



## Total Nitric Oxide Assay Kit

Catalog No. 917-020

192 Determination Kit

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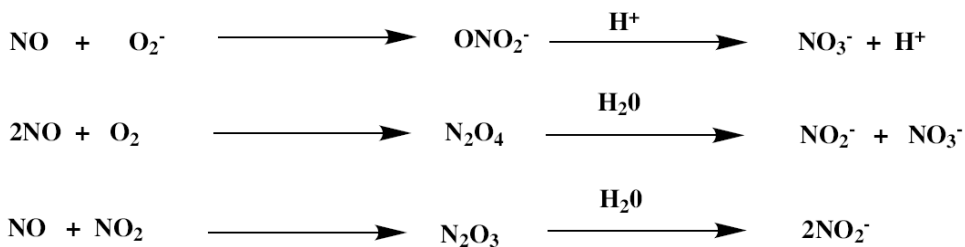
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## **Description**

Assay Designs™ Total Nitric Oxide Assay Kit is a complete kit for the quantitative determination of total Nitric Oxide (NO) in biological fluids. Please read the entire kit insert before performing this assay. The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme Nitrate Reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. The conversion of NO into nitrate and nitrite by the reactions outlined below, varies in each system. The interaction of NO in a system is measured by the determination of the total nitrate and nitrite concentrations in the sample. This kit allows for the total determination of both NO products in the sample by conversion of all the sample nitrate into nitrite, followed by the determination of the total concentration of nitrite in the sample.

## **Introduction**

Nitric oxide is a major mammalian secretory product that initiates host defense, homeostatic and developmental functions by either direct effect or intercellular signaling<sup>1</sup>. NO is the product of a five-electron oxidation of the amino acid L-arginine mediated by nitric oxide synthase<sup>2</sup>. As a direct effector, NO is thought to activate regulatory proteins, kinases and proteases that are directed by reactive oxygen intermediates<sup>3</sup>. As a messenger molecule, NO covalently interacts with target molecules based on redox potential rather than noncovalent complementarity<sup>1</sup>. Activation of the immune system is associated with an increase in macrophage NO production<sup>4</sup>. NO exerts a variety of homeostatic influences as an activator of soluble guanylyl cyclase<sup>5</sup>, a neuronal potentiator<sup>6</sup>, a peripheral nervous system neurotransmitter<sup>7</sup>, and a contraction regulator of both smooth muscle and vascular tissue<sup>8</sup>. In addition, NO has been linked to the formation of olfactory<sup>9</sup> and synaptic memories and remodeling<sup>10</sup>. The transient and volatile nature of NO makes it unsuitable for most convenient detection methods; however, two stable breakdown products, nitrate (NO<sub>3</sub>) and nitrite (NO<sub>2</sub>) can be easily detected by photometric means.



## Precautions

1. The Griess Reagents I and II are dissolved in 2M hydrochloric acid. **CAUTION:** Caustic; care should be taken in use. The Griess Reagents I and II contain organic chemicals. Care should be taken in handling these materials.
2. The water used for any dilutions of samples or reagents should be free of endogenous nitrate and nitrite. We suggest the use of deionized or distilled water. Take care to avoid nitrate/nitrite contamination of samples or buffers.
3. Do not mix components from different lots of kits.
4. Dispose of the contents of the plate with care. Attention should be taken in handling because of unknown effects of the contents.
5. Care should be used in the selection of disposable gloves and transfer pipettes as sources of contamination. Please refer to reference 11 concerning nitrate and nitrite contamination in these products.

## Materials Supplied

1. **Microtiter Plate, 2 Each, Catalog No. 80-0144**  
Break-apart microtiter plate. The plate is ready to use.
2. **Nitrate Reductase, 1 Bottle, Catalog No. 80-1516**  
One bottle containing 2 lyophilized vials of Nitrate Reductase.
3. **Nitrate Reductase Storage Buffer, 4 mL, Catalog No. 80-0255**  
A phosphate based buffer containing preservatives.
4. **Desiccated NADH, 2 Bottles, Catalog No. 80-0258**  
Vials of lyophilized reduced  $\beta$ -Nicotinamide adenine dinucleotide, Catalog No. 80-0256.  
**Store in the Dark.**
5. **Nitrate Standard, 0.5 mL, Catalog No. 80-0223**  
A solution of sodium nitrate at 1,000  $\mu$ mole/L in water with preservatives.
6. **Reaction Buffer 10x Concentrate, 30 mL, Catalog No. 80-0257**  
A HEPES based buffer containing detergent and preservatives.
7. **Griess Reagent I, 12 mL, Catalog No. 80-0227**  
A solution of sulfanilamide in 2M hydrochloric acid.
8. **Griess Reagent II, 12 mL, Catalog No. 80-0228**  
A solution of N-(1-Naphthyl)ethylenediamine in 2M hydrochloric acid.
9. **Plate Sealers, 2 each, Catalog No. 30-0012**
10. **Total Nitric Oxide Assay Layout Sheet, 1 each, Catalog No. 30-0219**

## Storage

All components of this kit are stable at 4°C until the kit's expiration date, **except the Nitrate Reductase enzyme which must be stored at -20°C prior to and after reconstitution.**

## **Materials Needed but Not Supplied**

1. Deionized or distilled water.
2. Precision pipets for volumes between 25  $\mu$ L and 1,000  $\mu$ L.
3. Repeater pipets for dispensing 25  $\mu$ L and 50  $\mu$ L.
4. Disposable beakers for diluting Reaction Buffer.
5. Graduated cylinders.
6. Ice bath or refrigerated container capable of maintaining 0°C.
7. A 37°C Incubator.
8. Microplate reader capable of reading between 540-570 nm.
9. Graph paper for plotting the standard curve.
10. 10,000 MWCO polysulfone filters, Sigma #M0911 or similar based on application needs.

## **Interferences**

Assay Designs' Total Nitric Oxide assay is compatible with nitrite and nitrate in samples in a wide range of matrices. Samples diluted into Reaction Buffer or water and passed through MWCO filters can be read directly from the standard curve. The Griess reaction involves the interaction of nitrite ions with two organic molecules and involves an oxidation and nucleophilic reaction. Buffer or sample components that may interfere with this oxidation and nucleophilic reaction may interfere with color formation. The conversion of nitrate to nitrite involves the enzyme Nitrate Reductase. Any sample or buffer component that may interfere with this enzyme will lower the conversion of sample nitrate to nitrite and therefore give rise to lower estimates of NO.

Examples of nucleophiles and antioxidants that may interfere with the assays are azide, ascorbic acid, sulphhydryl containing compounds such as cysteine, glutathione, DTT and  $\beta$ -mercaptoethanol. If concentrations of these materials are to be in excess of 10  $\mu$ M in the sample, a test of nitrate recovery should be made using the nitrate provided with the kit. 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 is a significant change in the nitrate concentrations found in the buffer containing the interfering compound the effect should be determined and suitable corrections made.

**Some tissue culture media, such as RPMI, contain high nitrate concentrations and should not be used as this will 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. Certain systems involving NO synthetase enzymes utilize high concentrations (0.5-1 mM) of NADPH which may inhibit the Griess color reaction slightly. Care should be taken to ensure that these are diluted sufficiently ( $\geq$  1:10) in Reaction Buffer to minimize any effect of NADPH. If samples may contain excessive amounts of NADPH this can be oxidized using Lactate Dehydrogenase (LDH) and pyruvic acid prior to color formation<sup>12</sup>. 10  $\mu$ L of 1,500U/mL LDH (Sigma, L-1378) in 30 mM sodium pyruvate (P-2256) is added to all wells after incubation with Nitrate Reductase and incubated at 37°C for 10 minutes prior to addition of the Griess reagents.

## **Sample Handling**

The dilutions recommended are to remove matrix interference in the assay. The optimal dilution for each experiment should be determined by the investigator.

### **Whole Organism Samples**

In studies on isolated organisms the environmental nitrite and nitrate must be taken into account. Any media or fluid that the organism is stored in should be analyzed separately. Adjustments to the nitrite and nitrate in the organism must take into account the turnover of environmental nitrite and nitrate by the organism.

### **Urine**

Fresh urine samples should be diluted at least 1:20 into Reaction Buffer, ultrafiltered through a 10,000 Molecular Weight Cut Off (MWCO) filter and used directly in the assay. If the samples need to be stored, either suitable antibiotics, such as penicillin or streptomycin at 100 U/mL, or 2-propanol at 6.5% (v/v) can be added prior to storing at -80°C.

### **Saliva**

Saliva samples should be diluted 1:2 - 1:100 into Reaction Buffer, ultrafiltered through a 10,000 MWCO filter and used directly in the assay. Typical saliva samples may contain a relatively high concentration of nitrate which is thought to be produced by oral bacteria.

### **Plasma**

Citrate plasma is recommended. Plasma should be used directly in the assay after a 1:2 - 1:20 dilution into Reaction Buffer and ultrafiltration through a 10,000 MWCO filter. EDTA or heparinized plasmas may be used in the assay after dilution into Reaction Buffer and ultrafiltration through a 10,000 MWCO filter, however they may not give reproducible results as the protein may precipitate during the Griess reaction.

### **Serum**

Serum should be diluted 1:2 - 1:20 into Reaction Buffer ultrafiltered through a 10,000 MWCO filter and used directly in the assay.

### **Culture supernates**

Avoid media containing nitrate salts. Samples should be diluted at least 1:2 in Reaction Buffer and ultrafiltered through a 10,000 MWCO filter and used directly in the assay.

## **Procedural Notes**

1. Do not mix reagents from different lot numbers or use reagents beyond the expiration date.
2. Allow all reagents, except the Nitrate Reductase enzyme, to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes; no difference in assay result is seen with either type of tube.
4. Pre-rinse the pipet tip with each reagent, use fresh pipet tips for each sample, standard, and reagent.
5. Add the reagents to the side of the well to avoid contamination.
6. The Nitrate Reductase enzyme must be kept at 0°C during use. The enzyme solution must be stored at -20°C both prior to use and after reconstitution.
7. **FOR RESEARCH USE ONLY, NOT FOR DIAGNOSTIC USE.**

## **Reagent Preparation**

### **1. Reaction Buffer**

Prepare the Reaction Buffer by diluting 10 mL of the supplied concentrate with 90 mL of deionized water. This can be stored at room temperature for 3 months.

### **2. NADH Reagent**

Reconstitute a NADH vial by adding 1 mL of water. Wait for 3 minutes and vortex prior to use. **Use on ice. Store unused NADH solution tightly capped at -20°C. Stable at -20°C for 45 days.**

**Final NADH Dilution.** Prior to use **take 0.9 mL of the NADH solution and add 1.8 mL of water. Vortex. Use on ice, and store tightly capped at -20°C. Stable at -20°C for 45 days.** Avoid repeated freeze/thaw cycles.

### 3. Nitrate Reductase Enzyme Reconstitution

**Enzyme Dilution I:** Reconstitute the Nitrate Reductase vial with 1 mL of Nitrate Reductase Storage Buffer. Vortex vigorously. Let sit **at room temperature** for 15 minutes. Vortex again. Let sit **at room temperature** for an additional 15 minutes. Vortex. **Use on ice, and store tightly capped at -20°C. Stable at -20°C for 3 months.** Avoid repeated freeze/thaw cycles.

**Final Enzyme Dilution:** Count the total number of wells needed for the samples and add 14 (for the complete standard curve in duplicate). Use the following formula to calculate the volume of **Enzyme Dilution I** and **Reaction Buffer** required for the **Final Enzyme Dilution**.

A. Volume of Enzyme Dilution I ( $\mu\text{L}$ ) =  
[Number of wells, incl. Standard Curve Wells + 2] x 10  $\mu\text{L}$

B. Volume of Reaction Buffer ( $\mu\text{L}$ ) =  
[Volume of Enzyme Dilution I in  $\mu\text{L}$ ( from A. above)] x 1.5  $\mu\text{L}$

Immediately before use, pipet the volume of Enzyme Dilution I from A. above and add to it the volume of Reaction Buffer from B. above. Vortex and **use on ice** within **15 minutes**.

### 4. Nitrate Standard

Allow the 1,000  $\mu\text{mole/L}$  Nitrate Standard solution to warm to room temperature. Label six 12 x 75 mm glass tubes #1 through #6. Pipet 900  $\mu\text{L}$  of Reaction Buffer into tube #1. Pipet 500  $\mu\text{L}$  of Reaction Buffer into tubes #2 - #6. Add 100  $\mu\text{L}$  of the 1,000  $\mu\text{mole/L}$  standard to tube #1. Vortex thoroughly. Add 500  $\mu\text{L}$  of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #6.

**The concentration of Nitrate standard in tubes #1 through #6 will be 100, 50, 25, 12.5, 6.25, and 3.125  $\mu\text{mole/L}$  respectively. See Total Nitric Oxide Assay Layout Sheet for dilution details.**

## **Assay Procedure**

1. Determine the number of wells to be used.
2. All standards and samples should be run in duplicate.
3. Pipet 200  $\mu\text{L}$  of Reaction Buffer into duplicate Blank wells.
4. Pipet 50  $\mu\text{L}$  of Standards #1 through #6 into duplicate wells.
5. Pipet 50  $\mu\text{L}$  of Reaction Buffer into duplicate wells to act as a Zero Standard.
6. Pipet 50  $\mu\text{L}$  of Samples into duplicate wells.
7. Pipet 25  $\mu\text{L}$  of **Final NADH dilution** into all Zero Standard, Standard and Sample wells.
8. Pipet 25  $\mu\text{L}$  of the Nitrate Reductase **Final Enzyme Dilution** into all Zero Standard, Standard and Sample wells.
9. Mix well by gently shaking or tapping the side of the plate and apply a plate sealer. Incubate for 30 minutes at 37°C.
10. Pipet 50  $\mu\text{L}$  of the Griess Reagent I into each well, except the Blank wells.
11. Pipet 50  $\mu\text{L}$  of the Griess Reagent II into each well, except the Blank wells.
12. Mix well by shaking or tapping the side of the plate.
13. Incubate the plate at room temperature for 10 minutes.
14. Read the optical density of each well at 540 -570 nm after blanking against the Blank wells.

## **Calculation of Results**

Several options are available for the calculation of the concentration of Total NO in the samples.

1. Calculate the average net Optical Density (OD) found for each standard and sample by subtracting the average Zero Standard OD from the average OD for each standard and sample.  
$$\text{Average Net OD} = \text{Average OD} - \text{Average Zero Standard OD}$$
2. Plot the Average Net OD for each Standard Concentration.
3. Plot the Average OD for each Sample and extrapolate Total NO concentration from the graph.



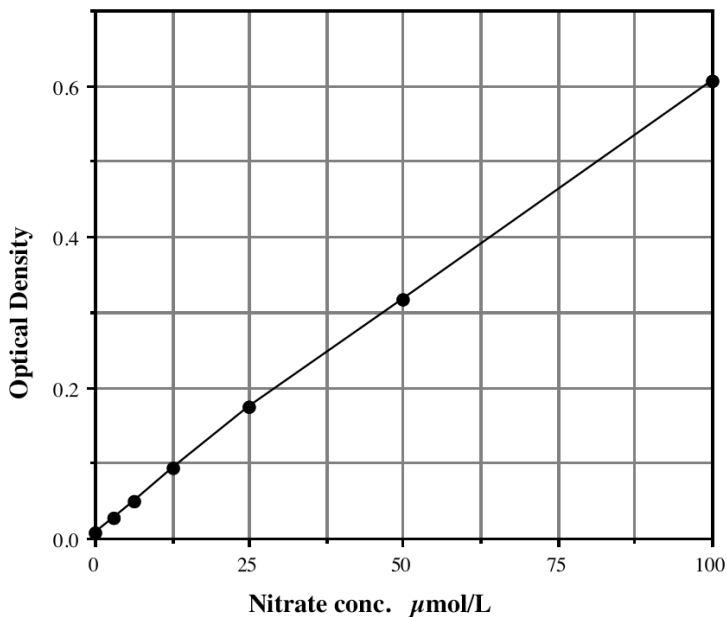
## Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average OD	Net OD	Nitrate ( $\mu\text{mole/L}$ )
Blank	0.038		
S1	0.644	0.606	100
S2	0.355	0.317	50
S3	0.212	0.174	25
S4	0.132	0.094	12.5
S5	0.086	0.048	6.25
S6	0.066	0.028	3.125
Zero	0.046	0.008	0

## Typical Standard Curve

A typical standard curve is shown below. The curve **must not** be used to calculate concentrations; each user must run a standard curve for each assay.



## Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols 13.

### Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty-four (24) wells run as the zero standard, and comparing to the average optical density for wells run with Standard #6. The detection limit was determined as the concentration measured at two (2) standard deviations from the zero along the standard curve.

OD for Zero Standard =	0.008 ± 0.004
OD for Standard #6 =	0.028 ± 0.002
Delta Optical Density (3.125-0 µmole/L) = 0.028 - 0.008 =	0.020
2 SD's of S0 =	0.0040
Sensitivity = $\frac{0.0040}{0.020} \times 3.125 \mu\text{mole/L} =$	0.625 µmole/L

### Linearity

A sample containing 75 µmole/L Nitrate was serially diluted 1:2 four times in the Reaction Buffer supplied in the kit and measured in the Nitrate assay. The data was plotted graphically as actual Nitrate concentration versus measured Nitrate concentration.

The line obtained had a slope of 1.011 with a correlation coefficient of 0.999.

### Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Nitrate and running these samples multiple times (n=8) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Total Nitrate in multiple assays (n=8). The precision numbers listed below represent the percent coefficient of variation for the concentrations of Nitrate determined in these assays, as calculated by a linear curve fitting program.

	Nitrate (µM/L)	Inter-assay (%CV)	Intra-assay (%CV)
Low	20.86		5.3%
Medium	50.92		3.1%
High	68.51		1.2%
Low	18.67	6.9%	
Medium	48.80	4.2%	
High	68.25	3.3%	

## Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Nitrate was spiked into samples which had been diluted with the kit Reaction Buffer. The diluted samples were ultrafiltered through a 10,000 MWCO filter and assayed in the kit. The following results were obtained:

Sample	% Recovery	Recommended Dilution
Tissue Culture Media	88%	1:2
Human Urine	104%	1:20
Human Saliva	100%	1:2-1:100
Human Serum	93%	1:2-1:20
Citrate Plasma	98%	1:2-1:20
Human EDTA Plasma	91%	1:2-1:20

Please note that the recommended dilutions for the Nitrate Assay take into account the normal levels of Nitrate in some samples. All samples should be diluted 1:2 in Reaction Buffer, but some samples may need to be diluted 1:2 to 1:200 to read on the Nitrate Standard Curve. See Sample Handling instructions on page 4 for full details.

## References

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## **LIMITED WARRANTY**

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**Material Safety Data Sheet (MSDS) available on our website or by fax.**

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Catalog No. 25-0551

March 10, 2008

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