Total Nitric Oxide Assay Kit

Catalog Number: EMSNOTOT

Product description

A complete kit for the quantitative determination of total nitric oxide (NO) in biological fluids. The kit uses the enzyme nitrate reductase to convert nitrate to nitrite. Nitrite is then detected as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. The interaction of NO in a system is measured by the determination of total nitrate and nitrite concentrations in the sample. This kit allows for the total determination of both NO products in the sample by conversion of all of the sample nitrate to nitrite, followed by the determination of the total concentration of nitrite in the sample.

Contents and storage

Upon receipt, store the kit at ≤ –20°C. Avoid repeated freeze-thaw cycles.

Description	Size
10X Reagent Diluent	30 mL
Nitrate Reductase Diluent	4 mL
Nitrate Reductase	2 vials
NADH, light-sensitive	2 vials
Nitrate Standard (1,000 µM)	0.5 mL
Griess Reagent I	12 mL
Griess Reagent II	12 mL

Additional required materials

- 96-well microtiter plates and plate sealers
- Deionized or distilled water.
- Precision pipettes (for volumes between 25 µL and 1,000 µL).
- Repeater pipettes (for dispensing 25 µL and 50 µL).
- Disposable beakers for diluting buffer concentrates.
- 12 × 75 mm glass tubes.
- Graduated cylinders.
- Ice bath or refrigerated container capable of maintaining 0°C.
- 37°C incubator.
- Microplate reader capable of reading between 540 – 570 nm.
- 10,000 MWCO polysulfone filters.

General guidelines

- Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Sample or buffer component that may interfere with the enzyme will lower the conversion of sample nitrate to nitrite and give rise to lower estimates of NO.
  - Nucleophiles and antioxidants that may interfere with the assays include azide, ascorbic acid, and sulfhydryl containing compounds such as β-mercaptoethanol, cysteine, DTT, and glutathione.
  - Test nitrate recovery using the nitrate provided with the kit if concentrations of these materials are >10 µM in the sample.
  - Add nitrate at concentrations similar to those used for the standard curve to the buffer containing the suspected interfering compound and compare results to a similar buffer without the suspected interfering compound. If there is a significant change, make the appropriate corrections.
- Do not use samples in tissue culture media with high nitrate concentrations (e.g. RPMI).
- Allow kit components **except the Nitrate Reductase enzyme** to come to room temperature for at least 30 minutes before use.

Prepare 1X Reagent Diluent

Dilute 10 mL of 10X Reagent Diluent with 90 mL of deionized water. Label as 1X Reagent Diluent.

The diluted buffer is stable for up to 3 months at room temperature.

Reconstitute and dilute NADH reagent

1. Add 1 mL of deionized water to a vial of NADH.
2. After 3 minutes, vortex prior to use. Use on ice.
3. Dilute 0.9 mL of NADH solution with 1.8 mL of deionized water in a new tube placed on ice.
4. Use diluted NADH solution on ice.
5. Store diluted and undiluted NADH solution tightly capped at –20°C in a manual defrost freezer.

   The solutions are stable for 45 days at ≤ –20°C.

Resuspend Nitrate Reductase Concentrate

6. Add 1 mL of Nitrate Reductase Diluent to a vial of Nitrate Reductase Concentrate.
7. Vortex vigorously and let sit at room temperature for 15 minutes.
8. Vortex again and let sit at room temperature for an additional 15 minutes.
9. Use enzyme on ice and store tightly capped at –20°C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.

   The diluted enzyme is stable for up to 3 months at ≤ –20°C.

Prepare Diluted Nitrate Reductase

1. Determine the number of wells needed for samples and add 14 wells for the complete standard curve (in duplicate).
2. Calculate the required volume of reconstituted Nitrate Reductase Concentrate using the following formula:

   Volume of Nitrate Reductase Concentrate (µL) =
   [Total number wells for samples and standards + 2] × 10 µL
3. Calculate the required volume of 1X Reagent Diluent using the following formula:

   Volume of 1X Reagent Diluent (µL) =
   [Volume Nitrate Reductase Concentrate (step 2)] × 1.5 µL
4. Add 1X Reagent Diluent (step 3) to a clean tube, and place on ice.
5. Immediately before use, add Nitrate Reductase Concentrate (step 2) to the tube containing 1X Reagent Diluent.
6. Vortex the tube and use on ice within 15 minutes.

Sample preparation guidelines

- All samples should be diluted at least 1:2 with 1X Reagent Diluent.
- NO synthetase enzyme systems using high concentrations (0.5 –1 mM) of NADPH, may inhibit the Griess color reaction slightly.
  - Dilute such samples are sufficiently (≥ 1:10) in 1X Reagent Diluent to minimize the effects of NADPH.
  - Oxidize NADPH with 10 µL of lactate dehydrogenase (1500 U/mL in 30 mM sodium pyruvate) after incubation with nitrate reductase (See “Nitrate assay procedure”, step 8), and incubate at 37°C for 10 minutes before adding the Griess reagents.

Whole Organism Samples

- Take environmental nitrite and nitrate levels into account.
- Adjustments to the nitrite and nitrate levels in the sample must take into account the turnover of environmental nitrite and nitrate by the organism.
- Analyze media or fluid that the organism is stored in separately.

Urine

- Fresh urine samples should be diluted at least 1:20 with 1X Reagent Diluent, and ultrafiltered through a 10,000 molecular weight cut off (MWCO) filter.
- Add antibiotics (e.g. penicillin or streptomycin at 100 U/mL), or 2-propanol at 6.5% (v/v) to the samples, and freeze at –80°C if samples will not be tested immediately.

Saliva

- Saliva samples should be diluted 1:2 – 1:100 into 1X Reagent Diluent, and ultrafiltered through a 10,000 molecular weight cut off (MWCO) filter.
- Typical saliva samples may contain relatively high concentrations of nitrate produced by oral bacteria.

Plasma

- Plasma should be diluted 1:2 – 1:20 in 1X Reagent Diluent and ultrafiltered through a 10,000 MWCO filter.
- Citrate plasma is recommended. EDTA or heparinized plasma may be used, but may not give reproducible results as the protein may precipitate during the Griess reaction.

Serum

Serum samples should be diluted 1:2 – 1:20 in 1X Reagent Diluent, and ultrafiltered through a 10,000 molecular weight cut off (MWCO) filter.

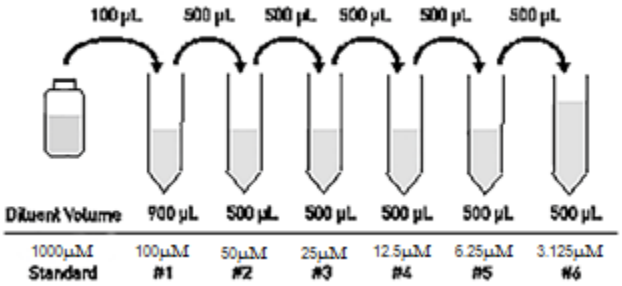
Culture Supernatant

Avoid media containing nitrates. Samples should be diluted at least 1:2 in 1X Reagent Diluent, and ultrafiltered through a 10,000 molecular weight cut off (MWCO) filter.

Dilute standards

Allow the Nitrate Standard solution to warm to room temperature before use.

- Label six 12 × 75 mm glass tubes #1 through #6.
- Add 900 µL 1X Reagent Diluent to Tube #1.
- Add 500 µL 1X Reagent Diluent to Tubes #2 to #6.
- Add 100 µL Nitrate Standard to Tube #1 and vortex thoroughly.
- Add 500 µL of Tube #1 to Tube #2 and vortex thoroughly.
- Continue to make serial dilutions of the standard as shown in the diagram below.



Nitrate assay procedure

Determine the number wells required for the assay.

Bring all reagents to room temperature for at least 30 minutes before opening. Run all standards and samples in duplicate.

1. Add 200 µL of 1X Reagent Diluent into the Blank wells.
2. Add 50 µL of Nitrate Standards #1 through #6 into the appropriate wells.
3. Add 50 µL of 1X Reagent Diluent into the Zero Standard wells.
4. Add 50 µL of the Samples into the appropriate wells.
5. Add 25 µL of diluted NADH into all Zero Standard, Standard, and Sample wells.
6. Add 25 µL of Diluted Nitrate Reductase into all Zero Standard, Standard, and Sample wells.
7. Tap the plate gently to mix the contents.
8. Seal the plate and incubate at 37°C for 30 minutes.
9. Add 50 µL of Griess Reagent I into each well, except the Blank wells.
10. Add 50 µL of Griess Reagent II into each well, except the Blank wells.
11. Tap the plate gently to mix the contents.
12. Incubate the plate at room temperature for 10 minutes.
13. Blank the plate reader against the Blank wells, and read the optical density at 540–570 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculations

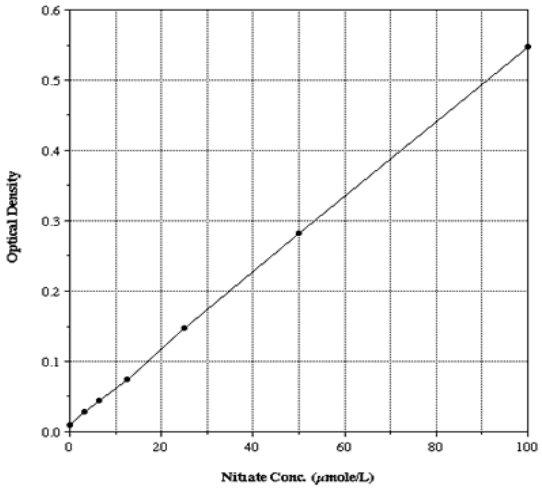
Analyze the data with a 4-parameter logistic curve-fitting program. If data reduction software is not available, the concentration of total NO can be calculated by the following method:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Zero Standard OD from the average OD for each standard and sample.

   Average Net OD = Average OD – Average Zero Standard OD
2. Plot the Average Net OD for each Standard Concentration.
3. Plot the Average OD for each Sample and interpolate Total NO Concentration from the graph.

Typical standard curve

A typical standard curve is shown below. This curve must not be used to calculate nitrate concentrations; a standard curve must be run with every assay.



Performance characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

Sensitivity

The minimum detectable dose of nitrate is 0.625 µM. This was determined by adding two standard deviations to the mean O.D. obtained from the average OD bound for 24 wells run as Zero Standard, compared to the average OD for wells run with Standard #6.

Linearity

A sample containing 75 µM nitrate was serially diluted in 1X Reagent Diluent over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a line with a slope of 1.011 and correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of nitrate and running these samples multiple times (n=8) in the same assay. Inter-assay precision was determined by measuring 3 samples with low, medium and high concentrations of total nitrate in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of nitrate determined in these assays as calculated by a 4-parameter logistic curve-fitting program.

Intra-assay	Nitrate (µM)	%CV
Low	20.86	5.3%
Medium	50.92	3.1%
High	68.51	1.2%

Inter-assay	Nitrate (µM)	%CV
Low	18.67	6.9%
Medium	48.80	4.2%
High	68.25	3.3%

Sample recovery

Nitrate was spiked into samples which had been diluted with 1X Reagent Diluent. The diluted samples were ultrafiltered through a 10,000 MWCO filter and assayed in the kit. The following results were obtained.

Sample	% Recovery	Recommended Dilution
Cell Culture Media	88%	1:2
Human Saliva	100%	1:2 to 1:100
Human Urine	104%	1:20
Human Serum	93%	1:2 to 1:20
Human Citrate Plasma	98%	1:2 to 1:20
Human EDTA Plasma	91%	1:2 to 1:10

The recommended dilutions for the assay take into account the normal nitrate levels in some samples. All samples are diluted 1:2 in 1X Reagent Diluent, but some samples need to be diluted 1:2 to 1:200 to read on the Nitrate Standard Curve.

Limited product warranty

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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

Manufacturer's address: Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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