

Nitric Oxide Fluorometric Assay Kit

(Catalog #K252-200; 200 assays; Store kit at -20°C)

For research use only

I. Introduction:

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune responses and apoptosis. Since most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions have been used as a quantitative measure of NO production. BioVision's Nitric Oxide Fluorometric Assay Kit provides an accurate and convenient 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 involves addition of DAN followed by NaOH, which converts nitrite into a fluorescent compound. Measurement of the fluorescence of this compound accurately determines the total nitric oxide production. The kit has been validated in culture media, plasma, and tissue homogenates. Some sample purification from these sources may be necessary.

II. Kit Contents:

| Component | K252-200 | 200 assays |
|-------------------|----------|-------------|
| 50X Assay Buffer | 1 vial | 2 ml |
| Enzyme Co-factors | 2 vials | Lyophilized |
| Nitrate Reductase | 2 vials | Lyophilized |
| Nitrate Standard | 1 vial | Lyophilized |
| DAN Reagent | 1 vial | 5 ml |
| Sodium Hydroxide | 1 vial | 5 ml |
| Microtiter Plate | 2 plates | 2 plates |
| Plate Cover | 2 covers | 2 covers |

III. Reconstitution of Reagents:

- Assay Buffer:** Dilute the 50X Assay Buffer to 100 ml with ddH₂O. The buffer should be used for dilution of samples as needed prior to assay. Store at 4°C.
- Enzyme Cofactors:** Reconstitute with 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freeze/thaw of the solution should be limited to 1 time.
- Nitrate Reductase:** Reconstitute to 1.2 ml of Assay Buffer. Keep on ice during use. Store at -20°C when not in use. Freeze/thaw of the solution should be limited to 1 time.
- Nitrate Standard:** Reconstitute with 1.0 ml of Assay Buffer. Vortex and mix well. Store at 4°C when not in use (**do not freeze!**). The reconstituted standard is stable for 4 months when stored at 4°C.
- Fluorometric Reagent DAN and NaOH:** Ready to use. Store at 4°C.

IV. Measurement of Nitrate + Nitrite:

- Prepare nitrate standard:** A nitrate standard curve must be performed in order to quantitate sample nitrate + nitrite concentrations. Mix 0.9-ml of Assay Buffer with 0.1-ml reconstituted nitrate standard and vortex. Use this diluted standard (200 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 μl of Assay Buffer to #1 and 500 μl of Assay Buffer to #2-8. Transfer 50 μl of Nitrate standard into #1 and mix thoroughly. The concentration of standard in tube #1 is 10 μM . 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 the process for tube #4-8. Do not store these standard for more than 24 hours.

| Tube# | Nitrate Conc. (μM) | Nitrate (pmol/well) |
|-------|---------------------------------|---------------------|
| 1 | 10 | 500 |
| 2 | 5 | 250 |
| 3 | 2.5 | 125 |
| 4 | 1.25 | 62.5 |
| 5 | 0.625 | 31.2 |
| 6 | 0.313 | 15.6 |
| 7 | 0.156 | 7.8 |
| 8 | 0.078 | 3.9 |

- Prepare nitrate standard curve:** Reserve 9 wells for each standard curve (If using a single-cell fluorometer, perform all reactions in a small test tube). Add 80 μl of Assay Buffer to the 1st standard well and 30 μl to each of the remaining 8 wells. Add 50 μl of the nitrate standard tube #8 to the 2nd standard well on the plate. Add 50 μl of tube #7 to the next standard well. Continue with the procedure for standard tube #6-#1.
- Aliquot 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).
- Add 10 μl of the Enzyme Cofactor mixture to each well.
- Add 10 μl of the Nitrate Reductase mixture to each well.
- Cover the plate with the plate cover and incubate at room temp. for 0.5-2 hrs.
- Add 10 μl of DAN Reagent to each well. Incubate for 10 min. at room temp.
- Add 20 μl of NaOH to each well.
- Read the plate in a fluorometer using Ex. = 375 nm and Em. = 415 nm. Alternatively, excitation and emission wavelengths of 360-365 and 430-450 nm, respectively, can also be used.

V. Calculations

- Plot standard curve:** Plot fluorescence vs. picomoles nitrate.
- Determine sample nitrate and nitrite concentrations:**

$$[\text{Nitrate} + \text{Nitrite}] \text{ (nM)} = \left(\frac{\text{fluorescence} - y\text{-intercept}}{\text{slope}} \right) \left(\frac{1}{\text{sample volume } (\mu\text{l})} \right) \times \text{dilution}^*$$

*Where dilution is the sample dilution done prior to addition of the sample to the plate.

VI. Related Products:

- Nitric Oxide Colorimetric Assay Kit
- Glutathione Fluorometric & Colorimetric Detection Kits
- DNA Damage Quantification Kit
- HDAC & HAT Fluorometric & Colorimetric Assay Kits
- Apoptosis Detection Kits and Reagents
- Cell Proliferation and Cytotoxicity Assays

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