Parameter™

Total NO/Nitrite/Nitrate Assay

Catalog Number KGE001

For the quantitative determination of Nitric Oxide concentrations in cell culture supernates, serum, plasma, and urine.

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

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

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INTRODUCTION

The biological activities of Nitric Oxide (NO) were first widely appreciated when it was identified as the endothelial-derived relaxing factor (EDRF) responsible for the potent vasodilating properties of stimulated endothelia (1 - 3). Since then, NO has been recognized as a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. It is a gaseous free radical with a short half-life *in vivo* of a few seconds or less. Therefore, the levels of the more stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), have been used in the indirect measurement of NO in biological fluids (4 - 7). Altered levels of NO have been shown to be associated with sepsis, reproduction, infection, hypertension, exercise, type 2 diabetes, hypoxia, and cancer (8 - 16).

Catalyzed by enzymes of the Nitric Oxide Synthase (NOS) family, NO is produced via the reaction of L-Arginine, NADPH, and O_2 to NO and Citrulline (17 - 20). Members of the NOS family include neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2) (21). As the name implies, nNOS is highly expressed in neurons of the central and peripheral nervous systems, and has also been described in other cell types including skeletal muscle myocytes, lung epithelial cells, and skin mast cells (22 - 29). eNOS is highly expressed by endothelial cells and may also be found in neurons, dermal fibroblasts, epidermal keratinocytes, thyroid follicular cells, hepatocytes, and smooth muscle cells (28, 30 - 34). iNOS is expressed in a wide range of cell types including chondrocytes, epithelial cells, hepatocytes, glial cells, and several cell types of the immune system (22, 35 - 37). In general, eNOS and nNOS are constitutively expressed and regulated by Ca^{2+}/Calmodulin, while iNOS is induced by endotoxin and inflammatory cytokines, and exhibits a relative insensitivity to Ca^{2+} (21, 38).

Because it is lipid soluble, NO is not stored but is synthesized *de novo* and freely diffuses across lipid membranes. NO has the potential to mediate its effects on target cells via several different mechanisms. For instance, NO-mediated activation of the enzyme Guanylyl Cyclase (GC) catalyzes the formation of the second messenger Guanosine 3’,5’-cyclic Monophosphate (cGMP). cGMP is implicated with a range of biological functions such as regulating smooth muscle contractility, cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, angiogenesis, and the activity of cyclic nucleotide-gated channels (39 - 47). NO also functions as an anti-tumor and anti-microbial agent via mechanisms that include its conversion to peroxynitrite ($ONOO^-$), the formation of S-nitrosothiols, and the depletion of arginine (35). Another putative role for NO includes the suppression of mitochondrial respiration through the inhibition of Cytochrome Oxidase (48). NO may also modify protein activity through post-translational nitrosylation via the attachment of an NO moiety to the thiol side chain of cysteine residues (49, 50).

R&D Systems’ Total Nitric Oxide kit has two assay options. Endogenous nitrite is measured in the first option. In the second option, nitrate is converted to nitrite using nitrate reductase and total nitrite is measured. To obtain the nitrate concentration, endogenous nitrite is subtracted from the total nitrite value.

PRINCIPLE OF THE ASSAY

This assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by 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 - 570 nm (51).

LIMITATIONS OF THE PROCEDURE

- **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 or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Reaction Diluent (1X) and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- 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.
- Pre-rinse the pipette tip when pipetting.
- Precautions should be taken to avoid the contamination of samples or buffers with outside sources of nitrites or nitrates. Possible sources of contamination include skin, saliva, food, drink, water, or laboratory supplies such as pipette tips, gloves, etc.
- To ensure accurate results, use of plate sealers during incubation steps is necessary to avoid assay contamination.

REAGENTS

Microplates (Part 892880) - Two 96 well polystyrene microplates (12 strips of 8 wells).

Nitrate Reductase (Part 892873) - 1 vial of lyophilized Nitrate Reductase.

Nitrate Reductase Storage Diluent (Part 892874) - 1.2 mL of a buffer containing glycerol.

NADH (Part 892875) - 2 vials of lyophilized reduced β-Nicotinamide adenine dinucleotide. **Must be stored in the dark.**

Nitrate Standard (Part 892876) - 0.5 mL of a Sodium Nitrate solution (2000 μmol/L) in buffer.

Nitrite Standard (Part 892886) - 0.5 mL of a Sodium Nitrite solution (2000 μmol/L) in buffer.

Reaction Diluent Concentrate (10X) (Part 892877) - 30 mL of a 10-fold concentrated buffer containing detergent.

Griess Reagent I (Part 892878) - 12 mL of Sulfanilamide in 2 N hydrochloric acid.

Griess Reagent II (Part 892879) - 12 mL of *N*-(1-Naphthyl) ethylenediamine in 2 N hydrochloric acid.

Plate Cover - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="7">Opened/ Reconstitued Reagents</td><td>Nitrate Reductase Storage Diluent</td><td rowspan="5">Store at 2 - 8° C for up to 1 month.*</td></tr>
<tr><td>Standards</td></tr>
<tr><td>Reaction Diluent (1X)</td></tr>
<tr><td>Griess Reagent I</td></tr>
<tr><td>Griess Reagent II</td></tr>
<tr><td>Nitrate Reductase</td><td rowspan="2">Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Diluted NADH</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, with the correction wavelength set at 690 nm
- Pipettes and pipette tips
- Deionized or distilled water
- 37° C incubator
- Vortex mixer
- 500 mL graduated cylinder
- 10,000 molecular weight cut-off filters for use in sample preparation, such as Millipore Microcon YM-10.
- Controls (optional; available from R&D Systems)

PRECAUTION

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

INTERFERENCE (See references 51 - 55)

The Griess reaction involves an oxidation and a nucleophilic reaction. Any reagents or substances that impact the Griess reaction will also likely alter color development or absorption. Examples of interfering substances are azide, ascorbic acid and sulfhydryl-containing compounds such as cysteine and glutathione. If concentrations of these compounds are expected to exceed 10 μM in the sample, nitrate/NO_3^- and nitrite/NO_2^- recovery should be determined using the nitrate and nitrite standards provided in the kit. Nitrate and nitrite concentrations similar to those used for the standard curve should be added to both the buffer containing the suspected interfering compound(s), and to an equivalent buffer that does not contain the suspected interfering compound. If significant differences are found in the buffer containing the interfering substance(s), the magnitude of the effect should be noted and suitable corrections made to the unknown samples.

In vivo, nitrite is converted to stable nitrate by hemoglobin. Thus nitrite levels are typically very low. With enzymatic conversion by nitrate reductase, all nitrite and converted nitrate is assayed, providing a comprehensive overview of total NO production. Problems with the enzyme assay include the presence of enzyme inhibitors and a failure to convert all nitrate to nitrite. Thus, strict controls should accompany all steps in the assay.

Proteins are also known to interfere with the Griess reaction. It is recommended that proteins be removed before analysis. Common deproteinizing protocols involve the use of $ZnSO_4$ or 35% sulfosalicylic acid, or ultrafiltration using 10,000 molecular weight cut-off filters. Anticoagulants are also a source of potential error.

The nitrate salt concentration must be considered when choosing a tissue culture media. Some media may contain unacceptably high levels of nitrate that may contribute to an over-estimation of NO production. A parallel situation may occur *in vivo*. Absorbed dietary nitrates may inflate NO estimates.

Absorbance interference is also a potential problem. Tissue culture media that uses phenol red as a pH indicator does not interfere with the Griess Reaction. Samples containing high protein levels may show considerable absorbance at 540 nm, so 10,000 MW filtration is recommended.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST). Centrifuge for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove plasma 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. Assay immediately or aliquot and store at ≤ -80° C. Avoid repeated freeze-thaw cycles. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

SAMPLE PREPARATION

Samples should be 10,000 MW filtered and then diluted prior to assay.

Serum and plasma samples require a 2-fold dilution. A suggested 2-fold dilution is 100 μL sample + 100 μL Reaction Diluent (1X).

Cell culture supernate and urine samples require at least a 5-fold dilution. A suggested 5-fold dilution is 50 μL sample + 200 μL Reaction Diluent (1X).

Note: *Some cell culture supernate and urine samples may contain high concentrations of nitrate and may require up to a 20-fold dilution.*

Note: *Samples containing 0.5 - 1.0 mM NADPH may require at least a 10 fold dilution in Reaction Diluent (1X). In samples containing > 1.0 mM NADPH, the NADPH should be oxidized prior to the Griess Reaction. See Reference 56 for an example of an appropriate oxidation procedure.*

REAGENT PREPARATION

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

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

Nitrate Reductase

Reconstitution - Reconstitute the Nitrate Reductase with 1.0 mL Nitrate Reductase Storage Diluent. 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 and use immediately.

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 5 μL.

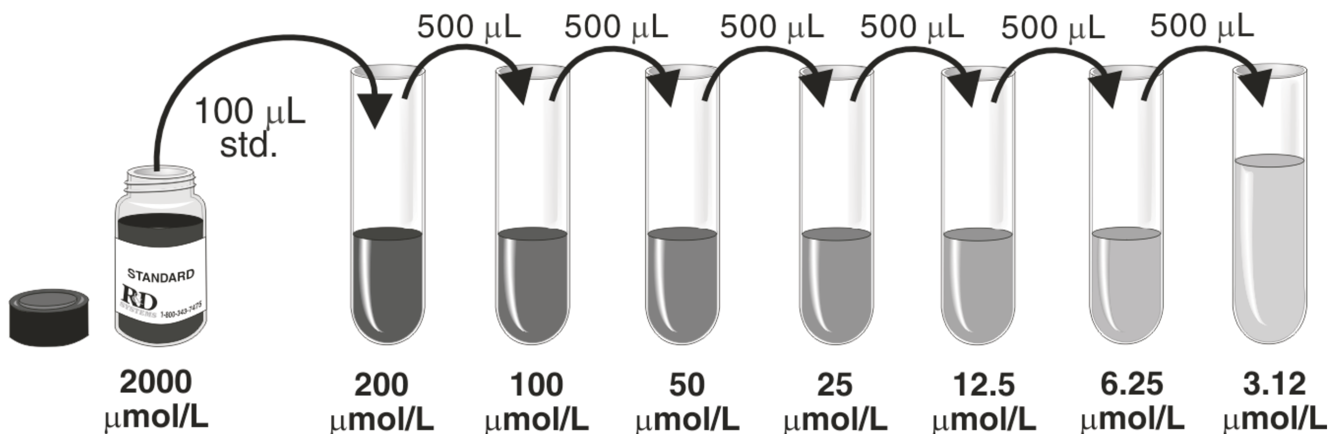
B. Reaction Diluent (1X) (μL) = volume from step A x 4.

C. Add volumes from steps A and B to a clean test tube and vortex.

D. Place on ice and use within 15 minutes of dilution.

NADH Reagent - Reconstitute the NADH with 5.0 mL deionized or distilled water. Allow the NADH to sit for 3 minutes with gentle agitation prior to use. **Use within 15 minutes or place on ice.**

Nitrite/Nitrate Standard - Pipette 900 μL of Reaction Diluent (1X) into the 200 μmol/L tube. Pipette 500 μL of Reaction Diluent (1X) into the remaining tubes. Use the appropriate 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 Diluent (1X) serves as the blank (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 50 µL of Reaction Diluent (1X) to the Blank wells.
4. Add 50 µL of Nitrite Standard or sample* to the remaining wells.
5. Add 50 µL of Reaction Diluent (1X) to all wells.
6. Add 50 µL Griess Reagent I to all wells.
7. Add 50 µL Griess Reagent II to all wells. Mix well by tapping the side of the plate gently.
8. Incubate for 10 minutes at room temperature.
9. Determine the optical density (OD) of each well using a microplate reader set at 540 nm (wavelength correction at 690 nm).

NITRATE REDUCTION ASSAY PROCEDURE

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 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 50 µL of Reaction Diluent (1X) to the Blank wells.
4. Add 50 µL of Nitrate Standard or sample* to the remaining wells.
5. Add 25 µL of NADH to all wells.
6. Add 25 µL of diluted Nitrate Reductase** to all wells. Mix well and cover with the adhesive strip provided.
7. Incubate for 30 minutes at **37° C**.
8. Add 50 µL of Griess Reagent I to all wells.
9. Add 50 µL of Griess Reagent II to all wells. Mix well by tapping the side of the plate gently.
10. Incubate for 10 minutes at room temperature.
11. Determine the optical density (OD) of each well using a microplate reader set at 540 nm (wavelength correction at 690 nm).

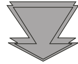
*Samples must be filtered and diluted. See Sample Preparation.

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

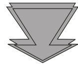
ASSAY PROCEDURE SUMMARY

Nitrite Assay

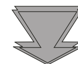
1. Prepare reagents, samples, and standards as instructed.



2. Add 50 μL Reaction Diluent (1X) to the Blank wells.

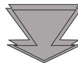


3. Add 50 μL Nitrite Standard or sample* to the remaining wells.



4. Add 50 μL Reaction Diluent (1X) to all wells.

5. Add 50 μL Griess Reagent I to each well.



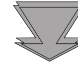
6. Add 50 μL Griess Reagent II to each well. Mix well by gently tapping the side of the plate.

7. Incubate for 10 minutes at RT.

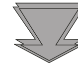
Determine the optical density (OD) of each well using a microplate reader set at 540 nm/690 nm.

Nitrate Reduction Assay

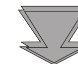
1. Prepare reagents, samples, and standards as instructed.



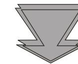
2. Add 50 μL Reaction Diluent (1X) to the Blank wells.



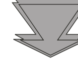
3. Add 50 μL Nitrate Standard or sample* to the remaining wells.



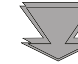
4. Add 25 μL NADH to all wells.



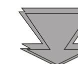
5. Add 25 μL diluted Nitrate Reductase to all wells. Mix well and cover with an adhesive strip.



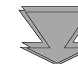
6. Incubate for 30 minutes at **37° C.**



7. Add 50 μL Griess Reagent I to each well.



8. Add 50 μL Griess Reagent II to each well. Mix well by gently tapping the side of the plate.



9. Incubate for 10 minutes at RT.

Determine the optical density (OD) of each well using a microplate reader set at 540 nm/690 nm.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

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

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the concentration versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If 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:

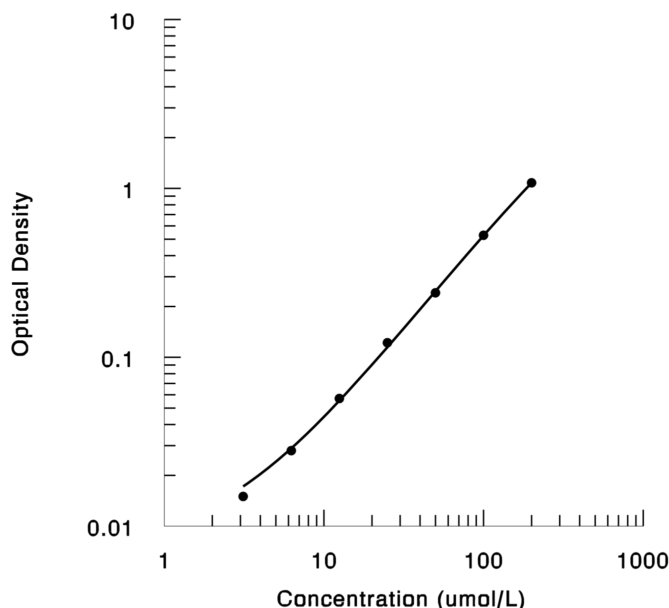
a. Measure the endogenous nitrite concentration (X μmol/L) using the Nitrite Assay Procedure.
b. Measure the total nitrite concentration (Y μmol/L) after the conversion of nitrate to nitrite using the Nitrate Reduction Assay Procedure.
c. Determine the nitrate concentration in the sample by subtracting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

   Nitrate concentration = (Y - X) μmol/L

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



μmol/L	O.D.	Average	Corrected
0	0.014 0.016	0.015	—
3.12	0.030 0.030	0.030	0.015
6.25	0.041 0.044	0.043	0.028
12.5	0.070 0.073	0.072	0.057
25	0.133 0.140	0.137	0.122
50	0.254 0.257	0.256	0.241
100	0.528 0.557	0.543	0.528
200	1.071 1.118	1.095	1.080

PRECISION

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

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

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (μmol/L)	30.0	77.2	138.0	31.5	79.9	138.7
Standard deviation	0.76	1.10	2.20	1.46	2.91	4.78
CV (%)	2.5	1.4	1.6	4.6	3.6	3.4

RECOVERY

The recovery of the nitrate standard spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Serum (n=10)	95	89 - 101%
Plasma (n=25)	98	87 - 110%

The recovery of the nitrite standard spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Serum (n=10)	109	98 - 118%
Plasma (n=20)	104	92 - 117%
Urine (n=10)	101	87 - 112%
Cell Culture Supernates (n=10)	107	91 - 115%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of the nitrate standard were serially diluted with the Reaction Diluent (1X) to produce samples with values within the dynamic range of the assay.

		Serum* (n=10)	Cell culture supernates* (n=10)	Heparin plasma* (n=10)	EDTA plasma* (n=10)	Citrate plasma* (n=5)	Urine* (n=10)
1:2	Average % of Expected	101	98	99	99	99	100
	Range (%)	96 - 105	96 - 102	92 - 105	96 - 103	95 - 101	96 - 103
1:4	Average % of Expected	101	99	98	98	99	102
	Range (%)	98 - 103	94 - 104	89 - 104	95 - 102	96 - 101	97 - 105
1:8	Average % of Expected	105	101	102	102	101	107
	Range (%)	101 - 109	93 - 111	92 - 109	99 - 108	95 - 105	99 - 119
1:16	Average % of Expected	112	106	108	111	106	108
	Range (%)	104 - 118	102 - 121	92 - 120	100 - 119	96 - 111	—

*Samples were diluted prior to assay. See Sample Preparation section.

SENSITIVITY

Fifty-two assays were evaluated and the minimum detectable dose (MDD) ranged from 0.09 - 0.78 μmol/L. The mean MDD was 0.25 μmol/L.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma - Samples were filtered and diluted and then evaluated for the presence of NO_2^- in the nitrite assay. All samples measured below the lowest standard, 3.12 μmol/L. Samples were also evaluated for the presence of total nitric oxide (NO_2^-/NO_3^-) in the nitrate reduction assay. Results are shown in the table below.

Sample Type	Mean (μmol/L)	Standard Deviation (μmol/L)	Range (μmol/L)
Serum* (n=25)	37	22	13 - 97
EDTA plasma* (n=25)	37	21	10 - 92
Heparin plasma* (n=25)	36	20	10 - 90
Citrate plasma* (n=8)	30	23	11 - 81
Urine* (n=10)	1396	877	369 - 2684
Cell culture supernates* (n=10)	502	414	11 - 850

*Samples were filtered and diluted prior to assay. See Sample Preparation section.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

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