NOS Activity Assay Kit

Catalog Number KA1345

50 assays

Version: 06

Intended for research use only

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Introduction

Principle of the Assay

The NOS Activity Assay Kit is a simple, sensitive and specific assay for nitric oxide synthase (NOS) activity. This kit is based on the biochemical conversion of L-arginine to L-citrulline by NOS.[1-6] This reaction, which represents a novel enzymatic process, involves a five-electron oxidation of a guanidino nitrogen of L-arginine to nitric oxide (NO), together with the stoichiometric production of L-citrulline (see figure 1). The reaction consumes 1.5 equivalents of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and also requires molecular oxygen, calcium, calmodulin, and tetrahydrobiopterin (BH_4).[7,8]

Measuring NOS activity by monitoring the conversion of arginine to citrulline is a standard assay for NOS activity in both crude and purified enzyme preparation. Advantages of the NOS Activity Assay Kit include the use of radioactive substrates ([^{3}H] arginine or [^{14}C] arginine) that enable sensitivity to the picomole level, as well as the specificity of the assay for the NOS pathway due to the direct enzymatic conversion of arginine to citrulline in eukaryotic cells. Additionally, the easy separation of neutrally charged citrulline from positively charged arginine allows multiple assays to be performed easily.

For routine assays, radioactive arginine is added to protein extracts or purified NOS samples. After incubation, the reactions are stopped with a buffer containing ethylenediaminetetraacetic acid (EDTA), which chelates the calcium required by nNOS (neuronal NOS; NOS I) and eNOS (endothelial NOS; NOS III) and, consequently, inactivates the enzyme. In the case of iNOS (inducible NOS; NOS II), the low pH of the Stop Buffer, pH 5.5, stops the enzyme-catalyzed reaction. Equilibrated Resin, which binds to the arginine, is added to the sample reactions and the reactions are then pipetted into spin cups. The citrulline, being ionically neutral at pH 5.5, flows through the cups completely. NOS activity is then quantitated by counting the radioactivity in the eluate.

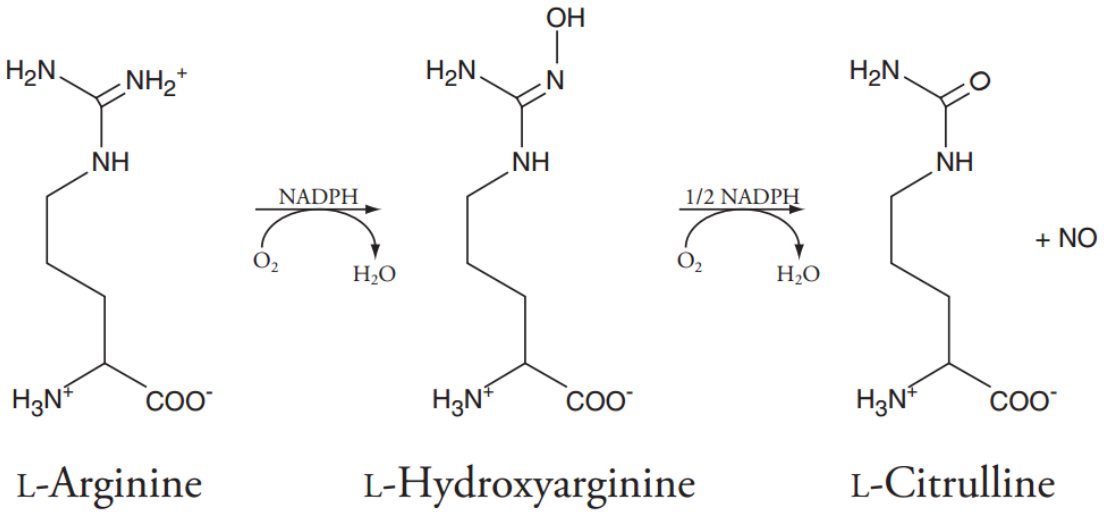

Figure 1. NOS catalyzes a 5-electron oxidation of a guanidino nitrogen of L-arginine to generate NO and L-citrulline. L-Hydroxyarginine is formed as an intermediate that is tightly bound to the enzyme. Both steps in the reaction are dependent on calcium and calmodulin.

General Information

Materials Supplied

List of component

Item	Quantity
iNOS Positive Control	50 µL
Calmodulin (1 µM)	200 µL
Reaction Buffer (2X)	1.25 mL
L-NNA (10 mM)	100 µL
Homogenization Buffer (10X)	50 mL
Stop Buffer	25 mL
Equilibrated Resin	5 mL
Calcium Chloride (6 mM)	400 µL
Spin Columns and Collection Tubes	50 sets

Storage Instruction

After opening kit, store individual components as stated below and used before the expiration date indicated on the outside of the box.

Item	Storage
iNOS Positive Control	-80°C
Calmodulin (1 µM)	-20°C
Reaction Buffer (2X)	-20°C
L-NNA (10 mM)	-20°C
Homogenization Buffer (10X)	Room Temperature
Stop Buffer	Room Temperature
Equilibrated Resin	Room Temperature
Calcium Chloride (6 mM)	Room Temperature
Spin Columns and Collection Tubes	Room Temperature

Materials Required but Not Supplied

- ✓ [^{3}H] Arginine monohydrochloride [40-70 Ci/mmol, 1 µCi/µL (Perkin Elmer, Item No. NET1123001MC)] -or- [^{14}C] arginine [>300 mCi/mmol, 100 µCi/ml (Perkin Elmer, Item No. NEC267E050UC)]
- ✓ Reduced nicotinamide adenine dinucleotide phosphate (NADPH*) (Sigma, Item No. N1630 or N7505)
- ✓ 10 mM Tris-HCl, pH 7.4
- ✓ Optional: magnesium acetate (Sigma, Item No. M2545) for assaying crude iNOS samples
- ✓ Scintillation fluid and vials
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable

*NADPH is not stable in solution; therefore, it is not included in the reaction buffer.

Precautions for Use

- ✓ This product is for research only - Not for human or veterinary diagnostic or therapeutic use.
- ✓ Please read these instructions carefully before carefully before beginning this assay.
- ✓ Safety Data

  The material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handing. Before use, the user must review the complete Material Safety Data Sheet.

Assay Protocol

Reagent Preparation

1. iNOS Positive Control
   The vial contains 50 µL of murine recombinant iNOS. Thaw the enzyme on ice before use. It is then ready to use as a control in the assay.
2. Calmodulin (1 µM)
   The vial contains 1 µM calmodulin. It is ready to use in the assay.
3. Reaction Buffer (2X)
   The vial contains 1.25 mL of 50 mM Tris-HCl, pH 7.4, containing 6 µM tetrahydrobiopterin (BH_4), 2 µM flavin adenine dinucleotide, and 2 µM flavin adenine mononucleotide.
4. Homogenization Buffer (10X)
   The vial contains 50 mL of 250 mM Tris-HCl, pH 7.4, containing 10 mM EDTA and 10 mM ethyleneglycol-bis(β-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA). See under Sample Preparation to determine how much Homogenization Buffer (1X) to make.
5. L-NNA (10 mM)
   The vial contains 100 µL of 10 mM L-NNA. L-NNA is a non-specific NOS inhibitor. It will eliminate all of the NOS activity. It is ready to use in the assay.
6. Stop Buffer (1X)
   The vial contains 25 mL of 50 mM N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES), pH 5.5, and 5 mM EDTA. It is ready to use in the assay.
7. Equilibrated Resin
   The vial contains 5 mL of resin. It is ready to use.
8. Calcium Chloride (6 mM)
   The vial contains 400 µL of 6 mM calcium chloride. It is ready to use to assay nNOS and eNOS.
9. Spin Columns and Collection Tubes
   Sufficient spin columns and collection tubes are provided for 50 total reactions.

Sample Preparation

✓ Preparation of Extracts from Tissues and Cultured Cells
The citrulline assay has been used to quantitate levels of NOS activity in tissue homogenates from numerous sources including blood vessels, immune cells, visceral organs, nervous tissue, and cultured cells. NOS enzymes are relatively unstable; therefore, tissues should be harvested quickly after animal euthanasia. If enzyme assays are to be conducted at a later time, it is best to freeze intact tissues or harvested cultured cells prior to homogenization. Wrap the tissues in aluminum foil, flash freeze the tissues in liquid nitrogen, and then store at -80°C.

✓ Extraction of Proteins from Tissues

Note: Because the level of NOS activity will vary between tissues, the volume of 1X Homogenization Buffer used may require optimization.

1. Prepare an appropriate volume of Homogenization Buffer (1X) (*i.e.*, a 1:10 dilution of the Homogenization Buffer (10X). Add 10-20 volumes [volume of buffer (mL): weight of tissue (g)] of ice-cold Homogenization Buffer (1X) to a tissue sample (for tissues with low NOS activity, start with 5-10 volumes of Homogenization Buffer (1X)).
2. Homogenize the tissue using a tissue grinder or an equivalent tissue homogenizer. Keep the tissue homogenate on ice.
3. Centrifuge at 10,000 x g for 15 minutes at 4˚C (or at full speed for five minutes in a microcentrifuge at 4°C).
4. Transfer the supernatant to fresh microcentrifuge tubes and keep the tubes on ice until use. This extract will be used in the NOS Activity Assay (see step 3 of Preparing the Reactions).

✓ Subcellular Tissue Distribution

The subcellular distribution of NOS is tightly regulated in tissues. eNOS is largely membrane associated as a result of N-terminal myristoylation.[9,10] nNOS is largely soluble in adult rat brain, yet in skeletal muscle, it is predominantly associated with membrane fractions.[11] The mechanism for membrane attachment of nNOS remains unclear. iNOS is a soluble enzyme. NOS activity in soluble and membrane-associated fractions can be separated by centrifuging the homogenized tissues at 100,000 x g for 60 minutes. The supernatant contains soluble NOS, while the pellet, which is resuspended in Homogenization Buffer, contains membrane-associated NOS.

✓ Extraction of Proteins from Tissue Culture Cells

Certain cultured cells, such as endothelial cells and activated macrophages, contain NOS, which can be measured using the citrulline assay. The proteins must first be extracted from the cells as follows:

1. Remove the culture media from the tissue culture cells.
2. Wash the tissue culture cells once with phosphate-buffered saline (PBS) and harvest the tissue culture cells in PBS containing 1 mM EDTA. Transfer the tissue culture cells to microcentrifuge tubes.
3. Spin the microcentrifuge tubes in a microcentrifuge at full speed for two minutes to pellet the cells.
4. Remove the supernatant from the pelleted cells by vacuum aspiration and then add 100-500 µL of the Homogenization Buffer (1X) to each microcentrifuge tube of pelleted cells. Sonicate briefly to disrupt the cells.
5. Spin the microcentrifuge tubes in a microcentrifuge at full speed for five minutes.
6. Separate the supernatant from the pellet and adjust the resulting protein sample to a concentration of 5-10 mg/mL.

Assay Procedure

- ✓ Performing the Assay
- Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts

  Note: Refer to Appendix for notes on the stability of radiolabeled arginine.

  Incubation of the citrulline assay reaction may be carried out for 10-60 minutes at 22-37°C depending on the tissue being used. High levels of nNOS in nervous tissues[12] and skeletal muscle[11] permit brief assays (10-15 minutes) of NOS with room temperature incubations. Lower levels of eNOS in vascular tissues require that assays be performed for prolonged periods (60 minutes) at room temperature.

  eNOS and nNOS require calcium for enzyme activity; therefore, it is essential to add calcium to experimental assays. A final free calcium concentration of 75 µM is required for optimal NOS activity.[12] When testing NOS activity from tissue extracts, addition of calmodulin to the reaction is not required. However, when testing purified NOS, the addition of calmodulin is required for nNOS and eNOS (for review, see Reference 1). Crude extracts of macrophage iNOS, such as the 100,000 x g supernatant solution, require 1 mM of Mg^{2+} for maximum activity, while purified iNOS shows no such dependence.

  NOS activity in the citrulline assay is defined as counts per minute (cpm) in an incubated test sample as compared to an appropriate blank. The following control reactions can serve as a blank: a reaction that includes 1 mM N^G-nitro-L-arginine (L-NNA, a competitive NOS inhibitor provided at a concentration of 10 mM), a reaction in which the extract is boiled prior to the assay, a reaction in which either NADPH or calcium (for nNOS and eNOS) is omitted, or a reaction that is incubated on ice. As for any quantitative enzyme assay, it is important to verify reaction conditions are such that the assay is linear with respect to time and protein concentration. Specific activity and substrate affinity of NOS can be assessed by carrying out replicate reactions in the presence of varying amounts of unlabeled arginine. The K_m (Michaelis constant) of NOS is in the range of 2-20 µM.[7,10,13] Appropriate concentrations of arginine for kinetic studies are 0.1-100 µM.

✓ Preparing the Reactions

1. Prepare and store the Reaction Mixture on ice by adding the following components to a microcentrifuge tube (500 µL or 1.5 mL):

   Note: The volumes given here yield sufficient Reaction Mixture for 10 reactions. The Reaction Mixture can be stored on ice for up to 24 hours. Inducible NOS (iNOS) is Ca^{2+} independent. When assaying iNOS, substitute 50 µL HPLC-grade water for $CaCl_2$ or add 50 µL of 8 mM $MgCl_2$ (see Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts).

   250 µL of Reaction Buffer (2X)

   50 µL of 10 mM NADPH [freshly prepared in 10 mM Tris-HCl, pH 7.4]

   10 µL of [^{3}H] arginine (1 µCi/µL) -or- 5 µL [^{14}C]arginine (100 µCi/mL)

   50 µL of $CaCl_2$ -or- 50 µL of HPLC-grade water -or- 50 µL $MgCl_2$

   40 µL of HPLC-grade water for [3H] arginine -or- 45 µL of HPLC-grade water for [^{14}C] arginine
2. Label 1.5 mL microcentrifuge tubes for the appropriate reaction below.
3. iNOS Positive Control – add 40 µL of Reaction Mixture, 5 µL of HPLC-grade water, and 5 µL of iNOS Positive Control to microcentrifuge tube.
4. Control Sample – add 40 µL of Reaction Mixture, 5 µL of calmodulin*, and 5 µL of tissue extract or NOS enzyme preparation to a microcentriguge tube.
5. Inhibition of Control Sample (optional) – To chemically inhibit all of the NOS activity in the control sample (background), add 5 µL of L-NNA to 40 µL of Reaction Mixture before adding the 5 µL of tissue extract or NOS enzyme preparation to a microcentrifuge tube. If needed, add 5 µL of calmodulin* to the mixture.
6. Incubate the reaction at 22-37°C for 10-60 minutes. (For initial experiments, the reaction should be allowed to proceed at room temperature for 30 minutes.)
7. Stop the reaction by adding 400 µL of Stop Buffer to each microcentrifuge tube.

*nNOS and eNOS required 0.1 µM calmodulin when assayed as purified enzymes. The addition of calmodulin to tissue extracts is not necessary, but it is recommended. If the addition of calmodulin is required, add calmodulin to a final concentration of 0.1 µM (i.e., add 5 µL to a 50 µL reaction).

- Processing the Samples

1. Thoroughly resuspend the Equilibrated Resin provided. Pipette 100 µL of the Equilibrated Resin into each reaction sample.
2. Place the spin column into collection tubes and transfer the reaction samples to spin columns.
3. Centrifuge the spin columns and collection tubes in a microcentrifuge at full speed for 30 seconds.
4. Remove the spin columns and collection tubes and transfer the eluate (i.e., the “flowthrough”) to scintillation vials. Add scintillation fluid to the vials and quantitate the radioactivity in a liquid scintillation counter.

- Additional Controls

1. Total Counts

   To determine total counts used in the reaction, transfer 40 µL of the Reaction Mixture to a scintillation vial.

Add 400 µL of Stop Buffer. Add scintillation fluid and quantify the radioactivity.

2. Background Counts

To determine background counts used in the reaction, transfer 40 µL of the Reaction Mixture to a microcentrifuge tube. Add 400 µL of Stop Buffer and 100 µL of Euilibrated Resin. Process the Control the same as described for the samples (*i.e.,* apply to spin columns and count the eluate).

✓ Quick Reference Protocol

Preparation of Extracts from Tissues and Cultured Cells

1. Extraction of Proteins from Tissues

- Add 10-20 volumes of ice-cold Homogenization Buffer (1X) to a tissue sample.
- Homogenize the tissue and spin the homogenate in a microcentrifuge for five minutes at 4°C (or 10,000 x g for 15 minutes at 4°C).
- Transfer the supernatant to a fresh tube and keep the tube on ice until use.

2. Extraction of Proteins from Tissue Culture Cells

- Remove the culture media from the tissue culture cells, wash the cells with PBS and harvest the cells in PBS containing 1 mM EDTA.
- Spin the cells at full speed for two minutes, remove the supernatant by vacuum aspiration and resuspend the cells in Homogenization Buffer.
- Sonicate or homogenize the cells.
- Spin the cells at full speed in a microcentrifuge for five minutes at 4˚C, remove the supernatant, and adjust the protein sample to a concentration of 5-10 mg/mL.

3. Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts

- Prepare the Reaction Mixture in a microcentrifuge tube and store on ice.
- Add the cell/tissue extract to 40 µL of the Reaction Mixture.
- Incubate the reaction at 22-37°C for 10-60 minutes.
- Add 400 µL of Stop Buffer and 100 µL of Equilibrated Resin to the reaction sample.
- Transfer the reaction sample to a spin column and spin at full speed for 30 seconds.
- Transfer the eluate to a scintillation vial, add scintillation fluid, and quantitate the radioactivity in a liquid scintillation counter.

Data Analysis

Calculation of Results

The percent citrulline formed in the reaction in relation to total possible counts can be determined as follows:

$$\% \text{ conversion} = \left(\frac{(\text{cpm reaction} - \text{cpm background*})}{\text{cpm total}} \right) \times 100$$

*The cpm from the “blank” as described on “Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts” can also be used here.

In the tissues assayed according to these protocols, citrulline is the major radiolabeled compound in the eluate. This can be readily verified using thin-layer chromatography (TLC). Separation of arginine and relevant metabolites is achieved using silica-gel chromatography plates developed with CH_3OH:NH_4OH (6:1). With this solvent system, arginine migrates at an R_f (the ratio of the distance traveled by a compound to the distance traveled by the solvent) of ~0.1 while citrulline migrates at an R_f of ~0.5.

Resources

Appendix

- Stability of Radiolabeled Arginine

  Prior to initiating the enzyme assays, it is essential to verify the purity of the radiolabeled arginine, otherwise a high blank value for the liquid scintillation counting will greatly reduce the sensitivity of the assay. To assess the blank value, a Reaction Mixture is applied to the Equilibrated Resin. Nonadherent radioactivity is eluted with Stop Buffer, the eluate is collected and the radioactivity is quantitated in a liquid scintillation counter.

1. Prepare a reaction mix by combining the following components in a microcentrifuge tube.

   20 µL of Reaction Buffer (2X)

   4 µL of 10 mM NADPH [freshly prepared in 10 mM Tris, pH 7.4]

   1-10 µL of [^{3}H] arginine (1 µCi/µL) or [^{14}C] arginine (100 µCi/mL)

   dH_2O to bring the total volume to 40 µL
2. Store the reaction mix on ice.
3. Perform the control experiments (*i.e.,* total counts and background counts) as described above using 10 µL of the Reaction Cocktail.

Greater than 90% of the applied radioactivity should be retained by the spin cup. This represents a relatively low blank value. If more than 10% of the radioactivity flows through the spin cup, it is important to purchase new arginine prior to conducting the assay. [^{3}H] Arginine is prone to radiolytic decay and may degrade significatly within two months; [^{14}C] arginine is more stable but more expensive.

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