Nitrate/Nitrite Fluorometric Assay Kit

Item No. 780051

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. After opening kit, store individual components 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 or RT
780016	Nitrite Standard	1 vial	4°C or RT
780070	DAN Reagent	1 vial	4°C
780068	Sodium Hydroxide (2.8 M)	1 vial	4°C or RT
700029	96-Well Solid Plate (white)	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

Fax: 734-971-3640

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 in the **Materials Supplied** section on page 3 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 fluorescence using excitation wavelengths of 360-365, or 375 nm and emission wavelengths of 430 or 415 nm, respectively
2. Adjustable pipettes and a repeating pipettor
3. A source of UltraPure water (Milli-Q, HPLC-grade, or equivalent)

INTRODUCTION

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 molecular oxygen, arginine, and NADPH to produce NO, citrulline, and $NADP^+$. This process requires five additional cofactors (FMN, FAD, Heme, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron; see Figure 1). Three distinct isoforms of NOS have been identified, as detailed in Figure 2, see page 6.

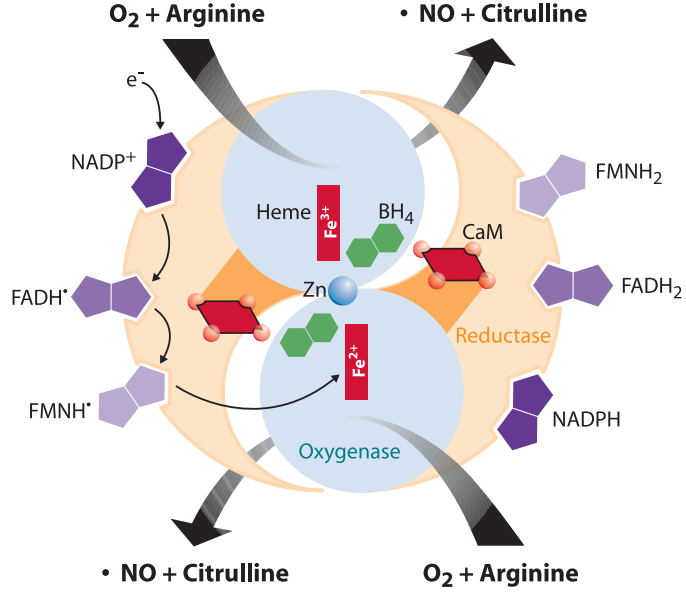


Figure 1. Nitric oxide synthesis

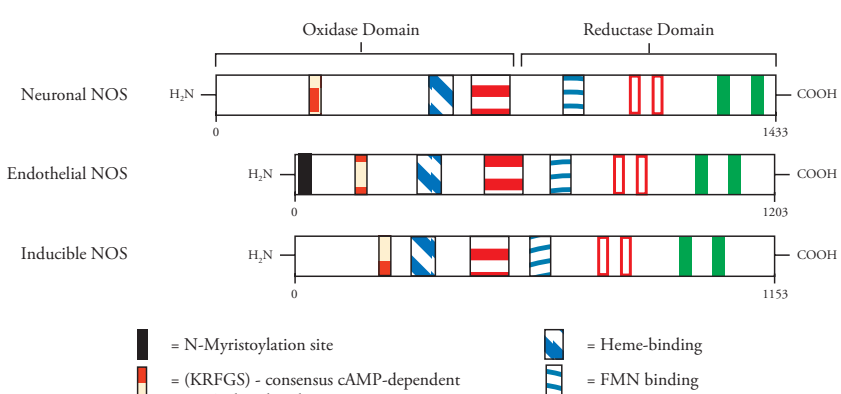


Figure 2. Nitric oxide synthase isoforms

About This Assay

NO is produced in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli.[1,2] 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:

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

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^-.

Cayman's 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, provided as an acidic solution, followed by NaOH which enhances the detection of the fluorescent product, 1(H)-naphthotriazole (see Figure 3). Measurement of the fluorescence of this compound accurately determines NO_2^- concentration.[3,4]

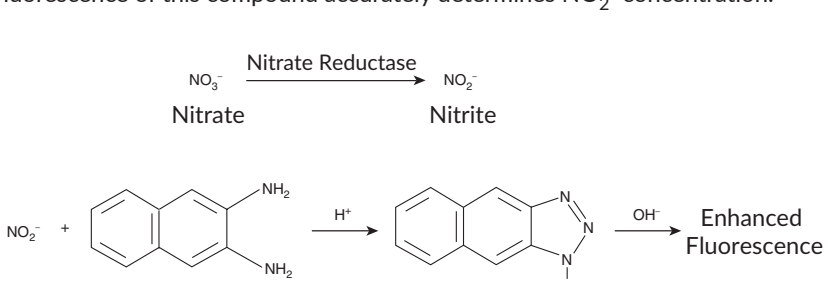


Figure 3. Chemistry of nitrate/nitrite detection

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Nitrate/Nitrite Assay Buffer - (Item No. 780022)**

   Dilute the contents of the Assay Buffer vial to 100 ml with UltraPure water (Milli-Q or equivalent). This Assay Buffer should be used for dilution of samples as needed prior to assay. This buffer will be stable for approximately two months at 4°C.

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

   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 one time.

3. **Enzyme Cofactors - (Item No. 780012)**

   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 one time.

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

   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 - (Item No. 780016)**

   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 - (Item Nos. 780070 and 780068)**

   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 15 for details).*

Pipetting Hints

- Use different tips to pipette the Assay Buffer, Standard, sample, and color development reagents.
- 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.

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. Store samples at -20°C or -80°C after collection.

Culture Medium

Some types of tissue culture medium 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 µl. 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 page 15) substituting the amount of media desired in place of Assay Buffer. For the measurement of nitrate plus nitrite, an incubation of one hour is necessary for the reaction to reach completion.

Plasma and Serum

Ultrafilter plasma and serum samples through a 10 or 30 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 µl of the filtrate. The conversion of nitrate to nitrite requires 1-2 hours for ≥95% conversion.

Tissue Homogenates

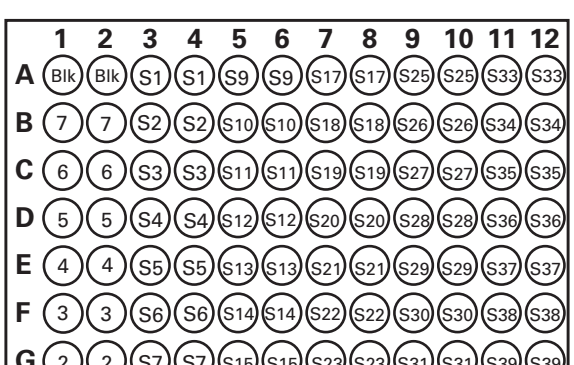
Homogenize the sample in PBS (pH 7.4) and centrifuge at 10,000 x g for 20 minutes. Centrifuge at 100,000 x g for 30 minutes (centrifugation at 100,000 x g is optional, but will increase filtration rates. Also, filtration of the solution through a 0.45 micron filter prior to ultrafiltration can increase the ultrafiltration rate). Ultrafilter tissue homogenates through a 10 or 30 kDa molecular weight cut-off filter (pre-rinsed with UltraPure water). Assay for nitrate and/or nitrite using 10 µl of the filtrate. The conversion of nitrate to nitrite requires two hours for ≥95% conversion.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, eight 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 26).

This kit provides sufficient cofactors and reagents to run two 96-well plates measuring total NO (NO_2^- + NO_3^-) in all the wells. If you wish to test some samples for 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.



Blk = Blank Wells
1-7 = Standards
S1-S40 = Sample Wells

Figure 2. Sample plate format

Nitrate Standard Preparation

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. The concentration of this stock standard is 200 µM. Use this diluted standard (200 µM) for the preparation of the nitrate standard curve as described below.

Obtain seven clean test tubes and number them #1 through #7. Aliquot 950 µl of Assay Buffer to tube #1 and 500 µl of Assay Buffer to tubes #2-7. Transfer 50 µl of Nitrate Standard as prepared above into tube #1 and mix thoroughly. The concentration of standard in tube #1 is 10 µM. (If using a single cell spectrofluorometer which requires a final volume of ~2 ml, do not make this dilution. Use the 200 µM stock standard for serial dilutions to make the standards 2-7. More information is provided below). Serially dilute the nitrate by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-7. We recommend that you store these diluted standards for no more than 1-2 hours. See Table 1 on page 16 for the nitrate concentrations of the serial dilutions.

Tube	Nitrate Concentration (tube) (µM)	Nitrate (per well) (pmol)	Final Nitrate Concentration (well) (µM)
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

Table 1. Nitrate concentrations
*The concentration is calculated for the final 130 µl assay volume after addition of DAN and NaOH.

Performing the Assay

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

   Reserve 16 wells for each standard curve *NOTE: Running the standard curve in triplicate will aid in obtaining better data.* Add 80 µl of Assay Buffer (or culture medium when applicable) to the two Blank wells and 30 µl to each of the remaining 14 wells. Add 50 µl of Nitrate Standard tube #7 to the second two standard wells on the plate. Add 50 µl of tube #6 to the next two standard wells. Continue with this procedure for standard tubes #5-#1.

2. **Aliquot the Samples**

   Add 10-20 µl of sample to the wells and adjust the volume to 80 µl with Assay Buffer. *NOTE: Plasma samples and tissue homogenates should be assayed with no more than 10 µl of undiluted sample per well (See page 11 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 µl of the Enzyme Cofactor Mixture (Item No. 780012) to each well.

4. **Aliquot the Nitrate Reductase**

   Add 10 µl of the Nitrate Reductase Mixture (Item No. 780010) 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 one hour when assaying tissue culture medium or two hours when assaying plasma and tissue samples.

6. **Aliquot the DAN**

   After the required incubation time, add 10 µl of DAN Reagent (Item No. 780070) to each well. Incubate for 10 minutes.

7. **Aliquot the NaOH**

   Add 20 µl of NaOH (Item No. 780068) to each well.

8. **Read the Plate**

   Read the plate in a fluorometer using an excitation wavelength of 360-365 nm and an emission wavelength of 430 nm. Alternatively, excitation and emission wavelengths of 375 and 417 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.

Nitrite Standard Preparation

Follow the Nitrate Standard Curve Preparation instructions on page 15 using the Nitrite Standard (Item No. 780016). 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 16 wells for each standard curve *NOTE: Running the standard curve in triplicate will aid in obtaining better data.* Add 100 µl of Assay Buffer to the first two Blank wells and 50 µl to each of the remaining 14 wells. Add 50 µl of Nitrite Standard tube #7 to the second two standard wells on the plate. Add 50 µl of tube #6 to the next two standard wells. Continue with this procedure for standard tubes #5-#1.

2. **Aliquot the Samples**

   Add 10-20 µl of sample to the wells and adjust the volume to 100 µl with Assay Buffer. *NOTE: Plasma samples and tissue homogenates should be assayed with no more than 10 µl of undiluted sample per well (See page 11 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 µl of DAN Reagent (Item No. 780070) to each well. Incubate for 10 minutes.

4. **Aliquot the NaOH**

   Add 20 µl of NaOH (Item No. 780068) to each well.

5. **Read the Plate**

   Read the plate in a fluorometer using an excitation wavelength of 360-365 nm and an emission wavelength of 430 nm. Alternatively, excitation and emission wavelengths of 375 and 415 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.

ANALYSIS

Plotting the Standard Curve

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 concentrations over 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. Examples of a nitrite and a nitrate standard curve are shown on page 21.

Representative Nitrate and Nitrite Standard Curves

The standard curves presented here are examples of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use these to determine the values of your samples.

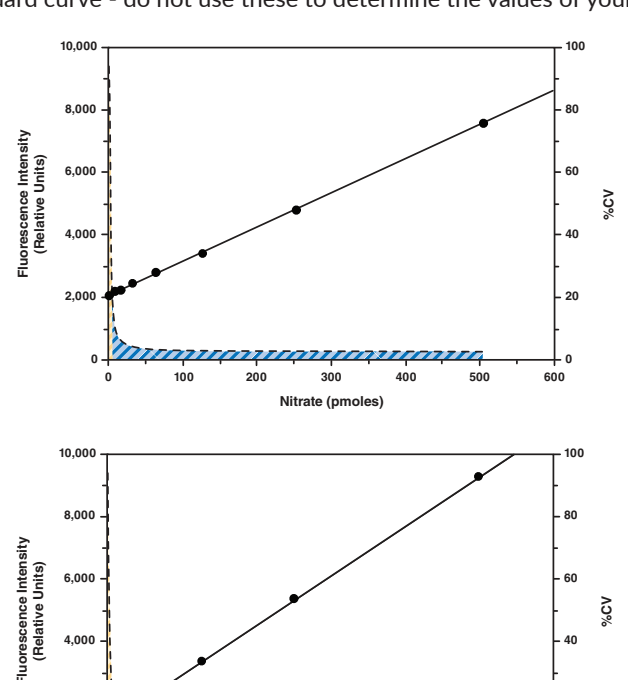


Figure 3. Nitrate and nitrite standard curves

Determination of sample nitrate or nitrite concentrations

$$[\text{Nitrate + Nitrite}]\ (\mu M) = \left(\frac{\text{fluorescence - y-intercept}}{\text{slope}}\right)\left(\frac{1}{\text{volume of sample used } (\mu l)}\right) \text{ x dilution}$$

$$[\text{Nitrite}]\ (\mu M) = \left(\frac{\text{fluorescence - y-intercept}}{\text{slope}}\right)\left(\frac{1}{\text{volume of sample used } (\mu l)}\right) \text{ x dilution}$$

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

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

Performance Characteristics

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 µl of sample, the detection limit for nitrite in the original sample is ~0.2 µM.

RESOURCES

Interferences

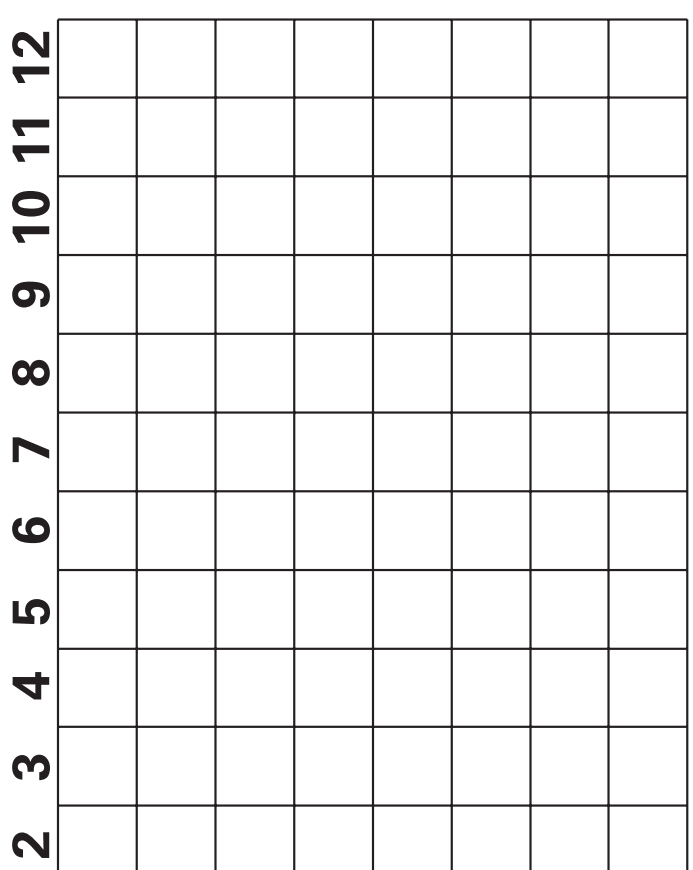
Fluorescence measurements are typically more susceptible to interference compared to absorbance measurements. For this reason, it is necessary to include proper controls (*i.e.*, preparing standard curves with tissue culture medium) that can account for agents that may quench the fluorescence. Known 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 µM 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 or 30 kDa molecular weight cut-off filters.

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
Poor standard curve and no sample detection	Instrument settings needs to be optimized	Check to make sure correct filters are being used; adjust gain
No fluorescence in nitrate standard curve	Cofactors and/or nitrate reductase have not been added; DAN and/ or NaOH have not been added	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
Non-linearity of nitrate standard curve at low concentrations	Background fluorescence due to the presence of nitrate in buffers or water; instrument sensitivity problems due to use of wrong filters or incorrect settings	Use UltraPure water when preparing buffers; troubleshoot possible instrument problems by using the nitrite standard; excess standard, buffer, DAN, and NaOH are supplied making it easy and convenient to use these reagents (rather than nitrate standards) for troubleshooting purposes

References

1. Moncada, S. The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.* **145**, 201-227 (1992).
2. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051-3064 (1992).
3. Miles, A.M., Chen, Y., Owens, M.W., *et al.* Fluorometric determination of nitric oxide. *Methods* **7**, 40-47 (1995).
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).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.