Data Sheet

Nitric Oxide Assay Kit, Fluorometric

482655

Pack Size: 192-288 Tests

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Not for use in diagnostic procedures. Not for human or animal consumption.

Background

Nitric oxide (NO), produced in trace quantities by neurons, endothelial cells, platelets, neutrophils and other cells, acts as a unique second messenger molecule. It readily diffuses through cell membranes to exert a variety of biological actions in mammalian cells. Excess generation of NO lead to the formation of peroxynitrite, destruction of iron-sulfur clusters, thiol nitrosylation, and nitration of protein tyrosine residues. The final products of NO in vivo are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportions of these two products are variable. Hence, the best index of total NO produced is the sum of both [NO_2^-] and [NO_3^-].

Principles of the assay

The Calbiochem® Nitric Oxide Assay Kit, Fluorometric provides an accurate and convenient method for the measurement of total [NO_2^-] and [NO_3^-] in a simple two-step process. The first step involves the conversion of nitrate to nitrite by the enzymatic action of nitrate reductase. The second step involves the addition of 2,3-diaminonaphthalene (DAN) followed by NaOH to convert nitrite to a fluorescent compound 1(H)-naphthotriazole. Measurement of fluorescence of 1(H)-naphthotriazole provides an accurate assay of [NO_2^-]

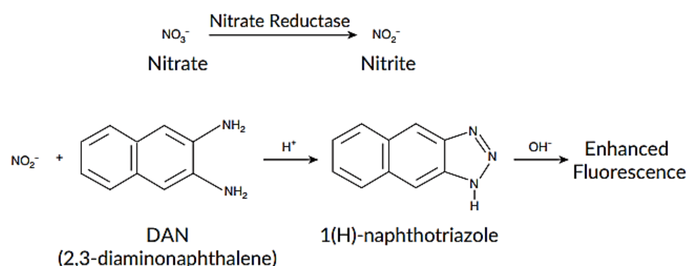


Figure 1: Principle of the Assay

Detection Method

Fluorometric

Format

96 Well Plate

Materials Provided

- Assay Buffer (KP0201): 1 vial
- Nitrate Reductase (KP0202): 2 vials
- Enzyme Co-factors (KP0203): 2 vials
- Nitrate Standard (KP0204): 1 vial
- Nitrite Standard (KP0205): 1 vial
- DAN Reagent (KP0206): 1 vial
- NaOH (2.8 M (KP0207): 1 vial
- 96-Well plate and Covers (KP0208): 3 plates, 3 plate covers

Materials Required (Not supplied)

- A fluorometric plate reader capable of measuring fluorescence (excitation = 365 nm; emission = 450 nm)
- An adjustable pipette
- Glass distilled or HPLC-Grade water

Warnings and Precautions

Useful Pipetting Tips

- To maintain precise times of incubation and for saving time, use of a repipettor is recommended.
- Always use different tips for pipetting assay buffer, standards, samples and color development reagents.
- Before pipetting, equilibrate the pipette tip in the reagent to be used (for example, fill the tip and expel the solution, repeat a couple of times).
- Do not touch the pipette tip to the reagents already in the well.

Storage and Stability

Upon arrival, store the entire contents of the kit at -20 °C until use. For storage information on individual components following initial thawing and reconstitution, please consult the section on Pre-Assay Preparation.

Pre-Assay Preparation

Preparation

Culture Medium

Culture medium such as RPMI 1640 may contain high levels of nitrate. It is best not to use these types of media, particularly when small changes in nitrate levels are measured. If it is absolutely necessary to use this type of medium, then cellular nitrate/nitrite levels can be quantitated by subtracting the level of nitrate/nitrite in the medium (in the absence of cells) from the total levels. Phenol red and fetal bovine serum (FBS) added to the medium can cause a significant reduction in fluorescence. Whenever possible these components should be avoided. The effect of media components on fluorescence intensity must be assessed by making the nitrate or nitrite standard curve in the presence of an equivalent amount of the phenol red or FBS. To obtain maximum signal response, it is best to use 10 or 20 µL sample volumes. Use of larger sample volumes (30 to 50% of the final reaction volume) can lead to quenching of fluorescence. To prepare a standard curve in the presence of media, simply prepare the nitrate or nitrite standard curve substituting the amount of media desired in the place of assay buffer. For the measurement of nitrate plus nitrite an incubation period of 1 hour is required for the reaction to reach completion.

Plasma or Serum

Ultrafilter plasma or serum samples through a 10 or 30 kDa cut-off filter using a commercially available centrifuge or microfuge ultrafiltration device. This procedure removes hemoglobin thereby avoiding the reduction in fluorescence intensity. Assay for nitrate and/or nitrite using a maximum of 10 µL filtrate. The conversion of nitrate to nitrite requires 1-2 hours (for ≥ 95% conversion).

Tissue Homogenates

Homogenize the sample in phosphate-buffered saline (PBS, pH 7.4) and centrifuge at 10,000 x *g* for 20 minutes. Centrifuge at 100,000 x *g* for 30 minutes (this second centrifugation is optional but will increase filtration rates). Ultrafilter the supernatant through a 10 or 30 kDa cut-off filter. Use 10 µL of the filtrate for nitrate and/or nitrite assay. The conversion of nitrate to nitrite requires about 2 hours for ≥ 95% conversion.

Reagent Preparation

Assay Buffer

Dilute the contents of the vial to 100 mL with HPLC-Grade water. Use this buffer for diluting samples, as needed, prior to assay. Store at 4 °C. This buffer will be stable for approximately 2 months at 4 °C.

Nitrate Reductase

Reconstitute the contents of vial with 1.2 mL of assay buffer. Keep on ice during use. Aliquot and store at -20 °C. Allow only one time freezing and thawing of this solution.

Enzyme Co-factors

Reconstitute the contents of this vial with 1.2 mL of assay buffer. Keep on ice during use. Aliquot and store at -20 °C. Allow only one time freezing and thawing of this solution.

Nitrate and Nitrite Standards

Remove the vial stopper slowly to minimize disturbance of the lyophilized powder. Reconstitute the lyophilized nitrate standard using 1.0 mL of Assay Buffer. The concentration of this solution is 2 mM. Swirl to ensure that powder clinging to the sides of the vial is dissolved. Vortex gently. Store all stock solutions at 4 °C; do not freeze after reconstitution. When stored under these conditions, the nitrate standard is stable for at least three months.

Fluorometric reagent (DAN)

Ready to use. Store at 4 °C. Do not add water or assay buffer to this vial.

NaOH

Ready to use. Store at 4 °C. Do not add water or assay buffer to this vial.

Protocol

Measurement of Nitrate + Nitrite

1. 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 900 µL of Assay Buffer. To this, add 100 µL of reconstituted nitrate standard and vortex. Use this diluted standard (200 µM) for the preparation of the nitrate standard curve as described below. Obtain 8 clean test tubes and label them as 1 through 8. Aliquot 950 µL of Assay Buffer to tube 1 and 500 µL of assay buffer to tubes 2 through 8. Transfer 50 µL of Nitrate Standard into test tube 1 and mix well. The concentration of standard in tube 1 is 10 µM. Serially dilute the nitrate standard by removing 500 µL volume from tube 1 and placing in tube 2. Mix well. Then remove 500 µL from tube 2 and place it in tube 3. Repeat the procedure with tubes 4 to 7. Mix well after each addition. DO NOT store the diluted standards for more than 1-2 hours.

   Table 1. Nitrate concentrations

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

   Note: The concentration is calculated for the final 130 µL assay volume after addition of DAN and NaOH.

2. Aliquoting the Standards for the standard curve

   - Reserve nine wells on the plate for each standard curve (for better data we recommend running standards in duplicate).

     Note: if you use a single cell spectrofluorometer, perform all reactions in small test tubes.

   - Add 80 µL of the assay buffer to the first standard well and 30 µL to each of the remaining eight. Add 50 µL of nitrate standard from test tube #8 to the second standard well on the plate. Then add 50 µL from test tube #7 to the third standard well. Continue this process for test tube #6, 5, 4, 3, 2, and 1.

3. Aliquoting the samples

   - Add 10-20 µL of sample to the wells and adjust the volume to 80 µL with Assay Buffer.

     Note: For plasma samples and tissue homogenates, use no more than 10 µL of undiluted plasma or filtrate per well.

   - Avoid any bubbles from entering the wells.

4. Aliquoting the Enzyme Co-Factors: Add 10 µL of the enzyme co-factors to each well.
5. Aliquoting the Nitrate Reductase: Add 10 µL of the nitrate reductase to each well.
6. Incubation

   Cover the plate with the plate cover and incubate at room temperature for 30 minutes. This incubation period should be increased to 1 hour when assaying tissue culture medium or 2 hours when assaying plasma and tissue samples.
7. Aliquoting DAN: After the required incubation period add 10 µL of DAN reagent to each well and incubate for 10 minutes.
8. Aliquoting NaOH: Add 20 µL of NaOH to each well.
9. Reading the plate

   Read the plate in a fluorometer using the 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). When using a single cell spectrofluorometer, dilute the sample to 2 to 3 mL to allow for the measurement of all the samples. Higher concentrations of nitrate and nitrite may require lower gain setting, whereas the gain may need to be increased for low concentrations of nitrate and nitrite.

Measurement of Nitrite

1. Preparation of nitrite standard curve and samples

   Follow the nitrate standard curve preparation instructions (see above) using the nitrite standard vial. If using a single cell fluorometer, perform all reactions in small test tubes.
2. Aliquoting the standards for the standard curve
   - Reserve nine wells on the plate for each standard curve (for better data we recommend running standards in duplicate).

     Note: if you use a single cell spectrofluorometer, perform all reactions in small test tubes.
   - Add 100 µL of the assay buffer to the first standard well and 50 µL to each of the remaining eight. Add 50 µL of nitrate standard from test tube #8 to the second standard well on the plate. Then add 50 µL from test tube #7 to the third standard well. Continue this process for test tube #6, 5,4,3,2, and 1.
3. Aliquoting the samples
   - Add 10-20 µL of sample to the wells and adjust the volume to 100 µL with Assay Buffer.

     Note: For plasma samples and tissue homogenates, use no more than 10 µL of undiluted plasma or filtrate per well.
   - Avoid any bubbles from entering the wells.
4. Aliquoting DAN

   After the required incubation period add 10 µL of DAN reagent to each well and incubate for 10 minutes.
5. Aliquoting NaOH

   Add 20 µL of NaOH to each well.
6. Reading the plate

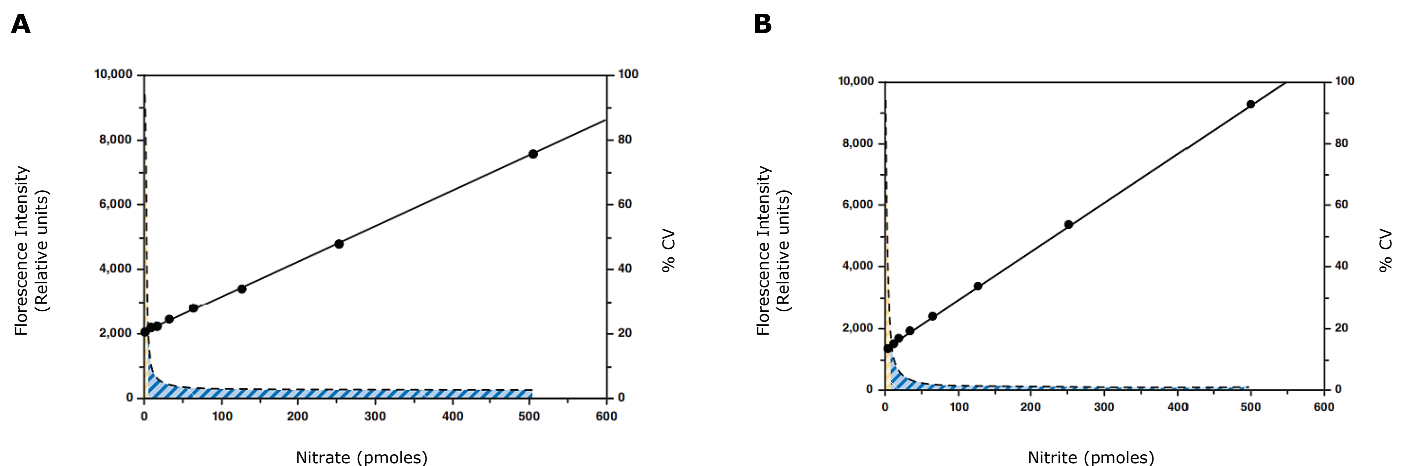
   Read the plate in a fluorometer using the excitation wavelength of 365 nm and an emission wavelength of 450 nm. Alternatively, excitation and emission wavelengths of 375 and 415 nm, respectively, can be used. Do not use emission wavelengths above 450 nm. When using a single cell spectrofluorometer, dilute the sample to 2 to 3 mL to allow for the measurement of all the samples. Higher concentrations of nitrate and nitrite may require lower gain setting, whereas the gain may need to be increased for low concentrations of nitrate and nitrite.

Standard Curve

Plotting the Standard Curve

- Make a plot of fluorescence vs. picomoles of nitrate or nitrite.
- The nitrate standard curve is used for determination of total nitrate plus 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 is often observed.
- Fluorescence measurements have the advantage of measuring a broad linear range. Hence, the standard curve is prepared using serial dilutions of a stock standard. It may be necessary to expand or reduce the scale in instances where extremely low or high levels or nitrate and/or nitrite are measured.

A typical standard curve is shown below:



C

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

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

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

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

Figure 2. (**A**) Nitrate and (**B**) Nitrite standard curves. (**C**) Determination of sample nitrate or nitrite concentration.

Sensitivity Notes

This fluorometric assay will detect as little as 30 nM nitrite in the final reaction mixture (< 4 pmol in 120 µL). When using 20 µL sample, the detection limit for nitrite in the original sample is 0.2 µM.

Plate Configuration

There is no specific recommended pattern for using the wells on the plate. However, nine wells will be required for the standard curve. For NO assay, when using tissue culture medium, the standard curve should also be prepared in the presence of the medium. If you plan to measure the total NO products (nitrate + nitrite), only the nitrate standard curve is required. If you wish to measure nitrite, then only the nitrite standard curve is needed. The remaining wells can be used for assay of samples.

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