Nitrate/Nitrite Colorimetric Assay Kit (LDH method)

Item No. 760871

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity	Storage
780022	Nitrate/Nitrite Assay Buffer	1 vial	4°C
760872	Nitrate Reductase (LDH method)	1 vial	-20°C
760874	Lactate Dehydrogenase Cofactor Preparation	1 vial	-20°C
780014	Nitrate Standard	1 vial	4°C or RT
760873	Lactate Dehydrogenase	1 vial	-20°C
780018	Griess Reagent R1	1 vial	4°C
780020	Griess Reagent R2	1 vial	4°C
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	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:	734-971-3641
E-Mail:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 specified 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 absorbances of 540 or 550 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of UltraPure water (Milli-Q, HPLC-grade, or equivalent)
4. NADPH

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 on page 7.

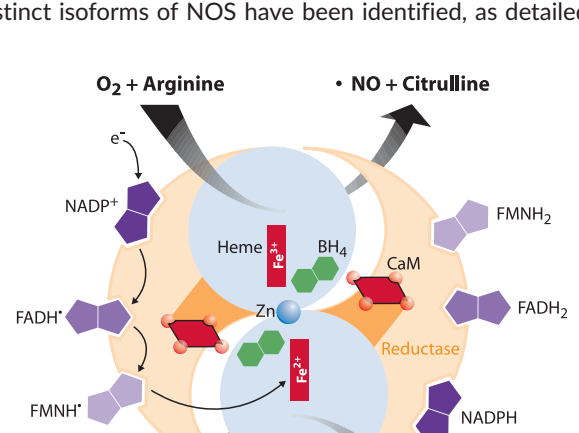


Figure 1. Nitric Oxide Synthesis

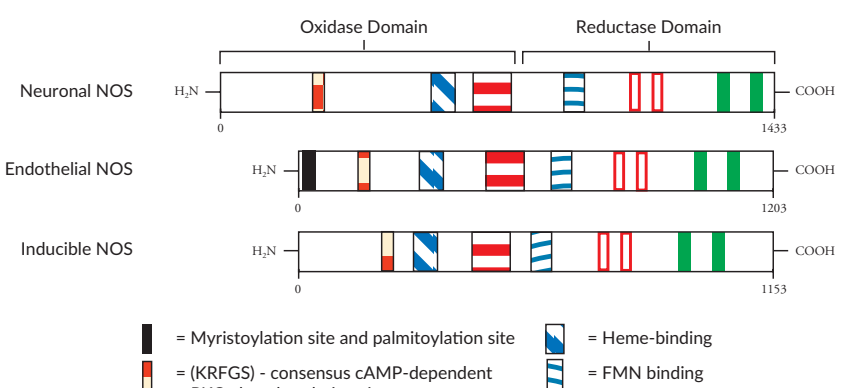


Figure 2. Nitric Oxide Synthase Isoforms

About This Assay

NADPH is an essential cofactor for the function of the NOS enzyme. In addition, nitrate reductase utilizes NADPH in the enzymatic reduction of nitrate to nitrite. Unfortunately, NADPH interferes with the chemistry of the Griess reaction, which is the most commonly used method for nitrite quantification. There are two ways to prevent this interference. Vanishingly small amounts of NADPH can be used in the nitrate reductase reaction in conjunction with a catalytic system for recycling spent $NADP^+$ back to NADPH. This is the system used in Cayman's Nitrate/Nitrite Colorimetric Assay Kit (Item No. 780001). It works well for the analysis of nitrate and nitrite in fluids such as plasma and urine, and is also available in a high sensitivity fluorometric version (Item No. 780051) for the detection of low levels of nitrite. However, this method cannot be used to analyze nitrate and nitrite from an *in vitro* NOS assay in which excess NADPH has been added.

The second way to handle the NADPH interference problem is to use an excess of NADPH. An additional step is added to the protocol to remove the excess NADPH. This is the avenue we have taken in the development of this nitrate/nitrite assay kit. This kit uses Lactate Dehydrogenase (LDH) to oxidize the excess NADPH. The kit is particularly well suited to measurements of NOS activity *in vitro*. High throughput screening applications using recombinant NOS preparations can be quickly and accurately assayed with the LDH method kit. This kit can also be used for the assay of nitrate and nitrite in urine, plasma, serum, and tissue culture medium.

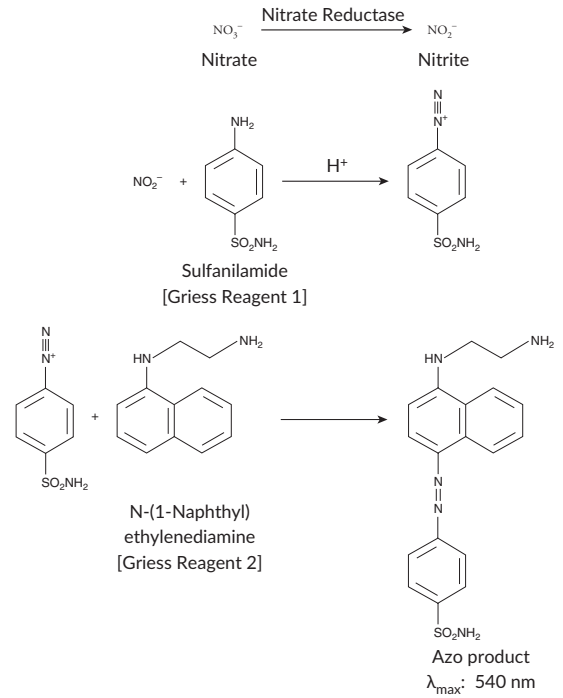


Figure 3. Chemistry of the Griess Reagents

PRE-ASSAY PREPARATION

Reagent Preparation

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

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

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

2. **Nitrate Reductase (LDH method) - (Item No. 760872)**

   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. **Lactate Dehydrogenase Cofactor Preparation - (Item No. 760874)**

   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. **Lactate Dehydrogenase - (Item No. 760873)**

   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.

6. **Griess Reagents R1 and R2 - (Item Nos. 780018 and 780020)**

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

7. **NADPH - (not included in this kit)**

   Prepare a 1 mM solution of NADPH (Sigma Item No. N-1630 or equivalent) in assay buffer. At least 1 ml of this solution will be required for the nitrate assays and must be made fresh each day.

NOTE: After reconstitution the standard must be further diluted prior to **Performing the Assay (see page 16 for details).*

Sample Preparation

Nitrate and nitrite are the stable end products of the reaction of Nitric Oxide (NO) with molecular oxygen. This kit is designed to measure the total accumulation of nitrate and nitrite in biological fluids and also derived from NO production by Nitric Oxide Synthase in controlled *in vitro* assays.

Urine

Urine can be used directly in the assay after dilution to the proper concentration in Assay Buffer. Urine contains relatively high levels of nitrate (200-2,000 µM), so dilutions of approximately 1:10 - 1:50 may be necessary.

Culture Media

Some types of tissue culture media contain very high nitrate levels (*ex.* 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. When samples cannot be diluted prior to assaying, all samples should be assayed with the same volume and the standard curve should be prepared in the presence of the same amount of culture medium. Sample dilutions, if required, would then be done using the medium.

Plasma and serum

Ultrafilter plasma or serum samples through a 10 or 30 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. The filters, supplied through Amicon or Millipore, should be pre-rinsed with UltraPure water prior to ultrafiltration of serum or plasma. Ultrafiltration will reduce background absorbance due to the presence of hemoglobin and improve color formation using the Griess reagents. Assay for nitrate/nitrite using a maximum of 40 µl of the filtrate.

Heparinized plasma may form a precipitate upon addition of Griess Reagent R1, thus making the sample unusable for analysis. Citrate or EDTA are recommended as anticoagulants for plasma preparation.

Tissue Homogenates

Homogenize the sample in PBS (pH 7.4) and centrifuge at 10,000 x g for 20 minutes to create the supernatant. Next, centrifuge the supernatant solution at 100,000 x g for 30 minutes. *NOTE: centrifugation at 100,000 x g is optional, but if performed it will increase filtration rates in the next step. If 100,000 x g is not available, centrifuge the supernatant longer at a lower force, i.e. 10,000 x g for one hour.* Ultrafilter using a 30 kDa molecular weight cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. The filters, supplied through Amicon or Millipore, should be pre-rinsed with ultrapure water prior to ultrafiltration of the sample. Assay the sample for nitrate/nitrite using a maximum of 40 µl of the filtrate.

Assay of *in vitro* assays NOS reactions

The conditions for an *in vitro* NOS assay using purified enzymes or tissue and cell homogenates, can be set up as required by your experimental design, but the following parameters should be observed.

a. **Sample volume**

   The amount of sample utilized in the assay for nitrate + nitrite can vary from <10 µl - 60 µl, depending on the activity of the enzyme. A convenient amount of sample for each assay will be 60 µl. Based on this amount of sample, a total volume for the NOS reaction should be at least 200 µl to allow for duplicate or triplicate analysis of the products.

b. **Stopping the NOS reaction**

   Heat inactivation is recommended for stopping the NOS reaction. The use of acid to quench the NOS reaction will lead to erroneous results for two reasons - NO will be released from nitrite under acidic conditions and nitrate reductase is inhibited by a variety of acids even when the pH has been adjusted to neutral. Following heat inactivation, centrifuge the samples to pellet the denatured protein.

c. **Controls**

   A heat-inactivated, zero-time point control should be included for each NOS preparation. This serves as a control to measure endogenous nitrate + nitrite in the NOS preparation, as well as determines the level of interference that may (or may not) be present in the sample. A control of this type should be made whenever the volume of NOS preparation used in the assay changes.

d. **Linearity of the assay**

The use of a single time-point assay to measure the activity of an enzyme will only be valid when the steady-state reaction is in a linear phase. Typically, this is done by stopping the reaction when <20% of the substrate(s) has been utilized. Another concern is the stability of the enzyme for extended periods of time. NOS is not a highly stable enzyme and activity often decreases during the assay, particularly at 37°C. The linearity of the assay can be assessed by measuring several time points for a single reaction condition (example - every minute for 5-10 minutes) and plotting the absorbance (obtained in the nitrate assay) as a function of time. Once linearity has been established, single time-point assays for NOS can be performed.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of nitrate standards, blanks, and samples to be measured in duplicate is given below in Figure 4. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	Blk	Blk	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-F = Standards
Blk = Blank Wells
S1-S41 = Sample Wells

Figure 4. Sample plate format

Pipetting Hints

- 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.

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

Performing the Assay

1. Pipette the following reagents into designated wells on the 96-well plate.

Well	Nitrate Standard (µl)	Assay Buffer (µl)	Final Nitrate Concentration (µM)*	Nitrate per well (nmoles)
A1, A2	0	60	0	0
B1, B2	5	55	5	1
C1, C2	10	50	10	2
D1, D2	15	45	15	3
E1, E2	20	40	20	4
F1, F2	25	35	25	5

*Based on a final concentration of 200 µl after the addition of all reagents.

The addition of other reagents to the standard curve are detailed in the next section.

2. Add 200 µl of water or Assay Buffer to the blank wells. Do not add any other reagents to these wells.
3. Add up to 60 µl of sample to the wells (40 µl for plasma, serum, or tissue homogenates). The final volume must be adjusted to 60 µl using the Assay Buffer solution.
4. Add 10 µl of the freshly prepared NADPH solution (1 mM) to each of the wells (standards and samples).
5. Add 10 µl of the Nitrate Reductase mixture (Item No. 760872) to each of the wells (standards and samples).
6. Incubate at room temperature for 40 minutes (60 minutes for plasma, serum, or tissue homogenates).
7. Add 10 µl of the cofactors solution (Item No. 760874) and 10 µl of the LDH solution (Item No. 760873) to each well (standards and samples).
8. Incubate at room temperature for 20 minutes.
9. After the required incubation time, add 50 µl of Griess Reagent R1 (Item No. 780018) to each of the wells (standards and samples).
10. Immediately add 50 µl of Griess Reagent R2 (Item No. 780020) to each of the wells (standards and samples).
11. Allow the color to develop for 10 minutes at room temperature.
12. Read the absorbance at 540 nm or 550 nm using a plate reader.

ANALYSIS

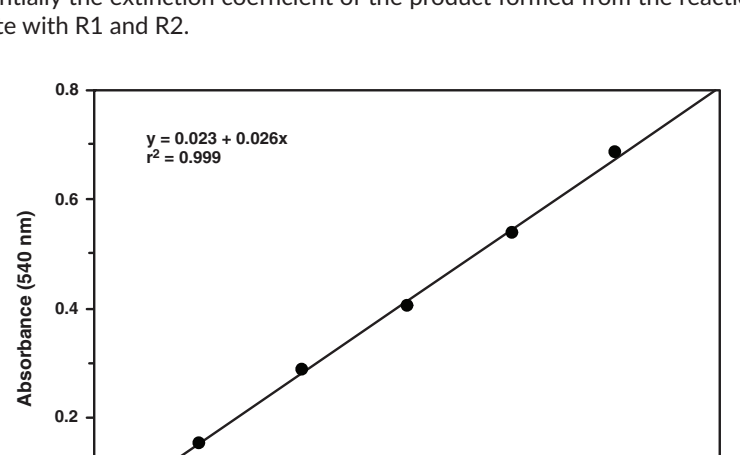
Calculations

Subtract the blanks

Average the absorbance value of the blank wells and subtract this from the absorbance values of all the other wells.

Plotting the standard curve

Make a plot of absorbance at 540 nm (or 550 nm) as a function of nitrate concentration and determine the equation of the line. The slope of the line is essentially the extinction coefficient of the product formed from the reaction of nitrite with R1 and R2.



Determination of sample nitrate or nitrite concentrations

$$[\text{Nitrate + Nitrite}]\ (\mu M) = \left[\frac{A_{540} - (\text{y-intercept})}{\text{slope}}\right]\left[\frac{200\ \mu l}{\text{volume of sample used } (\mu l)}\right] \times \text{dilution}$$

(where dilution is a sample dilution done prior to addition of the sample to the plate)

Performance Characteristics

Sensitivity:

When using the maximum amount of sample for the nitrate assay (60 µl), the detection limit is 2.5 µM. The detection limit for plasma is higher since only 40 µl of sample can be used.

RESOURCES

Interferences

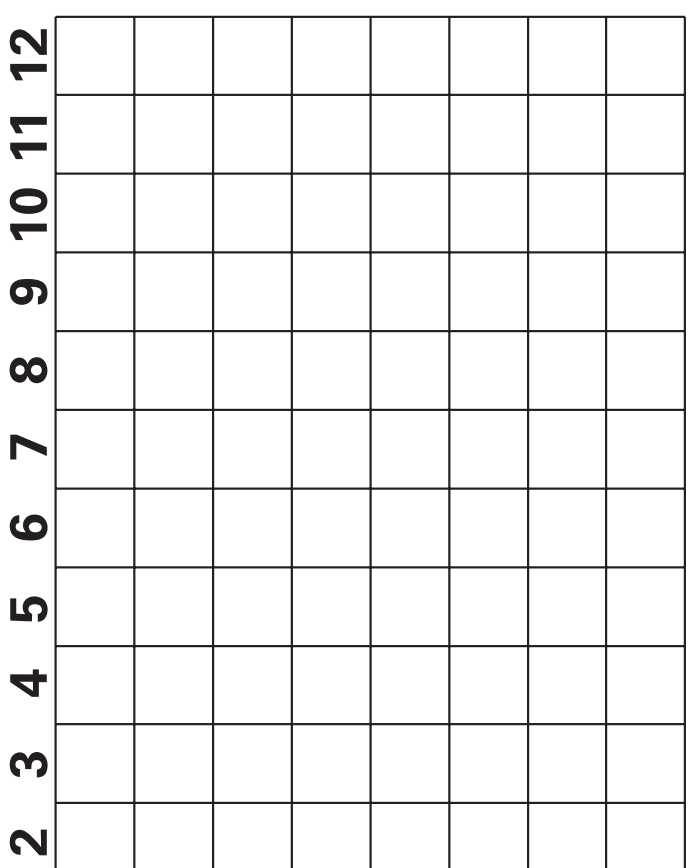
Antioxidants will interfere with the color development reaction. Azide, ascorbic acid, dithiothreitol, and mercaptoethanol will interfere with color development when present at concentrations as low as 100 µM. Alkyl amines, most sugars, lipids, or amino acids (except those containing thiol groups) do not interfere.[4] Phosphate concentrations greater than approximately 50 mM will interfere with the conversion of nitrate to nitrite.

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
No color development in nitrate standard curve	Substrates or enzymes (or both) not 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

References

1. Moncada, S. *Acta Physiol. Scand.* **145**, 201-227 (1992).
2. Nathan, C. *FASEB Journal* **6**, 3051-3064 (1992).
3. Green, L.C., Wagner, D.A., Glogowski, J., *et al. Anal. Biochem.* **126**, 131-138 (1982).
4. Nims, R.W., Darbyshire, J.F., Saavedra, J.E., *et al. Methods* **7**, 48-54 (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.