

Quantikine[®]

Human iNOS Immunoassay

Catalog Number DNS00

For the quantitative determination of human Inducible Nitric Oxide Synthase (iNOS) concentrations in cell lysates.

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 a short-lived molecule ($t_{1/2}$ = seconds) capable of diffusing across membranes and reacting with a variety of targets. A reaction with O_2 in aqueous solutions produces the relatively unreactive nitrate (NO_3^-) and nitrite (NO_2^-) ions as products (1 - 4). In the presence of superoxide (O_2^-), however, NO reacts extremely rapidly to produce the very reactive and toxic peroxynitrite ($ONOO^-$) which subsequently decomposes into additional highly reactive intermediates. NO ultimately exerts its biological effects by reacting either directly or through other reactive nitrogen intermediates with a variety of targets such as heme groups, Fe-S or Zn-S clusters, sulfhydryl groups or various other chemical substrates (2 - 8). This diversity of potential targets is reflected in the large number of different systems that utilize NO as a mediator and provides ample opportunity for abnormal regulation and development of pathological effects.

NO is produced by a group of enzymes called nitric oxide synthases (NOS) (9 - 13). 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 (14, 15). The three main isoforms share structural similarities and have nearly identical catalytic mechanisms (9 - 13). They all require a number of cofactors and prosthetic groups for activity including FAD, FMN, heme, calmodulin, and tetrahydrobiopterin. The homodimeric form is required for NO production, and the subunits have molecular masses of approximately 160 kDa (nNOS), 135 kDa (eNOS), and 130 kDa (iNOS). Three distinct domains are necessary for catalytic activity. Starting at the C-terminus there is a reductase domain, a calmodulin-binding domain, and an oxygenase domain. The reductase domain contains the FAD and FMN moieties and shares extensive amino acid (aa) homology with cytochrome P-450 reductase. This domain transfers electrons from NADPH to the oxygenase domain. The oxygenase domain actually catalyzes the conversion of arginine into citrulline and NO and contains the binding sites for heme, tetrahydrobiopterin, and arginine. Calmodulin binding is required for activity of all of the NOS isoforms.

Isolation of iNOS was first reported in macrophages where its activity was found to be inducible in response to stimuli such as proinflammatory cytokines or endotoxin. Expression of iNOS has now been reported in a large number of cell types, and in most circumstances, the enzyme is inducible (14, 15). The iNOS gene is under transcriptional control although activity is also influenced by a variety of other control mechanisms that affect mRNA stability, translation and degradation of the protein, and availability of substrate and cofactors (9, 10, 13, 14). This enzyme is found in the cytoplasm.

All NOS isoforms require bound calmodulin for activity; however, iNOS is unusual in that it binds calmodulin tightly even at very low Ca^{2+} concentrations (9, 10). iNOS activity is not responsive to changes in intracellular Ca^{2+} levels and thus this isoform is capable of a high output and long-lasting release of NO far exceeding that of the other isoforms. iNOS produces physiological concentrations of NO in the nanomolar range, whereas the Ca^{2+} -dependent isoforms produce picomolar concentrations of NO (16).

One of the best described functions of iNOS is its role in the macrophage-mediated response to infectious agents (17, 18). While there is evidence that NO plays a regulatory role in the immune system signaling cascade, macrophage-derived NO has been more clearly defined as an effector molecule that kills or inactivates target cells. The cytotoxic effect of NO has also been demonstrated against cancer cells as well as the macrophages that produce NO (19). These mammalian cell targets are killed by an apoptotic mechanism, but the exact signaling pathway leading to cell death is not understood.

Overproduction of NO by iNOS is implicated in a number of pathologies. Septic shock is caused by the overactivation of macrophages in response to bacterial infections in blood (15). The resulting overproduction of NO leads to a severe drop in blood pressure and subsequent dysfunction of multiple organs. NO generated by iNOS is present in a number of inflammatory conditions including rheumatoid arthritis, Crohn's disease and asthma (20).

The Quantikine Human iNOS Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human iNOS in cell lysates. It contains *E. coli*-expressed recombinant human iNOS and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human iNOS showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human iNOS kit can be used to determine relative mass values for naturally occurring iNOS.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for iNOS has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any iNOS present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for iNOS 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 iNOS 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.
- Do not mix or substitute reagents with those from other sources or lots.
- If samples generate values higher than the highest standard, 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.

MATERIALS PROVIDED

iNOS Microplate (Part 892265) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against iNOS.

iNOS Conjugate (Part 892266) - 21 mL of a mouse monoclonal antibody against iNOS conjugated to horseradish peroxidase with preservatives.

iNOS Standard (Part 892267) - 1 vial (160 U/vial) of recombinant human iNOS in a buffered protein base with preservatives; lyophilized.

Assay Diluent RD1-34 (Part 895265) - 11 mL of a buffered protein base with blue dye and preservatives.

Calibrator Diluent RD5-10 (Part 895266) - 21 mL of a buffered protein base with preservatives.

Cell Lysis Buffer 2 (Part 895347) - 2 vials (21 mL/vial) of a buffered solution with preservatives.

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

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

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-34	
	Calibrator Diluent RD5-10	
	Cell Lysis Buffer 2	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store for up to 1 month at ≤ -70° C.*
	Microplate Wells	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.*

*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.
- 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 rpm \pm 50 rpm.
- Centrifuge.
- Sterile PBS (for cell lysis procedure).
- Glycerol.
- Test tubes for serial dilution.
- Human iNOS Controls (optional; available from R&D Systems).

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 2 at 2 - 8° C.

1. Centrifuge cells at 300 x g for 5 minutes. Pour off supernate.
2. Wash cells 2 times in sterile PBS. After each wash, centrifuge at 300 x g for 5 minutes. Pour off supernate.
3. Lyse cells for 10 minutes at 2 - 8° C with Cell Lysis Buffer 2 (1 mL of buffer per 1×10^6 cells).
4. Centrifuge cells at 300 x g for 5 minutes.
5. Remove supernate and assay immediately or add 1% glycerol final concentration and store at $\leq -20^\circ$ C.
6. Dilute cell lysate 1:2 in Calibrator Diluent RD5-10 prior to assay.

REAGENT PREPARATION

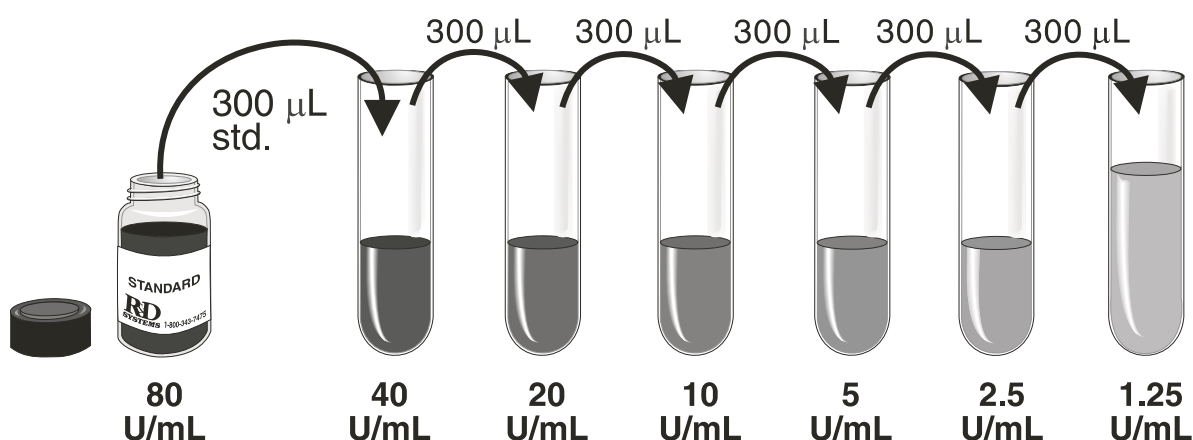
Bring all reagents to room temperature before use.

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 μ L of the resultant mixture is required per well.

iNOS Standard - Reconstitute the iNOS Standard with 2.0 mL of Calibrator Diluent RD5-10. This reconstitution produces a stock solution of 80 U/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 300 μ L of Calibrator Diluent RD5-10 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 80 U/mL standard serves as the high standard. The Calibrator Diluent serves as the zero standard (0 U/mL).



ASSAY PROCEDURE

Bring all other reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls 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, and reseal.
3. Add 100 μ L of Assay Diluent RD1-34 to each well.
4. Add 100 μ L of Standard, Control, 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 rpm \pm 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, 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 μ L of iNOS 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 μ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 μ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the 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.

*Samples require lysis. See Cell Lysis Procedure.

ASSAY PROCEDURE SUMMARY

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



2. Add 100 μ L Assay Diluent RD1-34 to each well.



3. Add 100 μ L Standard, Control or sample* to each well.
Incubate 2 hours on the shaker at RT.



4. Aspirate and wash 4 times.



5. Add 200 μ L Conjugate to each well.
Incubate 2 hours on the shaker at RT.



6. Aspirate and wash 4 times.



7. Add 200 μ L Substrate Solution to each well.
Protect from light. Incubate 30 minutes on the benchtop.



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

*Samples require lysis.

CALCULATION OF RESULTS

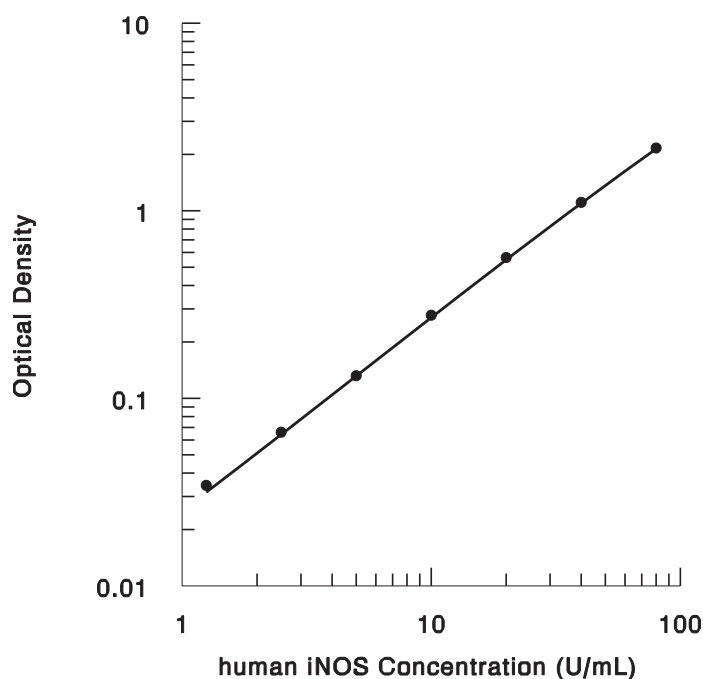
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 iNOS 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.

TYPICAL DATA

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



U/mL	O.D.	Average	Corrected
0	0.012 0.014 0.046	0.013	—
1.25	0.048 0.077	0.047	0.034
2.5	0.080 0.140	0.078	0.065
5	0.149 0.290	0.144	0.131
10	0.291 0.560	0.290	0.277
20	0.593 1.119	0.576	0.563
40	1.126 2.156	1.123	1.110
80	2.193	2.175	2.162

TECHNICAL HINTS

- 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 until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless 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.

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.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (U/mL)	8.01	26.8	52.1	9.05	27.3	56.2
Standard deviation	0.28	2.13	2.72	0.85	2.23	3.96
CV (%)	3.5	7.9	5.2	9.4	8.2	7.0

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of iNOS were serially diluted with the Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell lysates* (n=4)
1:2	Average % of Expected	103
	Range (%)	99 - 110
1:4	Average % of Expected	105
	Range (%)	100 - 109
1:8	Average % of Expected	84
	Range (%)	79 - 93

*Two samples were initially diluted 1:2 prior to assay.

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of iNOS ranged from 0.05 - 0.46 U/mL. The mean MDD was 0.15 U/mL.

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

CALIBRATION

This immunoassay is calibrated against *E. coli*-expressed recombinant human iNOS produced at R&D Systems.

1 U/mL of partially purified human iNOS is approximately equal to 1 ng/mL.

SAMPLE VALUES

Cell Lysates - DLD-2C2 cells were cultured in DMEM supplemented with 10% fetal calf serum. Cells were then grown one day past confluence and stimulated (with or without serum) for

24 hours with 10 ng/mL of IL-1 β , 10 ng/mL of TNF- α , and 25 ng/mL of IFN- γ .

A549 cells were cultured in MEM supplemented with 10% fetal calf serum, grown one day past confluence, and stimulated for 24 hours with 10 ng/mL of IL-1 β , 10 ng/mL of TNF- α , and 25 ng/mL of IFN- γ .

HepG2 cells were cultured in MEM supplemented with 5% fetal calf serum and stimulated for 24 hours with 50 ng/mL of PMA.

All cells were lysed according to the protocol.

Cell Lysates	Value (U/mL)
DLD-2C2 with serum	27.8
DLD-2C2 without serum	1.49
A549	22.7
HepG2	ND

ND = Non-detectable

SPECIFICITY

This assay recognizes recombinant and natural human iNOS. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-10 and assayed for cross-reactivity.

Preparations of the following factors at 50 ng/mL in a mid-range recombinant human iNOS control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
eNOS
nNOS

Recombinant mouse:
iNOS

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	A	B	C	D	E	F	G	H

NOTES