Nitric Oxide Synthase Colorimetric Non-Enzymatic Assay Refill Kit

Catalog Number: NOS39-K01R
96 Wells
For Research Use Only
v. 2.0 (08.09.22)

Eagle Biosciences, Inc.
20A NW Blvd., Suite 112, Nashua, NH 03063
Phone: 866-419-2019 Fax: 617-419-1110
www.EagleBio.com

INTRODUCTION

NOTE: This Nitric Oxide Synthase Colorimetric Assay Refill Kit possesses the entire necessary components to run this assay in the absence of cadmium beads. Cadmium is a recyclable resource and should be provided from another source to complete the functionality of this kit.

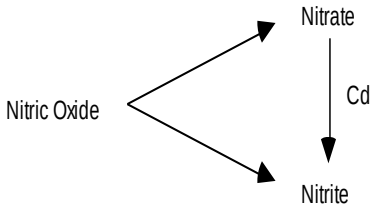
Nitric Oxide Synthase Colorimetric Assay Refill Kit allows you to measure total nitric oxide (NO) produced for *in vitro* experimental systems following conversion of nitrate to nitrite by metallic cadmium.

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 involves the conversion of nitrate to nitrite catalyzed by cadmium. This is then followed by the quantitation of nitrite using the griess reaction.

Nitric Oxide Synthase Colorimetric Assay Refill Kit is suitable for the quantitative determination of total nitric oxide in samples that have high protein concentrations. The cadmium catalyst proves far more robust when in the presence of harsh deproteinating reagents than the alternative, nitrate reductase.

PRINCIPLE OF ASSAY

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 metallic cadmium for quantitative conversion of nitrate to nitrite (figure 1) prior to quantitation of nitrate 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 (1 to 100µL depending on the [NO] in the sample). The completed reaction is read at 540 nm.

MATERIALS PROVIDED

1. **$ZnSO_4$ SOLUTION (NB88b):** 2mL of 30% (wt/vol) $ZnSO_4$ for sample deproteination.
2. **MICROCENTRIFUGE TUBES (NB88c):** 50 x 1.5-mL microcentrifuge tubes for overnight incubation of the samples.
3. **COLOR REAGENT #1 (NB88d):** 7 mL of Sulfanilamide (p-Aminobenzenesulfonamide) dissolved in 3N HCl.
4. **COLOR REAGENT #2 (NB88e):** 7 mL of N-(1-Naphthyl) ethylenediamine dihydrochloride dissolved in deionized H_2O.

5. **NITRITE STANDARD (NB88f):** 1.5 mL 500pmol/μL $NaNO_2$ (equivalent to 500 μM NO).
6. **MICROTITER PLATE (NB88g):** One optically clear 96 well microplate.
7. **MICROTITER PLATE TEMPLATE (NB88h):** One 96 well microplate template.
8. **REAGENT RESERVOIRS (NB88i):** Three plastic troughs for dispensing and pipetting reagents.
9. **CADMIUM BEAD HCl-WASH SOLUTION (NB88j):** 125 mL of 0.1 M HCl.
10. **CADMIUM BEAD NH_4OH-WASH SOLUTION (NB88k):** 125 mL of 0.1 M NH_4OH.

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with 540nm filter.
2. Precision pipettes (10 μL –1,000μL) and disposable tips.
3. Microcentrifuge tubes or equivalent for dilution, transferring and centrifugation of samples.
4. Vortex mixer.
5. Double Deionized Water (DD water).
6. Granulated/ shot cadmium beads 99.9%.

STORAGE CONDITIONS

- Recycled cadmium should be stored in a dedicated dry environment and washed prior to use.
- All other kit components should be stored at 4 °C. Alternatively, this kit exhibits good stability at RT for extended periods of time during shipping and preparations.

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 is not recommended as it may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

PROCEDURAL NOTES

1. Reagents can be used immediately upon removal from refrigeration.
2. Performance of the entire kit at once is not required. When performing this kit in part, please adhere to the following:
   -All unused components should be returned to their respective storage areas.
   -Create a standard curve for each performance of the assay. Sufficient stock solution of standard is provided for two standard curves.
3. Use fresh pipette tips when transferring or pipetting reagents from stock reagents.
4. Pipette tips should be pre-wetted or rinsed prior to dispensing reagent. To do this, adjust pipette to the volume desired and draw up reagent of choice into the tip 2-3 times before uptake for dispensing.
5. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.
6. Take note of the volume of each sample at the various stages of preparation and assay procedure to ensure proper dilution factor and NO concentration calculation.

REAGENT PREPARATION

- Cadmium beads should be washed just prior to use. Cadmium beads prepared days prior to its intended use should be stored in a sealed container under inert gas such as nitrogen or argon.

- All other kit components are supplied ready to use.

CADMIUM WASH PROCEDURE

1. Place the desired amount of Cd^{++} in an appropriately sized, sealable container or containers. Please consider that for every 0.5 g of Cd^{++} there will be 1.0 mL of wash solution applied.
2. Apply 1.0 mL of DD H_2O for every 0.5 g of Cd to be used. Seal container and mix by inversion five times.
3. Decant the remaining solution and repeat Step 2 one time prior to proceeding to Step 4.
4. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.
5. Apply 1.0 mL of HCl-Wash solution for every 0.5 g of Cd^{++} to be used. Seal container and mix by inversion five times.
6. Decant the remaining solution and repeat Step 5 one time prior to proceeding to Step 7.
7. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.
8. Apply 1.0 mL of NH_4OH-Wash solution for every 0.5 g of Cd^{++} to be used. Seal container and mix by inversion five times.
9. Decant the remaining solution and repeat Step 8 one time prior to proceeding to Step 10.
10. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.
11. The washed and dried Cd^{++} is now ready for use. If you are not using the washed Cd^{++} immediately then store the Cd^{++} under inert gas such as nitrogen or argon in a sealed container.

SAMPLE PREPARATION

This kit is designed for use with samples possessing high concentrations of protein such as serum, culture medium, or tissue homogenates. Each sample should be diluted according to its presence of NO metabolites (nitrate and nitrite). Pilot studies or other research should serve as a precedent for the optimal dilution.

1. Adjust 10-50 µL of sample volume to 190 µL with DD water.
2. Add 10 µL of 30% (wt/vol) $ZnSO_4$ to the 190 µL of diluted sample and vigorously mix and incubate at RT for 15 minutes.
3. Centrifuge sample at 3,000 x g for 5 minutes.
4. Transfer the resulting supernatant to a collection tube for sample storage at –20 °C or proceed to the Sample Incubation Procedure located below.

SAMPLE INCUBATION PROCEDURE

1. Place approximately 0.5 g of washed and dried Cd^{++} beads in a dedicated centrifuge tube for each representative sample.
2. Add the deproteinated and clarified sample directly to dedicated centrifuge tube with the Cd^{++} present. The volume of the sample will be approximately 200 µL.
3. Incubate the sample and Cd^{++} together for 24 hours.
4. After incubation, transfer the sample to a clean microcentrifuge tube for further clarification by centrifugation. Centrifuge for 5 minutes at 3,000 x g. Begin your assay within 1 hour for best results. The used Cd^{++} beads should be collected and washed as indicated in the reagent preparation section above.

STANDARD PREPARATION

The stock nitrite standard is provided as a 500 µM NO equivalents (500 pmol/µL of NH_2). Prepare the standards according to the following Table 1.

Table 1.

Standard	NO Equivalents (µM)	DD H_2O (mL)	Transfer Volume (mL)	Transfer Source	Final Volume (mL)
S_7	100	2.0	0.5	Stock	1.5
S_6	50	0.5	0.5	S_7	0.5
S_5	25	0.5	0.5	S_6	0.5
S_4	10	0.5	0.5	S_5	0.5
S_3	5	0.75	0.5	S_4	0.75
S_2	1	0.5	0.5	S_3	0.5
S_1	0.5	2.0	0.5	S_2	2.5
S_0	0	0.5	---	---	0.5

NOTE: The prepared standards do not require incubation with cadmium.

ASSAY PROCEDURE

1. Establish a plate layout accounting for each sample and standard. See **Scheme I** for a suggested plate layout.
2. Add 100 µL of sample and standard to their respective wells on the plate. **Note:** samples may require further dilution with DD water if the concentration exceeds the standard curve parameters.
3. Add 50 µL Color Reagent #1 and shake briefly.
4. Add 50 µL Color Reagent #2. Shake for 5 minutes at room temperature.
5. Read plate at 540 nm and proceed to the calculations section.

INTERPRETATION OF RESULTS

Sample concentrations can be determined using Y-intercept equation as demonstrated below.

Note: The standard curve is demonstrated in Nitric Oxide µM equivalents but can alternatively be demonstrated in pmol/mL of NH_2.

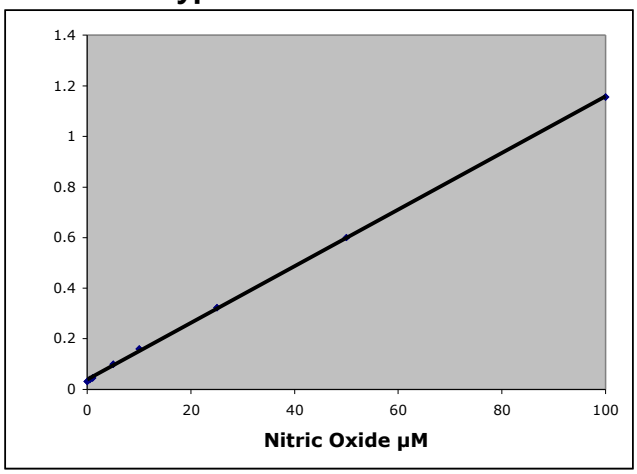
1 µM NO = pmol/mL NH_2

1. Average the O.D. values for each replicate of sample and standard.
2. Plot the standard curve using the standard concentration (X-axis) vs. the corresponding O.D. (Y-axis).
3. Determine the concentration of each sample by interpolation from the standard curve using the Y-intercept equation. Enter a sample O.D. value in place of "Y" and solve for "X" to determine the concentration.

   Y=mX+b where:

   Y= The ordinate or y-axis value (corresponds to the OD reading).
   X= The abscissa or x-axis value (corresponds to the NO concentration).
   m= Slope.
   b= Y axis intercept.

Typical Standard Curve



Scheme I. Suggested 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}

REFERENCES

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

WARRANTY INFORMATION

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.