KAMIYA BIOMEDICAL COMPANY

Human Nitric oxide synthase 1, Neuronal (NOS1) ELISA

For the quantitative determination of human NOS1 in serum, plasma, tissue homogenates and other biological fluids

Cat. No. KT-23660

For Research Use Only. Not for use in diagnostic procedures.

Product Information

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INTENDED USE

This ELISA kit is a sandwich enzyme immunoassay for the *in vitro* quantitative measurement of human NOS1 in serum, plasma, tissue homogenates and other biological fluids. For research use only. Not for use in diagnostic procedures.

BACKGROUND

Nitric oxide synthases (NOSs) are a family of eukaryotic enzymes that catalyze the production of nitric oxide (NO) from L-arginine. NO is an important cellular signaling molecule, having a vital role in many biological processes. NOS is an enzyme in the body that contributes to transmission from one neuron to another, to the immune system and to dilating blood vessels. It does so by synthesis of nitric oxide (NO) from the terminal nitrogen atom of arginine in the presence of NADPH and dioxygen (O2). NOS is the only known enzyme that binds flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4) and calmodulin.

Different members of the NOS family are encoded by separate genes. NOS is one of the most regulated enzymes in biology. There are three known isoforms, two are constitutive (cNOS) and the third is inducible (iNOS). Cloning of NOS enzymes indicates that, cNOS include both brain constitutive (NOS1) and endothelial constitutive (NOS3), the third is the inducible (NOS2) gene.

Neuronal NOS (NOS1) produces NO in nervous tissue in both the central and peripheral nervous system. Neuronal NOS also performs a role in cell communication and is associated with plasma membranes. NOS1 action can be inhibited by NPA (N-propyl-L- arginine) . This form of the enzyme is specifically inhibited by 7-nitroindazole.

COMPONENTS

Reagents	Quantity
Pre-coated, ready to use 96-well plate	1
Calibrator (lyophilized)	2
Calibrator Diluent	1 × 20 mL
Detection Reagent A	1 × 120 µL
Detection Reagent B	1 × 120 µL
Assay Diluent A (2X concentrate)	1 × 6 mL
Assay Diluent B (2X concentrate)	1 × 6 mL
TMB Substrate	1 × 9 mL
Stop Solution	1 × 6 mL
Wash Buffer (30X concentrate)	1 × 20 mL
Plate sealer for 96 wells	4

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Precision single and multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.

6. Container for Wash Solution

STORAGE

All reagents should be stored according to their label. The **Calibrators**, **Detection Reagent A, Detection Reagent B** and the **96-well plate** should be stored at -20°C upon receipt. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Opened test kits will remain stable until the expiration date, provided they are stored as above.

PRINCIPLE

The microtiter plate provided in this kit has been pre-coated with an antibody specific to NOS1. Calibrators and samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for NOS1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then the TMB substrate solution is added to each well. Only those wells that contain NOS1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of NOS1 in the samples is then determined by comparing the O.D. of the samples to the calibration curve.

SAMPLE COLLECTION AND STORAGE

Serum

Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000 x g. Assay freshly prepared serum immediately or aliquot samples and store them at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Plasma

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 x g at 2 - 8°C within 30 minutes of collection. Remove serum and assay immediately or aliquot samples and store them at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue Homogenate

The preparation of tissue homogenates will vary depending upon tissue type. As an example, rinse tissue with 1X PBS to remove excess blood, homogenize in 5~10 mL of 1X PBS and store overnight at ≤ -20°C. Perform two freeze-thaw cycles to break the cell membranes and centrifuge the homogenates for 5 minutes at 5000 x g. Remove the supernatant and assay immediately or aliquot and store at -20°C.

Other Biological Fluids

Centrifuge samples for 20 minutes at 1000 x g. Remove particulates and assay immediately or aliquot samples and store at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Before performing the assay, slowly bring samples to room temperature.
3. Avoid hemolysis as excessive hemolysis will impact the result.

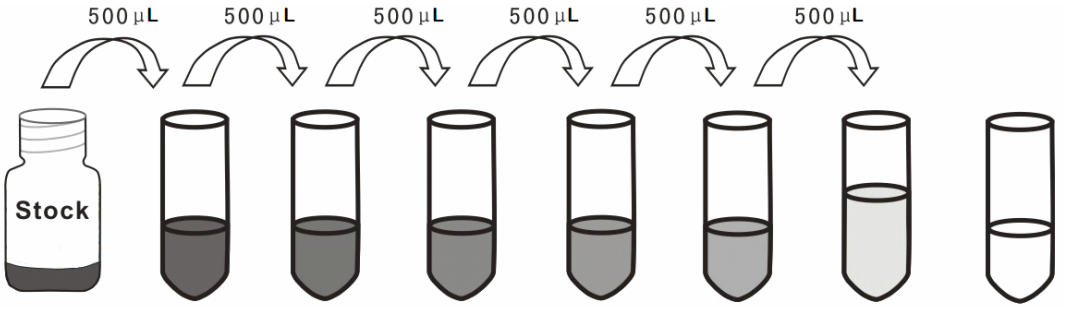
REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use.

Calibrators

Reconstitute the **Calibrator** with 1.0 mL of **Calibrator Diluent** and allow it to sit for 10 minutes at room temperature. Then mix uniformly but gently, avoid foaming. The concentration of the stock calibrator solution is now at 20 U/mL. Use the stock calibrator solution and the **Calibrator Diluent** to produce a dilution series. Pipette 500 µL of **Calibrator Diluent** into each tube. Then perform a serial dilution,

beginning with the stock calibrator solution, to create the other 6 calibrators (as shown below). Make sure to use a new pipette tip for each transfer and mix each tube thoroughly before the next transfer. The seven calibrators will have concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.312 U/mL, and the last tube filled with only **Calibrator Diluent** will serve as the blank at 0 U/mL.



Tube	1	2	3	4	5	6	7	8
U/mL	25	10	5	2.5	1.25	0.625	0.312	0

Assay Diluent A and B
Dilute 6 mL of Assay Diluent A or B Concentrate (2X) with 6 mL of deionized or distilled water to prepare 12 mL of Assay Diluent A or B. **The prepared working dilution can not be frozen.**

Detection Reagent A and B
Briefly spin or centrifuge the stock Detection Reagent A and Detection Reagent B before use. Dilute to the working concentration with working **Assay Diluent A or B,** respectively (1:100).

Wash Solution
Dilute 20 mL of Wash Solution Concentrate (30X) with 580 mL of deionized or distilled water to prepare 600 mL of Wash Solution (1X).

TMB Substrate
Aspirate the needed dosage of the solution with sterilized tips and do not return the residual solution to the vial.

Note:
1. Prepare the calibrators within 15 minutes of beginning the assay. Do not dissolve the reagents at 37°C.
2. Do not perform your serial dilutions directly in the wells.
3. Carefully reconstitute Calibrators or working Detection Reagent A and B according to the instruction. Avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to draw more than 10µl for pipetting.
4. The reconstituted Calibrators, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30X), warm to room temperature and mix gently until the crystals have completely dissolved.

ASSAY PROCEDURE

Estimate the sample NOS1 concentration before assaying. If the estimated values are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.

1. Determine wells to be used for diluted calibrators, blank and samples. Prepare 7 wells for calibrators, 1 well for blank. Add 100 µL each of dilutions of calibrators (see Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.

2. Remove the liquid from each well, do not wash.

3. Add 100 µL of the **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering with the Plate sealer.

4. Aspirate the solution and wash each well with 400 µL of 1X Wash Solution using a squirt bottle, multi-channel pipette, manifold dispenser or auto-washer, and let sit for 1~2 minutes. Remove the remaining liquid from all wells completely by sharply striking the plate on absorbent paper. Repeat wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 µL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering with the Plate sealer.

6. Repeat the aspiration/wash process five times as in step 4.

7. Add 90 µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Do not exceed 30 minutes). Protect from light. The solution will turn blue after the addition of Substrate Solution.

8. Add 50 µL of **Stop Solution** to each well. The liquid will turn yellow after the addition of Stop solution. Mix the liquid by gently tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drops of solution or fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Then, run the microplate reader and conduct measurements at 450 nm immediately.

⚠ **Note:**

1. **Assay preparation:** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Unused strips should be resealed and stored at 4°C until the expiration date.
2. **Sample or reagent additions:** Use freshly prepared Calibrators**.** Carefully add samples to wells and mix gently to avoid foaming. Do not touch the well walls if possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. After reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drops of solution or fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too dark, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Protect from light.

CALCULATION OF RESULTS

Average the duplicate readings for each calibrator, control, and sample and subtract the average zero calibrator optical density. Create a calibration curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a calibration curve by plotting the mean absorbance for each calibrator 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 NOS1 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. It is recommended to use some related software to do this calculation. If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

PERFORMANCE

Detection Range

The detection range is: 0.312 - 20 U/mL.

The calibration curve concentrations used for the ELISA's were 20, 10, 5, 2.5, 1.25, 0.625, 0.312 U/mL.

Sensitivity

The minimum detectable dose of human NOS1 is typically less than 0.16 U/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. It was determined by the mean O.D. value of 20 replicates of the zero calibrator plus three standard deviations.

Specificity

This assay has high sensitivity and excellent specificity for detection of human NOS1. No significant cross-reactivity or interference was observed.

MPORTANT NOTES

1. The final experimental results will be closely related to the operative skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
2. This assay is designed to eliminate interference by soluble receptors, ligands, binding proteins, and other factors present in biological samples. However, until all factors have been tested the possibility of interference cannot be eliminated.
3. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
4. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be tightly closed to prevent the evaporation or contamination by microorganisms.
5. There may be some foggy substance in the plate wells when first opened. This will have no effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
6. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. or greater at 450nm wavelength is acceptable for use in absorbance measurement.
7. The Stop Solution used with this kit is an acid. Wear eye, hand, face, and clothing protection when using this material.

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