BioVision

Nitric Oxide Colorimetric Assay Kit

(Catalog #K262-200; 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 Colorimetric Assay Kit provides an accurate and convenient measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is to convert nitrate to nitrite utilizing nitrate reductase. The second step involves addition of the Griess Reagents which convent nitrite into a deep purple azo compound. Measurement of the absorbance of the azo chromophore accurately determines the total nitric oxide production.

II. Kit Contents:

Component	K262-200	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
Griess Reagent R1	2 vials	2 x 5 ml
Griess Reagent R2	2 vials	2 x 5 ml
Microtiter Plate	2 plates	2 plates
Plate Cover	2 covers	2 covers

III. Reconstitution of Reagents:

1. **Assay Buffer:** Dilute the 50X Assay Buffer to 100 ml with ddH_20. The buffer should be used for dilution of samples as needed prior to assay. Store at 4°C.
2. **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.
3. **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.
4. **Nitrate Standard:** Reconstitute with 1.0 ml of Assay Buffer. Vertex 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.
5. **Griess Reagents R1 and R2:** Ready to use. Store at 4°C.

IV. Measurement of Nitrate + Nitrite:

1. **Nitrate standard curve:** 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 vertex. Use this diluted standard (200 µM) for the preparation of the nitrate standard curve as described below.

Well	Nitrate Standard (µl)	Assay Buffer (µl)	Final Nitrate Concentration (µM)*
A1	0	80	0
B1	5	75	5
C1	10	70	10
D1	15	65	15
E1	20	60	20
F1	25	55	25
G1	30	50	30
H1	35	45	35

*The concentration is calculated for the final 200 µl assay volume after addition of the Griess Reagents.

2. **Preparation of sample:**

The kit has been validated in urine, culture media, and plasma. No sample purification from these sources is necessary. Samples containing nitrate (with or without nitrite) can be assayed by addition of up to 80 µl of sample per well and should be done in duplicate. When using less than 80 µl of sample, the volume must be adjusted to 80 µl by adding Assay Buffer. In the event that the approximate concentration of nitrate or nitrite is completely unknown, we recommend that several different dilutions of the sample be made.

The absorbance of samples should be between 0.05 and 1.2 absorbance units, since the detector on the plate reader will give the most accurate values when the absorbance is in this range. The detection limit of the assay is approximately 1 µM nitrite. When using 80 µl of sample, this translates into 2.5 µM nitrate in the original sample.

3. **Assay procedure:**

1) Add 200 µl of Assay buffer to the blank wells. Do not add any other reagents to these wells.
2) Add 80 µl of samples or sample dilutions to each well.
3) Add 10 µl of the Enzyme Cofactor mixture to each well (standards and unknowns).
4) Add 10 µl of the Nitrate Reductase mixture to each well (standards and unknowns).
5) Cover the plate with plate cover and incubate at room temperature for 1-4 hrs.
6) Add 50 µl of Griess Reagent R1 to each well (standards and unknowns).
7) Immediately add 50 µl of Griess Reagent R2 to each well (standards and unknowns).
8) Allow the color to develop for 10 minutes at room temperature.
9) Read the absorbance at 540 nm using the plate reader.

V. Calculations

1) **Subtract the blank:** Subtract this absorbance value of the blank from the absorbance values of all other wells.
2) **Plot standard curve:** Plot absorbance at 540 nm as a function of nitrate concentration.
3) **Determine sample nitrate and nitrite concentrations:**

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

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