3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Anti-Nitric Oxide Synthase, Endothelial (1185-1205)
produced in rabbit, IgG fraction of antiserum

Catalog Number **N3893**

Synonym: Anti-eNOS

Product Description
Anti-Nitric Oxide Synthase, Endothelial is produced in rabbit using repeated injections of the synthetic peptide K-RHLRGAVPWAFDPPGPDTPGP corresponding to eNOS of bovine endothelial origin (amino acids 1185-1205 with N-terminally added lysine) conjugated to KLH as the immunogen. This sequence is highly conserved in human eNOS. Whole antiserum is purified to provide an IgG fraction of antiserum proteins.

Anti-Endothelial Nitric Oxide Synthase is specific for nitric oxide synthase (NOS) derived from endothelial tissue (eNOS, 135 kDa), and does not recognize NOS derived from brain (bNOS) and macrophages (iNOS).

Nitric Oxide Synthases (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 (macNOS), NOS2 or inducible NOS (iNOS). The NOS isoform found in endothelial cells is a 135 kDa protein, also called endothelial NOS or 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.

Anti-Endothelial Nitric Oxide Synthase (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.

Reagent
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
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.

Storage/Stability
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, or storage in "frost-free" freezers, is not recommended. If slight turbidity occurs upon prolonged storage, clarify by centrifugation before use.

Procedure
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* , Catalog Number L2654.
5. Interferon-γ (IFN-γ), mouse, recombinant, Catalog Number I4777.
6. Phenylmethylsulfonylfluoride (PMSF), Catalog Number P7626, 0.5M in ethanol.
7. Pepstatin A, Catalog Number P4265, 2 mg/ml in DMSO.
8. Leupeptin hemisulfate salt, Catalog Number L2884.
9. Aprotinin, Catalog Number A4529.
10. DTT, Catalog Number D9760.
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 (3x) buffer containing 2-mercaptoethanol.
16. Laemmli sample (1x) buffer containing 2-mercaptoethanol.

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 cold 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.
1. Rapidly dissect out whole brains (5 g) from Sprague-Dawley rats (4-5 rats, 250-300 g) and collect into ice cold homogenization buffer.
2. Homogenize tissue in 5x 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).
3. Homogenize briefly on ice with fine glass homogenizer.
4. Centrifuge homogenate at 1200 x *g* for 10 min at 4 °C. Collect supernatant.
5. Centrifuge supernatant at 15,000 x *g* for 20 min at 2-8 °C. Collect clear supernatant.
6. Remove 0.5 ml aliquot of supernatant for protein determination using the Bradford method with BSA as standard.
7. Add to supernatant 3x sample buffer to final dilution of 1x sample buffer.
8. Boil sample for 5 min. at 95 °C.
9. 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 μm).
6. Prestained HMW markers, Catalog Number C3312.
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 1185-1205 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 (1185-1205), Catalog Number N3893, at appropriate dilution in dilution buffer.
12. Secondary Antibody: AntiRabbit IgG- Alkaline Phosphatase, Catalog Number A9919, at appropriate dilution in dilution buffer.
13. Substrate: BCIP/NBT Tablets, Catalog Number B5655.
14. Electrophoresis and transfer apparatus.

Immunoblotting 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 dilution 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 (1185-1205), 10 µg/ml (final concentration) for 2 hours at room temperature or overnight at 2-8 °C.

Product Profile

Immunoblotting: a minimum working dilution of 1:10,000 is determined using a whole extract of bovine lung endothelial cells.

Immunohistochemical staining: a minimum working dilution of 1:100 is determined using acetone-fixed frozen tissue sections of mouse heart.

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

References

1. Dinerman, J. L., et al., *Proc. Natl. Acad. Sci. USA*, **91**, 4214 (1994).
2. Bredt, D. S., and Snyder, S. H., *Proc. Natl. Acad. Sci. USA*, **87**, 682 (1990).
3. Stuehr, J. D., and Griffith, 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. H., *Nature*, **372**, 504 (1994).
6. Snyder, S. H., *Nature*, **377**, 196 (1995).
7. Pollock, J. S., et al., *Histochem. J.*, **27**, 738 (1995).

MG,KAA,PHC 01/10-1