3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

ANTI-NITRIC OXIDE SYNTHASE, ENDOTHELIAL (eNOS) (596-609)
Developed in Rabbit
IgG Fraction of Antiserum

Product No. **N 2643**

Product Description

Anti-Endothelial Nitric Oxide Synthase (Anti-eNOS) is developed in rabbit using repeated injections of the synthetic peptide SGPYNSSPRPEQHK corresponding to eNOS of bovine endothelial origin (amino acids 596-609) conjugated to KLH as the immunogen. This sequence is identical in human eNOS (amino acids 594-607). Whole antiserum is fractionated and then further purified by ion-exchange chromatography to provide the IgG fraction of antiserum which is essentially free of other rabbit serum proteins.

Anti-eNOS is specific for nitric oxide synthase (NOS), derived from endothelial tissue, but does not recognize NOS derived from brain (bNOS) and macrophages (iNOS). By immunoblotting of endothelial cells extract, the staining of eNOS band is specifically inhibited with eNOS peptide (bovine, amino acids 596-609).

NOS are enzymes involved in the synthesis of nitric oxide (NO), a messenger molecule generated under physiological conditions by virtually all mammalian cells.[1,2,3] NO is formed from oxidation of L-arginine by NOS, releasing NO and L-citrulline. NO mediates diverse functions including vasodilation, synaptic neurotransmission and cytotoxicity. NO participates in signal transduction pathways by activating soluble guanylate cyclase. In addition, NO has been implicated as a pathogenic mediator in a variety of conditions, such as central nervous system (CNS) disease states, including focal cerebral ischemia, and the animal model of multiple sclerosis (MS), experimental allergic encephalomyelitis.[4] At least three distinct isoforms of NOS have been described which are the products of different mammalian genes. The NOS isoform found in neurons is a 150-160 kDa protein. It is also termed brain NOS (bNOS), NOS1, neuronal NOS (nNOS), neuronal constitutive NOS or Ca^{2+}-regulated NOS (cNOS, ncNOS). The NOS isoform characterized in macrophages is a 130 kDa protein, also known as macrophage NOS (mNOS), NOS2 or inducible NOS (iNOS). The NOS isoform found in endothelial cells is a 135 kDa protein, also called endothelial NOS, NOS 3 (eNOS, or ecNOS). Neuronal and endothelial NOS are constitutively expressed and are dependent on Ca^{2+}/ calmodulin for NO production. Inducible NOS is Ca^{2+} independent and is expressed in cytokine-activated macrophages and microglial cells in response to bacterial lipopolysaccharide (LPS) stimulation. NOS are complex enzymes forming homodimers under native conditions, and require three cosubstrates L-arginine, NADPH and O_2 and five cofactors FAD, FMN, tetrahydrobiopterin (BH4), heme and calmodulin. The C-terminal half of NOS possesses a high level of homology with NADPH-cytochrome P-450 reductase, where the predicted sites for binding NADPH and flavins are also located. However, the predicted heme and calmodulin binding sites of NOS are located within its N-terminal half. NOS isoforms may subserve a multiplicity of disparate biological functions.[1,5,6] For instance, bNOS is present also in skeletal muscle, where it is complexed with dystrophin and is absent in Duchenne's muscular dystrophy (DMD).[6] iNOS not only occurs in macrophages but in several other cell types including hepatocytes, chondrocytes, endothelial cells and fibroblasts. eNOS is not restricted to the endothelium of blood vessels but exists in the epithelium of several tissues, including the bronchial tree. It is also localized in neurons in the brain, especially the pyramidal cells of the hippocampus, where it may function in long-term potentiation. NOS seems to be a highly conserved enzyme between the various types. Human bNOS and eNOS share 52% amino acid identity, and rat and human bNOS share

93% amino acid identity. The production of isoform-specific antibodies to NOS[7] allows investigators to identify which isoform(s) is present in a specific cell or tissue. These antibodies are valuable for elucidating the expression of these isoforms in a variety cell types and tissues.

Working Dilutions
1. A minimum working dilution of 1:10,000 is determined by immunoblotting using a whole extract of bovine lung endothelial cells.
2. A minimum working dilution of 1:100 is determined by immunohistochemical staining of eNOS in acetone-fixed frozen tissue sections of mouse heart.

In order to obtain best results, it is recommended that each individual user determine their optimum working dilutions by titration assay.

Total Protein: is determined by absorbance at 280 nm ($E_{280}^{1\%}$ = 14.0).

Uses
Anti-eNOS may be used for the detection and localization of eNOS by immunoblotting using a whole extract of bovine lung endothelial cells and by immunohistochemical staining of eNOS in frozen tissue sections of mouse heart.

Reagents
The product is provided as a solution in 0.01 M phosphate buffered saline, pH 7.4, containing 15 mM sodium azide as a preservative.

Precautions and Disclaimer
Due to the sodium azide content a material safety data sheet (MSDS) for this product has been sent to the attention of the safety officer of your institution. Consult the MSDS for information regarding hazards and safe handling practices.

Storage
For continuous use, store at 2-8 °C for up to one month. For extended storage, solution may be frozen in working aliquots. Repeated freezing and thawing is not recommended. Storage in "frost-free" freezers is not recommended. If slight turbidity occurs upon prolonged storage, clarify by centrifugation before use.

Immunoblotting Procedure of Whole Cell Extract and Rat Brain Extract

Reagents and Equipment
1. Bovine endothelial lung cells culture.
2. Rats (Sprague-Dawley).
3. Macrophage cell line RAW264.7 (mouse), activated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS), (iNOS control).
4. Lipopolysaccharide (LPS), from *E.coli* (Product No. L 2654).
5. Interferon-γ (IFN-γ), mouse, recombinant (Product No. I 4777).
6. Phenylmethylsulfonylfluoride (PMSF) (Product No. P7626), 0.5M in EtOH.
7. Pepstatin A (Product No. P 4265), 2 mg/ml in DMSO.
8. Leupeptin (Product No. L 2884).
9. Aprotinin (Product No. A 4529).
10. DTT (Product No. D 9760).
11. Homogenization Buffer (Rat brain), Ice Cold: 20 mM Tris-HCl buffer pH 7.5 containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, and protease inhibitors: 2 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin A and 2 mM dithiothreitol (DTT).

Note: Add all protease inhibitors freshly before tissue homogenization.

12. Mechanical homogenizer.
13. Refrigerated High-Speed Centrifuge.
14. Protein assay kit .
15. Laemmli sample (3×) buffer containing 2-mer-captoethanol.
16. Laemmli sample (1×) buffer containing 2-mercaptoethanol.

Procedure
Preparation of Whole Cell Culture Extracts
1. Grow bovine endothelial cells to confluence in 10 cm plate containing 10% FCS in DMEM.
2. Grow RAW 264.7 macrophage cells to confluence in 10 cm plate containing 10% FCS in DMEM.
3. Activate RAW 264.7 cells by incubation with LPS (1 μg/ml) and IFN-γ (10 ng/ml) overnight at 37 °C.
4. Remove medium from culture dishes.
5. Rinse plates with ice PBS pH 7.4 (2 x 10 ml).
6. Add 1 ml/plate of (1x) boiling sample buffer and scrape cells.
7. Boil sample for 5 min. at 95 °C.
8. Aliquot samples of cells extracts and store at –70 °C.

Preparation of Rat Brain Extract

Note: All procedure steps are carried out on ice, unless noted otherwise. Rapidly dissect out whole brains (5 g) from Sprague-Dawley rats (4-5 rats, 250-300 g) and collect into ice cold homogenization buffer.

1. Homogenize tissue in 5 × volumes of ice cold homogenization buffer (w/v) using mechanical homogenizer at maximum speed (3 x 10 sec pulses with 1 min rest in between).
2. Homogenize briefly on ice with fine glass homogenizer.
3. Centrifuge homogenate at 1200 x g for 10 min at 4 °C. Collect supernatant.
4. Centrifuge supernatant at 15,000 x g for 20 min at 2-8 °C. Collect clear supernatant.
5. Remove 0.5 ml aliquot of supernatant for protein determination using the Bradford method with BSA as standard.
6. Add to supernatant 3× sample buffer to final dilution of 1× sample buffer.
7. Boil sample for 5 min. at 95 °C.
8. Aliquot sample of rat brain extract and store at –70 °C.

Immunoblotting Reagents and Equipment

1. Bovine endothelial cell extract.
2. Rat brain extract (bNOS control).
3. Activated-RAW 264.7 macrophage cells extract (iNOS control).
4. 7% polyacrylamide slab minigel with 5% stacking gel (80 x 80 x 1.5 mm).
5. Nitrocellulose membrane (0.45 mm).
6. Color Marker High Range (Product No. C 3312).
7. Blocking Buffer: 10% dry milk (w/v) in 10 mM phosphate buffered saline (PBS), pH 7.4.
8. Dilution Buffer: 1% BSA in PBS pH 7.4 containing 0.05% Tween-20.
9. Washing Buffer: PBS pH 7.4 containing 0.05% Tween-20.
10. NOS endothelial peptide (amino acids 596-609 with N-terminally added lysine). Dissolve in double distilled water at 0.5 mg/ml. Store aliquots at –20 °C.
11. Primary antibody: Anti-eNOS (596-609) (Product No. N 2643) at appropriate dilution in dilution buffer.
12. Secondary Antibody: Alkaline Phosphatase-Anti-Rabbit IgG, (Product No. A 9919) at appropriate dilution in dilution buffer.
13. Substrate: BCIP/NBT Tablets (Product No. B 5655).
14. Electrophoresis and transfer apparatus.

Procedure

Note: In order to obtain best results in different preparations it is recommended to optimize procedure conditions (antibody dilutions, incubation times, blocking conditions etc.), for a specific application.

1. Resolve whole cell extracts (250 μl/slab) and rat brain extract (250 μg/slab), on precast 7% polyacrylamide minigel.
2. Run SDS-PAGE at room temperature.
3. Perform transfer for 1 hour at room temperature to nitrocellulose membrane.
4. Block nitrocellulose membrane in blocking buffer for at least 1 hour at room temperature.
5. Incubate membrane with primary antibody dilutions for 2 hours at room temperature[a].
6. Wash membrane with washing buffer 4 x 5 min.
7. Incubate membrane with secondary antibody at recommended dilution in diluion buffer for 1 hour at room temperature.
8. Wash membrane with washing buffer 4 x 5 min. Wash 1 x 5 min. in deionized water.
9. Dissolve BCIP/NBT substrate tablet each in 10 ml deionized water. Incubate membrane with substrate solution.
10. Wash membrane thoroughly with deionized water.
11. Air-dry blots on filter paper.

(a) For specific inhibition of NOS endothelial (eNOS) band (135 kDa band) it is recommended to incubate prediluted antibody with eNOS peptide (596-609), 10 μg/ml (final concentration) for 2 hours at room temperature or overnight at 2-8 °C.

References

1. Dinerman, J., et al., Proc. Natl. Acad. Sci. USA, **91**, 4214 (1994).
2. Bredt, D., and Snyder, S., Proc. Natl. Acad. Sci. USA, **87**, 682 (1990).
3. Stuehr, J., and Griffiths, O., In: Advances in Enzymology and Related Areas of Molecular Biology, Vol. 65, Meister, A., (ed.), John Wiley & Sons, New York pp. 287-346 (1992).
4. Bagasra, O., et al., Proc. Natl. Acad. Sci. USA., **92**, 12041 (1995).
5. Snyder, S., Nature, 372, 504 (1994).
6. Snyder, S., Nature, 377, 196 (1995).
7. Pollock, J., et al., Histochemical J., **27**, 738 (1995).

02/02

Sigma brand products are sold through Sigma-Aldrich, Inc.
Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.