

Product Information

Anti-Nitric Oxide Synthase, Inducible

produced in rabbit, IgG fraction of antiserum

Catalog Number **N7782**

Synonyms: Anti-iNOS, Anti-macNOS

Product Description

Anti-Nitric Oxide Synthase, Inducible is produced in rabbit using as immunogen the synthetic peptide FSYGAKKGSALPEPKATRL corresponding to the C-terminus of iNOS of mouse macrophage origin (amino acids 1126-1144) conjugated to KLH. This sequence is highly conserved in rat macNOS and diverges from human macNOS. Whole antiserum is purified to provide an IgG fraction of antiserum.

Anti-Nitric Oxide Synthase, Inducible is specific for nitric oxide synthase (NOS), derived from activated mouse macrophage RAW 264.7 cells (iNOS, 130 kDa), but does not recognize NOS derived from brain tissue (bNOS) and endothelial cells (eNOS). By immunoblotting of an activated mouse macrophage RAW 264.7 cells extract, the staining of the iNOS band is specifically inhibited by the immunizing peptide.

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, the animal model of multiple sclerosis (MS), and 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 Ca²⁺-regulated NOS (cNOS, ncNOS). The NOS isoform characterized in macrophages is a 130 kDa protein, also known as macrophage NOS (macNOS; mNOS), 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 Ca²⁺/calmodulin for NO production. Inducible NOS is Ca²⁺ 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 O₂ 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 serve 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 NOS⁷ 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.

Reagent

Supplied 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

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.

Product Profile

Immunoblotting: a minimum working dilution of 1:10,000 is determined using a whole extract of mouse macrophage RAW 264.7 cells pre-activated with lipopolysaccharides (LPS) and interferon- γ (INF- γ).

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

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

Immunoblotting Procedure of Whole Cell Extract and Rat Brain Extract

Reagents and Equipment

1. Macrophage cell line RAW 264.7 (mouse), activated with interferon- γ (INF- γ) and lipopolysaccharide (LPS)
2. Rats (Sprague-Dawley)
3. Bovine endothelial lung cells culture (eNOS control)
4. Lipopolysaccharide (LPS), from *E. coli*, Catalog Number L2654
5. Interferon- γ (INF- γ), mouse, recombinant, Catalog Number I4777
6. Phenylmethanesulfonyl fluoride (PMSF), Catalog Number P7626, 0.5M in ethanol
7. Pepstatin A, Catalog Number P4265, 2 mg/ml in DMSO
8. Leupeptin, 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 mg/ml leupeptin, 25 mg/ml aprotinin, 10 mg/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 Extract

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 mg/ml) and INF- 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 minutes 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 second pulses with 1 minute rest in between).
3. Homogenize briefly on ice with fine glass homogenizer.
4. Centrifuge homogenate at 1200 x g for 10 minutes at 4 °C. Collect supernatant.
5. Centrifuge supernatant at 15,000 x g for 20 minutes 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 minutes at 95 °C.
9. Aliquot sample of rat brain extract and store at -70 °C.

Immunoblotting Reagents and Equipment

1. Bovine endothelial cell extract (eNOS control)
2. Rat brain extract
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 Markers, Catalog Number C1992 or C4861.
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. Inducible NOS peptide (mouse, amino acids 1126-1144). Dissolve in double distilled water at 0.5 mg/ml. Store aliquots at -20 °C
11. Primary antibody: Anti-iNOS, Catalog Number N7782, at appropriate dilution in dilution buffer; Secondary Antibody: Anti-Rabbit IgG (whole molecule)-Alkaline Phosphatase, Catalog Number A9919, at appropriate dilution in dilution buffer
12. Substrate: BCIP/NBT Tablets, Catalog Number B5655
13. 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 diluted primary antibody for 2 hours at room temperature.
6. Wash membrane with washing buffer 4 x 5 minutes.

7. Incubate membrane with diluted secondary antibody for 1 hour at room temperature.
8. Wash membrane with washing buffer 4 x 5 minutes. 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.

Note: For specific inhibition of NOS inducible (iNOS) band (130 kDa band) it is recommended to incubate prediluted antibody with iNOS peptide (1126-1144), (concentration 10 mg/ml), for 2 hours at room temperature or overnight at 2-8 °C.

References

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