QuantiChrom™ Nitric Oxide Assay Kit (DINO-250)

Quantitative Colorimetric Determination of Nitric Oxide at 540nm

DESCRIPTION

Nitric oxide (NO) is a reactive radical that plays an important role in many key physiological functions. NO, an oxidation product of arginine by nitric oxide synthase, is involved in host defense and development, activation of regulatory proteins and direct covalent interaction with functional biomolecules.

Simple, direct and automation-ready procedures for measuring NO are becoming popular in Research and Drug Discovery. Since NO is oxidized to nitrite and nitrate, it is common practice to quantitate total NO_2^-/NO_3^- as a measure for NO level. BioAssay Systems' QuantiChrom™ Nitric Oxide Assay Kit is designed to accurately measure NO production following reduction of nitrate to nitrite using improved Griess method. The procedure is simple and the time required for sample pretreatment and assay is reduced to only 40 min.

KEY FEATURES

Sensitive and accurate. Detection range 0.1 - 50 µM in 96-well plate.
Rapid and reliable. Using optimized Cd/Cu reagent, the time required for reduction of NO_3^- to NO_2^- is 15 min at >98.5% conversion rate.
Simple and high-throughput. The procedure involves mixing sample with two reagents, incubation for 5 min and reading the optical density. Can be readily automated to measure thousands of samples per day.

APPLICATIONS:

Direct Assays: NO in plasma, serum, urine, tissue/cells and foods.
Drug Discovery/Pharmacology: effects of drugs on NO metabolism.

KIT CONTENTS (250 tests in 96-well plates)

Reagent A:	14 mL
Reagent B:	14 mL
20x $ZnSO_4$:	1.0 mL 1.5 M solution
30x NaOH:	1.0 mL 1.65 M solution
1 x Glycine Buffer:	30 mL
3 x Activation Buffer:	50 mL
Cadmium granules:	15 g
Nitrite standard:	1.0 mL 1.0 mM nitrite

Storage conditions. Store Reagent A, B and Nitrite standard at 4°C. All other components can be stored at room temp. Shelf life: 12 months.

Precautions: reagents are for research use only. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Preparations. Dilute 20x $ZnSO_4$ and 30x NaOH to 1-fold with water. The final concentrations are 75 mM $ZnSO_4$ and 55 mM NaOH, respectively. Dilute Activation Buffer by mixing 1 volume of 3 x Activation Buffer with 2 volumes of distilled water. All diluted solutions can be stored at 4°C for 12 months.

Procedure using 96-well plate:

1. *Standards*. Prepare 600 µL 50 µM Premix by mixing 30 µL 1.0 mM Standard and 570 µL distilled water. Dilute standard in centrifuge tubes as shown below. Add 50 µL Glycine Buffer per tube. Transfer 100 µL diluted standards into wells of a clear-bottom 96-well plate.

No	Premix + H_2O + Gly Buffer	Vol (µL)	Nitrite (µM)
1	150µL + 0µL + 50 µL	200	50
2	120µL + 30µL + 50 µL	200	40
3	90µL + 60µL + 50 µL	200	30
4	60µL + 90µL + 50 µL	200	20
5	45µL + 105µL + 50 µL	200	15
6	30µL + 120µL + 50 µL	200	10
7	15µL + 135µL + 50 µL	200	5
8	0µL + 150µL + 50 µL	200	0

2. *Deproteination* is required for serum, plasma and other proteinaceous samples. Mix 100 µL sample with 80 µL 75 mM $ZnSO_4$ in 1.5-mL tubes. If precipitation occurs, centrifuge 5 min at 14,000 rpm. Transfer supernatant to a clean tube containing 120 µL 55 mM NaOH. Pellet protein precipitates again (dilution factor n = 3). Transfer 210 µL supernatant and mix with 70 µL Glycine Buffer in a 1.5-mL centrifuge tube.

   If solution remains clear in these steps, deproteination is not required. Directly transfer 210 µL sample (dilution factor n = 1) and mix with 70 µL Glycine Buffer in a 1.5-mL centrifuge tube.

3. *Activation of Cd*. The number of Cd granules to be used is 3 x the number of samples. Transfer Cd granules in a 50-mL centrifuge tube. Wash Cd three times with water. Remove residual water with a pipet. Add 200 µL diluted 1 x Activation Buffer per granule and incubate 5 min at room temperature. Swirl tube intermittently. Wash three times with water. Activated Cd should be used within 20 min.

   Note: cadmium is a toxic and expensive metal. Avoid direct contact (wear gloves). About 150 Cd granules are provided that are sufficient for about 50 samples. Used Cd granules should be stored or washed in 0.1 N HCl for at least 5 min. Washed 3 times with water and regenerated using the same activation procedure. Cd granules can be regenerated and used 7 times without loss of activity.

4. *Nitrate Reduction*. Dry the activated Cd granules on a filter paper (e.g. Kimwipes® EX-L). Add three Cd granules per sample (Step 2) and shake tubes intermittently. Incubate 15 min at room temperature. Transfer 2 x 100 µL samples (duplicate) into wells of the 96-well plate.

5. *Assay*. Add 50 µL Reagent A to all wells and tap plate lightly to mix. Add 50 µL Reagent B and mix. Incubate 5 min at room temperature. Read OD at 500-570nm (peak 540 nm). Signal is stable for > 60 min.

Procedure using Cuvet:

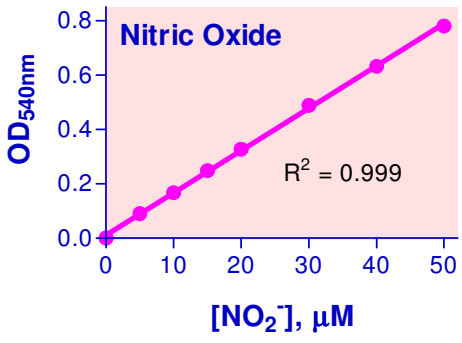
Prepare standards and samples as described for the 96-well procedure. After the reduction step, mix 500 µL diluted standard and deproteinated sample with 250 µL Reagent A and 250 µL Reagent B. Measure OD_{540nm} in the cuvet.

CALCULATION

Subtract blank OD (water, #8) from the standard OD values and plot the OD against standard concentrations. Determine the slope using linear regression fitting. The NO concentration of Sample is calculated as

$$= \frac{OD_{SAMPLE} - OD_{BLANK}}{\text{Slope}} \times n \ (\mu M)$$

OD_{SAMPLE} and OD_{BLANK} are optical density values of the sample and water, respectively. n is the dilution factor (see Step 2).
Conversions: 1 mg/dL NO equals 333 µM, 0.001% or 10 ppm.



Calibration curve in 96-well plate assay

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting devices, centrifuge tubes and table centrifuge.

Procedure using 96-well plate:

Clear bottom 96-well plates (e.g. Corning Costar) and plate reader.

Procedure using cuvette:

Cuvets and spectrophotometer for measuring optical density at 540 nm.

GENERAL CONSIDERATIONS

Antioxidants and nucleophiles (e.g. β-mercaptoethanol, glutathione, dithiothreitol and cysteine) may interfere with this assay. Avoid using these compounds during sample preparation.

Samples that need deproteination include serum, plasma, whole blood, cell culture media containing FBS, tissue or cell homogenates. Urine and saliva do not need deproteination.

EXAMPLE

Rat serum and fresh human urine was analyzed using the 96-well protocol. The NO levels were 28.7 ± 0.3 and 6.4 ± 0.2 μM, respectively. Coefficient of Variance < 4%. Day-to-day variation < 8%.

LITERATURE

1. Cortas NK, Wakid NW (1990). Determination of inorganic nitrate in **serum and urine** by a kinetic cadmium-reduction method. Clin Chem. 36:1440-3.

2. Ridnour LA, Sim JE, Hayward MA, Wink DA, Martin SM, Buettner GR, Spitz DR (2000). A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in **cell culture media**. Anal Biochem. 281:223-9.

3. Sen NP, Donaldson B (1978). Improved colorimetric method for determining nitrate and nitrate in **foods**. J Assoc Off Anal Chem. 61:1389-1394.