NOS*detect* Assay Kit

Instruction Manual

Catalog #204500

Revision C

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204500-12

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NOS*detect* Assay Kit

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity	Storage conditions
Cerebellum extract, rat brain[a]	—	5 × 20 µl	–80°C
Calmodulin	1 µM	250 µl	–20°C
Reaction buffer[b]	2×	1.25 ml	–20°C
N_{ω}-nitro-L-arginine methyl ester HCl	10 mM	40 µl	–20°C
Homogenization buffer[b]	10×	50 ml	Room temperature
Stop buffer[b]	1×	25 ml	Room temperature
Equilibrated resin	—	5 ml	Room temperature
Calcium chloride ($CaCl_2$)	6 mM	500 µl	Room temperature
Elution buffer[b]	1×	20 ml	Room temperature
Spin cups and cup holders[c]	—	50	Room temperature

[a] This positive control is a homogenate of rat brain tissue resuspended in homogenization buffer.

[b] See *Preparation of Media and Reagents*.

[c] Sufficient spin cups and cup holders are provided for 50 total reactions.

STORAGE CONDITIONS

Cerebellum Extract, Rat Brain: –80°C
Calmodulin Solution: –20°C
***N*$_{\omega}$-Nitro-L-arginine Methyl Ester HCl:** –20°C
2× Reaction Buffer: –20°C
All Other Components: Room Temperature

ADDITIONAL MATERIALS REQUIRED

[^{3}H]Arginine monohydrochloride [40–70 Ci/mmol, 1 µCi/µl (Amersham, Arlington Heights, Illinois, Catalog #TRK698)] or [^{14}C]arginine monohydrochloride[>300 mCi/mmol, 50 µCi/ml (Amersham, Arlington Heights, Illinois, Catalog #CFB63)]
Reduced nicotinamide adenine dinucleotide phosphate (NADPH)[‖] (Sigma, St. Louis, Missouri)
10 mM Tris-HCl (pH 7.4)
Scintillation fluid and vials

[‖] NADPH is not stable in solution; therefore, it is not included in the reaction buffer.

Revision C

INTRODUCTION

The Stratagene NOS*detect* Assay Kit is a simple, sensitive and specific assay for nitric oxide synthase (NOS) activity. The NOS*detect* assay 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 guanidinonitrogen 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)[7] and also requires molecular oxygen, calcium, calmodulin and tetrahydrobiopterin (BH_4).[8]

Measuring NOS activity by monitoring the conversion of arginine to citrulline is currently the standard assay for NOS activity in both crude and purified enzyme preparations. Advantages of the NOS*detect* 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 intact tissues or protein extracts. After incubation, the reactions are stopped with a buffer containing ethylenediaminetetraacetic acid (EDTA), which chelates the calcium required by NOS and, consequently, inactivates the NOS. 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. The NOS activity is then quantitated by counting the radioactivity in the eluate.

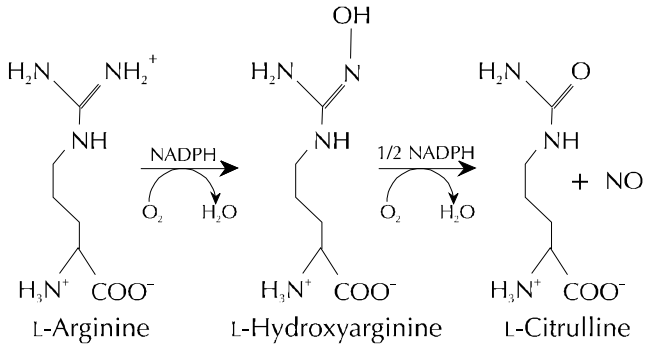

FIGURE 1 NOS catalyzes a 5-electron oxidation of an amidine nitrogen of L-arginine to generate NO and L-citrulline. L-Hydroxyarginine is formed as an intermediate that is tightly bound to the enzyme. Both steps in the reaction are dependent on calcium and calmodulin.

PROTOCOL

Measurement of Nitric Oxide Synthase Activity

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. Nitric oxide synthase activity is 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 1× homogenization buffer used may require optimization.*

1. Prepare an appropriate volume of 1× homogenization buffer (i.e., a 1:10 dilution of the 10× homogenization buffer). Add 10–20 volumes [weight of tissue (g)/volume of buffer (ml)] of ice-cold 1× homogenization buffer to a tissue sample (for tissues with low NOS activity, start with 10 volumes of 1× homogenization buffer).

2. Homogenize the tissue using a tissue grinder or an equivalent tissue homogenizer. Keep the 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. This is the extract that will be used in the NOS activity assay (i.e., step 3 of *Preparing the Reactions* in *Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts*).

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.[9,10] The mechanism for membrane attachment of neuronal NOS (nNOS or NOS-I) remains unclear. Nitric oxide synthase activity in soluble and membrane-associated fractions can be separated by centrifuging the homogenized tissues at 100,000 × *g* for 60 minutes. The supernatant contains soluble NOS, while the pellet, which is resuspended in homogenization buffer, contains membrane-associated NOS. Literature shows nNOS is largely soluble in adult rat brain, yet in skeletal muscle, it is predominantly associated with membrane fractions.[11]

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 2 minutes to pellet the cells.

4. Remove the supernatant from the pelleted cells by vacuum aspiration and then add 100–500 µl of 1× homogenization buffer to each microcentrifuge tube of pelleted cells. Homogenize the pelleted cells with repeated pipetting to disrupt the cells.

5. Spin the microcentrifuge tubes in a microcentrifuge at full speed for 5 minutes.

6. Separate the supernatant from the homogenate and adjust the resulting protein sample to a concentration of 5–10 µg/µl.

Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts

Note *Refer to* Appendix: Stability of Radiolabeled Arginine *for notes on the purification and stability of radiolabeled arginine.*

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[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).

Endothelial NOS and nNOS require calcium for enzyme activity; therefore, it is essential to add calcium to experimental assays. A final free calcium concentration of 750 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 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_{ω}-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 for any quantitative enzyme assay, it is important to verify reaction conditions are such 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.[7, 10, 13] Appropriate concentrations of arginine for kinetic studies are 0.1–100 µM.

Preparing the Reactions

Note *Prepare a control reaction with the provided rat cerebellum extract at step 3 below. Thaw the rat cerebellum extract just before using and keep the thawed extract on ice. Do not refreeze the thawed extract.*

1. Prepare the reaction mixture on ice by adding the following components to a microcentrifuge tube:

   Notes *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 is Ca^{2+} independent.

   250 µl of 2× reaction buffer (See *Preparation of Media and Reagents*)
   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 [^{14}C]arginine (50 µCi/ml)
   50 µl of 6 mM $CaCl_2$
   40 µl of dH_2O

2. Store the reaction mixture on ice.

   Note *To chemically inhibit the control reaction, add 5 µl of the inhibitor N_{ω}-nitro-L-arginine methyl ester HCl to the reaction mixture **before** adding the tissue extract or NOS enzyme preparation.*

3. Combine 40 µl of the reaction mixture with 1–10 µl of tissue extract for the experimental reaction. Combine 40 µl of the reaction mixture with 5 µl of the provided rat cerebellum extract for the control reaction.

   Notes *Recombinant NOS may also be used for a control reaction. (Calmodulin must be added to reactions using recombinant NOS.)*

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

4. Incubate the reaction samples at 22–37°C for 10–60 minutes. (For initial experiments, the reaction should be allowed to proceed at room temperature for 30 minutes.)

5. Stop the reactions by adding 400 µl of stop buffer to the reaction sample (See *Preparation of Media and Reagents*).

Processing the Samples

1. Thoroughly resuspend the equilibrated resin provided. Pipet 100 µl of the equilibrated resin into each reaction sample.

2. Transfer the reaction samples to spin cups and place the spin cups into cup holders.

3. Centrifuge the spin cups and holders in a microcentrifuge at full speed for 30 seconds.

4. Remove the spin cups from the cup holders 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.

5. If determining the ratio of unreacted arginine to citrulline is desired, place the spin cups in fresh microcentrifuge tubes and add 400 µl of elution buffer[§] to the spin cup.

6. Spin the microcentrifuge tubes (with the spin cups in them) in a microcentrifuge at full speed for 30 seconds.

7. Remove the spin cups and transfer the eluate to scintillation vials. Add scintillation fluid to the vials and quantitate the radioactivity in a liquid scintillation counter.

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.

APPENDIX: STABILITY OF RADIOLABELED ARGININE

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 2× reaction buffer
   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 (50 µCi/ml)
   dH_2O to bring the total volume to 40 µl

2. Store the reaction mix on ice.

3. Prepare a reaction sample by combining 100 µl of well-resuspended equilibrated resin and 10 µl of the reaction cocktail in a microcentrifuge tube.

4. Add 400 µl of stop buffer to the reaction sample, mix the contents of the reaction sample and transfer the reaction sample to a spin cup. Place the spin cup into a spin cup holder.

5. Centrifuge the spin cup and holder in a microcentrifuge at full speed for 30 seconds.

6. Collect 100 µl of the eluate from the spin cup and quantitate 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. [^{3}H]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*detect* assay kit[14] 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, Richmond, California, Catalog #731-1550)].

2. Wash the column with 5 ml of distilled water.

3. Elute the arginine with two 2-ml washes of elution buffer.

4. Lyophilize the arginine and resuspend the arginine in 2% (v/v) ethanol.

PREPARATION OF MEDIA AND REAGENTS

Elution Buffer 0.5 M NH_4Cl	**10× Homogenization Buffer** 250 mM Tris-HCl (pH 7.4) 10 mM EDTA 10 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)
2× Reaction Buffer 50 mM Tris-HCl (pH 7.4) 6 µM tetrahydrobiopterin (BH_4) 2 µM flavin adenine dinucleotide 2 µM flavin adenine mononucleotide	**Stop Buffer** 50 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) (pH 5.5) 5 mM EDTA

REFERENCES

1. Bredt, D. S. and Snyder, S. H. (1994) *Annu Rev Biochem* 63:175-95.
2. Ignarro, L. J. (1991) *Biochem Pharmacol* 41(4):485-90.
3. Marletta, M. A. (1993) *J Biol Chem* 268(17):12231-4.
4. Moncada, S. and Higgs, A. (1993) *N Engl J Med* 329(27):2002-12.
5. Nathan, C. and Xie, Q. W. (1994) *J Biol Chem* 269(19):13725-8.
6. Schmidt, H. H., Lohmann, S. M. and Walter, U. (1993) *Biochim Biophys Acta* 1178(2):153-75.
7. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. and Nathan, C. F. (1991) *Proc Natl Acad Sci* U S A 88(17):7773-7.
8. Schmidt, H. H., Pollock, J. S., Nakane, M., Gorsky, L. D., Forstermann, U. et al. (1991) *Proc Natl Acad Sci U S A* 88(2):365-9.
9. Busconi, L. and Michel, T. (1993) *J Biol Chem* 268(12):8410-3.
10. Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H. et al. (1991) *Proc Natl Acad Sci U S A* 88(23):10480-4.
11. Kobzik, L., Reid, M. B., Bredt, D. S. and Stamler, J. S. (1994) *Nature* 372(6506):546-8.
12. Knowles, R. G., Palacios, M., Palmer, R. M. and Moncada, S. (1989) *Proc Natl Acad Sci U S A* 86(13):5159-62.
13. Bredt, D. S. and Snyder, S. H. (1990) *Proc Natl Acad Sci U S A* 87(2):682-5.
14. Salter, M., Knowles, R. G. and Moncada, S. (1991) *FEBS Lett* 291(1):145-9.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

STRATAGENE

An **Agilent Technologies** Division

NOS*detect* Assay Kit

Catalog #204500

QUICK-REFERENCE PROTOCOL

Preparation of Extracts from Tissues and Cultured Cells

Extraction of Proteins from Tissues

- Add 20 volumes of ice-cold 1× homogenization buffer to a tissue sample
- Homogenize the tissue and spin the homogenate in a microcentrifuge for 5 minutes at 4°C
- Transfer the supernatant to a fresh tube and keep the tube on ice until use

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 2 minutes, remove the supernatant by vacuum aspiration and resuspend the cells in homogenization buffer
- Spin the cells at full speed for 5 minutes, separate the supernatant from the homogenate and adjust the protein sample to a concentration of 5–10 µg/µl

Measurement of Nitric Oxide Synthase Activity in Enzyme Extracts

- Prepare the reaction mixture in a microcentrifuge tube and store on ice
- Add the 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 cup 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
- To determine the ratio of unreacted arginine to citrulline, add 400 µl of elution buffer to the spin cup, place the spin cup in a microcentrifuge tube 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