DATA SHEET

Rat endothelial Nitric Oxide Synthase(eNOS)ELISA Kit Catalog No. CSB-E08323r

(96 T)

This immunoassay kit allows for the in vitro quantitative determination of **rat eNOS** concentrations in **serum, plasma and other biological fluids**._ **Expiration date six** months from the date of manufacture

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

Nitric oxide (NO) is produced by a group of enzymes called nitric oxide synthases (NOS). These enzymes catalyze the production of NO and L-citrulline from L-arginine, O2, and NADPH derived electrons. Mammalian systems contain three well-characterized isoforms of the enzyme: neuronal NOS (nNOS, also called NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The cell types that express eNOS include vascular endothelial cells, cardiomyocytes and others. In blood vessels, NO produced by the eNOS in endothelial cells functions as a vasodilator thereby regulating blood flow and pressure. Mutant eNOS knockout mice have blood pressure that is 30% higher than wild-type littermates. Within cardiomyocytes, eNOS affects Ca2+ currents and contractility. Expression of eNOS is usually reported to be constitutive, though modest degrees of regulation occur in response to factors such as shear stress, exercise, chronic hypoxia, and heart failure. The unique N-terminal sequence of eNOS is about 70 residues long and functions to localize the enzyme to membranes. Upon myristoylation at one site and palmitoylation at two other sites within this segment, the enzyme is exclusively membrane-bound. Palmitoylation is a reversible process that is influenced by some agonists and is essential for membrane localization. Within the membrane, eNOS is targeted to the caveolae, small invaginations characterized by the presence of proteins called caveolins. These regions serve as sites for the sequestration of signaling molecules such as receptors, G proteins and protein kinases. The oxygenase domain of eNOS contains a motif that binds to caveolin-1, and calmodulin is believed to competitively displace caveolin resulting in eNOS activation. Bound calmodulin is required for activity of eNOS, and this binding occurs in response to transient increases in intracellular Ca2+. Thus, eNOS occurs at sites of signal transduction and produces short pulses of NO in response to agonists that elicit Ca2+ transients. Physiological concentrations of eNOS-derived NO are in the picomolar range . Within the cardiovascular system, eNOS generally has protective effects. Studies with nNOS and eNOS knockout mice clearly indicate that eNOS plays a protective role in cerebral is chemia by preserving cerebral blood flow. During inflammation and atherosclerosis, low concentrations of NO prevent apoptotic death of endothelial cells and preserve the integrity of the endothelial cell monolayer. NO also acts as an inhibitor of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation.

PRINCIPLE OF THE ASSAY

The microtiter plate provided in this kit has been pre-coated with an antibody specific to eNOS. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for eNOS and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain eNOS, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of eNOS in the samples is then determined by comparing the O.D. of the samples to the standard curve.

DETECTION RANGE

0.31 IU/ml-20 IU/ml. The standard curve concentrations used for the ELISA's were 20 IU/ml, 10 IU/ml, 5 IU/ml, 2.5 IU/ml, 1.25 IU/ml, 0.63 IU/ml, 0.31 IU/ml.

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SPECIFICITY
This assay recognizes recombinant and natural rat eNOS. No significant cross-reactivity or interference was observed.

SENSITIVITY
The minimum detectable dose of rat eNOS is typically less than 0.08 IU/ml.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

MATERIALS PROVIDED

Reagent	**Quantity**
Assay plate	1
Standard	2
Sample Diluent	1 x 20 ml
Biotin-antibody Diluent	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Biotin-antibody	1 x 120µl
HRP-avidin	1 x 120µl
Wash Buffer (25×concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml

STORAGE
1. Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag. The test kit may be used throughout the expiration date of the kit, provided it is stored as prescribed above. Refer to the package label for the expiration date.
2. Opened test plate should be stored at 2-8°C in the aluminum foil bag with desiccants to minimize exposure to damp air. The kits will remain stable until the expiring date shown, provided it is stored as prescribed above.
3. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

REAGENT PREPARATION
Bring all reagents to room temperature before use.
1. **Wash Buffer** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. **Standard** Centrifuge the standard vial at 6000-10000rpm for 30s. Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. This reconstitution produces a stock solution of 20 8 IU/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (20 IU/ml). The **Sample Diluent** serves as the zero standard (0 IU/ml). Prepare fresh for each assay. Use within 4 hours and discard after use.
3. **Biotin-antibody** Centrifuge the vial before opening. Dilute to the working concentration using **Biotin-antibody Diluent**(1:100), respectively.
4. **HRP-avidin** Centrifuge the vial before opening. Dilute to the working concentration using **HRP-avidin Diluent**(1:100), respectively.
Precaution: The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

OTHER SUPPLIES REQUIRED
_ Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
_ Pipettes and pipette tips.
_ Deionized or distilled water.
_ Squirt bottle, manifold dispenser, or automated microplate washer.
_ An incubator which can provide stable incubation conditions up to 37°C±0.5°C.

SAMPLE COLLECTION AND STORAGE
_ **Serum** Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 g. Remove serum and assay immediately or aliquot and store samples at -20°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.
_ **Plasma** Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.
Note: Grossly hemolyzed samples are not suitable for use in this assay.

ASSAY PROCEDURE
Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate. All the reagents should be added directly to the liquid level in the well. The pipette should avoid contacting the inner wall of the well.

1. Add 100µl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37°C. **Biotin-antibody** working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash: Fill each well with Wash Buffer (200µl) and let it stand for 2 minutes, then remove the liquid by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel. Complete removal of liquid at each step is essential to good performance.
5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
6. Repeat the aspiration and wash five times as step 4.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 10-30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well when the first four wells containing the highest concentration of standards develop obvious blue color. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

Using the professional soft "Curve Exert 1.3" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the eNOS concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

_ The kit should not be used beyond the expiration date on the kit label.
_ Do not mix or substitute reagents with those from other lots or sources.
_ It is important that the Standard Diluent selected for the standard curve be consistent with the samples being assayed.
_ If samples generate values higher than the highest standard, dilute the samples with the appropriate Standard Diluent and repeat the assay.
_ Any variation in Standard Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
_ This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

_ Centrifuge vials before opening to collect contents.
_ When mixing or reconstituting protein solutions, always avoid foaming.
_ To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
_ When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
_ To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
_ Substrate Solution should remain colorless or light blue until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless or light blue to gradations of blue.
_ Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.