

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 azochromophore 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:

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.

Enhancer: Reconstitute with 1.1 ml distilled water. Store at +4°C.

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.

Nitrate and Nitrite Standards: Reconstitute with 100µl 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.

Griess Reagents R1 and R2: Ready to use. Store at +4°C.

VI. Nitric Oxide Assay Protocol:

1. Standard Curve Preparation:

Mix 5 µl of the 100 mM reconstituted Nitrite or Nitrate standard with 495 µl of Assay Buffer to generate 1 mM standard working solution. Add 0, 2, 4, 6, 8, 10 µl of standard into a series of wells. Adjust volume to 85 µl with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of Nitrite or Nitrate Standard.

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. Sample Preparations:

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. Include a sample blank for each sample. If the approximate nitrate/nitrite concentration is completely unknown, we recommend that several dilutions be made. Urine can have high nitrate content 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 0.2-2 mM and 1-20 µM while typical normal serum levels are ~20 µM and ~2 µM for nitrate and nitrite, respectively (various disease states elevate 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 re-diluted and rerun.

3. Assay procedure:

- To each sample blank (85µl adjusted volume) add 115 µl Assay Buffer (Note: sample blanks do not contain any of the following additions).
- Add 5µl of the Nitrate Reductase mixture to each well (standards and unknowns).
- Add 5µl of the enzyme cofactor to each well (standards and unknowns).
- Cover the plate and incubate at room temperature for 1 hr to convert nitrate to nitrite.
- Add 5 µl of the enhancer to each well and incubate 10 min. (standards and unknowns).
- Add 50 µl of Griess Reagent R1 to each well (standards and unknowns).
- Add 50 µl of Griess Reagent R2 to each well (standards and unknowns).
- Develop the color for 10 min. at room temperature. The color is stable for about an hour.
- Read the absorbance at 540 nm using a plate reader.

4. **Calculations:** Plot absorbance at 540 nm as a function of nitrate and/or nitrite concentration (It is possible to use plate readers with filters rather far from 540 nm; e.g., 570 nm). The sensitivity of the assay will be reduced approximately 35% in this case.

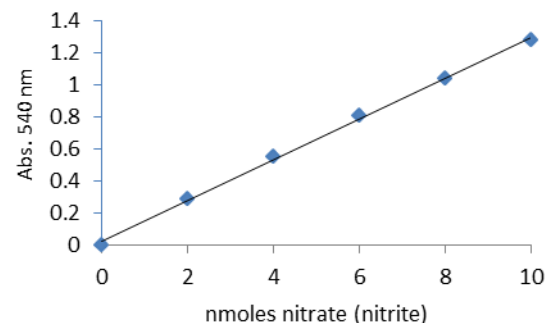
$$\text{Nitrate/nitrite concentration} = \frac{(\text{sample abs.} - \text{blank abs.})}{(\text{slope of standard curve})} \times (\mu\text{l of sample})$$

or

$$C = Sa/Sv = \text{nmol}/\mu\text{l or mM nitrate (nitrite)}$$

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 (nitrite) in the sample.



GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none"> • Use of ice-cold assay buffer • Omission of a step in the protocol • Plate read at incorrect wavelength • Use of a different 96-well plate 	<ul style="list-style-type: none"> • Assay buffer must be at room temperature • Refer and follow the data sheet precisely • Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	<ul style="list-style-type: none"> • Use of an incompatible sample type • Samples prepared in a different buffer • Samples were not deproteinized (if indicated in datasheet) • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples 	<ul style="list-style-type: none"> • Refer data sheet for details about incompatible samples • Use the assay buffer provided in the kit or refer data sheet for instructions • Use the 10 kDa spin cut-off filter or PCA precipitation as indicated • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	<ul style="list-style-type: none"> • Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used 	<ul style="list-style-type: none"> • Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	<ul style="list-style-type: none"> • Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots 	<ul style="list-style-type: none"> • Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	<ul style="list-style-type: none"> • Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range 	<ul style="list-style-type: none"> • Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
<p>Note# The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.</p>		