

# Nitrate/Nitrite Fluorometric Assay Kit

*Catalog No. 850-012-KI01 • 2 x 96 wells*

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## CONTENTS OF THE KIT

Number	Item	Quantity
1	Assay Buffer	1 vial
2	Nitrate Reductase	2 vials
3	Enzyme Cofactors	2 vials
4	Nitrate Standard	1 vial
5	Nitrite Standard	1 vial
6	DAN Reagent	1 vial
7	Sodium Hydroxide	1 vial
8	96 Well Plate	3 plates
9	Plate Cover	3 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at 41 62 926 8989 or We cannot accept any returns without prior authorization.

## PRECAUTIONS

- Please read these instructions carefully before beginning this assay.
- For research use only. Not for human or diagnostic use.

## WARRANTY AND LIMITATION OF REMEDY

ALEXIS Biochemicals Corp. makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery. Buyer's exclusive remedy and ALEXIS Biochemicals Corp's sole liability hereunder shall be limited to refund of the purchase price of, or at ALEXIS Biochemicals Corp's option, the replacement of, all material that does not meet our specifications. ALEXIS Biochemicals Corp. shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling. Said refund or replacement is conditioned on Buyer giving written notice to ALEXIS Biochemical Corp. within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within said thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

## IF YOU HAVE PROBLEMS

Our technical support staff may be reached by phone (USA) (800) 658-0065, (Europe) 41 61 926 8989, (UK) 44 1949 836111. In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit.

## STORAGE AND STABILITY

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

## ADDITIONAL ITEMS REQUIRED

1. A fluorometric plate reader with the capacity to measure fluorescence using excitation wavelengths of 375 or 360-365 nm and emission wavelengths of 415 or 430-450 nm, respectively.
2. An adjustable pipettor.
3. A source of pure water. Glass distilled water or HPLC-grade water is acceptable.

## NITRIC OXIDE BACKGROUND

Nitric Oxide (NO) is synthesized in biological systems by the enzyme Nitric Oxide Synthase (NOS). NOS is a remarkably complex enzyme which acts on a pair of substrates (molecular oxygen and arginine) to produce two products (NO and citrulline). This process requires five essential cofactors (FMNH<sub>2</sub>, FADH, NADPH, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron; see Figure 1 below). Three distinct isoforms of NOS have been identified, as detailed in Figure 2 (See below).

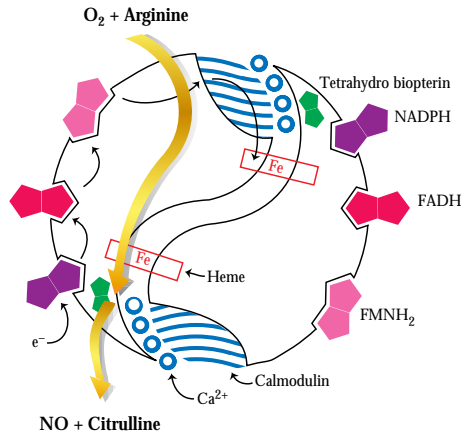


Figure 1. Nitric Oxide Synthesis

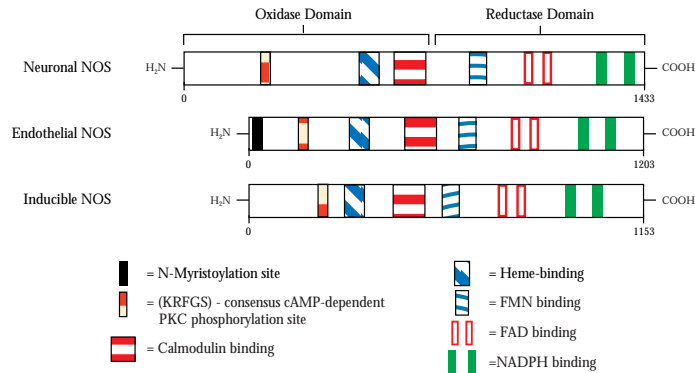
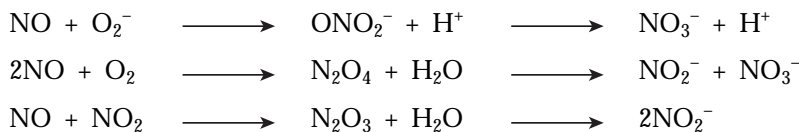


Figure 2. Nitric Oxide Synthase Isoforms

## ABOUT THIS ASSAY

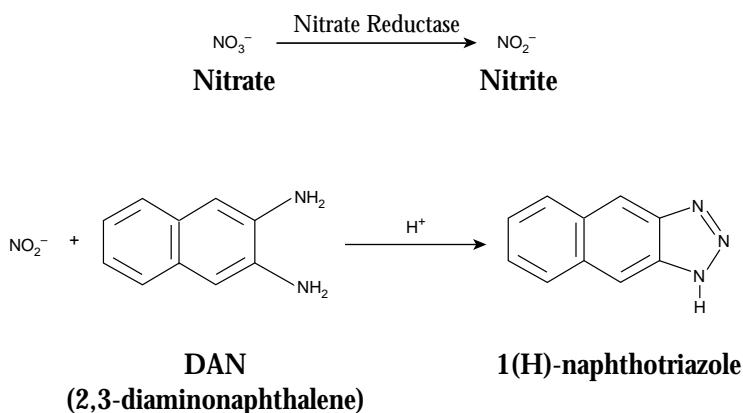
Nitric Oxide (NO) is produced in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli.<sup>1,2</sup> This NO is scavenged rapidly ( $t_{1/2} = 4$  seconds) and acts in a paracrine fashion to transduce cellular signals. NO interacts with the HEME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

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



The final products of NO *in vivo* are nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ). The relative proportion of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both  $\text{NO}_2^-$  and  $\text{NO}_3^-$ .

The ALEXIS Corp. Nitrate/Nitrite Fluorometric Assay Kit provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of DAN followed by NaOH which converts nitrite into a fluorescent compound (see Figure 3). Measurement of the fluorescence of this compound accurately determines  $\text{NO}_2^-$  concentration.<sup>4,5</sup>



*Figure 3. Chemistry of Nitrate/Nitrite Detection*

## PRE-ASSAY PREPARATION

### Reconstitution of the Reagents

Some of the kit components are in lyophilized form and need to be reconstituted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used to reconstitute the vial components.

#### 1. Assay Buffer

Dilute the contents of the Assay Buffer vial to 100 ml with HPLC-grade water. This Assay Buffer should be used for dilution of samples as needed prior to assay. Store at 4°C when not in use.

#### 2. Nitrate Reductase (vial #2)

Reconstitute the contents of the vial with 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of this solution should be limited to 1 time.

#### 3. Enzyme Cofactors (vial #3)

Reconstitute the contents of the vial with 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freezing and thawing of this solution should be limited to 1 time.

#### 4. Nitrate standard (vial #4)

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

#### 5. Nitrite standard (vial #5)

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

#### 6. Fluorometric reagents DAN and NaOH (vials #6 and #7)

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

\*NOTE: After reconstitution the standards must be further diluted prior to performing the assay (see page 4 for details).

## Plate Configuration

There is no specific pattern for using the wells on the plate. However, nine wells will be needed for the standard curve. For assays done using tissue culture media, the standard curve(s) should be done in the presence of this media. If you plan to measure total NO products (nitrate + nitrite), only the nitrate standard curve is required. If only nitrite is being measured, then only the nitrite standard curve is needed. The remaining wells on the plate can then be used for the assay of your samples. We suggest you record the contents of each well on the template sheet provided (See page 10).

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

### PIPETTING HINTS

- It is recommended that a repeating pipettor be used to deliver substrate, enzymes, and color development reagents to the wells. This saves time and helps to maintain more precise times of incubation.
- Use different tips to pipet the Assay Buffer, standard, sample, and color development reagents.
- Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e., fill the tip and expel the contents; repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the well.

## Sample Preparation

The kit has been validated in culture media and plasma. Some sample purification from these sources is necessary using the special instructions below.

### 1. Culture Media

Some types of tissue culture media contain very high nitrate levels (e.g., 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. Phenol red and fetal bovine serum can cause a significant reduction in the intensity of the fluorescence. Whenever possible, these components should be excluded from culture media. The effect of media components on the intensity of the fluorescence must be assessed by making the nitrite or nitrate standard curve in the presence of the amount of media to be used in the assay. To obtain maximum signal response, it is best to limit the amount of sample to 10 or 20  $\mu\text{l}$ . Higher volumes of sample can be used (30-50% of the final reaction volume) however, the fluorescence can be significantly quenched under these conditions. To make the standard curve in the presence of media, simply prepare the nitrate or nitrite standard curve (See pages 6-7) substituting the amount of media desired in place of Assay Buffer. For the measurement of nitrate plus nitrite, an incubation of 1 hour is necessary for the reaction to reach completion.

### 2. Plasma or Serum

Ultrafilter plasma and serum samples through a 10 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. This procedure will remove hemoglobin, which causes a drastic reduction in the intensity of the fluorescence. Assay for nitrate and/or nitrite using a maximum of 10  $\mu\text{l}$  of the filtrate. The conversion of nitrate to nitrite requires 1-2 hours for  $\geq 95\%$  conversion.

### 3. Tissue Homogenates

Homogenize the sample in PBS (pH 7.4) and centrifuge at 100,000 x g for 20 minutes. Ultrafilter tissue homogenates through a 10 kDa molecular weight cut-off filter. Assay for nitrate and/or nitrite using 10  $\mu\text{l}$  of the filtrate. The conversion of nitrate to nitrite requires 2 hours for  $\geq 95\%$  conversion.

## MEASUREMENT OF NITRATE + NITRITE

### Preparation of nitrate standard curve

A nitrate standard curve must be performed in order to quantitate sample nitrate + nitrite concentrations. In a clean test tube place 0.9 ml of Assay Buffer. To this, add 0.1 ml of reconstituted nitrate standard and vortex. Use this diluted standard (200  $\mu\text{M}$ ) for the preparation of the nitrate standard curve as described below.

Obtain eight clean test tubes and number them #1 through #8. Aliquot 950  $\mu\text{l}$  of Assay Buffer to tube #1 and 500  $\mu\text{l}$  of Assay Buffer to tubes #2-8. Transfer 50  $\mu\text{l}$  of Nitrate Standard as prepared above into tube #1 and mix thoroughly. The concentration of standard in tube #1 is 10  $\mu\text{M}$ . Serially dilute the nitrate by removing 500  $\mu\text{l}$  from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500  $\mu\text{l}$  from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. We recommend that you store these diluted standards for no more than 1-2 hours. See Table 1 for the nitrate concentrations of the serial dilutions.

Tube Number	Nitrate Concentration ( $\mu\text{M}/\text{tube}$ )	Nitrate ( $\text{pmol}/\text{well}$ )	Nitrate Concentration ( $\mu\text{M}/\text{well}$ )
1	10	500	3.85
2	5	250	1.92
3	2.5	125	0.96
4	1.25	62.5	0.48
5	0.625	31.3	0.24
6	0.313	15.6	0.12
7	0.156	7.8	0.06
8	0.078	3.9	0.03

*Table 1. Nitrate Concentrations*

### Performing the Assay

#### 1. Aliquot the Standards for the Standard Curve

Reserve nine wells for each standard curve (*NOTE*: running the standard curve in duplicate will aid in obtaining better data). (If using a single-cell spectrofluorometer, perform all reactions in small test tubes). Add 80  $\mu\text{l}$  of Assay Buffer to the first standard well and 30  $\mu\text{l}$  to each of the remaining eight. Add 50  $\mu\text{l}$  of nitrate standard tube #8 to the second standard well on the plate. Add 50  $\mu\text{l}$  of tube #7 to the next standard well. Continue with this procedure for standard tubes #6-#1.

#### 2. Aliquot the Samples

Add 10-20  $\mu\text{l}$  of sample to the wells and adjust the volume to 80  $\mu\text{l}$  with Assay Buffer. *NOTE*: Plasma samples and tissue homogenates should be assayed with no more than 10  $\mu\text{l}$  of undiluted sample per well (See page 5 for complete information on sample preparation). Caution should be taken when pipetting plasma samples to ensure that no bubbles enter the well.

#### 3. Aliquot the Enzyme Cofactors

Add 10  $\mu\text{l}$  of the Enzyme Cofactor mixture (vial #3) to each well.

#### 4. Aliquot the Nitrate Reductase

Add 10  $\mu\text{l}$  of the Nitrate Reductase mixture (vial #2) to each well.

#### 5. Incubate the Plate

Cover the plate with the plate cover and incubate at room temperature for 30 minutes. This incubation time should be increased to 1 hour when assaying tissue culture medium or 2 hours when assaying plasma and tissue samples.

#### 6. Aliquot the DAN

After the required incubation time, add 10  $\mu\text{l}$  of DAN Reagent (vial #6) to each well. Incubate for 10 minutes.

## 7. Aliquot the NaOH

Add 20  $\mu\text{l}$  of NaOH (vial #7) to each well.

## 8. Read the Plate

Read the plate in a fluorometer using an excitation wavelength of 375 nm and an emission wavelength of 415 nm. Alternatively, excitation and emission wavelengths of 360-365 and 430-450 nm, respectively, can be used. (Any emission wavelength above 450 nm cannot be used.) It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. Higher concentrations of nitrate and nitrite may require the use of lower gain settings whereas the gain may need to be increased for low concentrations of analyte.

# MEASUREMENT OF NITRITE

## Preparation of Nitrite Standard Curve and Samples

Follow the Nitrate Standard Curve preparation instructions on page 6 using the Nitrite Standard (vial #5). If using a single-cell spectrofluorometer, perform all reactions in small test tubes.

## Performing the Assay

### 1. Aliquot the Standards for the Standard Curve

Reserve nine wells for each standard curve (*NOTE:* running the standard curve in duplicate will aid in obtaining better data). Add 100  $\mu\text{l}$  of Assay Buffer to the first standard well and 50  $\mu\text{l}$  to each of the remaining eight. Add 50  $\mu\text{l}$  of nitrate standard tube #8 to the second standard well on the plate. Add 50  $\mu\text{l}$  of tube #7 to the next standard well. Continue with this procedure for standard tubes #6-#1.

### 2. Aliquot the Samples

Add 10-20  $\mu\text{l}$  of sample to the wells and adjust the volume to 100  $\mu\text{l}$  with Assay Buffer. *NOTE:* Plasma samples and tissue homogenates should be assayed with no more than 10  $\mu\text{l}$  of undiluted sample per well (See page 5 for complete information on sample preparation). Caution should be taken when pipetting plasma samples to ensure that no bubbles enter the well.

### 3. Aliquot the DAN

Add 10  $\mu\text{l}$  of DAN Reagent (vial #6) to each well. Incubate for 10 minutes.

### 4. Aliquot the NaOH

Add 20  $\mu\text{l}$  of NaOH (vial #7) to each well.

### 5. Read the Plate

Read the plate in a fluorometer using an excitation wavelength of 375 nm and an emission wavelength of 415 nm. Alternatively, excitation and emission wavelengths of 360-365 and 430-450 nm, respectively, can be used. (Any emission wavelength above 450 nm cannot be used.) It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. Higher concentrations of nitrate and nitrite may require the use of lower gain settings whereas the gain may need to be increased for low concentrations of analyte.

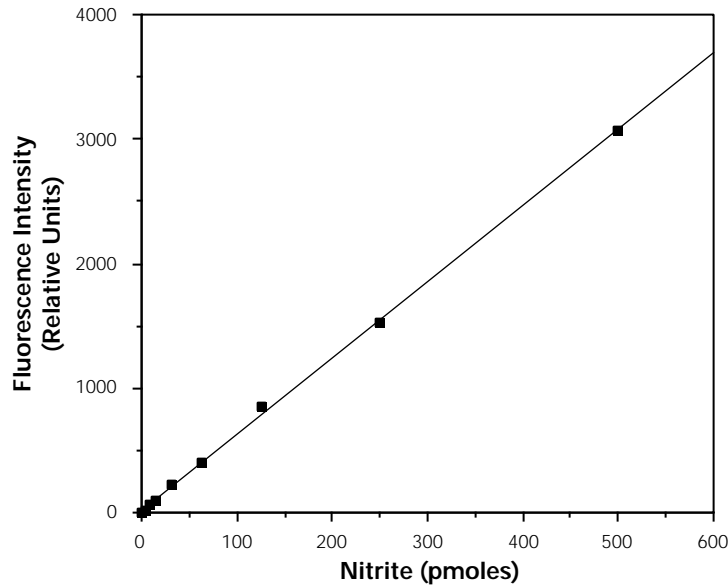
# CALCULATIONS

## Plotting the standard curves

Make a plot of fluorescence vs. picomoles nitrate OR nitrite. The nitrate standard curve is used for determination of total nitrate + nitrite concentration, whereas the nitrite standard curve is used for the determination of nitrite alone. In theory these two standard curves should be identical however, in practice a small discrepancy often occurs.

Fluorescence measurements have the advantage of measuring a broad linear range. For this reason, the standard curve has been made using serial dilutions of a stock standard. Therefore, it may be necessary to expand or reduce the scale in instances where extremely low or high levels of analyte are measured. An example of a nitrite standard curve is shown on the next page.

## Standard Curve



### Determination of sample nitrate or nitrite concentrations

$$[\text{Nitrate} + \text{Nitrite}] \text{ (nM)} = \left( \frac{\text{fluorescence} - \text{y-intercept}}{\text{slope}} \right) \left( \frac{1}{\text{volume of sample used (ml)}} \right) \times \text{dilution}$$

$$[\text{Nitrite}] \text{ (nM)} = \left( \frac{\text{fluorescence} - \text{y-intercept}}{\text{slope}} \right) \left( \frac{1}{\text{volume of sample used (ml)}} \right) \times \text{dilution}$$

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

Where dilution is a sample dilution done prior to addition of the sample to the plate (or tube).

## PERFORMANCE CHARACTERISTICS

### Interferences

Fluorescence measurements are typically more susceptible to interference compared to most absorbance measurements. For this reason, it is necessary to include proper controls (i.e., preparing standard curves with tissue culture media) that can account for agents that may quench the fluorescence. Known interfering agents include: hemoglobin, fetal calf serum, bovine serum albumin, DTT, NADPH, and phenol red. The NADPH concentration in this assay is kept below 1  $\mu\text{M}$  to essentially eliminate this interference. Whenever possible, other known interfering reagents should be eliminated from tissue culture media. Removal of most proteins from plasma, serum, or tissue homogenates is done using the 10 kDa molecular weight cut-off filters.

### Sensitivity

This fluorometric assay will detect as little as 30 nM nitrite in the final reaction mixture (<4 pmol in 0.12 ml). When using 20  $\mu\text{l}$  of sample, the detection limit for nitrite in the original sample is  $\sim 0.2 \mu\text{M}$ .

## TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates.

Cause: Poor pipetting/technique.

Problem: No fluorescence in nitrate standard curve.

Cause: Cofactors and/or nitrate reductase have not been added. DAN and/or NaOH have not been added.

Solution: Add DAN and/or NaOH if they have not been added. If the cofactors and/or nitrate reductase have not been added, you will need to do a new standard curve. If you have not added one of these reagents to the sample wells, you will need to repeat the experiment.

Problem: The nitrate standard curve is not linear at high concentrations.

Cause: Incomplete conversion of nitrate to nitrite at high nitrate concentrations.

Solution: Use only the points in the linear portion at the lower nitrate concentrations for making the standard curve. Allow the enzymatic reaction to incubate longer next time.

## REFERENCES

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2. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051-3064 (1992).
3. Green, L.C., Wagner, D.A., Glogowski, J., *et al.* Analysis of nitrate, nitrite, and [<sup>15</sup>N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138 (1982).
4. Misko, T.P., Schilling, R.J., Salvemini, D., *et al.* A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* **214**, 11-16 (1993).
5. Miles, A.M., Chen, Y., Owens, M.W., *et al.* Fluorometric determination of nitric oxide. *Methods* **7**, 40-47 (1995).

PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES