Nitric Oxide Colorimetric Detection Kit

Kit #9136, 2 plates

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1. Introduction

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. Nitrosylation 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].

With a half-life *in vivo* of a few seconds or less, 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 (NO_3^-) and nitrite (NO_2^-)[10].

ICT's Nitric Oxide Colorimetric Detection Kit has two assay options. The first option is designed to quantitatively measure endogenous Nitrite. In the second option, Nitrate is converted to Nitrite using Nitrate Reductase and Total Nitric Oxide is measured. To obtain the Nitrate concentration, endogenous Nitrite is subtracted from the Total Nitric Oxide value. The kit has been designed to work in a variety of samples. Sample types validated include: water, buffers, serum, plasma, urine, saliva, and tissue culture medium (TCM). 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 540 – 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. This kit is for research use only. Not for use in diagnostic procedures.

2. Kit Contents

- 2 Clear Half-Area 96-well Microwell Plates #267
- 1 vial Nitrate Standard (200 µL) #6628: Sodium Nitrate at 2,000 µM in a special stabilizing solution. Calibrated to NIST Standard Reference Material.
- 1 vial Nitrite Standard (200 µL) #6629: Sodium Nitrite at 2,000 µM in a special stabilizing solution. Calibrated to ISO/IEC 17025.
- 1 bottle Nitric Oxide Assay Buffer, 1X (60 mL) #6630: A buffer containing detergents and stabilizers.
- 1 vial NADH Concentrate, 2X (1.2 mL) #6631: Reduced ß-nicotinamide adenine dinucleotide (NADH) as a stable solution.
- 1 bottle Nitrate Reductase #6632: Nitrate Reductase (NR) as a stable solid stored in a desiccator.
- 1 vial Enzyme Stabilization Buffer (1 mL) #6633: A buffer containing special stabilizers for NR.
- 1 bottle Color Reagent A (5 mL) #6634: A solution of Sulfanilamide in acid. **Warning! Contains Hydrochloric Acid ≤ 10%**
- 1 bottle Color Reagent B (5 mL) #6635: A solution of N-(1-Naphthyl) ethylenediamine in acid. **Warning! Contains Hydrochloric Acid ≤ 10%**

3. Required Materials

- 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.

4. 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.

5. Safety Data Sheets (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

6. 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.

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

7. Detection Equipment

The assay can be analyzed with a plate reader:

- 96-well plate reader capable of reading optical absorbance at 540-570 nm. Set plate parameters for a 96-well Corning Costar 3695 plate.
- 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.

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.

9. Nitrate Reductase (NR)

1. Allow the desiccator to warm to room temperature.
2. Add 550 µL of Enzyme Stabilization Buffer to the vial.
3. 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 Nitric Oxide Assay Buffer, 1X. See Table 1: Nitrate Reductase Dilution Table. For example, if using 1 plate, add 275 µL reconstituted NR to 825 µL Nitric Oxide Assay Buffer, 1X for a total volume of 1.1 mL.

10. NADH

Prepare NADH by diluting one part of NADH Concentrate, 2X with an equal part of Nitric Oxide Assay Buffer, 1X. See Table 2: NADH Dilution Table. For example, if using 1 plate, add 550 µL NADH Concentrate, 2X to 550 µL Nitric Oxide Assay Buffer, 1X for a total volume of 1.1 mL

11. Standard Preparation

1. Nitrate and Nitrite Standards are prepared identically by labeling seven test tubes as #1 through #7.
2. Briefly vortex to mix and then spin the vial of standard in a microcentrifuge to ensure contents are at the bottom of the vial.
3. Pipet 360 µL of Nitric Oxide Assay Buffer, 1X into tube #1 and 200 µL into tubes #2 to #7.
4. Carefully add 40 µL of either the Nitrite (NO_2^-) or Nitrate (NO_3^-) Standard to tube #1 and vortex completely.
5. Take 200 µL of the solution in tube #1 and add it to tube #2 and vortex completely.
6. Repeat this for tubes #3 through #7.
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 (See Table 3: Nitrate and Nitrite Standards Preparation).

- **Use all Standards within 2 hours of preparation.**

12. Sample Types

Nitric Oxide (NO), Nitrate (NO_3^-), and Nitrite (NO_2^-) are identical across species and this kit will measure NO from all

Table 1: Nitrate Reductase Dilution Table
For extended periods of time (>2 hours) store reconstituted NR on ice.

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

Table 2: NADH Dilution Table
Do not store diluted NADH.

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

Table 3: Nitrate and Nitrite Standards Preparation							
	Std 1	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Std 7**
Sample Diluent Vol (µL)	360	200	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	40	200	200	200	200	200	200
Final Conc (µM)	200	100	50	25	12.5	6.25	3.125

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. Most cell lysates and tissue homogenates should also be compatible. 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%, although samples containing these detergents should be diluted at least 1:2 with Nitric Oxide Assay Buffer, 1X. Samples containing visible particulate should be centrifuged and filtered prior to use.

- **Samples containing SDS or azide are not compatible with the assay.**
- **All samples must be filtered through a 10,000 MWCO spin filter to remove protein prior to use.**

13. Sample Preparation

Serum, plasma, saliva, or urine
Dilute sample with Nitric Oxide Assay Buffer, 1X and filter through a 10,000 MWCO device following the manufacturer's recommendations. Collect the filtrates and either further dilute with Nitric Oxide Assay Buffer, 1X 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.

14. Assay Protocol

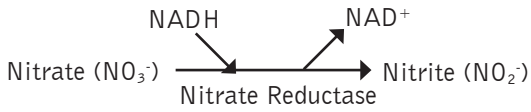
Use the appropriate standards for either the Nitrite (NO_2^-) or Total Nitric Oxide (NO) determination. All samples should be diluted and filtered through a 10,000 MWCO filter prior to using.

15. Nitrite (NO_2^-) Determination Protocol

1. Use the plate layout sheet on the back page (Figure 4) to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3695 plate.
2. Pipet 50 µL of samples or Nitrate standards into duplicate wells in the plate.
3. Pipet 50 µL of Nitric Oxide Assay Buffer, 1X 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 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.

16. Total Nitric Oxide (NO_2^- + NO_3^-) Determination Protocol

1. Use the plate layout sheet on the back page (Figure 4) to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3695 plate.
2. Pipet 50 µL of samples or Nitrate standards into duplicate wells in the plate.
3. Pipet 50 µL of Nitric Oxide Assay Buffer, 1X into duplicate wells as the Zero standard.
4. Add 10 µL of prepared NADH to each well using a repeater pipet (Section 10).
5. Add 10 µL of prepared NR to each well using a repeater pipet (Section 9).
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 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.



17. Calculation of Results

1. Average the duplicate optical density readings for each standard and sample.
2. Subtract the mean ODs for the Zero standard.
3. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader.
4. The concentrations obtained should be multiplied by the dilution factor to obtain sample values.
5. Nitrite (NO_2^-) concentrations are calculated from the data obtained from the Nitrite Protocol standard curve data (Section 15) utilizing the curve fitting routine supplied with the plate reader.
6. Total NO concentrations are calculated from the data obtained from the Total Nitric Oxide Protocol (nitrite + nitrate) standard curve data (Section 16) utilizing the curve fitting routine supplied with the plate reader.

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7. Nitrate (NO_3^-) concentrations are obtained by subtracting the NO_2^- concentrations of each sample from the Total NO concentrations.

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

18. Validation Data: Sensitivity

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the zero and standard #7 (low standard). 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.

19. Validation Data: Limit of Detection

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 Nitrite and 3.0 μM in the Total Nitric Oxide assays.

20. Linearity

Linearity was determined by taking two human urine samples with known Nitrite and Total NO Concentrations and mixing them in the ratios given; see Table 6: Sample Linearity Data. The measured concentrations were compared to the expected values based on the ratios used. Figures 2 and 3 illustrate linear plots of observed versus expected concentrations for Nitrite and Total NO, respectively.

21. References

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Table 4: Typical Data - Nitrite
Always run your own standard curve for calculation of results. Do not use this data.

Sample	Mean OD	Net OD	Nitrite Conc. (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

Table 5: Typical Data - Total Nitric Oxide
Always run your own standard curve for calculation of results. Do not use this data.

Sample	Mean OD	Net OD	Total Nitric Oxide Conc. (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

Figure 1: Typical Standard Curves
Always run your own standard curves for calculation of results. Do not use this data.

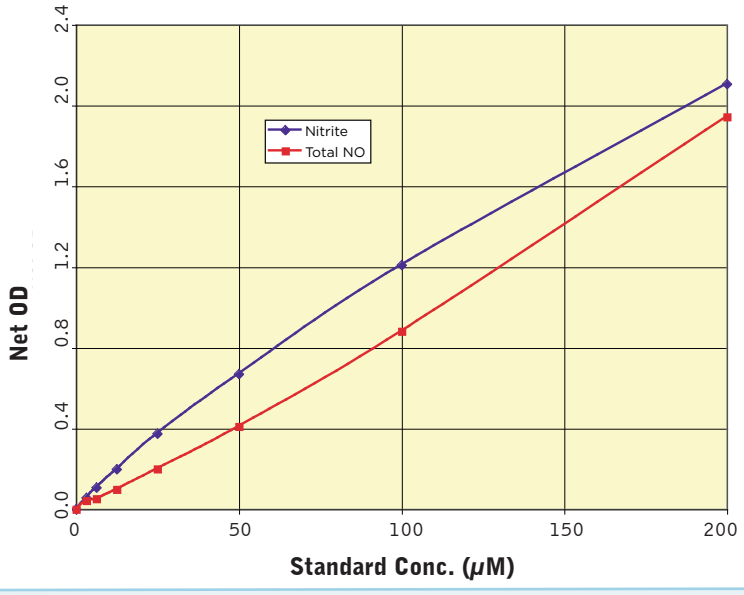

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Table 6: Sample Linearity Data

High Urine Sample	Low Urine Sample	Observed Conc. (μM)		Expected Conc. (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%

Figure 2: Nitrite Sample Linearity Curve

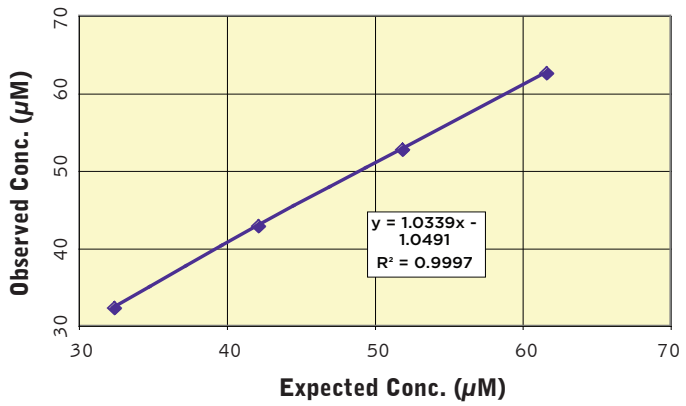

Figure 3: Total NO Sample Linearity Curve

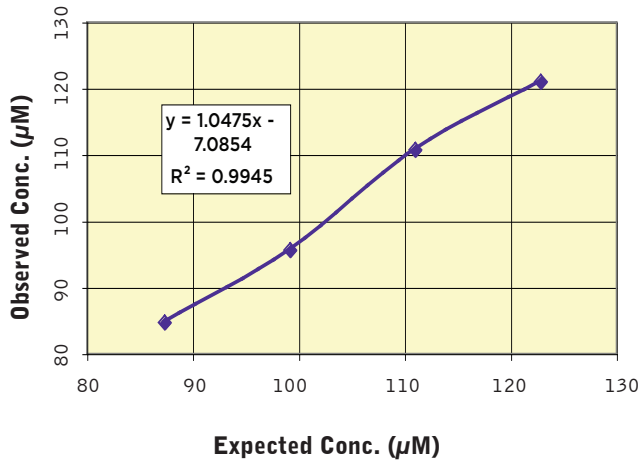

Table 7: Intra Assay Precision

Three samples were 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 Conc. (µM)	%CV	Total NO Conc. (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

Table 8: Inter Assay Precision

Three samples were 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 Conc. (µM)	%CV	Total NO Conc. (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

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Figure 4: Plate Layout Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Thank you for using this kit. If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.

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