

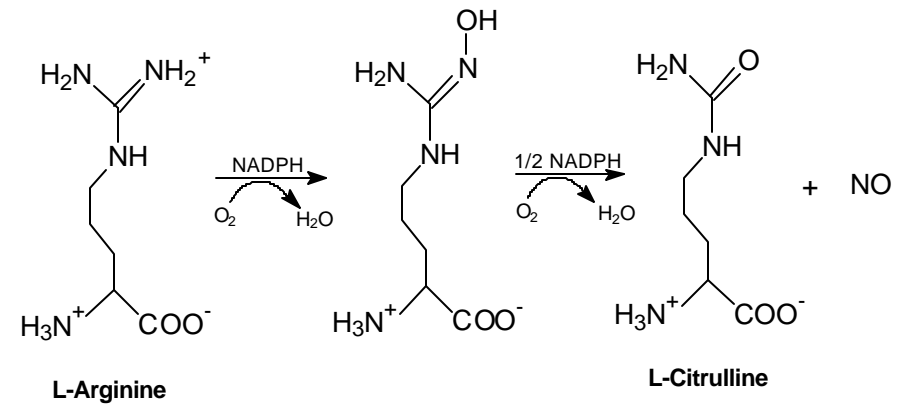


BIOXYTECH[®] Nitric Oxide Synthase Assay Kit

**Quantitative Assay
for the
Determination of NOS Activity**

Catalog No. 22112

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

**For Research Use Only.
Not For Use In Diagnostic Procedures.**

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INTRODUCTION

BIOXYTECH[®]'s Nitric Oxide Synthase (NOS) Assay Kit is an assay for nitric oxide synthase (NOS) activity. The NOS assay kit is based on the biochemical conversion of L-arginine to L-citrulline by NOS. This reaction involves a five-electron oxidation of a guanidinonitrogen of L-arginine to nitric oxide (NO), together with the stoichiometric production of L-citrulline. The reaction consumes 1.5 equivalents of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and also requires molecular oxygen, calcium, calmodulin and tetrahydrobiopterin.

Measuring NOS activity by monitoring the conversion of arginine to citrulline is a widely accepted assay for NOS activity in both crude and purified enzyme preparations. Advantages of the NOS assay kit include the use of radioactive substrates (³H-arginine or ¹⁴C-arginine), and an easy method for separating neutrally charged citrulline from positively charged arginine.

For routine assays, radioactive arginine is added to tissue or cellular extracts. After incubation, the reactions are stopped with a buffer containing EDTA, which chelates the calcium required by NOS and, consequently, inactivates the NOS. Equilibrated resin, which binds to arginine, is added to the samples and they are centrifuged in spin cups. The citrulline, being ionically neutral at pH 5.5, passes through the spin cup column. The NOS activity is then reported by quantitating the radioactivity in the eluate.

Materials Provided

Part 1, Room Temperature Storage

- Homogenization Buffer, 10X, 250 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM EGTA, 50 mL
- Stop Buffer, 1X, 50 mM HEPES, pH 5.5, 5 mM EDTA, 25 mL (stops cNOS reactions)
- Equilibrated Arginine-binding Resin, 5 mL
- Calcium Chloride (CaCl₂), 6 mM, 400 μL
- Elution Buffer, 1X, 0.5 M NH₄Cl, 20 mL
- Spin Cups and Cup Holders, sufficient for 50 reactions

Part 2, -70° C Storage

- Cerebellum Extract, Rat Brain Homogenate Resuspended in Homogenization Buffer, 4 x 20 μL
- Calmodulin, 1 μM, 200 μL
- Reaction Buffer, 2X, 50 mM Tris-HCl, pH 7.4, 6 μM Tetrahydrobiopterin, 2 μM FAD, 2 μM FMN, 1.25 mL
- NADPH, Tetrasodium Salt, 25 mg
- N^G-Nitro-L-arginine Methyl Ester, HCl, (L-NAME, HCl), 10 mM, 40 μL

Materials Required But Not Provided

- ³H-Arginine or ¹⁴C-arginine [~64 Ci/mM, 1 μCi/μL (Amersham, Arlington Heights, Illinois, Catalog #TRK698)]
- 10 mM Tris-HCl, pH 7.4
- Scintillation fluid and vials
- Deionized (or distilled) water

SAMPLE PREPARATION

Preparation of Extracts from Tissues and Cultured Cells

The citrulline assay has been used to quantify levels of NOS activity in tissue homogenates from numerous sources including blood vessels, immune cells, visceral organs, nervous tissue and cultured cells. Nitric oxide synthase is relatively unstable; therefore, tissues should be harvested quickly after animal euthanasia. If enzyme assays are to be performed 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 in liquid nitrogen and then store at -70°C.

Extraction of Proteins from Tissues

1. Prepare an appropriate volume of ice cold 1X homogenization buffer (i.e. a 1:10 dilution of the 10X homogenization buffer). Add 20 mL of ice cold 1x homogenization buffer per gram of tissue.
2. Homogenize the tissue using a tissue grinder or an equivalent tissue homogenizer. Keep tissue homogenate on ice.
3. Pipet 1 mL aliquots of the tissue homogenate into microcentrifuge tubes and spin the tubes in a microcentrifuge at full speed for 5 minutes at +4°C.
4. Transfer the supernatant to fresh microcentrifuge tubes and keep the tubes on ice until use.

Subcellular Tissue Distribution

The subcellular distribution of NOS is tightly regulated in tissues. Endothelial NOS (eNOS or NOS-III) is largely membrane associated as a result of N-terminal myristoylation. nNos is found primarily in the cytoplasmic fractions in adult rat brain, yet in

skeletal muscle, it is predominately associated with membrane fractions. Nitric oxide synthase 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. Wash the tissue culture cells once with phosphate-buffered saline (PBS); Harvest the cells in PBS containing 1 mM EDTA, and transfer them to microcentrifuge tubes.
2. Pellet the cells by spinning in a microcentrifuge at full speed for 2 minutes at +4°C.
3. Remove the supernatant from the pelleted cells by vacuum aspiration.
4. Resuspend the pellet in 100 µL of 1x homogenization buffer and disrupt the cells by repeated pipetting.
5. Spin the homogenates in a microcentrifuge at full speed for 5 minutes.
6. Separate the supernatant from the pellet and use supernatant and/or pellet for NOS assay (depending upon the known distribution of NOS). Protein concentration in the range of 5-10 µg/µL is recommended for this assay.

ASSAY PROCEDURE

Note: Read section on the stability and purity of radiolabeled arginine before proceeding

Incubations 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 and skeletal muscle permit brief assay periods (10 - 15 minutes) at room temperature. Lower levels of eNOS in vascular tissues require that assays be performed for prolonged periods (60 minutes).

Endothelial NOS and nNOS require calcium for enzyme activity; therefore, it is essential to add calcium to the assay medium. A final free calcium concentration of 75 µM is required for optimal NOS activity. 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 may be required depending on the type of NOS in question (for review, see Reference 1).

Nitric oxide synthase 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 methyl ester, HCl (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 is omitted or a reaction that is incubated on ice. As in any quantitative enzyme assay, it is important to optimize reaction conditions so that the assay is linear with respect to time and tissue concentration. The NOS in the rat cerebellum extract provided in this kit is linear for at least a 30-minute reaction. 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. Appropriate concentrations of arginine for kinetic studies are 0.1 - 100 µM.

1. Prepare a reaction stock mix by adding the following components and store on ice. *Note: The volumes given here yield sufficient reaction mix for 10 reactions. The reaction mix can be stored on ice for up to 24 hours.*

250 µL	2x Reaction Buffer
50 µL	10 mM NADPH (freshly prepared in 10 mM Tris-HCl, pH 7.4)
10 µL	³ H-Arginine or ¹⁴ C-arginine (1 µCi/µL)
50 µL	6 mM CaCl ₂ (for eNOS and nNOS)
40 µL	H ₂ O

2. Add 40 µl of reaction mix to each microcentrifuge tube.
3. For the negative controls, add 5 µl of the NOS inhibitor, N^G-Nitro-L-arginine methyl ester, HCl, to each tube.
4. Add 1-10 µL of tissue extract to each tube containing reaction mix. For the control reaction, use 5 µL of the rat cerebellum extract provided. If you are assaying purified eNOS or nNOS, the addition of calmodulin to a final concentration of 0.1 µM is required.
Note: The rat cerebellum extract should be thawed just before use and should be kept on ice. Do not refreeze the thawed extract.
5. Incubate the reaction sample at 22 - 37°C for 10 to 60 minutes. (For initial experiments, the reactions should be carried out at room temperature for 30 minutes.)
6. Stop the reaction by adding 400 µl of stop buffer to the reaction sample.
7. Thoroughly resuspend the equilibrated resin. Pipet 100 µl of the equilibrated resin into each reaction sample.
8. Transfer the samples to spin cups and place the spin cups into cup holders.
9. Centrifuge the spin cups and holders in a microcentrifuge at full speed for 30 seconds.
10. Remove the spin cups from the cup holders and transfer the eluate to scintillation vials. Add scintillation fluid and quantify the radioactivity in a liquid scintillation counter.

If determining the ratio of unreacted arginine to citrulline (optional)

1. Place the spin cups in fresh microcentrifuge tubes and add 400 μL of elution buffer to spin cup.
2. Spin the microcentrifuge tubes (with the spin cups in them) in a microcentrifuge at full speed for 30 seconds.
3. Remove the spin cup and transfer the eluate to scintillation vials. Add scintillation fluid to the vials and quantify the radioactivity in a scintillation counter.
4. This value for the quantity of unreacted arginine can be compared with the value for the citrulline produced.

Stability and Purification of Radiolabeled Arginine

Stability Testing

Prior to initiating the enzyme assays, it is essential to verify the purity of the radiolabeled arginine; otherwise, the resistant high blank value will greatly reduce the sensitivity of the assay. To assess the blank value, a reaction mixture is applied to the equilibrated resin. Non-adherent radioactivity is eluted with stop buffer, the eluate is collected and the radioactivity is quantified in a scintillation counter.

1. Prepare the reaction mixture by combining the following components and place on ice:

20 μL	2x reaction buffer
4 μL	10 mM NADPH (freshly prepared in 10 mM Tris-HCl, pH 7.4)
1-10 μL	^3H -Arginine or ^{14}C -arginine (1 $\mu\text{Ci}/\mu\text{L}$)
H_2O	to bring the total volume to 40 μL

2. Prepare a reaction sample by combining 100 μL of well-resuspended equilibrated resin and 10 μL of the above reaction mixture in a microcentrifuge tube.
3. Add 400 μL of stop buffer to the reaction sample, mix, and transfer the reaction sample to a spin cup. Place the spin cup into a spin cup holder.
4. Centrifuge the spin cup and holder in a microcentrifuge at full speed for 30 seconds.
5. Collect 100 μL of the eluate from the spin cup, add scintillation fluid, and quantify the radioactivity in a liquid scintillation counter.

Greater than 95% of the applied radioactivity should be retained by the spin cup. This represents a relatively low blank value. If more than 5% of the radioactivity flows through the spin cup, it is important to purify the arginine prior to conducting the assay. ^3H -Arginine is prone to radiolytic decay and must be purified every 2 months, while ^{14}C -Arginine is more stable but much more expensive.

Purification of Radiolabeled Arginine

Radioactive arginine can be purified with the equilibrated resin included in the NOS assay kit as follows:

1. Apply the radioactive arginine to 0.5 mL of equilibrated resin in a disposable spin column [e.g., a Poly-Prep® chromatography column (Bio-Rad, Catalog #731-1550)].
2. Wash the column with 5 mL of distilled water.
3. Elute the arginine with 2 mL washes of elution buffer.
4. Lyophilize the arginine and resuspend the arginine in 2% (v/v) ethanol.

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