot and store at ≤ -80° C.** Avoid repeated freeze-thaw cycles.

Saliva - Collect 2 - 3 mL of saliva in a polypropylene tube. Vortex briefly and centrifuge for 6 minutes at 14,000 rpm in microcentrifuge tubes. Collect the aqueous layer only (no pellet) and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge at 1000 x g within 30 minutes of collection. Prepare samples as directed in Sample Preparation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma is recommended. EDTA or heparinized plasma may be used after dilution in Reaction Buffer and ultrafiltration through a 10,000 Molecular Weight cutoff (MWCO) filter, however, they may not give reproducible results as the protein may precipitate during the Griess reaction.*

Serum - Use a serum separator tube (SST). Centrifuge for 10 minutes at approximately 1000 x g. Remove serum. Prepare samples as directed in Sample Preparation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

All samples require at least a 2-fold dilution into Reaction Buffer (1X). A suggested 2-fold dilution is 100 µL sample + 100 µL Reaction Buffer (1X). **After dilution, samples must be ultrafiltered through a 10,000 MWCO filter to eliminate proteins.**

REAGENT PREPARATION

Bring all reagents (except Nitrate Reductase) to room temperature before use. Use deionized or distilled water when reconstituting or diluting reagents to avoid nitrite/nitrate contamination.

Reaction Buffer (1X) - Dilute 30 mL of Reaction Buffer Concentrate (10X) into deionized or distilled water to prepare 300 mL of Reaction Buffer (1X).

NADH Reagent

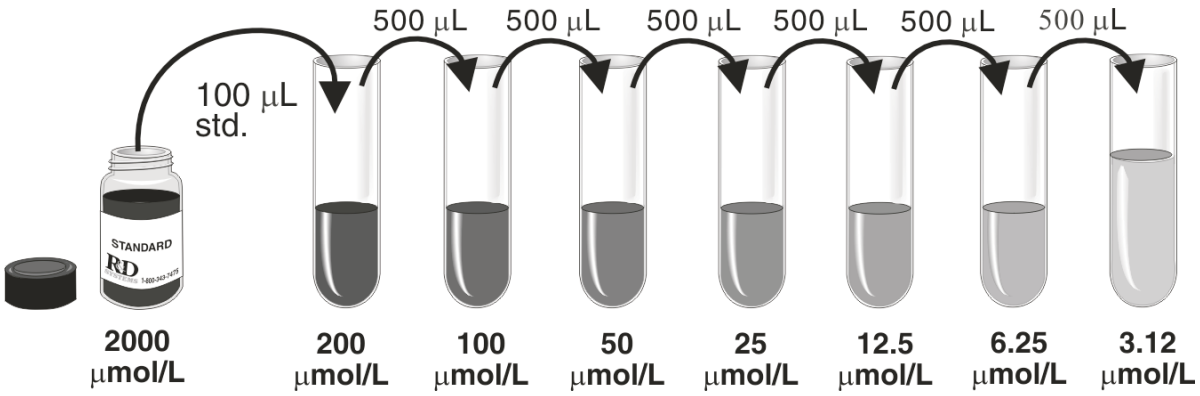
1. **Reconstitution** -
   Reconstitute the NADH with 1.0 mL deionized or distilled water. Allow the NADH to sit for 3 minutes with gentle agitation prior to use. **Keep tightly capped on ice for the duration of the assay**.
2. **Dilution** -
   Immediately before use, dilute 900 µL of NADH with 1.8 mL of deionized or distilled water. **Keep on ice for the duration of the assay.**

Nitrate Reductase

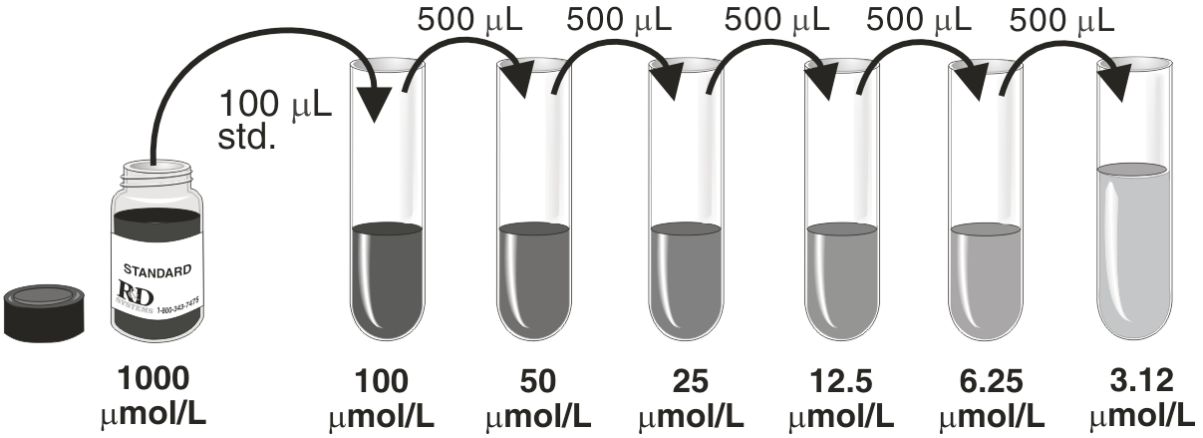
1. **Reconstitution** -
   Reconstitute the Nitrate Reductase with 1 mL Nitrate Reductase Storage Buffer. Vortex vigorously and allow to sit for 15 minutes at room temperature. Vortex again and allow to sit for an additional 15 minutes at room temperature. Vortex again. **Keep on ice for the duration of the assay.**
2. **Dilution** -
   Immediately before use, dilute the Nitrate Reductase using the following equation. Determine the number of standard and sample wells to be used (do not include blank wells). All samples and standards should be assayed in duplicate.

   a. Nitrate Reductase (µL) = (# wells + 2) x 10 µL.
   b. Reaction Buffer (µL) = volume from step a x 1.5.
   c. Add volumes from steps a and b to a tube, vortex.
   d. Place on ice and use within 15 minutes of dilution.

Nitrite Standard - Pipette 900 µL of Reaction Buffer (1X) into the 200 µmol/L tube. Pipette 500 µL of Reaction Buffer (1X) into the remaining tubes. Use the 2000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 200 µmol/L standard serves as the high standard and the Reaction Buffer (1X) serves as the zero standard (0 µmol/L).



Nitrate Standard - Pipette 900 µL of Reaction Buffer (1X) into the 100 µmol/L tube. Pipette 500 µL of Reaction Buffer (1X) into the remaining tubes. Use the 1000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 100 µmol/L standard serves as the high standard and the Reaction Buffer (1X) serves as the zero standard (0 µmol/L).



NITRITE ASSAY PROCEDURE

Bring reagents to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

This assay procedure measures the concentration of endogenous nitrite present in the sample.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the storage bag.
3. Add 200 µL of Reaction Buffer (1X) to the Blank wells.
4. Add 50 µL of Reaction Buffer (1X) to the zero standard wells.
5. Add 50 µL of Nitrite Standard or sample* to the remaining wells.
6. Add 50 µL of Reaction Buffer (1X) to all standard and sample wells.
7. Add 50 µL Griess Reagent I to each well except the Blank wells.
8. Add 50 µL Griess Reagent II to each well except the Blank wells. Mix well by tapping the side of the plate gently.
9. Incubate for 10 minutes at room temperature.
10. Determine the optical density (OD) of each well using a microplate reader set at 540 nm.

*Samples must be diluted and ultrafiltered. See Sample Preparation.

NITRATE REDUCTION ASSAY PROCEDURE

The reconstituted NADH and Nitrate Reductase should be kept on ice throughout the duration of the assay. Bring all other reagents to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

This assay procedure measures total nitrite by converting nitrate to nitrite. To determine the nitrate concentration in the sample, the endogenous nitrite concentration measured from the Nitrite Assay Procedure must be subtracted from the converted nitrite concentration measured in this assay procedure.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the storage bag.
3. Add 200 µL of Reaction Buffer (1X) to the Blank wells.
4. Add 50 µL of Reaction Buffer (1X) to the zero standard wells.
5. Add 50 µL of Nitrate Standard or sample* to the remaining wells.
6. Add 25 µL of NADH** into all standard and sample wells.
7. Add 25 µL of Nitrate Reductase** into all standard and sample wells. Mix well and cover with the adhesive strip provided.
8. Incubate for 30 minutes at **37° C**.
9. Add 50 µL of Griess Reagent I to all wells except Blank wells.
10. Add 50 µL of Griess Reagent II to all wells except Blank wells. Mix well by tapping the side of the plate gently.
11. Incubate for 10 minutes at room temperature.
12. Determine the optical density (OD) of each well using a microplate reader set at 540 nm.

*Samples must be diluted and ultrafiltered. See Sample Preparation.

**NADH and Nitrate Reductase must be diluted. See Reagent Preparation.

CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a linear curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the nitrite or total nitrite concentration on the x-axis.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Calculate the concentration of nitrite corresponding to the mean absorbance from the Nitrite standard curve.

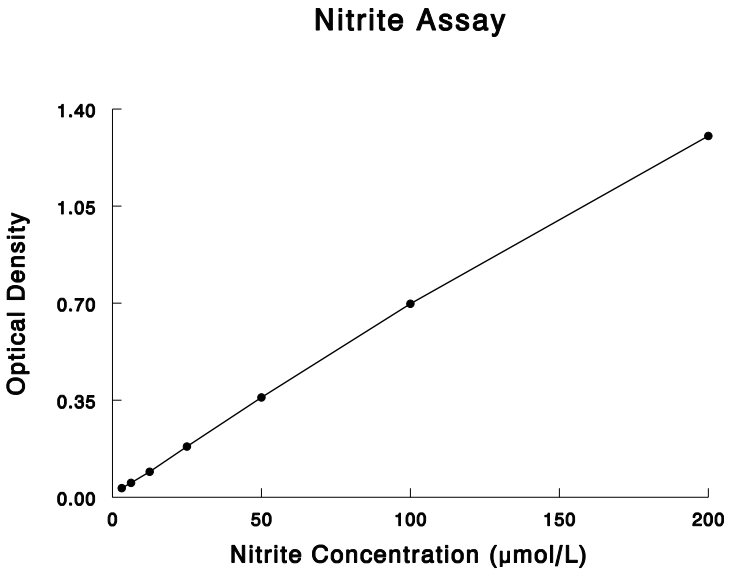
To determine the concentration of nitrate in the sample:

a. Measure the endogenous nitrite concentration (X μmol/L) using the Nitrite Assay Procedure.
b. Measure the total nitrite concentration (Y μmol/L) after the conversion of nitrate to nitrite using the Nitrate Reduction Assay Procedure.
c. Determine the nitrate concentration in the sample by subtracting the endogenous nitrite concentration from the total nitrite concentration.

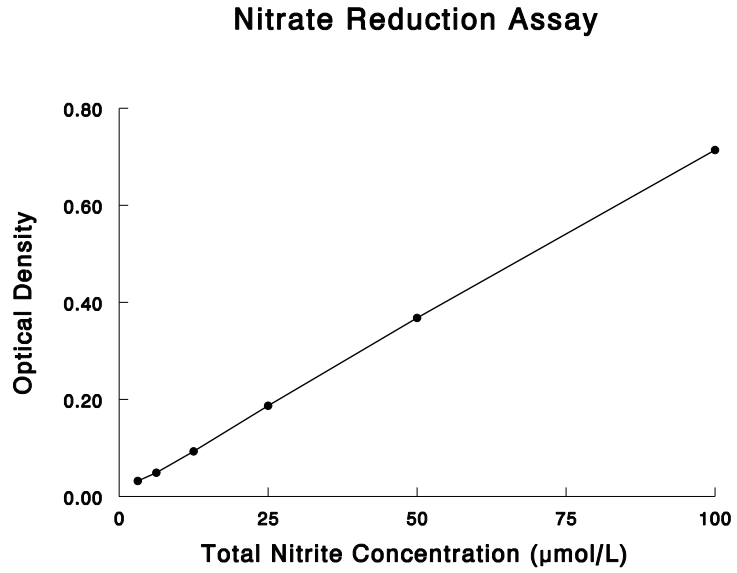
$$\text{Nitrate concentration} = (Y - X)\ \mu\text{mol/L}$$

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



µmol/L	O.D.	Average	Corrected
0	0.005 0.009	0.007	—
3.12	0.039 0.041	0.040	0.033
6.25	0.060 0.058	0.059	0.052
12.5	0.098 0.100	0.099	0.092
25	0.191 0.190	0.190	0.183
50	0.368 0.366	0.367	0.360
100	0.706 0.705	0.706	0.698
200	1.304 1.317	1.310	1.303



µmol/L	OD	Average	Corrected
0	0.081 0.079	0.080	—
3.12	0.113 0.111	0.112	0.032
6.25	0.128 0.129	0.128	0.048
12.5	0.166 0.180	0.173	0.093
25	0.264 0.269	0.266	0.186
50	0.448 0.447	0.448	0.368
100	0.793 0.795	0.794	0.714

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested eight times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in eight separate assays to assess inter-assay precision.

Nitrite Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	8	8	8	8	8	8
Mean (μmol/L)	8.0	25.1	64.1	7.7	25.7	64.6
Standard deviation	0.19	0.43	0.51	0.59	0.75	0.84
CV (%)	2.4	1.7	0.8	7.7	2.9	1.3

Nitrate Reduction Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	8	8	8	8	8	8
Mean (μmol/L)	8.7	19.2	25.5	10.5	16.9	21.9
Standard deviation	0.13	0.23	0.20	0.36	0.47	1.2
CV (%)	1.5	1.2	0.8	3.4	2.8	5.5

RECOVERY

The recovery of nitrite and nitrate spiked into samples in various matrices was evaluated.

Nitrite Assay

Sample Type*	Average % Recovery	Range
Cell Culture Media	100	100 - 101%
Human Serum	112	109 - 118%
Human Plasma (Citrate)	118	112 - 123%
Human Plasma (EDTA)	89	82 - 96%
Human Plasma (Heparin)	117	102 - 130%
Human Urine	110	110 - 111%
Human Saliva	96	92 - 101%

Nitrate Reduction Assay

Sample Type*	Average % Recovery	Range
Cell Culture Media	88	86 - 90%
Human Serum	93	84 - 106%
Human Plasma (Citrate)	98	97 - 99%
Human Plasma (EDTA)	91	86 - 102%
Human Plasma (Heparin)	96	93 - 99%
Human Urine	104	90 - 117%
Human Saliva	100	94 - 106%

*Samples were spiked, diluted and ultrafiltered prior to assay as directed in Sample Preparation.

SENSITIVITY

The sensitivity of the Nitrite Assay is typically less than 0.22 μmol/L.

The sensitivity of the Nitrate Assay is typically less than 0.54 μmol/L.

Sensitivity was determined by adding two standard deviations to the mean absorbance value of sixteen zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, Reaction Buffer (1X) spiked with nitrite and nitrate was assayed using serial 2-fold dilutions.

Nitrite Assay

Dilution	Observed (µmol/L)	Expected (µmol/mL)	% Observed / Expected
Neat	131	—	—
1:2	66.9	65.5	102
1:4	35.0	32.7	107
1:8	17.4	16.4	106
1:16	8.0	8.2	98

Nitrate Assay

Dilution	Observed (µmol/L)	Expected (µmol/mL)	% Observed / Expected
Neat	80.1	—	—
1:2	42.5	40.0	106
1:4	21.4	20.0	107
1:8	11.5	10.0	115
1:16	6.06	5.00	121

REFERENCES

1. Moncada, S. *et al.* (1989) Biochem. Pharm. **38**(11):1709.
2. Schreck, R. and P.A. Baeuerle (1991) Trends Cell Biology **1**:39.
3. Nathan, C. (1992) FASEB J. **6**:3051.
4. Stuehr, D.J. and M.A. Marletta (1985) Proc. Natl. Acad. Sci. USA **82**:7738.
5. Ignarro, L.J. (1990) Pharmacol. Toxicol. **67**:1.
6. Brendy, D.S. *et al.* (1990) Nature **347**:768.
7. Garthwaite, J. (1991) Trends Neuroscience **14**:60.
8. Conner, E.M. and M.B. Grisham (1995) Methods **7**:3.
9. Kendrich, K.M. *et al.* (1997) Nature **388**:670.
10. Nowad, R. (1992) J. NIH Res. **4**:49.
11. Miles, A.M. *et al.* (1996) Methods Enzymology **268**:105.
12. Makelo, S. *et al.* (1997) Clin. Chem. **43**:2418.
13. Grisham, M.B. *et al.* (1995) Comp. Meth. Enz. **7**:84.

Manufactured for R&D Systems by Assay Designs, Inc.

06.05 751508.0 6/05