Colorimetric Assay for
Nitric Oxide

Catalog Number: A22075 (AOXRE Catalog Number 22075)

For Research Use Only

Store at 2 to 8°C.

INTRODUCTION

This kit employs immunoaffinity purified Nitrate Reductase (NaR) enabling the measure of total nitric oxide (NO) produced in *in vitro* experimental systems.

Nitric oxide can be spectrophotometrically assayed by measuring the accumulation of its stable

degradation products, nitrate and nitrite. The ratio of these two products in biological fluids, tissue culture media, etc. may vary substantially. Hence, for accurate assessment of the total nitric oxide generated, one must monitor both nitrate and nitrite. An excellent solution to this problem is the enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase, followed by quantitation of nitrite using Griess Reagent.

In addition to providing all necessary components in a microtiter format, this kit employs affinity purified nitrate reductase and NADH, thereby circumventing some of the potential problems reported for NO measurement using NADPH dependent nitrate reductases.

PRINCIPLES OF PROCEDURE

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This kit employs the NADH-dependent enzyme nitrate reductase for conversion of nitrate to nitrite prior to the quantitation of nitrite using Griess reagent — thus providing for accurate determination of total NO production.

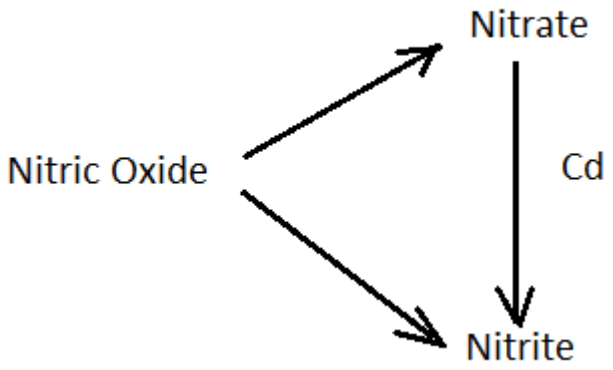


Figure 1: The reduction of nitrate to nitrite

This kit can be used to accurately measure as little as 1 pmol/µL (~1µM) NO produced in aqueous

solutions. Very little sample is required (5 to 85 µL depending on the [NO] in the sample. The completed reaction is read at 540 nm.

MATERIALS PROVIDED

Component	Description	Volume	Storage
Nitrate Reductase	Lyopholized enzyme to convert nitrate to nitrite	1 Unit	-20°C
Nitrate Reductase Buffer	Buffer used reconstitute the Nitrate Reductase	1.5 mL	4°C
MOPS Buffer	Buffer used to dilute standards and samples	25 mL	4°C
NADH	Lyopholized NADH	2 mg	RT
Color Reagent #1	Griess reagent #1	7 mL	4°C
Color Reagent #2	Griess reagent #2	7 mL	4°C
Nitrate Standard	500 µM NO standard solution	1.5 mL	4°C
Microtiter Plate	96-well microplate	1 plate	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 540 nm filter
2. Adjustable micropipettes (1 – 1000 µL) and tips
3. Deionized water
4. Test tubes
5. Vortex mixer
6. Plate shaker

STORAGE & STABILITY

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Reconstituted Nitrate Reductase is stable for 6 months if stored at -20°C; one year if stored at -80°C.
4. Diluted Nitrate Standards may be stored at 4°C for later use.
5. Reconstituted NADH should be stored at -20°C. Avoid freeze-thaw cycles! Store in the dark.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. For best results, read the plate within 20 minutes of the color reaction.
2. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the
microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE NOTES

1. If the [NO] in a sample is low, increase the Sample volume and decrease the Buffer volume.
2. Samples containing very high levels of protein (resulting in >1 mg/well in this assay) may produce a precipitate that may interfere with the accurate measurement of NO. If a precipitate is visible after the addition of Color Reagent #1, remove excess proteins (e.g. by boiling and centrifuging or diluting the samples) prior to performing the assay. Alternatively, the reaction may be performed in a conical micro plate. Then, prior to reading the absorbance values, centrifuge the plate and transfer 85 µL of supernatant from each well to the corresponding well of a flat-bottomed plate.

Note: If it is necessary to analyze samples with high protein concentrations (e.g. 85 µL/well of
undiluted serum) our non-enzymatic NO kit (Cat. No. NB88) can be used.

REAGENT PREPARATION

1. **Nitrate Reductase:** Reconstitute with 1.0 mL of Nitrate Reductase Buffer and incubate at room
temperature (RT) for 20 minutes, with light vortexing at 0, 10, and 20 minutes.
2. **NADH:** Add 1.28 mL deionized water to the vial to obtain a 2mM working solution.

STANDARD CURVE PREPARATION

The Nitrate Standard is provided as a 500 µM stock solution. Use the following table to construct an eight-point standard curve.

Table 1: Standard Curve Preparation

Standard	Nitrate Conc. (µM)	Vol. of H_2O (mL)	Transfer Vol. (mL)	Transfer Source	Final Volume (mL)
S7	100.0	2.0	0.5	Stock	2.0
S6	50.0	0.5	0.5	S7	0.5
S5	25.0	0.5	0.5	S6	0.5
S4	10.0	0.75	0.5	S5	0.75
S3	5.0	0.5	0.5	S4	0.5
S2	1.0	2.0	0.5	S3	2.0
S1	0.5	0.5	0.5	S2	1.0
S0	0	0.5	-	-	0.5

ASSAY PROCEDURE

1. Add 85 µL of Standards and Samples (depending on [NO], samples may need to be diluted in MOPS Buffer) to the microplate in duplicate. See **Scheme I** for a sample plate layout.
2. Add 10 µL of the reconstituted Nitrate Reductase to each well.
3. Add 10 µL of NADH working solution to each well and shake the plate for 20 minutes at RT.
4. Add 50 µL of Color Reagent #1 to each well and shake briefly
5. Add 50 µL of Color Reagent #2 to each well and shake for 5 minutes at RT.
6. Read the plate at 540 nm in the plate reader.

Scheme I: Sample Plate Layout

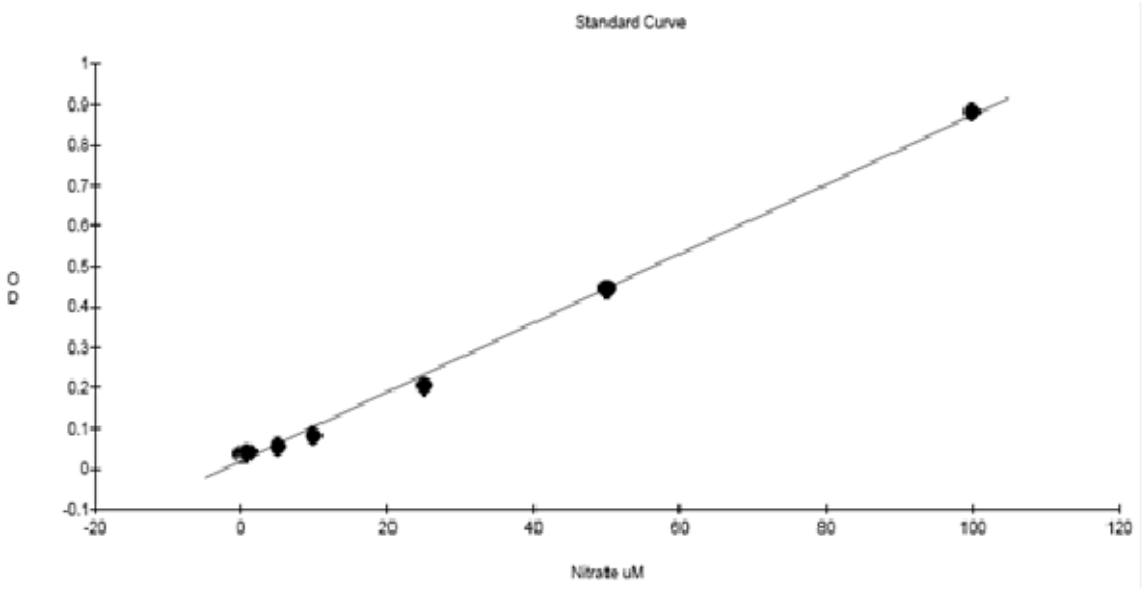
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S0	S0	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36

E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

CALCULATIONS

1. Average all duplicate well absorbance values.
2. Subtract the average absorbance values for the blank wells (S0) from all other well pairs.
3. Plot a standard curve using the corrected absorbance values of each Standard (y-axis) versus the
Standard concentration (x-axis).
4. Determine the concentration of each unknown using the equation of the line.

Figure 1: Typical Standard Curve



REFERENCES

1. Schmidt, H. H., *et. al.*; (1995) *Biochemica* **2**:22-23

OxisResearch™

2946 Scott Blvd
Santa Clara, CA 95054, U.S.A.
E-mail: sales@lumiquick.com
Telephone: 408-855-0061
www.lumiquick.com, www.aoxre.com
Made in the U.S.A.