NO_2/ NO_3 Assay Kit - C II (Colorimetric) - Griess Reagent Kit - Technical Manual

Technical Manual (Japanese version) is available at http://www.dojindo.co.jp/manual/nk05.pdf

General Information

Nitric oxide (NO), a gaseous paramagnetic radical, is a very important and versatile messenger in biological systems. NO is synthesized from L-arginine by NO synthase (NOS). It has been identified as an endothelial derived relaxation factor(EDRF) and antiplatelet substance. It serves as a neurotransmitter derived from neutrophile and a cytotoxic substance from an activated macropharge. Although NO's molecular action in the biological system is very versatile, the most important role of NO is the activation of guanylate cyclase.

The Griess assay is one of the most popular and simplest methods used to detect the NO concentration. The Griess assay machanism is summarized as the azo coupling between diazonium species, which are produced from sulfanilamide with NO_2, and naphthylethylenediamine(Fig.1). The NO_2/NO_3 Assay Kit-CII contains these dyes, Nitrate Reductase, Enzyme Co-factors, Buffer Solution and NO_2, NO_3 Solutions as standards. Therefore, total NO metabolites (Scheme 1) are easily detectable using this kit. The suitable NO_2 detection range is from 10 to 100 µmol/l.

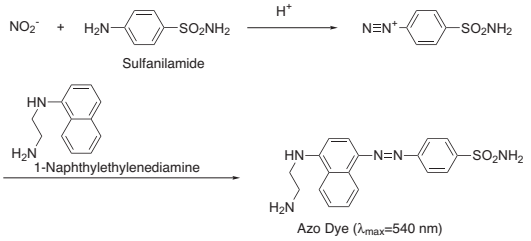
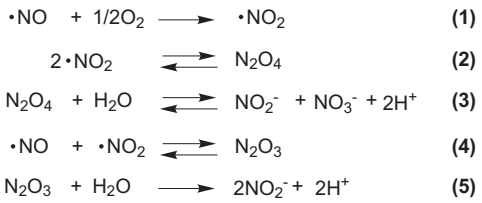


Fig. 1 Coloring reaction scheme of NO_2^- detection



Scheme 1 NO reaction and its metabolites

Contents of The Kit

- $NaNO_2$ Standard Solution (100 µmol/l)	x 1	-Griess Reagent A	x 1
- $NaNO_3$ Standard Solution (100 µmol/l)	x 1	-Griees Reagent B	x 1
- Buffer Solution (20 mmol/l, pH 7.6)	x 1		
- Nitrate Reductase (Dissolve with 1.2 ml of Buffer Solution prior to use)			x 1
- Enzyme Co-factors (Dissolve with 1.2 ml of Buffer Solution prior to use)			x 1

Storage

Store the kit at 0-5 °C. The solutions of Nitate Reductase and Enzyme Co-factors, should be stored at 0-5 °C and used up within 2 weeks.

Precaution

- This kit contains glass vials. Please handle them carefully.
- Both Nitrate Reductase and Enzyme Co-factors solutions should be kept in ice bath during your experiment.
- NO_2 and NO_3 Standard Solution should be used up within 2 months once the vial is opened.
- This kit is able to determine nitrate and nitrite when the concentration range from 10 to 100 µmol/l. Dilute a sample if it contains high concentrations of nitrate and nitrite. The total volume of sample should be no less than 80 µl. Add Buffer Solution to a sample as necessary .
- Prepare a calibration curve for each experiment. NO_2 calibration curve should not be used to determine the NO_3 concentration, and NO_3 calibration curve should not be used to determine the NO_2 concentration.
- Do not mix Nitrate Reductase with Enzyme Co-factors solution prior to use.
- Since inside of each vial of Nitrate Reductase and the Enzyme Co-factors is under reduced pressure, add 1.2 ml Buffer Solution into the vial with a syringe in order to avoid the dispersal of the powder. Please do not open the rubber septum at the beginning.

Required Equipment and Materials

- Microplate reader (530 - 580 nm filter: Optimum at 540 nm)
- 96-well microplate
- 10 µl, 100-200 µl pipettes, multichannel pipettes

General Protocol for 96-well Microplate Assay

Preparation of NO_2^- Calibration Curve

1) Add $NaNO_2$ Standard Solution and Buffer Solution to each well as follows.

Well	$NaNO_2$ Standard Solution(µl)	Buffer Solution(µl)	Final conc. of NO_2^-(µmol/l)
A1	0	80	0
B1	20	60	25
C1	40	40	50
D1	80	0	100

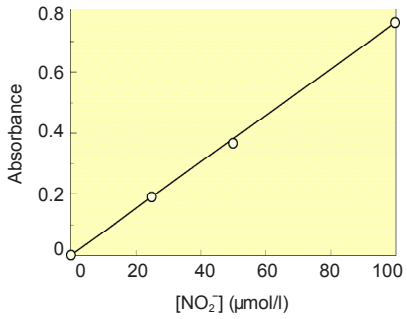


Fig.2 Calibration Curve of Nitrite Solution

2) Add 20 µl of Buffer Solution to each well (total volume is 100 µl/well).
3) Add 50 µl of Griess Reagent A to each well, and mix, incubate the plate for 5 minutes at room temperature.
4) Add 50 µl of Griess Ragent B to each well, and mix. After 10 minutes of incubation at room temperature, measure the absorbance of each well at 540 nm with a microplate reader.
5) Determine the absorbance of each well with subtracting blank(well A1) .
6) Plot the concentrations of $NaNO_2$ solution as X-axis and the absorbance values as Y-axis to prepare a calibration curve.

Preparation of NO_3^- +NO_2^- Calibration Curve

1) Add $NaNO_3$ Standard Solution and Buffer Solution to each well as follows.

Well	$NaNO_3$ Standard Solution(µl)	Buffer Solution(µl)	Final conc. of NO_3(µmol/l)
E1	0	80	0
F1	20	60	25
G1	40	40	50
H1	80	0	100

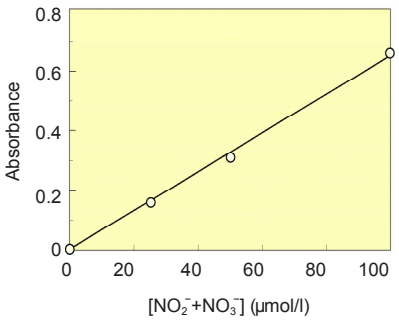


Fig.3 Calibration Curve of [NO_2^- + NO_3^-]

2) Add 10 µl of the Nitrate Reductase solution and 10 µl of the Enzyme Co-factors solution to each well, and mix.
3) Incubate the plate at room temperature for 2 hours.
4) Add 50 µl of Griess Reagent A to each well, and mix. Then, incubate the plate for 5 minutes at room temparature.
5) Add 50 µl of Griess Ragent B to each well, and mix. After 10 minutes of incubation at room temperature, measure the absorbance of each well at 540 nm with a microplate reader.
6) Determine the absorbance of each well with subtracting blank(well E1) .
7) Plot the concentrations of $NaNO_3$ solution as X-axis and the absorbance values as Y-axis to prepare the calibration curve

Preparation of Sample Solution

Cell or tissue culture medium:

Centrifuge the medium at 1000*xg* for 15minutes, and use the supernatant as a sample solution.
A cell culture medium that contains NO_3^- as a component (such as RPMI1640) cannot be used suitable for [NO_3^- +NO_2^-] detection.However, NO_2^- in the medium can be determined using this kit.

Serum:

Serum or plasma sample should be treated with Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (7000*xg*, 4°C, 20minutes) to remove hemoglobin and protein.

Determination of NO_2^- Concentration in Sample Solution

1) Add 80 µl of a sample solution to a well.
2) Add 20 µl of Buffer Solution to each well (total volume is 100 µl/well).
3) Add 50 µl of Griess Reagent A to each well, and mix. Then, incubate the plate for 5minutes at room temperature.
4) Add 50 µl of Griess Reagent B to each well, and mix. After incubation for 10minutes at room temperature, measure the absorbance at 540 nm with a microplate reader.
5) Subtract the absorbance of the blank solution (well A1) from the absorbance of the well.
6) Determine the concentration of nitrite in the sample solution using the calibration curve.

Determination of NO_3^- +NO_2^- Concentration in Sample Solution

1) Add 80 µl of a sample solution to a well.
2) Add 10 µl of the Nitrate Reductase solution and 10 µl of the Enzyme Co-factors solution to each well, and mix.
3) Incubate the plate at room temparature for 2 hours.
4) Add 50 µl of Griess Reagent A to each well, and mix. Then, incubate the plate for 5minutes at room temperature.
5) Add 50 µl of Griess Reagent B to each well, and mix. After 10minutes of incubation at room temperature, measure the absorbance at 540 nm with a microplate reader.
6) Subtract the absorbance of the blank solution (well E1) from the absorbance of the well.
7) Determine the concentration of [NO_3^- +NO_2^-] in the sample solution using the calibration curve.

Determination of Nitrate Concentration in Sample Solution

Nitrate concentration can be obtained by the following equation.

$[NO_3^-] = [NO_2^-] + NO_3^-] - [NO_2^-]$

References

1) S. Archer, *FASEB. J.*, **1993**, *7*, 349.
2) W. R. Tracy, J. Linden, M. J. Prach, R. A. Johns, *J. Pharmacol. Exp. Ther.*, **1990**, *252*, 922.
3) J. S. Pollock, U. Forstermann, J. A. Mitchell, T. D. Warner, H. H. H. Schmidt, M. Nakane, F. Murad, *Proc. Natl. Acad. Sci. U.S.A.*, **1991**, *88*, 10480.
4) H. H. H. Schmidt, T. D. Warner, M. Nakane, U. Forstermann, F. Murad, *Mol. Pharmacol.*, **1992**, 615.

If you need more infomation, please contact Dojindo technical service.

Dojindo Laboratories
2025-5 Tabaru, Mashiki-machi, Kamimashiki-gun, Kumamoto
861-2202, Japan Phone: +81-96-286-1515 Fax: +81-96-286-1525
E-mail: info@dojindo.co.jp Web: www.dojindo.co.jp

Dojindo Molecular Technologies,Inc.
Tel: +1-301-987-2667 Web:http://www.dojindo.com/
Dojindo EU GmbH
Tel: +49-89-3540-4805 Web: http://www.dojindo.eu.com/
Dojindo China Co., Ltd
Tel: +86-21-6427-2302 Web:http://www.dojindo.cn/