KAMIYA BIOMEDICAL COMPANY

Nitric Oxide Fluorometric Assay

For the quantitative determination of total nitric oxide (NO) in culture media, plasma and tissue homogenates

Cat. No. KT-152

For Research Use Only. Not For Use in Diagnostic Procedures.

PRODUCT INFORMATION

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PRODUCT

The **K-*ASSAY*®** Nitric Oxide Fluorometric Assay is for the quantitative determination of total nitric oxide (NO) in culture media, plasma and tissue homogenates.

PRINCIPLE

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. Since NO is rapidly converted to nitrite (NO_2^-) and nitrate (NO_3^-), the total concentration of nitrite and nitrate is used as a quantitative measure of NO production. The **K-*ASSAY*®** Nitric Oxide Fluorometric Assay provides an accurate and convenient measurement of total nitrate/nitrite concentration in a simple two-step process. In the first step, nitrate is converted to nitrite by nitrate reductase. In the second step, nitrite reacts with the fluorescent probe DAN (2, 3 diaminonaphthalene). NaOH enhances the fluorescent yield. The fluorescent intensity is proportional to the total nitric oxide production. The kit has been tested with culture media, plasma and tissue homogenates.

COMPONENT

• Assay Buffer	1 bottle	30 mL
• Enzyme Cofactors (blue cap)	1 vial	Lyophilized
• Enhancer (purple cap)	1 vial	Lyophilized
• Nitrate Reductase (green cap)	1 vial	Lyophilized
• Nitrate Calibrator (yellow cap)	1 vial	Lyophilized
• Nitrite Calibrator (orange cap)	1 vial	Lyophilized
• DAN Reagent (red cap)	1 vial	1 mL
• Sodium Hydroxide (clear cap)	1 vial	1 mL
• Microtiter Plate	2 plates	
• Plate Cover	2 covers	

PROTOCOLS

Preparation of Reagents

1. Assay Buffer: The assay buffer is ready to use as supplied. Store at 4°C.
2. Enzyme Cofactors: Reconstitute with 1.2 mL of Assay Buffer. Aliquot desired amount and store at –20°C. Keep on ice during use. Freeze/thaw should be limited to 1 time.
3. Enhancer: Reconstitute with 1.2 mL of Assay Buffer. Keep on ice during use. Store at -20°C.
4. Nitrate Reductase: Reconstitute with 1.2 mL of Assay Buffer. Aliquot desired amount and store at –20°C. Keep on ice during use. Freeze/thaw should be limited to 1 time.
5. Nitrate/Nitrite Calibrators: Reconstitute with 1.0 mL of Assay Buffer. Vortex to generate 10 mM calibrator each. Store at 4°C when not in use (do not freeze!). The reconstituted calibrators are stable for 4 months when stored at 4°C.
6. Fluorometric DAN Reagent and NaOH: Ready to use. Store at 4°C.

Measurement of Nitrate + Nitrite:

1. Prepare Calibrators:
    - Add 5 µL of the reconstituted 10 mM nitrate/nitrite calibrators to 995 µL assay buffer.
    - Vortex to generate 50 µM working calibrator solution.
    - Add 0, 4, 8, 12, 16, 20 µL of the working calibrator to 6 consecutive wells to generate 0, 200, 400, 600, 800, 1,000 pmoL/well calibrator.
    - Bring the volume to 75 µL with Assay Buffer.

*Note: DAN Reagent reacts with nitrite, not nitrate. For routine total nitrite/nitrate assay, you may prepare a nitrate calibration curve only. However, if you need to measure nitrite and nitrate concentrations seperately, you may prepapre a nitrite calibration curve in the absence of Nitrate Reducatse in the calibration curve and assay samples. Nitrate = Total – Nitrite.

2. Prepare Samples:
   - Samples containing high protein concentration may need to be filtered through a 10Kd MW cut-off filter prior to assay.
   - Add 0-75 µL of sample to the wells and adjust the volume to 75 µL with Assay Buffer.

*Note: Typical urine nitrite and nitrate levels are in the 0.2-2 mM and 1-20 µM range respectively. Typical normal serum levels are 0-20 µM and 0-2 µM respectively with various disease states elevating these levels significantly. Plasma samples or tissue homogenates should be assayed with no more than 10 µL of undiluted sample.

3. Add 5 µL of the Enzyme Cofactor mixture to all wells.
4. Add 5 µL of the Nitrate Reductase to nitrate assay wells (unknowns and calibrators), add 5 µL of buffer in place of Nitrate Reductase (unknowns and calibrators) when you determine nitrite separately.
5. Cover the plate with the plate cover and incubate at room temperature for 1-4 hrs. 1 hour = ~90% conversion of Nitrate to Nitrite, 2 hours= ~95% conversion, 4 hours = ~99% conversion.
6. Add 5 µL of Enhancer to each well. Incubate 30 minutes to quench interfering compounds.
7. Add 5 µL of DAN Reagent to each well. Incubate for 10 minutes at room temperature.
8. Add 5 µL of NaOH to each well. Incubate for 10 minutes at room temperature.
9. Read the plate in a fluorometer using Ex.= 360 nm and Em.= 450 nm.

CALCULATIONS

1. Plot calibration curve: Plot fluorescence vs. picomoles nitrate.
2. Determine sample nitrate and nitrite concentrations:

$$\text{C= [Nitrate + Nitrite] (µM)} = \left(\frac{fluorescence - y\ \text{int}\,ercept}{slope}\right)\left(\frac{1}{sample\ volume(\mu L)}\right) \text{X}\ dilution\ *$$

OR

C = Sa/Sv, where Sa is the amount of samples as read from calibration curve (in pmole), and Sv is the volume of sample added to the well (in µL), multiplied by the dilution* factor.

*Dilution is the sample dilution done prior to addition of the sample to the well.

STORAGE

Store unopened kit at -20℃.

FOR RESEARCH USE ONLY

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