KAMIYA BIOMEDICAL COMPANY

Nitrate/Nitrite Assay

For the quantitative colorimetric determination of nitrate/nitrite in urine, plasma, serum, saliva, cell lysate and tissue culture medium

Cat. No. DN-006

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

PRODUCT INFORMATION

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INTENDED USE
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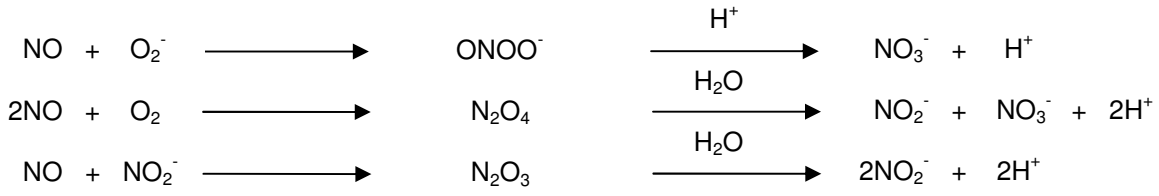
INTRODUCTION
The Nitrate/Nitrite Assay is a simple and sensitive assay for nitrate and nitrite determination based on the commonly used two-step assay method with no need to use lactate dehydrogenase (LDH). The sensitivity of the assay is equivalent to that of the three-step LDH assay method, but more sensitive than that of the commonly used two-step method. This new method involves the addition of two cofactors for the nitrate reductase reaction. With the help of additional cofactors, the reductase reaction to convert nitrate to nitrite is accelerated and simultaneously the excess NADPH is degraded to NADP. Thus, the reductase reaction can be completed within 30 minutes and colorimetric determination can be directly measured by the addition of Griess Reagent without the use of lactate dehydrogenase. This kit is fast, simple and can be used to assay nitrite and nitrate in urine, plasma, serum, saliva, cell lysate and tissue culture medium.

PRINCIPLE
Nitric oxide (NO) is a key molecule that, either directly or through intracellular signaling, stimulates host defenses in the immune system, maintains blood pressure in the cardiovascular system and modulates neural transmission in the brain. NO is an activator of soluble guanylyl cyclase, which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) and leads to vasodilatation and inhibition of leukocyte and platelet activation. As the biologically active component of endothelium-derived relaxing factor, NO plays critical roles in the maintenance of vascular homeostasis. NO acts as a neurotransmitter in the central and peripheral nervous systems and, therefore, is critical in the pathogenesis of stroke and other neurodegenerative disorders. As a signal transducer in mammalian systems, NO covalently interacts with target molecules based on redox potential. Finally, as a highly reactive chemical, NO directly regulates the activity of many proteins, such as kinases and proteases.

Nitric oxide synthase (NOS) catalyses the oxidation of the terminal guanidino nitrogen of the amino acid L-arginine to produce NO and L-citrulline. Three distinct forms of NOS have been described: neuronal NOS, endothelial NOS and inducible NOS. Neuronal and endothelial NOS are regulated by physiological changes in intracellular calcium concentrations, whereas inducible NOS appears to be regulated in a cell-specific manner. Monitoring cellular NO production is a useful tool for determining NOS activity. However, the volatile nature of NO makes it unsuitable for most detection methods.



In the cell, NO undergoes a series of reactions with several molecules present in biological fluids and is eventually metabolized to nitrite (NO_2^-) and nitrate (NO_3^-). Thus, the best index of total NO production is the sum of both nitrite and nitrate, commonly quantified in a two-step assay.

The first step in the measurement of NO is the conversion of nitrate to nitrite by the use of NADH or NADPH-dependent nitrate reductase. Subsequently, the converted nitrite can be quantified by the addition of Griess Reagent, which converts nitrite into a purple azo compound. Accurate concentration of nitrite can be determined by photometric measurement of the colored azo compound. In this two-step assay method, NADPH is commonly used as an essential

cofactor for nitrate reduction. However, excess NADPH interferes with the subsequent Griess reaction, which limits the sensitivity of the two-step assay. In order to remove this penalizing interference, the two-step assay method has been improved by the addition of an additional step. The three-step assay (LDH assay method) includes a lactate dehydrogenase reaction after the reductase reaction in order to degrade the excess NADPH. Although the additional step improves the sensitivity when compared to the two-step assay, the LDH assay is tedious and time-consuming. By the addition of two cofactors to the two-step assay, **KAMIYA BIOMEDICAL COMPANY's** Nitrate/Nitrite Assay combines high sensitivity and fast processing of samples in a two-step assay method.

COMPONENTS

Kit components can be stored at 4°C for 6 months. Upon reconstitution, the Nitrate Reductase and Cofactors should be stored at -20°C (stable up to 2 months).

Reagent	Quantity
Assay Buffer (10X)	1 bottle, 30 mL
Nitrate Reductase	4 vials, lyophilized
Cofactors	4 vials, lyophilized
Nitrate Calibrator	2 vial, 1.5 mL
Nitrite Calibrator	2 vial, 1.5 mL
Griess Reagent A	1 bottle, 12 mL
Griess Reagent B	1 bottle, 12 mL
Microtiter Plate, 96-well	2 plates
Plate sealers	4 covers

Materials Required But Not Provided

- Distilled water (HPLC-grade recommended)
- Multi-channel pipettor
- Microplate spectrophotometer with a 540 nm filter for samples and 620 nm filter for reference.
- Rocking platform (for nitrate assay only)
- 10,000 Dalton micropore filter (for samples rich in protein)

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only, not for human or diagnostic use.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.

PROTOCOLS

1. Assay Buffer: Prepare the amount of 1X Assay Buffer for the assay as follows: For every 10 mL of 1X Assay Buffer required, dilute 1 mL of 10X Assay Buffer in 9 mL of distilled water. The 1X Assay Buffer should be used for the reconstitution of Nitrate Reductase, dilution of samples and preparation of calibration curves for nitrate and nitrite assays. Excess 1X Assay Buffer can be stored at 4°C for one week.

2. Nitrate Reductase: Four vials are provided in lyophilized form. Each vial is sufficient for 50 reactions. Reconstitute the contents of each vial with 0.6 mL ice-cold 1X Assay Buffer. Store solution at -20°C when not in use. Freezing and thawing of reconstituted Nitrate Reductase should be limited to one time.

3. Cofactors: Four vials are provided in lyophilized form. Each vial is sufficient for 50 reactions. Reconstitute the contents of the vial with 1.2 mL of ice-cold distilled water. Store solution at -20°C when not in use. Freezing and thawing of reconstituted Cofactors should be limited to one time.

4. Nitrate Calibrator: Each vial contains a solution of 200 µM $NaNO_3$. **Do not** add water or Assay Buffer to this vial. The Nitrate Calibrator should be stored at 4°C.

5. Nitrite Calibrator: Each vial contains a solution of 200 µM $NaNO_2$. **Do not** add water or Assay Buffer to this vial. The Nitrite Calibrator should be stored at 4°C.

6. Griess Reagent: Griess reagent is composed of 2 solutions: Griess Reagent A and Griess reagent B. **Do not** add water or Assay Buffer to these bottles. Store at 4°C.

Sample Preparation

We recommend measuring the nitrate or nitrite concentrations in the samples in duplicate.

1. **Urine Sample**: Urine may be used directly after proper dilution with 1X Assay Buffer, but in some cases, precipitation will form after the addition of Griess Reagent. In this case, the sample should be filtered through a 10,000 Dalton micropore filter (e.g. Millipore UFC801008) prior to assay.

2. **Plasma, Serum and Saliva**: Citrate, EDTA or heparin may be used as an anticoagulant for the collection of plasma. All samples require at least a 2-fold dilution with 1X Assay Buffer followed by centrifugation to remove particulate matter. After the above treatments, samples must be filtered through a 10,000 Dalton micropore filter (e.g. Millipore UFC801008) prior to assay.

3. **Culture Media**: The use of a media containing high levels of intrinsic nitrate/nitrite (e.g. RPMI) must be avoided for cell culture. We recommend adapting the cells to a media poor in nitrate/nitrite (e.g. MEM), as well as phenol red. Cellular nitrite/nitrate production can be quantified by subtracting the level of nitrite/nitrate present in the media from the nitrite/nitrate level present during cell growth. The effect of components in the media on color development can be addressed by preparing nitrate or nitrite calibrators in the presence of a fixed volume of the culture media.

4. **Cell Lysate:** Cell lysate contains a high number of large proteins that can interfere with the Nitrate Reductase or Griess reaction. The sample should be filtered through a 10,000 Dalton micropore filter prior to the assay to remove high molecular weight proteins.

DETERMINATION OF NITRATE

Prior to starting the nitrate determination, make sure that all of the reagents needed are prepared and cooled on ice. For your samples, we recommend using duplicate wells for measuring the nitrate concentration, whereas one well can be used for each nitrate calibrator concentration.

Preparation of Nitrate Calibration Curve

The calibration curve for nitrate is prepared by the addition of reagents to the plate wells in the following way:

Well	Nitrate Cal. (µL)	1X Assay Buffer (µL)	Final Nitrate Conc. (µM)*
A1	0	70	0
B1	5	65	5
C1	10	60	10
D1	15	55	15
E1	20	50	20
F1	25	45	25
G1	30	40	30
H1	35	35	35

* Concentrations indicated are for those in the final 200 µL assay volume, after addition of the Griess Reagent.

Nitrate Assay Procedure

1. Add 70 µL of 1X Assay Buffer to the blank wells.

2. Add up to 70 µL of sample or diluted sample to the sample wells. If the sample volume is less than 70 µL, adjust the final volume to 70 µL using the 1X Assay Buffer.

   Note: The pH of the sample should be approximately neutral since the enzyme becomes inactivated at high and low pH values. Sample volumes for plasma, serum or tissue homogenates are recommended to be less than 40 µL.

3. Add 20 µL of the reconstituted Cofactors solution to each well (blanks, calibrators, samples).

4. Add 10 µL of the reconstituted Nitrate Reductase solution to each well.

5. Cover the plate with a plate sealer and mix on a rocking platform set at 150 rpm for one minute.

6. Incubate the plate for 30 minutes at room temperature (RT). Avoid light exposure during incubation. It is not necessary to shake the plate during incubation.

7. Add 50 µL of the Griess Reagent A to each well.

8. Immediately add 50 µL of Griess Reagent B to each well.

9. Allow the color to develop for 20 minutes at RT.

10. Read the absorbance at 540 nm with a reference wavelength of 620 nm using the plate reader.

DETERMINATION OF NITRITE

Prior to starting the nitrite determination, make sure that all of the reagents needed are prepared and cooled on ice. For your samples, we recommend using duplicate wells for measuring the nitrite concentration, whereas one well can be used for each nitrite calibrator concentration.

Preparation of Nitrite Calibration Curve

Nitrite concentrations may be measured directly without performing the Nitrate Reductase reaction. The nitrite calibration curve is prepared as follows:

Well	Nitrite Cal. (µL)	1X Assay Buffer (µL)	Final Nitrite Conc. (µM)*
A1	0	100	0
B1	5	95	5
C1	10	90	10
D1	15	85	15
E1	20	80	20
F1	25	75	25
G1	30	70	30
H1	35	65	35

* Concentrations indicated are for those in the final 200 µL assay volume, after addition of the Griess Reagent.

Nitrite Assay Procedure

1. Add 100 µL of 1X Assay Buffer to the blank wells.

2. Add up to 100 µL of sample to the sample wells. If the sample volume is less than 100 µL, adjust the final volume to 100 µL using the 1X Assay Buffer.

3. Add 50 µL of the Griess Reagent A to each well (blanks, calibrators and samples).

4. Immediately add 50 µL of Griess Reagent B to each well.

5. Allow the color to develop for 10 minutes at RT.

6. Read the absorbance at 540 nm with a reference wavelength of 620 nm using the plate reader.

CALCULATIONS

1. Subtract the blanks: Average the absorbance values of the blank wells and the sample wells, followed by subtracting the average blank value from the mean absorbance values of the samples.

2. Plotting the calibration curves: Make a plot of absorbance as a function of nitrate or nitrite concentration. The nitrate assay converts nitrate to nitrite, then measures total nitrite. Therefore, the nitrate calibration curve is used for determination of total nitrate and nitrite concentration in the sample, whereas the nitrite calibration curve is used only for the nitrite concentration. The nitrate and nitrite calibration curves are supposed to be identical; in practice, however, a small discrepancy often occurs.

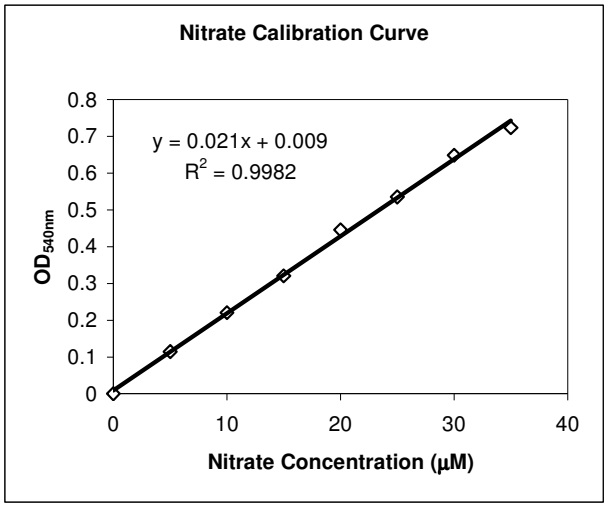
3. Determine nitrate and nitrite concentration:

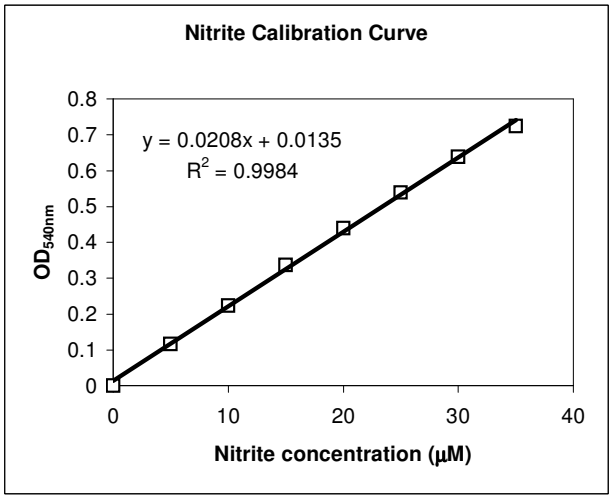
   The calibration curves are used as follows:
   (a.) Measure the endogenous nitrite concentration of a sample using the nitrite assay procedure and the calibration curve of nitrite.
   (b.) Measure the total nitrate/nitrite concentration of a sample using the nitrate assay procedure and the calibration curve of nitrate.
   (c.) Determine the endogenous nitrate concentration of a sample by subtracting the endogenous nitrite concentration obtained in (a) from the total nitrate/nitrite concentration obtained in (b).

PERFORMANCE CHARACTERISTICS

Typical Calibration Curves

The nitrate and nitrite calibration curves are provided for demonstration only. Calibration curves should be generated for each set of samples assayed.





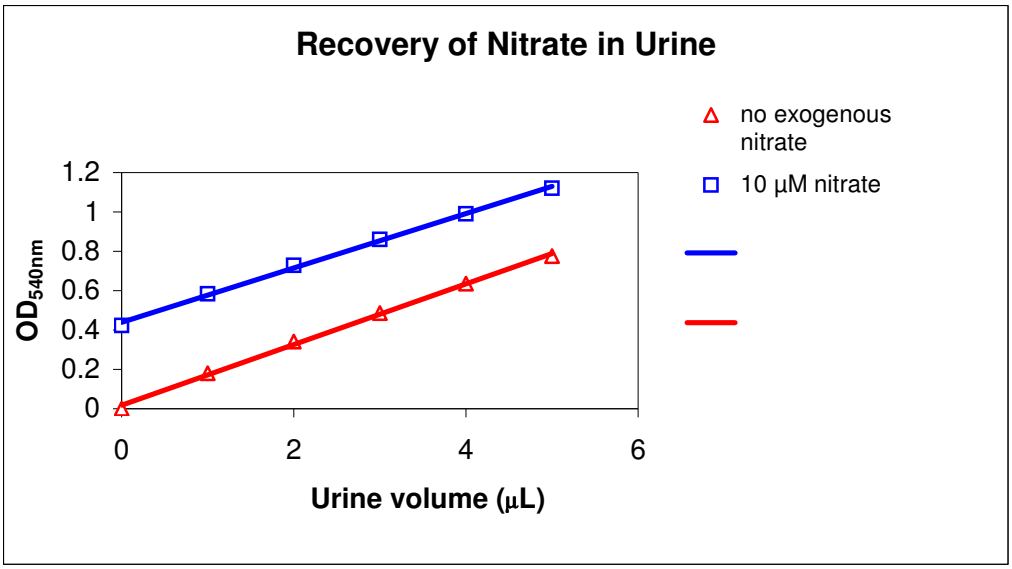
Nitrate/Nitrite Recovery

Samples may contain proteins or metal ions that could alter the nitrate reductase and/or Griess reactions. Redox agents such as ascorbic acid, dithiothreitol and mercaptoethanol will interfere with color development. Phosphate concentrations higher than 50 mM will alter the Nitrate Reductase activity. In these cases, nitrate and nitrite concentrations in the samples would be biased. The percentage of nitrate and nitrite recovery from a sample can be determined by adding a fixed amount of nitrite and nitrate calibrators to the sample, as shown below. Then, use kit procedures to determine nitrate and nitrite concentrations in the samples.

Typical Recovery Data

Human urine samples were collected, centrifuged and filtered through a 10,000 Dalton micropore filter. The amount of nitrate in various volumes of the filtrate (0-5 μL) was measured in the presence or absence of exogenous nitrate (final concentration: 10 μM) according to the nitrate assay procedure. Percent recovery is determined as follows:

1. Subtract the absorbance of the sample containing exogenous nitrate from the absorbance of the sample without exogenous nitrate.
2. Divide this value by the absorbance obtained with the 10 μM nitrate calibrator and multiply by 100.
3. Divide the nitrate/nitrite concentration of the sample determined from the corresponding calibration curve by the percentage of recovery corresponding to the amount of sample used for the assay.



Urine (µL)	OD_{540} without nitrate	OD_{540} with 10 µM nitrate	Recovery
0	0.000	0.423	100.0%
1	0.180	0.584	95.5%
2	0.342	0.729	91.5%
3	0.486	0.860	88.4%
4	0.636	0.990	83.7%
5	0.775	1.121	81.8%

Example for the 1 µL urine sample:

1. Subtract the OD_{540} with 10 µM nitrate from the OD_{540} without nitrate.
   0.584 - 0.180 = 0.404
2. Divide this value by the OD_{540} with 10 µM nitrate in absence of urine.
   0.404 / 0.423 = 0.955
3. Multiply this number by 100 to obtain the percentage of recovery.
   0.955 x 100 = 95.5%
4. Divide the [nitrate] determined by the nitrate calibration curve for OD_{540} without nitrate by the percentage of recovery obtained for 1 µL urine.
   7.86 x 100 / 95.5 = 8.23 µM

Linearity and Sensitivity

Linearity of the nitrate and nitrite calibration curve can be obtained at concentrations between 2 to 55 µM. The detection limit of the nitrate and nitrite assays were typically less than 1 µM.

TROUBLESHOOTING

Problem: Erratic values or dispersion of duplicates.

Cause: 1. Poor pipetting technique.
2. Bubbles in well.
3. Incorrect addition of reagents.

Solution: 1. Use a multi-channel pipettor when possible and make sure it is calibrated.
2. Make sure there are no bubbles in the wells prior to absorbance reading.
3. Make sure the correct amount of reagents are added in the right order.

Problem: No color development in the nitrate calibration curve.

Cause: 1. Incorrect addition of reagents.

Solution: 1. Repeat assay making sure the correct volume of reagents are added to all wells.

Problem: The nitrate calibration curve is not linear at high concentration of nitrate.

Cause: 1. Concentration of nitrate is more than 55 µM.
2. Enzyme and Cofactor activity is lost due to repeated freezing and thawing.
3. Plate reader settings are not optimal.

Solution: 1. Use the curve in the linear portion at lower nitrate concentrations.
2. Avoid freezing and thawing of the enzyme and Cofactors, and make sure the kit has not expired.
3. Verify the wavelength and filter settings in the plate reader.

Problem: The nitrite calibration curve is not linear at high concentration of nitrite.

Cause: 1. Plate reader settings not optimal.
2. Nitrite concentration is too high.

Solution: 1. Verify the wavelength and filter settings in the plate reader.
2. Use the curve in the linear portion at lower nitrite concentrations.

FOR RESEARCH USE ONLY

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