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Colorimetric Assay for
Nitric Oxide

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INTRODUCTION

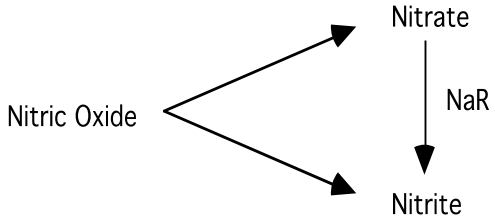
This kit employs immunoaffinity purified Nitrate Reductase (NaR) enabling the measure of total nitric oxide (NO) produced in *in vitro* experimental systems.

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.



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	Cat. No.
Nitrate Reductase	Lyopholized enzyme to convert nitrate to nitrite	1 Unit	-20°C	NB98a
Nitrate Reductase Buffer	Buffer used reconstitute the Nitrate Reductase	1.5 mL	4°C	NB98a-1
MOPS Buffer	Buffer used to dilute standards and samples	25 mL	4°C	NB98b
NADH	Lyopholized NADH	2 mg	4°C	NB98c
Color Reagent #1	Griess reagent #1	7 mL	4°C	NB98d
Color Reagent #2	Griess reagent #2	7 mL	4°C	NB98e
Nitrate Standard	500 µM NO standard solution	1.5 mL	4°C	NB98f

Microtiter Plate	96-well microplate	1 plate	4°C	NB98g

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)
S_7	100.0	2.0	0.5	Stock	2.0
S_6	50.0	0.5	0.5	S_7	0.5
S_5	25.0	0.5	0.5	S_6	0.5
S_4	10.0	0.75	0.5	S_5	0.75
S_3	5.0	0.5	0.5	S_4	0.5
S_2	1.0	2.0	0.5	S_3	2.0
S_1	0.5	0.5	0.5	S_2	1.0
S_0	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.

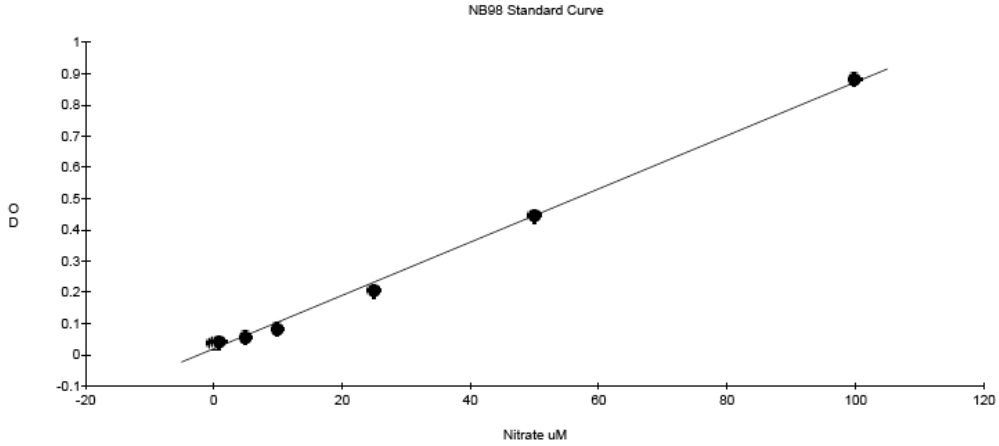
Scheme I: Sample Plate Layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S_0	S_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}

CALCULATIONS

1. Average all duplicate well absorbance values.
2. Subtract the average absorbance values for the blank wells (S_0) 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

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