StressMarq Biosciences INC.

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StressXpress®

Nitric Oxide Detection Kit

Catalog# SKT-212 (2 x 96 well kit)

Colorimetric detection of nitrate and nitrite

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GENERAL INFORMATION

Materials Supplied

Catalog Number	Reagent	Quantity	Description
SKC-212A	Clear 96 well Plates	2 Plates	-
SKC-212B	Nitrate Standard (Calibrated to NIST Standard Reference Material Lot Number 912a)	200 µL	Sodium Nitrate at 2,000 µM in a special stabilizing solution.
SKC-212C	Nitrite Standard (Calibrated to ISO/IEC 17025)	200 µL	Sodium Nitrite at 2,000 µM in a special stabilizing solution.
SKC-212D	Assay Buffer	60 mL	A buffer containing detergents and stabilizers.
SKC-212E	NADH Concentrate	1.2 mL	Reduced ß-nicotinamide adenine dinucleotide (NADH) as a stable solution.
SKC-212F	Nitrate Reductase	1 Vial	Nitrate Reductase (NR) as a stable solid stored in a desiccator.
SKC-212G	Enzyme Stabilization Buffer	1 mL	-
SKC-212H	Color Reagent A	5 mL	A solution of Sulfanilamide in acid. CAUTION:CAUSTIC
SKC-212I	Color Reagent B	5 mL	A solution of N-(1-Naphthyl) ethylenediamine in acid. CAUTION:CAUSTIC

If any of the items listed above are damaged or missing, please contact our Customer Service department at (250) 294-9065. We cannot accept any returns without prior authorization.

WARNING: Not for human or animal disease diagnosis or therapeutic drug use.

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The Color Reagents A and B are both acid solutions and should be handled like any laboratory acid.

Storage

All components of this kit should be stored at 4°C until the expiration date of the kit.

Once reconstituted, the Nitrate Reductase must be stored at -20°C.

Materials Needed But Not Supplied

- Distilled or deionized water free of detectable nitrate or nitrite.
- 10,000 Molecular Weight Cut Off (MWCO) polysulfone filters (Corning Spin-X UF 500, 431478) or similar product.
- Repeater pipets using disposable tips for addition of Color Reagents A & B, NADH and Nitrate Reductase.
- 96 well plate reader capable of reading optical absorption at 540-570 nm.
- Software for converting optical density (OD) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

Please read this booklet completely prior to using the product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

Background

Nitric oxide (NO) is a diffusible, transient, reactive molecule that has physiological effects in the picomolar-to-micromolar range. Acting through soluble guanylate cyclase activation, NO is an important physiological regulator of the cardiovascular, nervous, and immunological systems (1). NO is bio-available by two routes. It can be endogenously generated by constitutive or induced enzymes like Nitric Oxide Synthase or it can be orally ingested as nitrates / nitrites for rapid uptake into circulation and subsequent conversion (2).

The reactive nature of nitric oxide allows it to act as a cytotoxic factor when released during an immune response by cells such as macrophages. The reactivity also allows NO to be easily converted to a toxic radical that can produce nitrosative damage to cells, organelles and molecules such as DNA. Nitrosaylation however can be a regulated post-translational modification in cell signaling (3). The balance and dynamics of the regulatory/damage facets of NO are major forces in mitochondrial signaling and dysfunction (4). NO is linked not only to coronary heart disease, endothelial dysfunctions, erectile dysfunction, and neurological disorders, but also diabetes, chronic periodontitis, autism, cancer, and assorted age-related diseases (5-9).

The physical properties of Nitric Oxide make it challenging for direct detection methods. However, colorimetric methods can be applied to measure its stable break-down products nitrate (-NO3) and nitrite (-NO2) (10).

Assay Overview

The Nitric Oxide *StressXpress*® Colorimetric Detection Kit is designed to quantitatively measure Nitrate and Nitrite present in a variety of samples. Nitric Oxide content is derived from the sum of Nitrate (-NO3) and Nitrite (-NO2). Please read the complete kit insert before performing this assay. Both Nitrate and Nitrite standards are provided to generate standard curves for the assay and all samples should be read off the appropriate standard curve. For Nitrite detection, samples are mixed with the Color Reagents A and B and incubated at room temperature for 5 minutes. The colored product is read at 550 – 570 nm. The concentration of Nitrite in the sample is calculated, after making a suitable correction for any dilution of the sample, using software available with most plate readers.

Total Nitric Oxide content is measured after the sample is incubated with Nitrate Reductase and NADH. The reductase in combination with NADH reduces Nitrate to Nitrite. After a 20 minute incubation at room temperature, Color Reagents A and B are added and incubated at room temperature for 5 minutes. The colored product is read and calculated as with the Nitrite determination above. The concentration of Nitrate in the sample is calculated by subtracting the measured Nitrite concentration from the Total Nitric Oxide concentration for the sample.

This kit uses Nitrate and Nitrite Standard solutions calibrated to the US National Institute for Science and Technology Standard Reference Materials and ISO/IEC standards.

Sample Types

Sample Types Validated:

Water, Buffers, Serum, Plasma, Urine, Saliva and TCM

NO, Nitrate and Nitrite is identical across species and this kit will measure NO from all sources. We determined NO in human samples and the end user should evaluate recoveries of NO in samples from other species being tested. The kit will measure NO in cell culture medium, however many media contain nitrate salts. Care needs to be taken in the selection of media when NO measurement is to be done.

If samples need to be stored after collection, we recommend storing them at -70°C or lower, preferably after being frozen in liquid nitrogen. This assay has been validated for serum, plasma, urine, and saliva, as well as water and buffer samples. Tris, HEPES, and PBS buffers are compatible at pH 7.2, as is EDTA at ≤ 10 mM. Detergents such as Triton X-100, Tween 20 and CHAPS are compatible at concentrations of ≤ 0.1%. Most cell lysates and tissue homogenates should also be compatible. Samples containing these detergents should be diluted at least 1:2 with the Assay Buffer. Samples containing SDS or azide are not compatible with the assay. Samples containing visible particulate should be centrifuged prior to filtration and using.

Sample Preparation

All samples must be filtered through a 10,000 MWCO spin filter to remove protein.

Serum, plasma, saliva, or urine

Dilute sample with Assay Buffer and filter through a 10,000 MWCO device following the manufacturer's recommendations. Collect the filtrates and either further dilute with Assay Buffer as appropriate or use directly in the assay. For serum and plasma, the recommended final dilution is ≥1:4. For urine and saliva, the recommended final dilution is ≥1:8.

Reagent Preparation

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine NO concentrations. Ensure that all samples have reached room temperature and have been diluted and filtered through a 10,000 MWCO filter prior to running them in the kit.

Nitrate Reductase (NR)

Allow the desiccator to warm to room temperature. Add 550 µL of Enzyme Stabilization Buffer to the vial. Vortex gently and allow to sit at room temperature for 5 minutes. For extended periods of time (>2 hours) store reconstituted NR on ice. Store any unused reconstituted NR at -20°C.

Prepare NR for use in the assay by taking one part of reconstituted NR and adding to three parts of Assay Buffer. See Table below.

Nitrate Reductase Dilution Table

	1/2 Plate	One Plate	Two Plates
Reconstituted NR	150 µL	275 µL	500 µL
Assay Buffer	450 µL	825 µL	1.5 mL
Total Volume	600 µL	1.1 mL	2 mL

For extended periods of time (>2 hours) store reconstituted NR on ice.

NADH Preparation

Prepare NADH by diluting one part of NADH Concentrate with an equal part of Assay Buffer.

NADH Dilution Table

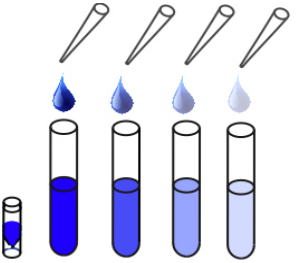
	1/2 Plate	One Plate	Two Plates
NADH Concentrate	300 µL	550 µL	1 mL
Assay Buffer	300 µL	550 µL	1 mL
Total Reaction Mix Volume	600 µL	1.1 mL	2 mL

Do not store diluted NADH.

Standard Preparation

Nitrate and Nitrite Standards are prepared identically by labeling seven test tubes as #1 through #7. Briefly vortex to mix and then spin the vial of standard in a microcentrifuge to ensure contents are at bottom of vial. Pipet 360 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #7. Carefully add 40 µL of either the -NO2 or -NO3 Standard to tube #1 and vortex completely. Take 200 µL of the solution in tube #1 and add it to tube #2 and vortex completely. Repeat this for tubes #3 through #7. The concentration of Nitrate or Nitrite in tubes 1 through 7 will be 200, 100, 50, 25, 12.5, 6.25 and 3.125 µM.

Use all Standards within 2 hours of preparation.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
Sample Diluent Volume (µL)	360	200	200	200	200	200	200
Addition	Stock	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
Volume of Addition (µL)	40	200	200	200	200	200	200
Final Concentration (µM)	200	100	50	25	12.5	6.25	3.125

Assay Protocol

Use the appropriate standards for either Nitrite (-NO2) or Nitrate (-NO3) determination. All samples should be diluted and filtered through a 10,000 MWCO filter prior to using.

Nitrite Determination Protocol

1. Use the plate layout sheet on page 22 to aid in proper sample and standard identification.

2. Pipet 50 µL of samples or Nitrite standards into duplicate wells in the plate.

3. Pipet 50 µL of Assay Buffer into duplicate wells as the Zero standard.

4. Add 25 µL of the Color Reagent A to each well using a repeater pipet.

5. Add 25 µL of the Color Reagent B to each of well using a repeater pipet.

6. Incubate at room temperature for 5 minutes.

7. Read the optical density at 540-570 nm. These readings are for the Nitrite determination.

Total Nitric Oxide Determination Protocol

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.

2. Pipet 50 µL of samples or Nitrate standards into duplicate wells in the plate.

3. Pipet 50 µL of Assay Buffer into duplicate wells as the Zero standard.

4. Add 10 µL of prepared NADH to each well using a repeater pipet.

5. Add 10 µL of prepared NR to each well using a repeater pipet.

6. Incubate at room temperature for 20 minutes.

7. Add 25 µL of the Color Reagent A to each well using a repeater pipet.

8. Add 25 µL of the Color Reagent B to each of well using a repeater pipet.

9. Incubate at room temperature for 5 minutes.

10. Read the optical density at 540-570 nm. These readings are for the Total Nitric Oxide determination.

Calculation of Results

Average the duplicate optical density readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean ODs for the zero standard. The concentrations obtained should be multiplied by the dilution factor to obtain sample values.

Nitrite (-NO2) concentrations are calculated from the data obtained from the Nitrite Protocol standard curve data utilizing the curve fitting routine supplied with the plate reader.

Total NO concentrations are calculated from the data obtained from the Total Nitric Oxide Protocol (nitrite + nitrate) standard curve data utilizing the curve fitting routine supplied with the plate reader.

Nitrate (-NO3) concentrations are obtained by subtracting the -NO2 concentrations of each sample from the Total NO concentrations. See Below:

$$\text{Nitrate } (-NO_3) = \text{Total NO} - \text{Nitrite } (-NO_2)$$

Typical Data - Nitrite

Sample	Mean OD	Net OD	Nitrite Concentration (µM)
Zero	0.038	0	0
Standard 1	2.144	2.106	200
Standard 2	1.248	1.210	100
Standard 3	0.708	0.670	50
Standard 4	0.412	0.374	25
Standard 5	0.236	0.198	12.5
Standard 6	0.145	0.107	6.25
Standard 7	0.095	0.057	3.125
Sample 1	0.639	0.601	43.9
Sample 2	1.484	1.446	124.4

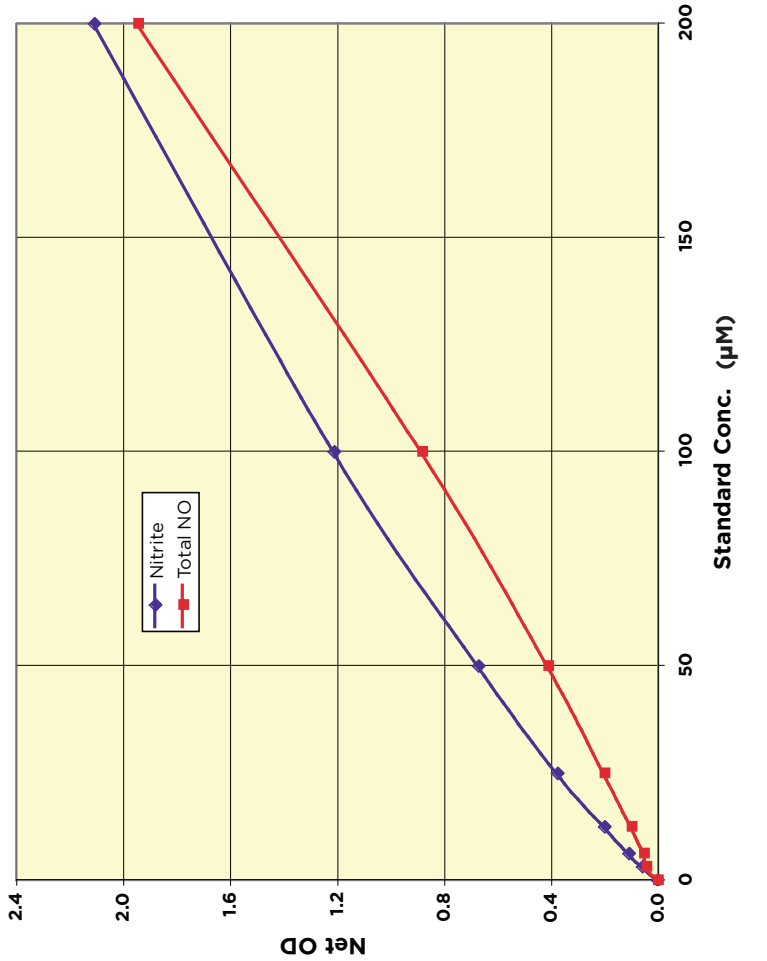
Typical Data - Total Nitric Oxide

Sample	Mean OD	Net OD	Total Nitric Oxide Concentration (µM)
Zero	0.040	0	0
Standard 1	1.984	1.944	200
Standard 2	0.921	0.881	100
Standard 3	0.450	0.410	50
Standard 4	0.240	0.200	25
Standard 5	0.138	0.098	12.5
Standard 6	0.092	0.052	6.25
Standard 7	0.083	0.043	3.125
Sample 1	1.058	1.018	113.1
Sample 2	1.420	1.380	147.9

Always run your own standard curve for calculation of results.

Do not use these data.

Typical Standard Curves



Validation Data

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the zero and standard #7. The detection limit was determined at two (2) standard deviations from the zero along the standard curve.
Sensitivity was determined as 2.63 µM in the Nitrite and 1.02 µM in the Total Nitric Oxide assays.

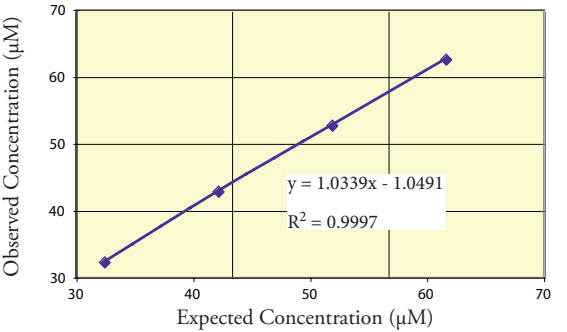
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the zero and a low concentration human sample.
The Limit of Detection was determined as 0.94 µM in the Nitirite and 3.0 µM in the Total Nitric Oxide assays.

Linearity

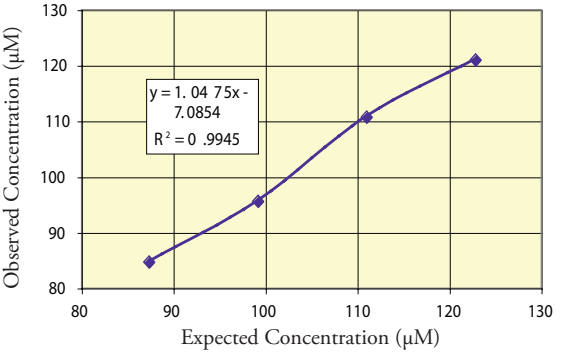
Linearity was determined by taking two human urine samples with known Nitrite and Total NO Concentrations and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Urine Sample	Low Urine Sample	Observed Concentration (µM)		Expected Concentration (µM)		% Recovery	
		Nitrite	Total NO	Nitrite	Total NO	Nitrite	Total NO
80%	20%	62.5	121.0	61.6	122.8	101.5%	98.5%
60%	40%	52.7	110.7	51.9	111.0	101.5%	99.7%
40%	60%	42.8	95.6	42.2	99.2	101.6%	96.4%
20%	80%	32.2	84.7	32.4	87.3	99.5%	97.0%
				Mean Recovery		101.0%	97.9%

Nitrite Linearity



Total NO Linearity



Intra Assay Precision

Three samples were further diluted in Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Nitrite or Total NO concentrations were:

Sample	Nitrite Concentration (µM)	%CV	Total NO Concentration (µM)	%CV
1	45.1	4.4	70.5	6.8
2	73.3	9.1	107.4	4.4
3	132.7	1.3	157.8	1.8

Inter Assay Precision

Three samples were further diluted in Assay Buffer and run in duplicates in twenty assays run over multiple days by three operators. The mean and precision of the calculated Nitrite or Total NO concentrations were:

Sample	Nitrite Concentration (µM)	%CV	Total NO Concentration (µM)	%CV
1	44.1	3.1	68.8	7.4
2	66.4	4.0	112.1	5.7
3	126.7	6.3	154.4	4.1

References

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Warranty and Limitation of Remedy

StressMarq Biosciences Inc. makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. StressMarq **warrants only** to the original customer that the material will meet our specifications at the time of delivery. StressMarq will carry out its delivery obligations with due care and skill. Thus, in no event will StressMarq have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if StressMarq is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of StressMarq, its directors or its employees.

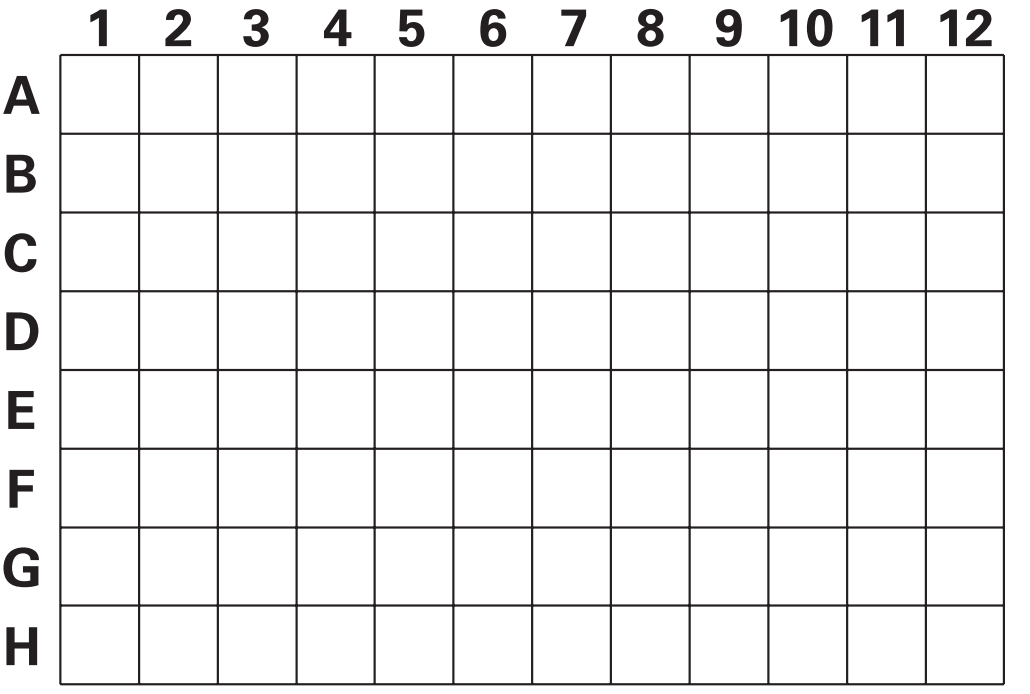
Buyer's **exclusive remedy** and StressMarq's sole liability hereunder shall be limited to a refund of the purchase price, or at StressMarq's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to StressMarq within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog.

Contact Information

Technical Service Contact Information
Phone: 250-294-9065
Fax: 250-294-9025
E-Mail:techsupport@stressmarq.com
Hours: M-F 9:00 AM to 5:00 PM PST
In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).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