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 1 nmol nitrite/well, or 10 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 100 µl of Assay Buffer. Vortex and mix well to generate a 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 10 kDa 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 (85 µl adjusted volume) add 115 µl Assay Buffer (Note: sample blanks do not contain any of the following additions)
- Add 5 µl of the Nitrate Reductase mixture to each well (standards and unknowns)
- Add 5 µl 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.

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

or

C = Sa/Sv = nmol/µl 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)

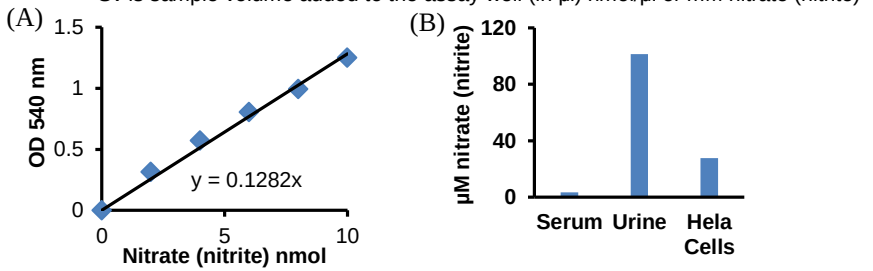

Figure: (A) Standard Curve. (B) Total nitrite/nitrate was measured using human serum (1 µl) and urine (1 µl) and Hela cell lysate (1 µl). Assays were performed following the kit protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		

BioVision Incorporated
155 S. Milpitas Boulevard, Milpitas, CA 95035 USA
Tel: 408-493-1800 | Fax: 408-493-1801
www.biovision.com | tech@biovision.com