Nitric Oxide Colorimetric Detection Kit

Catalog Number EIANOC (192 tests)

Rev 1.0

For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The Nitric Oxide Colorimetric Detection Kit is designed to detect and quantify the level of nitric oxide (NO) in various samples. The physical properties of NO make direct detection methods challenging, but colorimetric methods are effective for measuring its stable break-down products, nitrate ($^{-}NO_3$) and nitrite ($^{-}NO_2$).

This assay measures the level of nitrates and nitrites in serum, plasma, urine, saliva, water, buffered solutions (Tris, HEPES, or PBS at pH 7.2; EDTA at ≤10 mM), cell and tissue lysates, or tissue culture medium. The assay was characterized with human samples, but can be used to test samples from other species.

Nitric oxide is a diffusible, transient, reactive molecule that has physiological effects in the pM to µM range. NO can be endogenously generated by constitutive or induced enzymes like nitric oxide synthase, or it can be ingested as nitrates and nitrites for rapid uptake into circulation and subsequent conversion.

Contents and storage

Kit and components are shipped at –20°C. Upon receipt, store the kit at –20°C. Once open, store the kit at 4°C and use within 2 weeks.

Components	Quantity
Nitrate Standard; 2,000 µM sodium nitrate in a special stabilizing solution	200 µL
Nitrite Standard; 2,000 µM sodium nitrite in a special stabilizing solution	200 µL
Clear 96-well Plate	2 plates
Assay Buffer; buffer with detergents and stabilizers	60 mL
NADH Concentrate; reduced β-nicotinamide adenine dinucleotide	1.2 mL
Nitrate Reductase	1 vial
Enzyme Stabilization Buffer	1 mL
Color Reagent A; sulfanilamide in an acidic solution, CAUSITC	5 mL
Color Reagent B; N-(1-naphthyl)ethylenediamine in an acidic solution, CAUSITC	5 mL

Materials required but not supplied

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 540–570 nm
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solution
- 10,000 MWCO polysulfone filters (e.g., Corning 431478)

Procedural guidelines

Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

Sample preparation guidelines

- Collect samples in pyrogen/endotoxin-free tubes.
- Freeze samples after collection if samples will not be tested immediately. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well (do not vortex) prior to analysis.
- Avoid the use of hemolyzed or lipemic sera.
- If large amounts of particulate matter are present in the sample, centrifuge or filter sample prior to analysis.
- The assay is compatible with tissue culture media, but the appropriate media must be selected, as many media contain nitrate salts.
- Samples containing SDS or azide **are not** compatible with the assay.

Dilute samples

Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

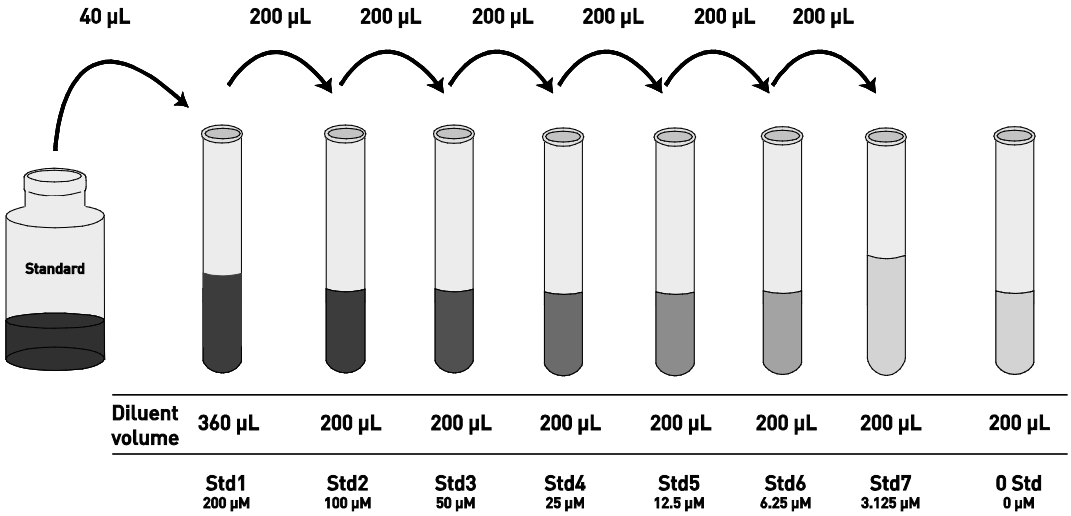
- **Filter all samples** through a 10,000 MWCO spin filter to remove protein after dilution.
- Dilute samples containing ≤0.1% Tween™ 20, Triton™ X-100, or CHAPS 1:2 in Assay Buffer.
- Dilute **serum** and **plasma** samples ≥1:4 in Assay Buffer.
- Dilute **urine** and **saliva** samples ≥1:8 in Assay Buffer.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

Note: The Nitrate Standard is calibrated to NIST Standard Reference Material Lot No. 3185.
The Nitrite Standard is calibrated to ISO/IEC 17025.

1. Add 40 µL Nitrate or Nitrite Standard to one tube containing 360 µL Assay Buffer and label as 200 µM nitrate or nitrite.
2. Add 200 µL Assay Buffer to each of 7 tubes labeled as follows: 100, 50, 25, 12.5, 6.25, 3.125, and 0 µM nitrate or nitrite.
3. Make serial dilutions of the standard as described below in the dilution diagram. Mix thoroughly between steps.
4. **Use the standards within 2 hours of preparation**.



Reconstitute Nitrate Reductase

1. Allow the Nitrate Reductase to reach room temperature in the sealed bag before opening.
2. Add 550 µL of the Enzyme Stabilization Buffer to the vial of Nitrate Reductase.
3. Vortex gently and incubate for 5 minutes at room temperature.
4. If not using reconstituted Nitrate Reductase for an extended period of time (>2 hours) store on ice. **Store unused reconstituted Nitrate Reductase at –20°C**.

Prepare Nitrate Reductase solution

Dilute reconstituted Nitrate Reductase with three volumes of Assay Buffer. Store Nitrate Reductase solution on ice if it is not used for an extended period of time.

Reagent	½ plate	1 plate	2 plates
Reconstituted Nitrate Reductase	150 µL	275 µL	500 µL
Assay Buffer	450 µL	825 µL	1.5 mL
Total volume	600 µL	1.1 mL	2 mL

Prepare NADH solution

Dilute NADH Concentrate with one volume of Assay Buffer. **Do not store diluted NADH**.

Reagent	½ plate	1 plate	2 plates
NADH Concentrate	300 µL	550 µL	1 mL
Assay Buffer	300 µL	550 µL	1 mL
Total volume	600 µL	1.1 mL	2 mL

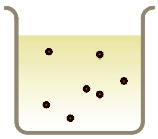
Assay procedure

Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use. **Total assay time is 5 minutes (nitrite determination) 25 minutes (total nitric oxide determination).**

IMPORTANT! Perform a standard curve with each assay.

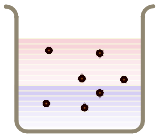
Use the appropriate standards for either nitrite or nitrate (total NO) determination.

Nitrite determination



Add sample

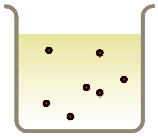
Add 50 µL of nitrite standards or diluted, filtered samples (see page 2) to the appropriate wells.



Add color reagent

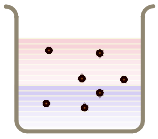
a. Add 25 µL Color Reagent A into each well.
b. Add 25 µL Color Reagent B into each well.
c. Incubate for 5 minutes at room temperature.

Total nitric oxide determination



Add sample

a. Add 50 µL of nitrate standards or diluted, filtered samples (see page 2) to the appropriate wells.
b. Add 10 µL of NADH solution into each well.
c. Add 10 µL of Nitrate Reductase solution into each well.
d. Incubate for 20 minutes at room temperature.



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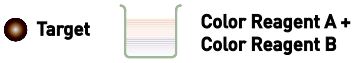

Read the plate and generate the standard curve

1. Read the absorbance at 540–570 nm.
2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: Dilute samples producing signals greater than that of the highest standard in Assay Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Guidelines for calculating NO concentration

- Total NO (nitrite + nitrate) concentrations are calculated from data obtained from the **total nitric oxide determination** procedure.
- Nitrite concentrations are calculated from data obtained from the **nitrite determination** procedure.
- Nitrate concentrations are obtained by subtracting the nitrite concentration of each sample from the total NO concentration (i.e., [nitrate] = [total NO] – [nitrite]).

Performance characteristics

Standard curve (example)

The following data were obtained for the various standards.

Standard Nitrite/Total NO (µM)	Nitrite	Total NO
	Optical Density (540–570 nm)	Optical Density (540–570 nm)
200	2.144	1.984
100	1.248	0.921
50	0.708	0.450
25	0.412	0.240
12.5	0.236	0.138
6.25	0.145	0.092
3.125	0.095	0.083
0	0.038	0.040

Intra-assay precision

Samples were assayed in replicates of 20 to determine precision within an assay.

Sample	Nitrite		Total NO	
	Mean (µM)	%CV	Mean (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

CV = Coefficient of Variation

Inter-assay precision

Samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Sample	Nitrite		Total NO	
	Mean (µM)	%CV	Mean (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

CV = Coefficient of Variation

Linearity of dilution

Linearity was determined by assaying high and low concentration NO samples mixed in the ratios shown in the following table.

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

Sensitivity

The analytical sensitivity of the assay is 2.63 µM nitrite, and 1.02 µM total NO. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

Limited product warranty

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