BioVision rev. 10/08 **For research use only**

Nitric Oxide Colorimetric Assay Kit

(Catalog #K262-200; 200 assays; Store kit at –20°C)

I. Introduction:

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. NO is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production. BioVision's Nitric Oxide Colorimetric Assay Kit provides an accurate, convenient measure of total nitrate/nitrite in a simple two-step process. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess Reagents to convert nitrite to a deep purple azo compound. The amount of the azo chromophore accurately reflects nitric oxide amount in samples. The detection limit of the assay is approximately 0.1 nmole nitrite/well, or 1 µM.

II. Kit Contents:

Components	K262-200	Cap Code	Part Number
Assay Buffer	30 ml	WM	K262-200-1
Enzyme cofactor	Lyophilized	Blue	K262-200-2
Enhancer	Lyophilized	Purple	K262-200-3
Nitrate Reductase	Lyophilized	Green	K262-200-4
Nitrate Standard	Lyophilized	Yellow	K262-200-5
Nitrite Standard	Lyophilized	Orange	K262-200-6
Griess Reagent R1	10 ml	NM	K262-200-7
Griess Reagent R2	10 ml	Amber NM	K262-200-8
Microtiter Plate	2 each	----	K262-200-9
Plate Cover	2 each	----	K262-200-10

III. Reconstitution of Reagents:

1. **Enzyme cofactor:** Reconstitute with 1.1 ml of Assay Buffer. Aliquot desired amount and store at -20°C. Keep on ice during use. Store at –20°C.
2. **Enhancer:** Reconstitute with 1.1 ml distilled water. Store at +4°C.
3. **Nitrate Reductase:** Reconstitute to 1.1 ml with Assay Buffer. This dissolves slowly, so gently vortex 2-3 times over 15 minutes. Keep on ice during use. Aliquot and store at -20°C.
4. **Nitrate and Nitrite Standards:** Reconstitute with 100 µl of Assay Buffer. Vortex and mix well to generate 100 mM standard. 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.

VI. Measurement of Nitrate + Nitrite:

1. **Nitrate standard curve:** Mix 5 µl of the 100 mM reconstituted standard with 495 µl of Assay Buffer to generate 1 mM standard working solution.

   Note: The reagents react with nitrite, not nitrate. For routine total nitrite/nitrate assay, you may prepare a nitrate standard curve only. However, if you desire to measure nitrite, nitrate concentration separately, you may prepare a nitrite standard curve in the absence of Nitrate Reductase in the standard and assay samples. Nitrate = Total – Nitrite.
2. **Preparation of samples:** Up to 85 µl of sample can be added per assay and should be done in duplicate. When using less than 85 µl of sample, adjust volume to 85 µl with Assay Buffer. If the approximate nitrate/nitrite concentration is completely unknown, we recommend that several dilutions be made. Urine can have high nitrate content and a 10 fold dilution should be used. Serum proteins will have a slight (~10%) effect on apparent nitrite levels. For best results serum filtrate from a 10Kd cutoff filter(BioVision Cat. 1997-25) should be used. Typical urine levels are 0.2-2mM and 1-20µM respectively. Typical normal serum levels are ~20µM and ~2µM for nitrate and nitrite respectively with various disease states elevating these levels significantly. The absorbance of samples should be in the linear range of the standard curve (0-10 nmol/well). If they fall outside this range, they should be rediluted and rerun.
3. **Assay procedure:**
   1) Add 85 µl sample and 115 µl Assay Buffer as blank well.
   2) Add 0, 2, 4, 6, 8, 10 µl of standard to each well and adjust to 85 µl.
   3) Add 85 µl of sample or dilution to each unknown well.
   4) Add 5 µl of the Nitrate Reductase mixture to each well (standards and unknowns).
   5) Add 5 µl of the enzyme cofactor to each well (standards and unknowns).
   6) Cover the plate and incubate at room temperature for 1 hr to convert nitrate to nitrite.
   7) Add 5 µl of the enhancer to each well and incubate 10 min.
   8) Add 50 µl of Griess Reagent R1 to each well (standards and unknowns).
   9) Add 50 µl of Griess Reagent R2 to each well (standards and unknowns).
   10) Develop the color for 10 min. at room temperature. The color is stable for about an hour.
   11) Read the absorbance at 540 nm using a plate reader.

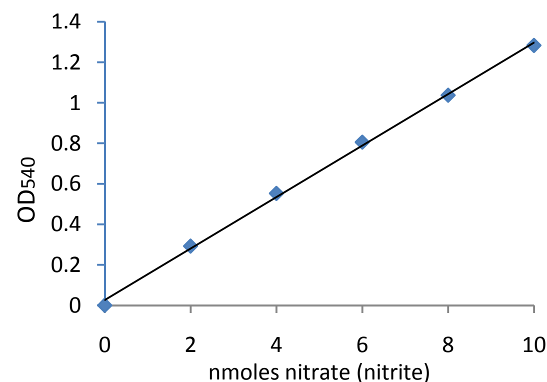
V. Calculations:

1. **Plot standard curve:** Plot absorbance at 540 nm as a function of nitrate concentration. It is possible to use plate readers with filters rather far from 540nm (i.e., 570nm). The sensitivity of the assay will be reduced approximately 35% in this case.
2. **Determine sample nitrate and nitrite concentrations:**

   Nitrate/nitrite concentration = $[\frac{sample\ abs. - blank\ abs.}{slope\ \ of\ std\ curve}]/(ul\ of\ sample)$

   = nmol/ul or mM nitrate (nitrite) in sample

   Or C = Sa/Sv. Where Sa is sample amount from standard curve (in nmol). Sv is sample volume added to the assay well (in µl), nmol/µl or mM nitrate in the sample.



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