Quantikine®

Human eNOS Immunoassay

Catalog Number DEN00

For the quantitative determination of human endothelial nitric oxide synthase (eNOS) concentrations from endothelial cells.

This package insert must be read in its entirety before using this product.

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

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INTRODUCTION

Nitric oxide (NO) is produced by a group of enzymes called nitric oxide synthases (NOS) (1 - 5). These enzymes catalyze the production of NO and L-citrulline from L-arginine, O_2, 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 names reflect characteristics of the activity or the original tissues in which the enzymes were first described, but it is now known that each of these isoforms is expressed in a variety of tissues and cell types (6, 7).

The cell types that express eNOS include vascular endothelial cells, cardiomyocytes and others (5, 6). In blood vessels, NO produced by the eNOS in endothelial cells functions as a vasodilator thereby regulating blood flow and pressure (8, 9). Mutant eNOS knockout mice have blood pressure that is 30% higher than wild-type littermates (8). Within cardiomyocytes, eNOS affects Ca^{2+} currents and contractility (10). 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 (11, 12).

The unique N-terminal sequence of eNOS is about 70 residues long and functions to localize the enzyme to membranes (5, 13). Upon myristoylation at one site and palmitoylation at two other sites within this segment, the enzyme is exclusively membrane-bound (5). Palmitoylation is a reversible process that is influenced by some agonists and is essential for membrane localization (14). 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 (11). Bound calmodulin is required for activity of eNOS, and this binding occurs in response to transient increases in intracellular Ca^{2+} (1, 2). Thus, eNOS occurs at sites of signal transduction and produces short pulses of NO in response to agonists that elicit Ca^{2+} transients. Physiological concentrations of eNOS-derived NO are in the picomolar range (15).

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 ischemia by preserving cerebral blood flow (8). During inflammation and atherosclerosis, low concentrations of NO prevent apoptotic death of endothelial cells and preserve the integrity of the endothelial cell monolayer (16, 17). NO also acts as an inhibitor of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation (9, 18, 19). Therefore, eNOS-related pathologies usually result from impaired NO production or signaling. Altered NO production and/or bioavailablility have been linked to such diverse disorders as hypertension, hypercholesterolemia, diabetes, and heart failure (12).

The eNOS Quantikine Immunoassay kit is a 4.5 hour solid phase ELISA designed to measure eNOS from endothelial cells. It contains *E. coli*-expressed, recombinant human eNOS and antibodies raised against the recombinant enzyme. It has been shown to accurately quantitate recombinant human eNOS. Results obtained using natural human eNOS showed linear curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that the Quantikine eNOS Immunoassay can be used to determine relative mass values for natural eNOS.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for eNOS has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any eNOS present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for eNOS is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of eNOS bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- If samples fall outside the dynamic range of the assay, dilute the samples with Calibrator 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.
- Other enzymes or proteins present in biological samples do not necessarily interfere with the measurement of synthases in samples. Until all proteins have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

REAGENTS

eNOS Microplate (Part 890702) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against eNOS.

eNOS Conjugate (Part 890703) - 21 mL of polyclonal antibody against eNOS conjugated to horseradish peroxidase, with preservative.

eNOS Standard (Part 890704) - 40 ng of recombinant human eNOS in a buffered protein base with preservative, lyophilized.

Assay Diluent RD1W (Part 895117) - 11 mL of a buffered protein base with preservative.

Calibrator Diluent RD5K (Part 895119) - 21 mL of a buffered protein base with preservative.

Cell Lysis Buffer (Part 890713) - 2 vials (21 mL/vial) of buffer with preservative.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="10">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="9">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1W</td></tr>
<tr><td>Calibrator Diluent RD5K</td></tr>
<tr><td>Cell Lysis Buffer</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

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.
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Saline solution for cell lysis.
- Centrifuge

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

CELL LYSIS PROCEDURE

Keep Cell Lysis Buffer at 2 - 8° C.

1. Centrifuge cells at 300 x g for 5 minutes. Pour off supernate.
2. Wash cells 2 times in saline solution. After each wash, centrifuge at 300 x g for 5 minutes. Pour off supernate.
3. Lyse cells at 2 - 8° C with Cell Lysis Buffer (1 mL of buffer per 1 x 10^6 cells).

   Note: *Lysis is typically immediate, however, each investigator should determine this empirically for their cell type and conditions.*
4. Centrifuge cells at 300 x g for 5 minutes.
5. Remove supernate and assay immediately or aliquot and store at ≤ -20° C for up to 24 hours.

REAGENT PREPARATION

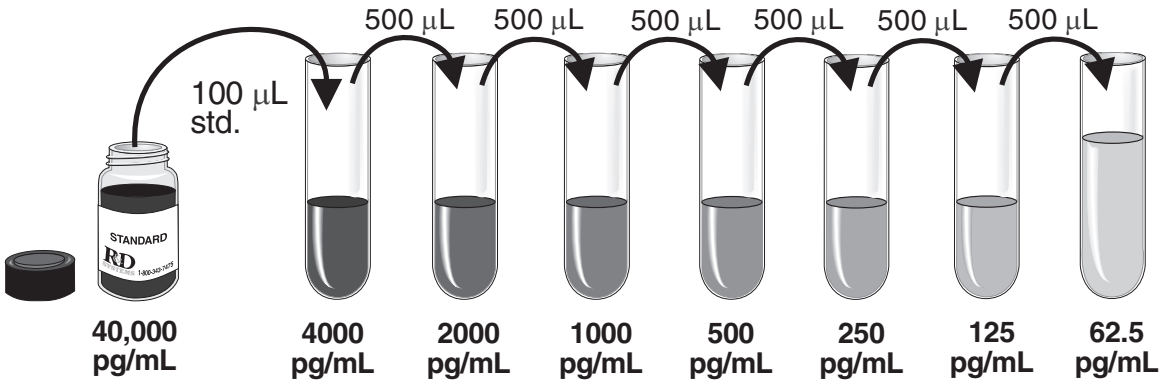
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm 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.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

eNOS Standard - Reconstitute the eNOS Standard with 1 mL of deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5K to the 4000 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5K into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 100 µL of Standard or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of eNOS Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop**. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Cells require lysis. See Cell Lysis Procedure.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 100 µL Assay Diluent RD1W to each well.

3. Add 100 µL Standard or sample* to each well. Incubate 2 hrs. RT on the shaker.

4. Aspirate and wash 3 times.

5. Add 200 µL Conjugate to each well. Incubate 2 hrs. RT on the shaker.

6. Aspirate and wash 3 times.

7. Add 200 µL Substrate Solution to each well. Incubate 30 min. RT **on the benchtop**. **Protect from light.**

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min. λ correction 540 or 570 nm

*Cells require lysis

CALCULATION OF RESULTS

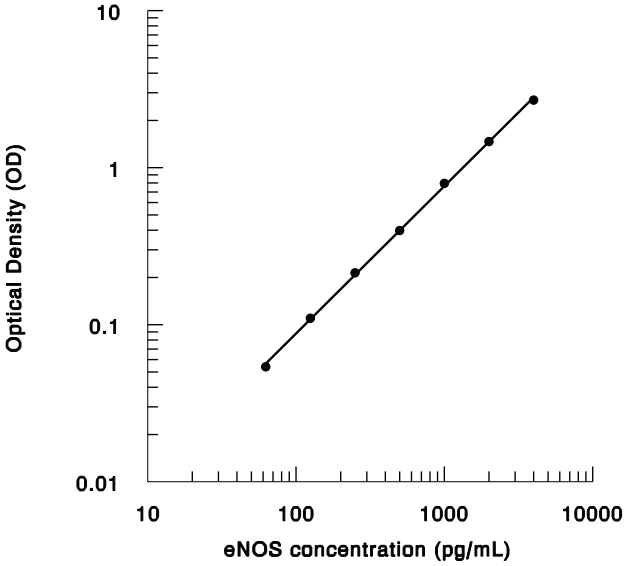
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the eNOS concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding eNOS concentration. If samples have been diluted, the concentration read from the standard curve must multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	O.D.	Average	Corrected
0	0.040 0.040	0.040	-
62.5	0.095 0.094	0.094	0.054
125	0.147 0.152	0.150	0.110
250	0.248 0.261	0.254	0.214
500	0.430 0.447	0.438	0.398
1000	0.821 0.849	0.835	0.795
2000	1.498 1.514	1.506	1.466
4000	2.757 2.713	2.735	2.695

TECHNICAL HINTS

- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	127	619	1280	129	621	1275
Standard deviation	6.2	22.9	51.8	9.6	27.5	45.7
CV (%)	4.9	3.7	4.0	7.4	4.4	3.6

LINEARITY

To assess the linearity of the assay, five cell lysate samples containing high concentrations of eNOS were diluted with Calibrator Diluent RD5K to produce samples with values within the dynamic range of the assay.

1:2	Average % of Expected	103
	Range (%)	100-108
1:4	Average % of Expected	111
	Range (%)	105-119
1:8	Average % of Expected	97
	Range (%)	94-101

SENSITIVITY

The minimum detectable dose of eNOS is typically less than 25 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Human endothelial cells (1 x 10^6 cells/mL) were cultured in EGM (endothelial growth media) supplemented with 2% fetal bovine serum, 10 ng/mL hEGF, 1.0 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphoteracin B, and 3 mg/mL BBE (bovine brain extract). Cells were lysed and assayed for levels of natural human eNOS.

Cell Type	(pg/mL)
HUVEC	5137
HMVEC	1396

CALIBRATION

This immunoassay is calibrated against highly purified, *E. coli*-expressed, recombinant human eNOS produced at R&D Systems.

SPECIFICITY

This assay recognizes recombinant and natural human eNOS. Recombinant human eNOS was prepared at 50 ng/mL in Calibrator Diluent RD5K and in a mid-level control. No significant cross-reactivity or interference was observed.

REFERENCES

1. Gorren, A.C.F. and B. Mayer (1998) Biochem. (Moscow) **63**:734.
2. Stuehr, D.J. (1999) Biochim. Biophys. Acta **1411**:217.
3. Ludwig, M.L. and M.A. Marletta (1999) Structure **7**:R73.
4. Werner, E.R. *et al*. (1998) Proc. Soc. Exptl. Biol. Med. **219**:171.
5. Hemmens, B. and B. Mayer (1998) Meth. Mol. Biol. **100**:1.
6. Taylor, B.S. *et al*. (1998) Biochem. (Moscow) **63**:905.
7. Titheradge, M.A. (1999) Biochim. Biophys. Acta **1411**:437.
8. Huang, P.L. and E.H. Lo (1998) Prog. Brain. Res. **118**:13.
9. Moncada, S. (1999) J. Roy. Soc. Med. **92**:164.
10. Drexler, H. (1999) Circulation **99**:2972.
11. Kone, B.C. (1999) Sem. Nephrol. **19**:230.
12. Harrison, D.G. (1997) J. Clin. Invest. **100**:2153.
13. Liu, J. *et al*. (1997) J. Cell Biol. **137**:1525.
14. Michel, T. and O. Feron (1997) J. Clin. Invest. **100**:2146.
15. Anggard, E. (1994) Lancet **343**:1199.
16. Haendeler, J. *et al.* (1999) Vitamins Hormones **57**:49.
17. Lopez-Farre, A. *et al*. (1998) Int. J. Biochem. Cell Biol. **30**:1095.
18. Wever, R.M.F. *et al*. (1998) Circulation **97**:108.
19. Lloyd-Jones, D.M. and K.D. Bloch (1996) Annu. Rev. Med. **47**:365.