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ProductInformation

Nitric Oxide Synthase Detection System, Isotopic

Catalog Number **NOS1**

TECHNICAL BULLETIN

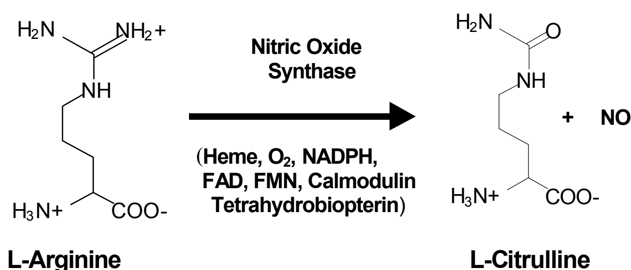
Product Description

The Nitric Oxide Synthase Detection System is a simple and specific assay for the measurement of nitric oxide synthase (NOS) activity. Nitric oxide (NO), formed from the amino acid L-arginine via the action of NOS, is a unique cell signaling molecule, that functions as both an intracellular and an extracellular messenger. NO has been implicated in inflammation, neurotransmission, cytotoxicity, and vasodilation.[1-3] Many effects of NO are mediated by its ability to activate the enzyme guanylyl cyclase. The resulting increase in intracellular cyclic GMP levels regulates protein kinase G activity, protein phosphorylation, and many biological processes.[4] In contrast, the cytotoxic and inflammatory effects of NO are independent of cyclic GMP and may result from its interaction with metal ions, thiol groups, and other free radicals that, in turn, can mutate DNA and inhibit several key enzymes involved in energy metabolism.[5]

Three distinct isoforms of NOS have been identified, referred to as neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Moreover, splice variants have been shown to exist for all of these isoforms. NO formed by nNOS (also known as cNOS, bNOS, NOS-1, or Type 1 NOS) in the central nervous system may be important in information storage associated with learning and memory. In contrast, NO synthesized by iNOS (also known as NOS-2 or Type II NOS) may participate in antimicrobial activity, cytotoxicity, and/or inflammatory responses. Lastly, NO formed by eNOS (also known as NOS-3 or Type III NOS) in endothelial cells is responsible for blood pressure regulation by endothelial-dependent vasodilators. Most cells and tissues possess one or more isoforms of NOS. The endothelial and neuronal isoforms are constitutive and highly regulated by Ca^{2+} and calmodulin. The inducible isoform of NOS is expressed *de novo* after exposure to bacterial endotoxins and/or any one of several inflammatory cytokines.

The NOS reaction involves a five-electron oxidation of the guanidine nitrogen of L-arginine with molecular oxygen to give the stoichiometric production of NO and L-citrulline (see Figure 1).

Figure 1.
Biochemical conversion of L-arginine to L-citrulline



The NOS enzyme itself requires five cofactors ($FMNH_2$, FADH, NADPH, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron).

This assay system is based on the enzymatic conversion of radiolabeled (^3H or ^{14}C) arginine to radiolabeled citrulline and is the standard assay for measuring NOS activity in cell lysates, tissue homogenates, and purified enzyme preparations.[6,7] The system is highly specific to the NOS enzyme and can detect L-citrulline in the picomole range.[6] In order to determine reaction specificity, a specific NOS inhibitor, L-NMMA, is included in the kit.

Components

The kit is sufficient for 50 tests.

Box 1 (Catalog Number NOS1-PART1)
Storage Temperature –70 °C

L-NMMA, 250 µM solution Catalog Number M6432	40 µl
Rat Cerebellum Extract Catalog Number C3474	5 vials
Calmodulin Catalog Number P4230	200 µl
2× Reaction Buffer Catalog Number R1524	1.25 ml

Box 2 (Catalog Number NOS1-PART2)
Store at Room Temperature

Item	Quantity
10× Homogenization Buffer Catalog Number H9783	50 ml
Stop Buffer Catalog Number S5309	20 ml
Equilibrated Resin Catalog Number R1399	5 ml
Calcium Chloride Catalog Number C3349	300 μl
Elution Buffer Catalog Number E5775	20 ml
Spin columns and caps Catalog Number S3563	50 units
Microcentrifuge tubes, 2 ml Catalog Number T2795	50 units

Reagents and Equipment Required But Not Provided

- [^{3}H] Arginine monohydrochloride, 40–70 Ci/mmol, 1 μCi/μl or
  [^{14}C] Arginine monohydrochloride, >300 mCi/mmol, 50 μCi/μl (GE Healthcare Life Sciences, formerly Amersham Biosciences, Catalog Numbers TRK698 and CFB63, respectively)
- β-Nicotinamide adenine dinucleotide 2′-phosphate reduced (NADPH, Catalog Number N1630)
- 10 mM Tris-HCl, pH 7.4 (prepare from 1 M stock, Catalog Number T2663)
- Microcentrifuge tubes (Catalog Number T9661)
- Microcentrifuge, e.g., Eppendorf® microcentrifuge 5417 Series (Catalog Numbers Z365998 and Z366005 or the refrigerated Z366013 and Z366021) or equivalent
- Scintillation Fluid, Sigma-Fluor™ High Performance LSC Cocktail (Catalog Number S4023 or equivalent) and vials (Catalog Number M1901 or equivalent)
- Hank's Balanced Salt Solution (Catalog Number H6648) or Dulbecco's Phosphate Buffered Saline (Catalog Number D8537)
- Protease Inhibitor Cocktail, optional (Catalog Number P8340)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Prior to the preparation of tissue or cell extracts, prepare 1× Homogenization Buffer by diluting an appropriate volume of the 10× Homogenization Buffer (Catalog Number H9783) 10-fold with ultrapure water.

Preparation of Extracts from Tissues
This assay system can be used to test NOS activity in homogenates of tissues. NOS activity has been demonstrated in a variety of tissues including blood vessels, neural tissue, and skeletal muscle.

1. Add 5–20 volumes of ice-cold 1× Homogenization Buffer to the tissue sample (5–20 ml/g weight of tissue). Since the level of NOS activity may vary greatly between tissues, it may be necessary to optimize the amount of homogenization buffer added. It is recommended to start with a low volume of buffer.
   Note: Given the susceptibility of the NOS enzyme to proteases, it may be advisable to add a Protease Inhibitor Cocktail (Catalog Number P8340) to the 1× Homogenization Buffer prior to homogenization.
2. Homogenize the tissue on ice using a tissue grinder or homogenizer.
3. Transfer the homogenate to a microcentrifuge tube(s) and spin at full speed in a microcentrifuge for 5 minutes at 4 °C. For large volumes use an appropriate centrifuge according to the above instructions.
4. Transfer the supernatant to another tube for further testing of either the supernatant or cell pellet in the NOS detection system.

Tissue homogenates can be processed further by subcellular fractionation to concentrate NOS activity. However, the subcellular distribution of the NOS enzyme is isoform and tissue dependent. For example, eNOS is predominantly associated with the membrane.[8] Alternatively, nNOS is primarily in the soluble fraction of brain, but is membrane bound in skeletal muscle.[9,10] The soluble and membrane bound NOS enzyme can be separated by centrifuging at 100,000 × *g* for 60 minutes at 4 °C. If you are uncertain which fraction is likely to contain the NOS enzyme, it is recommended to test both fractions initially to determine its localization.

Preparation of Extracts from Cultured Cells

This assay system can be used to determine NOS activity in cultured cells such as endothelial cells or induced macrophages. The cells should be processed as follows:

1. Remove the medium from the cells.
2. Wash the cells with Hank's Balanced Salt Solution (Catalog Number H6648) or Dulbecco's Phosphate Buffered Saline (Catalog Number D8537).
3. Harvest the cells according to a standard protocol (i.e., trypsinization, cell scraping, etc.).
4. Transfer the cells to a centrifuge tube and spin to pellet the cells.
5. Remove the supernatant from the pelleted cells and add 100–500 μl of 1× Homogenization Buffer. It may be advisable to add a Protease Inhibitor Cocktail (Catalog Number P8340) to the 1× Homogenization Buffer prior to homogenization. Homogenize the cells with repeated pipetting or with a homogenizer.
6. Spin the cell homogenate in a microcentrifuge at full speed for 5 minutes.
7. Separate the supernatant (extract) from the insoluble fraction and test the appropriate fraction for activity.

As described in the previous section, extracts from cultured cells can be further purified by subcellular fractionation. Optimal cell number/volume of homogenization buffer must be determined for each cell line.

Storage/Stability

This kit is supplied in 2 parts/boxes.

Store Box 1 (Catalog Number NOS1-PART1) and its contents at –70 °C.

Store Box 2 (Catalog Number NOS1-PART2) and its contents at room temperature.

Procedure

The radiolabeled substrate, ^{3}H or ^{14}C arginine, is incubated for 10–60 minutes with a cell lysate or tissue homogenate in a reaction buffer containing the necessary cofactors. The reaction is stopped by the addition of EDTA, which chelates all available calcium. A resin is added to the mixture, which binds all unreacted arginine. The mixture is passed through a spin column allowing the flow through of radiolabeled citrulline. Radioactivity in the flow-through (unbound) fraction is then counted and NOS activity can be determined. Unreacted arginine can be eluted from the resin with the Elution Buffer included in the kit.

The assay reaction is linear over 60 minutes at room temperature using the Rat Cerebellum Extract positive control (Catalog Number C3474) included in the kit. Different enzyme sources may require optimization of the assay incubation time and temperature. For example, eNOS is generally found at lower levels and thus, may require longer incubation times at a higher temperature (37 °C). Alternatively, nNOS is relatively abundant in neural tissue such as the brain and activity can be determined with shorter incubation times at a lower temperature. It may be necessary to optimize the general assay protocol with respect to incubation time and temperature for individual biological test samples.

Both eNOS and nNOS require the presence of calcium and calmodulin for activity. Calcium is included as part of the reaction cocktail to ensure sufficient calcium levels for activity. Depending on the source of the enzyme, additional calmodulin may be required (provided in the kit). Generally, in cell lysates and crude tissue extracts there is enough residual calmodulin to drive the reaction. However, it is necessary to add calmodulin when testing purified enzyme preparations.

While assay design is at the discretion of the investigator, it is strongly urged to include the following controls to allow proper interpretation of assay data from the kit. First, to ensure that the radiolabeled arginine substrate has not broken down, it is recommended that the background radioactivity due to arginine break down be determined with each assay (see Determining Purity of Radiolabeled Arginine section). Second, inclusion of the supplied Rat Cerebellum Extract as a positive control is recommended. Third, non-enzymatic or non-NOS-induced radiolabeled citrulline production should be determined by one of several methods. For example, the enzyme extract can be boiled prior to testing. Alternatively, a NOS inhibitor such as L-NMMA (included in the kit) can be added to the tissue extract. Key components such as NADPH and/or calcium can be omitted from the reaction buffer. At least one of these controls should be included to determine background conversion.

Please read all instructions carefully prior to initiating the assay.

1. Immediately prior to beginning the assay, prepare a 10 mM NADPH solution in 10 mM Tris-HCl, pH 7.4. Discard any unused NADPH solution.
2. Prepare the Reaction Cocktail on ice by adding the following reagents to a tube. The volumes given are for a single reaction. Determine the volume of each reagent needed by multiplying the volume by the total number of reactions.

   4 μl H_2O
   25 μl 2× Reaction Buffer (Catalog Number R1524)
   5 μl 10 mM NADPH solution
   5 μl 6 mM $CaCl_2$ (Catalog Number C3349)
   1 μl [^{3}H] arginine (1 μCi/μl) or [^{14}C] arginine (50 μCi/μl)

3. Add 40 μl of the Reaction Cocktail prepared in step 2 to a separate microcentrifuge tube for each sample or control.
4. To the control tube, measuring non-specific radiolabeled citrulline production, add 5 μl of the 250 μM L-NMMA solution (Catalog Number M6432) to a final concentration of 25 μM or another NOS inhibitor prior to the addition of cell or tissue extract.
5. As necessary, add 5 μl of Calmodulin (Catalog Number P4230) to the appropriate sample or control tubes prior to the addition of the biological sample.
6. Add 1–10 μl of the cell or tissue extract to the appropriate sample or control tubes. Add 5 μl of the Rat Cerebellum Extract (Catalog Number C3474) to the positive control tubes.
   Note: The Rat Cerebellum Extract is extremely viscous and thus, before use, should be spun down in a centrifuge and immediately pipetted out as it has a tendency to creep up the sides of the vial with time.
7. Incubate all tubes at 22–37 °C for 10–60 minutes. An incubation time of 30 minutes at room temperature is a reasonable condition for the initial activity screen.
8. Stop the reaction by adding 400 μl of Stop Buffer (Catalog Number S5309) to each tube.
9. Thoroughly resuspend the Equilibrated Resin (Catalog Number R1399) by gentle agitation of the bottle. Add 100 μl of the Equilibrated Resin suspension to each tube and mix thoroughly. Frequent mixing of the resin while dispensing is necessary to ensure even distribution of the resin in each assay sample.
10. Transfer the entire contents of each tube to a separate spin column (Catalog Number S3563) and place each spin column in a separate microcentrifuge tube.
    Note: Before placing the spin column in the microcentrifuge tube, uncap the column and break off the plastic tip at the bottom end of the column.
11. Centrifuge the spin columns for 30 seconds in a microcentrifuge at full speed.
12. Remove the spin columns from the microcentrifuge tube and transfer the flow through liquid into a scintillation vial. Add 6 ml of scintillation fluid to each vial and quantitate the radioactivity in a liquid scintillation counter.

At the user's discretion, the ratio of unreacted arginine to citrulline may be determined by performing steps 13–15.

13. Place each spin column from step 12 in a fresh microcentrifuge tube and add 400 μl of Elution Buffer (Catalog Number E5775) to the spin columns.
14. Centrifuge the spin columns in a microcentrifuge at full speed for 30 seconds.
15. Remove the spin column and transfer each eluate to a separate scintillation vial. Add 6 ml of scintillation fluid and count in a liquid scintillation counter.

<u>Determining Purity of Radiolabeled Arginine</u>
Radiolabeled arginine has the potential to break down over time. If breakdown occurs, background levels of unbound radioactivity can decrease the sensitivity of the assay. Determining the purity of the arginine prior to an assay is highly recommended.

To determine background levels, simply process the Reaction Cocktail as described previously, but without the addition of the biological sample. Briefly:

1. Prepare the Reaction Cocktail on ice, as described in step 2 of the assay protocol.
2. Add 40 µl of the prepared Reaction Cocktail to 400 µl of Stop Buffer.
3. Add 100 µl of Equilibrated Resin. Mix thoroughly and transfer the entire mix to a spin column.
4. Centrifuge the spin column in a microcentrifuge for 30 seconds at full speed.
5. Transfer the flow through liquid into a scintillation vial, add 6 ml of scintillation liquid, and quantitate the radioactivity in a liquid scintillation counter.
6. Quantitate the total radioactivity added to the reaction by counting a 40 µl aliquot of the Reaction Cocktail prepared in step 1.

The amount of unbound radioactivity in the flow through liquid from step 5 should be <5% of the total counts added from step 6 (i.e., 95% of the radioactivity should be retained by the resin). If the flow through contains >5% of the total radioactivity added, this could be indicative of breakdown of the radiolabeled substrate. The radiolabeled substrate can be purified or new substrate purchased. Methods for purification of the radiolabeled substrate have been published.[11]

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