



Human eNOS/NOS3 ELISA Kit

Catalog Number: NOS31-K01

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

v. 2.0 (30 OCT 23)

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INTENDED USE

The Eagle Bioscience's Human eNOS/NOS3 ELISA Assay Kit is intended for the measurement of nitric oxide synthase 3 in cell culture supernatants. The Human eNOS/NOS3 ELISA Assay Kit is for research use only and should not be used for diagnostic procedures.

For further information about this kit, its application, or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc at www.EagleBio.com or at 866-411-8023.

PRINCIPLE OF THE ASSAY

The Eagle Bioscience Human NOS3 Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human NOS3 with a 96-well strip plate that is pre-coated with antibody specific for NOS3. The detection antibody is a biotinylated antibody specific for NOS3. The capture antibody is a monoclonal antibody from mouse and the detection antibody is a biotinylated polyclonal antibody from goat. The kit includes Human NOS3 protein as standards.

To measure Human NOS3, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbound ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human NOS3 in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human NOS3 in the sample.

ASSAY BACKGROUND

Endothelial NOS (eNOS), also known as nitric oxide synthase 3 (NOS3) or constitutive NOS (cNOS), is an enzyme that in humans is encoded by the NOS3 gene located in the 7q35-7q36 region of chromosome 7. Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. Nitric oxide is synthesized from L-arginine by nitric oxide synthases. Variations in this gene are associated with susceptibility to coronary spasm. Alternative splicing and the use of alternative promoters results in multiple transcript variants.

PRECAUTIONS

1. Read this manual in its entirety in order to minimize the chance of error.
2. Confirm that you have the appropriate non-supplied equipment available.
3. Confirm that the species, target antigen, and sensitivity of this kit are appropriate for your intended application.
4. Confirm that your samples have been prepared appropriately based upon recommendations (see Sample Preparation) and that you have sufficient sample volume for use in the assay.
5. When first using a kit, appropriate validation steps should be taken before using valuable samples. Confirm that the kit adequately detects the target antigen in your intended sample type(s) by running control samples.



6. If the concentration of target antigen within your samples is unknown, a preliminary experiment should be run using a control sample to determine the optimal sample dilution (see Sample Preparation).
7. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
8. Before using the kit, spin tubes to bring down all components to the bottom of the tubes.
9. Don't let the 96-well plate dry out since this will inactivate active components on the plate.
10. Don't reuse tips and tubes to avoid cross-contamination.
11. Avoid using the reagents from different batches together.
12. The kit should not be used beyond the expiration date on the kit label. Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding. Variations in sample collection, processing, and storage may cause sample value differences.

MATERIALS PROVIDED

Description	Quantity	Volume	Storage of opened / Reconstituted material
Anti-Human NOS3 Pre-coated 96-well Strip Microplate	1	12 strips of 8 wells	Return unused wells to the foil pouch. Reseal along the entire edge of the zip-seal. May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.
Human NOS3 Standard	2	10 ng/tube	Discard the NOS3 stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours.
Human NOS3 Biotinylated Antibody (100X)	1	100ul	May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.
Avidin-Biotin-Peroxidase Complex (100X)	1	100ul	
Sample Diluent	1	30mL	
Antibody Diluent	1	12 ml	
Avidin-Biotin-Peroxidase Diluent	1	12 ml	
Color Developing Reagent (TMB)	1	10 ml	
Stop Solution	1	10 ml	
Wash Buffer (25X)	1	20 ml	



MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading absorbance at 450 nm.
- Incubator.
- Automated plate washer (optional) Pipettes and pipette tips capable of precisely dispensing 0.5 µl through 1 ml volumes of aqueous solutions. Multichannel pipettes are recommended for a large numbers of samples.
- Deionized or distilled water.
- 500 ml graduated cylinders.
- Test tubes for dilution

Component	Preparation
All Reagents	Bring all reagents to room temperature (18-25°C) prior to use. Please DO NOT equilibrate unused plate well strips to room temperature. They should be sealed and stored in the original packaging. The assay can also be done at room temperature however we recommend doing it at 37°C for best consistency with our QC results. Also, the TMB incubation time estimate (15-25 min) is based on incubation at 37°C.
Wash Buffer	Prepare 500 ml of Working Wash Buffer by diluting the supplied 20 ml of Wash Buffer (25 x) with 480 ml of deionized or distilled water. If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved
Biotinylated Anti-Human NOS3 antibody	It is recommended to prepare this reagent immediately prior to use by diluting the Human NOS3 Biotinylated antibody (100x) 1:100 with Antibody Diluent. Prepare 100 µl by adding 1 µl of Biotinylated antibody (100x) to 99 µl of Antibody Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.
Avidin-Biotin-Peroxidase Complex	It is recommended to prepare this reagent immediately prior to use by diluting the Avidin-Biotin-Peroxidase Complex (100x) 1:100 with Avidin-Biotin-Peroxidase Diluent. Prepare 100 µl by adding 1 µl of Avidin-BiotinPeroxidase Complex (100x) to 99 µl of Avidin-Biotin-Peroxidase Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.
Human NOS3 Standard	It is recommended that the standards be prepared no more than 2 hours prior to performing the experiment. Use one 10 ng of lyophilized Human NOS3 standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 10 ng/ml using 1ml of sample diluent. Allow the standard to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions.
Samples	Dilute the sample so that the expected range of concentrations fall within the detection range of this kit. If the expected range of concentration is unknown, a pilot test should be conducted to decide the optimal dilution ratio for your samples.



DILUTION OF HUMAN NOS3 STANDARD

1. Number tubes 1-8. Final Concentrations to be
 - a. # 1: 5,000.00 pg/ml
 - b. # 2: 2,500.00 pg/ml
 - c. # 3: 1,250.00 pg/ml
 - d. # 4: 625.00 pg/ml
 - e. # 5: 312.50 pg/ml
 - f. # 6: 156.25 pg/ml
 - g. # 7: 78.13 pg/ml
 - h. # 8: Sample Diluent serves as the zero standard (0 pg/ml)
2. To generate standard #1, add 500 μ l of the reconstituted standard stock solution of 10 ng/ml and 500 μ l of sample diluent to tube #1 for a final volume of 1000 μ l. Mix thoroughly.
3. Add 300 μ l of sample diluent to tubes # 2-7.
4. To generate standard # 2, add 300 μ l of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 μ l. Mix thoroughly.
5. To generate standard # 3, add 300 μ l of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 μ l. Mix thoroughly.
6. Continue the serial dilution for tube # 4-7.

SAMPLE PREPARATION AND STORAGE

These sample collection instructions and storage conditions are intended as a general guideline, and the sample stability has not been evaluated.

Sample dilution ratios should be determined by a pilot study (run a serial dilution of samples and see which dilution ratio results in the idea O.D., near the middle of the standard range). In general, high concentration samples can be diluted by 1:100, mid concentration samples 1:10, low concentration samples 1:2 or neat.

Cell Culture Supernatants

Clear sample of particulates by centrifugation, assay immediately, or store samples at -20°C.

SAMPLE COLLECTION NOTES

1. Eagle Biosciences recommends that samples are used immediately upon preparation.
2. Avoid repeated freeze/thaw cycles for all samples.
3. In the event that a sample type not listed above is intended to be used with the kit, it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Due to factors including cell viability, cell number, or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18-25°C) before performing the assay without the use of extra heating.



7. Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
8. Eagle Biosciences is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer supplied samples used with the kit.

SAMPLE DILUTION GUIDELINE

The user needs to estimate the concentration of the target protein in the sample and use an appropriate dilution factor so that the diluted target protein concentration falls in the range of O.D. values of the standard curve. Dilute the sample using provided diluent buffer. Pilot tests using a dilution series of each sample type are necessary. The sample must be mixed thoroughly with Sample Diluent.

ASSAY PROCEDURE

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1. Prepare all reagents and working standards as directed previously.
2. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.
3. Add 100 µl of the standard, samples, or control per well. Add 100 µl of the Sample Diluent into the zero well. At least two replicates of each standard, sample, or control is recommended.
4. Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).
5. Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
6. Add 100 µl of the prepared 1x Biotinylated Anti-Human NOS3 antibody to each well.
7. Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 minutes at 37°C).
8. Wash the plate 3 times with the 1x wash buffer:
 - a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
 - b. Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).
 - c. Repeat steps a-b 2 additional times.
 - d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.



9. Add 100 μ l of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 minutes at 37°C).
10. Wash the plate 5 times with the 1x wash buffer:
 - a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
 - b. Add 300 μ l of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).
 - c. Repeat steps a-b 4 additional times.
 - d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.
11. Add 90 μ l of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or 15-25 minutes at 37°C). (The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)
12. Add 100 μ l of Stop Solution to each well. The color should immediately change to yellow.
13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

ASSAY PROTOCOL NOTES

1. Solutions: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
2. Applying Solutions: All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
3. Assay Timing: The interval between adding samples to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.
4. Incubation: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
5. Washing: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.



6. Controlling Substrate Reaction Time: After the addition of the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. The excessively strong color will result in inaccurate absorbance readings.
7. Reading: The microplate reader should be preheated and programmed prior to use. Prior to taking O.D. readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.
8. Reaction Time Control: Control reaction time should be strictly followed as outlined.
9. Stop Solution: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
10. To minimize the external influence on the assay performance, operational procedures, and lab conditions (such as room temperature, humidity, incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

CALCULATION OF RESULTS

Eagle Biosciences recommends an online data analysis tool.

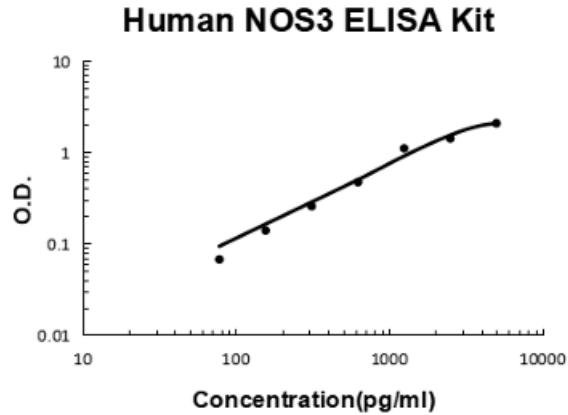
To analyze using manual methods, follow the process below: Average the duplicate readings for each standard, sample, and control. Subtract the average zero standard O.D. reading. It is recommended that a standard curve be created using computer software to generate a four-parameter logistic (4-PL) curve-fit. A free program capable of generating a four-parameter logistic (4-PL) curve-fit can be found online at: www.myassays.com/four-parameter-logistic-curve.assay.

Alternatively, plot the mean absorbance for each standard against the concentration. The measured concentration in the sample can be interpolated by using linear regression of each average relative O.D. against the standard curve generated using curve fitting software. This will generate an adequate but less precise fit of the data. For diluted samples, the concentration reading from the standard curve must be multiplied by the dilution factor.

TYPICAL CALIBRATOR CURVE

The highest O.D. value might be higher or lower than in the example. The experiment result is statistically significant if the highest O.D. value is no less than 1.0

Concentration	0	78	156	312	625	1,250	2,500	5,000
O.D.	0.022	0.089	0.161	0.278	0.492	1.121	1.431	2.091



PERFORMANCE AND CHARACTERISTICS

Intra-Assay

Three samples of known concentration were tested on one plate to assess intra-assay precision.

Sample	1	2	3
N	16	16	16
Mean (pg/ml)	112	365	1362
Standard deviation	3.47	10.22	43.58
CV %	3.1%	2.8%	3.2%

Inter-Assay

Three samples of known concentration were tested in separate assays to assess inter-assay precision.

Sample	1	2	3
N	24	24	24
Mean (pg/ml)	125	344	1399
Standard deviation	4.13	15.48	53.16
CV %	3.3%	4.5%	3.8%

REPRODUCIBILITY

We ensure reproducibility by testing three samples with differing concentrations of NOS3 in ELISA kits from four different production batches/lots.

Lots	Lot 1 (pg/ml)	Lot 2 (pg/ml)	Lot 3 (pg/ml)	Lot 4 (pg/ml)	Mean (pg/ml)	Standard Deviation	CV %
Sample	112	127	118	123	120	4.68	3.9%
Sample 2	365	359	381	377	371	19.27	5.2%
Sample 3	1362	1369	1489	1546	1442	31.71	2.2%

*number of samples for each test n=16.



WARRANTY INFORMATION

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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