Technical Bulletin

Nitric Oxide Synthase (NOS) Activity Assay Kit (Colorimetric)

Catalog Number MAK407

Product Description

Nitric oxide synthases (NOS) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. In the presence of NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, calmodulin and heme, NOS catalyzes a five-electron oxidation of the guanidino nitrogen of L-arginine with molecular oxygen to generate NO and L-citrulline. There are three isoforms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). nNOS accounts for the production of NO in the central nervous system, where NO participates in cell communication and information storage. eNOS produces NO in blood vessels and is involved in regulation of vascular function. In contrast to other isoforms, iNOS is expressed *de novo* under oxidative stress conditions and produces large amounts of NO as part of the body's defense mechanism.

The Nitric Oxide Synthase (NOS) Activity Assay Kit provides an accurate and convenient method to assay NOS activity in a variety of samples. In this assay, nitric oxide generated by NOS undergoes a series of reactions and reacts with Griess Reagent 1 and 2 to generate a colored product with a strong absorbance at 540 nm. The assay is simple, sensitive and high-throughput adaptable and can detect as low as 5 μU of NOS activity.

The kit is suitable for the detection of NOS activity in purified recombinant protein and tissue or cell extracts.

Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- NOS Assay Buffer — 25 mL
  Catalog Number MAK407A
- NOS Dilution Buffer — 1.5 mL
  Catalog Number MAK407B
- NOS Substrate — 500 μL
  Catalog Number MAK407C
- NOS Cofactor 1 — 1 vial
  Catalog Number MAK407D
- NOS Cofactor 2 (25x) — 100 μL
  Catalog Number MAK407E
- Nitrate Reductase — 1 vial
  Catalog Number MAK407F
- NOS (Positive Control) — 4 μL
  Catalog Number MAK407G
- Enhancer — 1 vial
  Catalog Number MAK407H
- Nitrite Standard — 1 vial
  Catalog Number MAK407I
- Griess Reagent 1 — 10 mL
  Catalog Number MAK407J
- Griess Reagent 2 — 10 mL
  Catalog Number MAK407K

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (including multichannel pipettor)
- 96 well clear flat-bottom plate. Cell culture or tissue culture treated plates are **not** recommended.
- Spectrophotometric multiwell plate reader
- Refrigerated microcentrifuge capable of RCF ≥10,000 × *g*
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Protease Inhibitor Cocktail (Catalog Number P8340 or equivalent)
- Bicinchoninic Acid Kit for Protein Determination (Catalog Number BCA1 or equivalent)

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on dry ice. Store kit at -80 °C, protected from light. Once opened, store each kit component per their respective temperatures outlined in the Preparation Instructions section below.

Preparation Instructions

Briefly centrifuge small vials at low speed prior to opening.

NOS Assay Buffer: Bring to room temperature prior to use. Chill an appropriate amount of NOS Assay Buffer for use in Sample Preparation. Store at 2-8 °C or at -20 °C.

NOS Dilution Buffer: Ready to use. Store at 2-8 °C or at -20 °C.

NOS Substrate: Ready to use. Divide into aliquots and store at -20 °C. Avoid repeated freeze/thaw cycles. Keep on ice while in use.

NOS Cofactor 1: Reconstitute vial with 110 µL of purified water to make a 10 mM stock solution. Aliquot and store at -20 °C. Freeze/thaw cycles should be limited to 1 time.

NOS Cofactor 2: Divide into aliquots and store at -20 °C Avoid repeated freeze/thaw cycles.

Nitrate Reductase: Reconstitute vial with 1.1 mL of NOS Assay Buffer. Aliquot and store at -20 °C. Avoid repeated freeze/thaw cycles. Keep on ice while in use.

NOS (Positive Control): Aliquot and store at -80 °C. Freeze/thaw cycles should be limited to 1 time. **During use, keep the solution on ice at all times since the enzyme loses activity at higher temperature.**

Enhancer: Reconstitute vial with 1.2 mL of NOS Assay Buffer. Keep on ice during use. Store at -20 °C.

Nitrite Standard: Reconstitute vial with 1 mL of NOS Assay Buffer. Vortex and mix well to generate a 10 mM Nitrite Standard. Store at 2-8 °C when not in use (**Do not freeze**). The reconstituted Nitrite Standard is stable for four months when stored at 2-8 °C.

Griess Reagents 1 and Griess Reagent 2: Ready to use. Store at 2-8 °C.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Tissue

1. Rinse the tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80 °C) to a pre-chilled tube.

2. Add 200 µL of cold NOS Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice.
3. Transfer the tissue homogenate to a cold microfuge tube.
4. Centrifuge the tissue homogenate at 10,000 × *g* at 4 °C for 10 minutes.
5. Transfer the clarified supernatant to a fresh pre-chilled tube and keep on ice.
6. Measure the protein concentration using BCA or preferred method.
7. Use lysates immediately to assay NOS activity. If immediate use is not possible, snap freeze the lysate and store at -80 °C.

Cells

1. Add 100-200 µL of cold NOS Assay Buffer containing protease inhibitor cocktail (not provided) to fresh or frozen cells (2-5 × 10^6 cells).
2. Homogenize to disrupt the cells.
3. Centrifuge the cell homogenate at 10,000 × *g* at 4 °C for 10 minutes.
4. Transfer the clarified supernatant to a fresh pre-chilled tube and keep on ice.
5. Measure the protein concentration using BCA or preferred method.
6. Use lysates immediately to assay NOS activity. If immediate use is not possible, snap freeze the lysate and store at -80 °C.

Standard Curve Preparation

Prepare a 50 µM working Nitrite Standard solution by adding 5 µL of reconstituted 10 mM Nitrite Standard to 995 µL of NOS Assay Buffer. Prepare Nitrite Standards according to Table 1.

Table 1.
Preparation of Nitrite Standards

Well	50 µM Nitrite Standard	NOS Assay Buffer	Nitrite (pmol/well)
1	0 µL	60 µL	0
2	5 µL	55 µL	250
3	10 µL	50 µL	500
4	15 µL	45 µL	750
5	20 µL	40 µL	1000
6	25 µL	35 µL	1250

Sample(s)

Add 30-60 µL (200-400 µg protein) of cell/tissue homogenate or purified protein into desired wells in a 96-well plate. Adjust the total volume in each well to 60 µL with NOS Assay Buffer. For unknown samples, test different amounts of sample to ensure the readings are within the Standard Curve range.

Positive Control

Dilute NOS (Positive Control) 1:20 in NOS Dilution Buffer just prior to use. Add 5-10 µL of diluted NOS enzyme into desired well(s). Adjust the total volume of Positive Control wells to 60 µL/well with NOS Assay Buffer.

Sample Background Control (Optional)

For samples having background, prepare parallel sample well(s) as Sample Background Control(s). Use the same amount of tissue/cell homogenate or purified enzyme as in the sample well. Adjust the final volume to 300 µL with NOS Assay Buffer.

Assay Reaction

1. Prepare 1 mM working solution of NOS Cofactor 1 by diluting 10 mM stock solution 1:10 with purified water to make 1 mM working solution just prior to use. Make as much as needed. Keep on ice while in use. Working solution can be stored at 2-8 °C for 6-8 hours.

2. Prepare a 1× working solution of NOS Cofactor 2 by diluting the 25× stock solution 1:25 with purified water. Keep on ice while in use.
3. Prepare enough reaction mix for the number of Standards, Positive Control and Sample(s) wells to be analyzed. For each well, prepare 40 µL of reaction mix according to Table 2, mix well.

Table 2.
Preparation of Reaction Mix

Reagent	Volume
Working Solution NOS Cofactor 1 (1 mM)	10 µL
Working Solution NOS Cofactor 2 (1×)	20 µL
NOS Substrate	5 µL
Nitrate Reductase	5 µL

4. Add 40 µL of the Reaction Mix into each Standard, Positive Control, and Sample(s) wells.
5. Mix well and incubate at 37 °C for 1 hour.
6. After reaction incubation, add 95 µL of NOS Assay Buffer to each Standard, Positive Control, and Sample(s) wells.
7. Subsequently add 5 µL of Enhancer into each Standard, Positive Control, and Sample(s) well.
8. Mix and incubate at room temperature for 10 minutes.
9. Add 50 µL of Griess Reagent 1 and 50 µL of Griess Reagent 2 to Standard, Positive Control and Sample(s) wells.
10. Mix and incubate for 10 minutes.

Measurement

Read absorbance at 540 nm (A_{540}) using a microplate reader.

Results

1. Subtract 0 Standard reading from all readings.
2. Plot the Nitrite Standard Curve.
3. If Sample Background Control is significant, subtract the Sample Background Control reading from the Sample readings.
4. Apply ΔA_{540} to the Standard Curve to get B pmoles of nitrite generated during the reaction.

Nitric Oxide Synthase Specific Activity (pmol/min/µg or µU/µg or mU/mg) =

$$\frac{B}{T \times C}$$

where:

B = Nitrite amount in sample well from the Standard Curve (pmol)

T = Reaction time in minutes (60 minutes)

C = Amount of protein (µg)

Unit Definition: One unit of NOS activity is the amount of enzyme required to yield 1.0 µmol of nitric oxide per minute at 37 °C.

Figure 1.
Typical Nitrite Standard Curve.

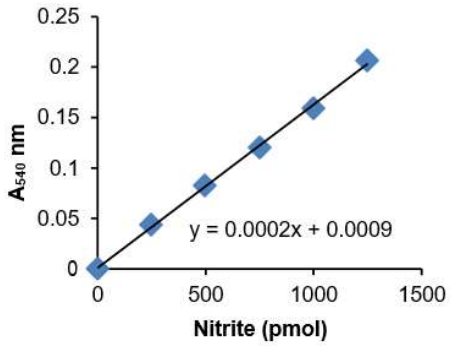


Figure 2.
Measurement of NOS Positive Control activity (10 μL).

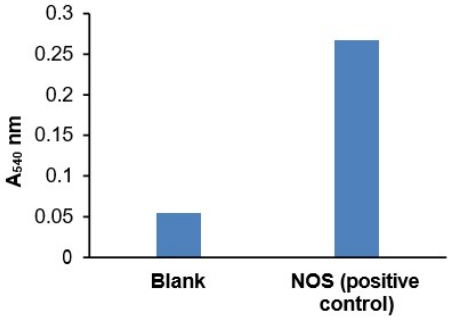
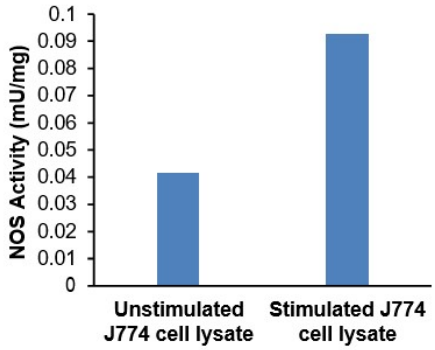


Figure 3.
Detection of endogenous NOS activity in J774.1A cell lysate (225 μg) stimulated with or without 200 ng/mL LPS and 100 ng/mL murine IFN-γ. Assays were performed following the kit protocol.



Frequently Asked Questions

What is the function of the enhancer?

The Enhancer stabilizes the NOS catalyzed reaction by mediating the electron transport between NADPH and tetrazolium dye.

Can this kit work with live cells?

No, this is an endpoint assay where the cells need to be lysed.

Can the kit work on bacteria or yeast cells?

The kit has been standardized for mammalian cells only.

Can the kit be used on muscle tissue homogenates specifically?

The kit can be used with most tissue extracts, provided that the tissues are well homogenized and contains >5μU of NOS activity.

Can frozen samples be used with this assay?

Fresh samples are always preferred over frozen samples. However, frozen samples can also be used, provided:

- they were frozen right after isolation,
- did not undergo multiple freeze/thaw cycles,
- and have been frozen for a relatively short period.

It is recommended to aliquot samples prior to freezing.

What is the exact volume of sample required for this assay?

There is no specific recommended volume for each sample to be used since activity is based on sample concentration and quality. Perform pilot experiments with multiple sample volumes to determine the optimal volume which gives a reading within the linear range of the standard curve.

Can an alternate buffer be used for sample preparation (cell lysis, sample dilutions etc.)?

The Nitric Oxide Synthase (NOS) Activity Assay Kit assay buffers are optimized for the reactions they are designed for. The buffers not only contain detergents for efficient lysis of cells/tissue, but also contain proprietary components required for the further reactions. Therefore, it is highly recommended to use the buffers provided in the kit for the best results.

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