Nitric Oxide Quantitation Kit

(version A4)

Catalog No. 40020

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Overview

The Nitric Oxide Quantitation Kit provides a simple and sensitive assay for monitoring nitric oxide production based on nitrate and nitrite determination. The kit uses a two-step assay method that eliminates the need to use lactate dehydrogenase (LDH). The sensitivity of the assay is equivalent to that of the three-step LDH method and more sensitive than that of the commonly used two-step assay method. This new method involves the addition of two cofactors to the nitrate reductase reaction. These cofactors accelerate the conversion of nitrate to nitrite while simultaneously degrading excess NADPH 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 need for lactate dehydrogenase treatment. This kit is fast and simple and can be applied to the assay of nitrite and nitrate determination in urine, plasma, serum, saliva, cell lysate and tissue culture medium.

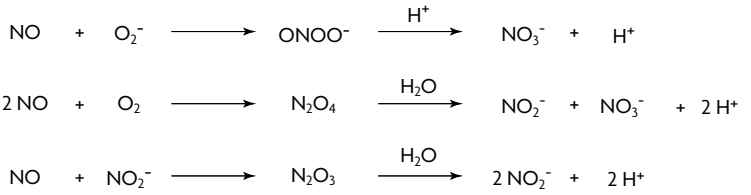
	Format	Catalog No.
Nitric Oxide Quantitation Kit	2 x 96-well plates	40020

See the Active Motif products related to the Nitric Oxide assay in Appendix, Section B.

Introduction

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[1]. NO is an activator of soluble guanylyl cyclase, which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), leading to vasodilatation and inhibition of leukocyte and platelet activation[2, 3]. As the biologically active component of endothelium-derived relaxing factor, NO has critical roles in the maintenance of vascular homeostasis[3]. 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[4]. As a signal transducer in mammalian systems, NO covalently interacts with target molecules based on redox potential[5]. Finally, as a highly reactive chemical, NO directly regulates the activity of many proteins, such as kinases and proteases[6].

Nitric oxide synthase (NOS) catalyses the oxidation of the terminal guanidino nitrogen of the amino acid L-arginine to produce NO and L-citrulline[7]. 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-colored 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, Active Motif's Nitric Oxide Quantitation Kit combines high sensitivity and fast processing of samples in a two-step assay method (Figure 1).

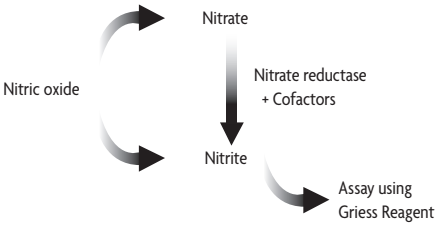


Figure 1: The principle of the Nitric Oxide Quantitation Kit. Nitric oxide is converted to nitrate and nitrite in the cell. Nitrate in the sample is converted to nitrite in presence of nitrate reductase and cofactors. Then, nitrite is assayed using Griess Reagent.

Kit Performance and Benefits

The Nitric Oxide Quantitation Kit is for research use only. Not for use in diagnostic procedures.

Assay Time: Nitrite assay: 30 minutes
Nitrate assay: 90 minutes

Detection Limit: < 1 µM of nitrite/nitrate

Range of Detection: This procedure provides quantitative results from 2 to 55 µM of nitrite/nitrate.

Kit Contents

Kit components can be stored at 4°C for 6 months. Upon reconstitution, the Nitrate Reductase and Cofactors should be stored at -20°C. Refer to the table below and the section Buffer Preparation and Recommendations in this manual for further details.

Reagent	Quantity	Storage/Stability
10X Assay Buffer	30 ml	4°C for 6 months
Nitrate Reductase	4 vials	Lyophilized: 4°C for 6 months Reconstituted: -20°C for 2 months
Cofactors	4 vials	Lyophilized: 4°C for 6 months Reconstituted: -20°C for 2 months
Nitrate Standard	2 x 1.5 ml	4°C for 6 months
Nitrite Standard	2 x 1.5 ml	4°C for 6 months
Griess Reagent A	12 ml	4°C for 6 months
Griess Reagent B	12 ml	4°C for 6 months
96-well plates	2	
Plate sealers	4	

Additional materials required

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

Buffer Preparation and Recommendations

Preparation of 1X Assay Buffer

Prepare the amount of 1X Assay Buffer required 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. This should be used for reconstitution of Nitrate Reductase, dilution of samples and preparation of standard curves for nitrate and nitrite assays. Excess 1X Assay Buffer can be stored at 4°C for one week.

Preparation of Nitrate Reductase

The Nitrate Reductase is provided lyophilized in four vials. Each vial is sufficient for 50 reactions. Reconstitute the contents of each vial with 0.6 ml ice-cold 1X Assay Buffer. Store at –20°C . Freezing and thawing of reconstituted Nitrate Reductase should be limited to one time.

Preparation of Cofactors

The Cofactors are provided lyophilized in four vials. Each vial is sufficient for 50 reactions. Reconstitute the contents of each vial with 1.2 ml ice-cold distilled water. Store at –20°C . Freezing and thawing of the reconstituted Cofactors should be limited to one time.

Preparation of Nitrate Standard

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

Preparation of Nitrite Standard

This vial contains a solution of 200 µM $NaNO_2$. **Do not** add water or Assay Buffer to this vial. The nitrite standard should be stored at 4°C.

Preparation of Griess Reagent

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

Sample Preparation and Recommendations

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

Urine

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

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 any particles. After the above treatment, samples must be filtered through a 10,000 Dalton micropore filter (*e.g.* a Millipore UFC801008) prior to assay.

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 standards in the presence of a fixed volume of the culture media.

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.

Preparation of Nitrate Standard Curve

Prior to starting the nitrate assay, 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 standard concentration.

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

Well	Nitrate Standard (µl)	1X Assay Buffer (µl)	Final Nitrate Concentration (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 1X Assay Buffer to the blank wells.
2. Add up to 70 µl 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 must be approximately neutral as the enzyme is 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 reconstituted Cofactors solution to each well (blanks, standards and samples).
4. Add 10 µl reconstituted Nitrate Reductase solution to each well.
5. Cover plate and mix on a rocking platform set at 150 rpm for one minute.
6. Incubate the plate for 30 minutes at room temperature. Avoid light exposure during incubation. It is not necessary to shake the plate during incubation.
7. Add 50 µl Griess Reagent A to each well.
8. Immediately add 50 µl Griess Reagent B to each well.
9. Allow the color to develop for 20 minutes at room temperature.
10. Read absorbance on a spectrophotometer at 540 nm with a reference wavelength of 620 nm.

Preparation of Nitrite Standard Curve

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 standard concentration.

Nitrite concentrations can be directly measured without performing the Nitrate Reductase reaction. The standard curve for nitrite is prepared by the addition of reagents to the plate wells in the following way:

Well	Nitrite Standard (µl)	1X Assay Buffer (µl)	Final Nitrite Concentration (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 1X Assay Buffer to the blank wells.
2. Add up to 100 µl 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 Griess Reagent A to each well (blanks, standards and samples).
4. Immediately add 50 µl Griess Reagent B to each well.
5. Allow the color to develop for 10 minutes at room temperature.
6. Read absorbance on a spectrophotometer at 540 nm with a reference wavelength of 620 nm.

Calculations

Subtract the blanks

Average the absorbance values of the sample wells. Subtract the blank value from the mean values of the samples.

Plot the standard curves

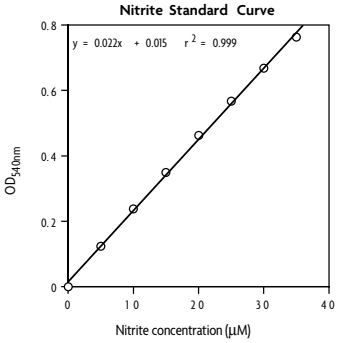
Plot absorbance as a function of nitrate or nitrite concentration. The nitrate assay converts nitrate to nitrite, then measures total nitrite. Therefore, the nitrate standard curve is used for determination of total nitrate and nitrite concentration in the sample, whereas the nitrite standard curve is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

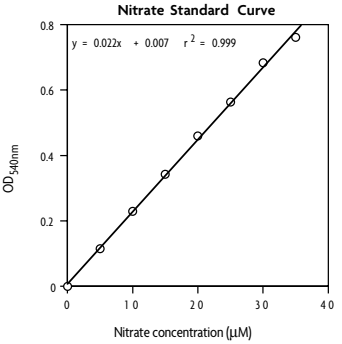
Determine nitrate and nitrite concentrations

The standard curves are used as follows:

1. Measure the endogenous nitrite concentration of a sample using the nitrite assay procedure and the standard curve of nitrite.
2. Measure the total nitrate/nitrite concentration of a sample using the nitrate assay procedure and the standard curve of nitrate.
3. Determine the endogenous nitrate concentration of a sample by subtracting the endogenous nitrite concentration obtained in (1) from the total nitrate/nitrite concentration obtained in (2).

Typical Standard Curves





These nitrate and nitrite standard curves are provided for demonstration only. Standard curves should be generated for each set of samples assayed.

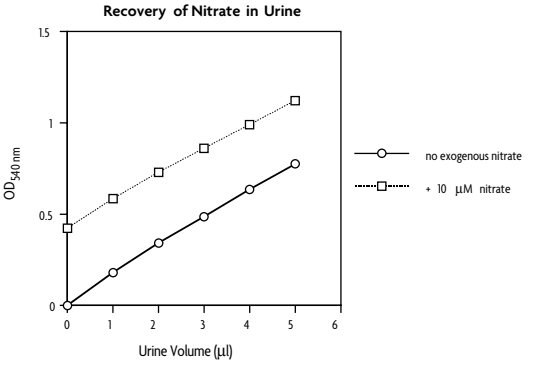
Nitrate/Nitrite Recovery

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 standards to the sample, as shown below. Then, use kit procedures to determine nitrate and nitrite concentrations in samples.

Typical recovery data

A human urine sample was 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) by the nitrate assay procedure. The percentage of recovery is determined according to the following procedure:

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 standard and multiply by 100.
3. Divide the nitrate/nitrite concentration of the sample determined from the corresponding standard 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	0.423	100
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 standard 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

References

1. Moncada S., Palmer R.M. and Higgs E.A. (1991) *Pharmacol. Rev.* 43: 109-42.
2. Arnold W.P., Mittal C.K., Katsuki S. and Murad F. (1977) *Proc. Natl. Acad. Sci.* USA 74: 3203-7.
3. Lloyd-Jones D.M. and Bloch K.D. (1996) *Annu. Rev. Med.* 47: 365-75.
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5. Nathan C. and Xie Q. (1994) *Cell* 78: 915-8.
6. Schreck R. and Bauerle P.A. (1991) *Trends Cell Biology* 1: 39.
7. Palmer, R.M.J., Ashton, D.S. and Moncada S. (1988) *Nature*, 333: 664-666.

Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
Erratic values or dispersion of duplicates	Poor pipetting technique	Use a multi-channel pipettor when possible and make sure it is calibrated
	Bubbles in well	Make sure there are no bubbles in the wells prior to absorbance reading
	Incorrect addition of reagents	Make sure the correct amount of reagents are added in the right order
No color development in the nitrate standard curve	Incorrect addition of reagents	Repeat assay making sure the correct volumes of reagents are added to all wells
Nitrate standard curve is not linear at high concentration of nitrate	Concentration of nitrate is more than 55 µM	Use the curve in the linear portion at lower nitrate concentrations
	Enzyme and Cofactor activity is lost due to repeated freezing and thawing	Avoid freeze and thawing of the enzyme and Cofactors, and make sure the kit has not expired
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
Nitrite standard curve is not linear at high concentration of nitrite	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
	Nitrite concentration is too high	Use the curve in the linear portion at lower nitrite concentrations

Section B. Related products

TransAM™ Kits	Format	Catalog No.
TransAM NFκB p65	1 x 96-well plate 5 x 96-well plates	40096 40596
TransAM NFκB p50	1 x 96-well plate 5 x 96-well plates	41096 41596
TransAM CREB	1 x 96-well plate 5 x 96-well plates	42096 42596
TransAM pCREB	1 x 96-well plate 5 x 96-well plates	43096 43596
TransAM AP-1 c-Fos	1 x 96-well plate 5 x 96-well plates	44096 44596
TransAM AP-1 FosB	1 x 96-well plate 5 x 96-well plates	45096 45596
TransAM AP-1 c-Jun	1 x 96-well plate 5 x 96-well plates	46096 46596
TransAM HIF-1	1 x 96-well plate 5 x 96-well plates	47096 47596
TransAM PPARγ	1 x 96-well plate 5 x 96-well plates	40196 40696
TransAM p53	1 x 96-well plate 5 x 96-well plates	41196 41696

Antibodies	Application	Format	Catalog No.
Caspase 3 mAb	WB, IP	100 µg	40924
Pro-Caspase 3 mAb	WB, IP	100 µg	40925
Caspase 7 mAb	WB	100 µg	40929
Caspase 8 mAb	WB	100 µg	40930
Caspase 9 pAb	WB	100 µg	40931
Caspase 14 mAb	WB	100 µg	40932
I-FLICE pAb	WB	100 µg	40922
DRAK1 pAb	WB	100 µg	40923
DRAK2 pAb	WB	100 µg	40926
DcR3 mAb	WB	100 µg	40933
DR4 mAb	WB	100 µg	40934
DR5 mAb	WB	100 µg	40935
DR6 pAb	WB	100 µg	40927
OPG mAb	WB	100 µg	40938
TRADD mAb	WB	100 µg	40939
TRANCE/RANKL/OPGL mAb	WB	100 µg	40940
D4-GDI mAb	WB, IP	100 µg	40941
TAC1 pAb	WB	100 µg	40936
TROY pAb	WB	100 µg	40937
DAP-3 mAb	WB	100 µg	40963
DAP-5 mAb	WB	100 µg	40964

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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