

Human Nitric oxide (NO) ELISA

Cat No: K12-1511**Ver 1.1**

Principle:

This is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Nitric oxide in samples. Standards or Samples are added to the microtiter well which is pre-coated with Human Nitric oxide monoclonal Antibody. Biotinylated Human Nitric oxide antibodies are added to the microplate to form a complex. Subsequently Streptavidin-HRP conjugate is pipetted. After incubation and a washing step TMB Substrate are added. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color. The concentration of the Human Nitric oxide in the samples is directly proportional to the yellow color developed in the wells.

Intended Use:

This kit is used to assay the level of Human Nitric oxide in Human serum, plasma and other biological samples. The Kit is For Laboratory / Research Use Only.

Materials provided in the kit:

1. Anti-Human Nitric oxide Coated Microtitre Plate (96 wells) - 1 no
2. Biotinylated Human Nitric oxide Antibody - 1 ml
3. Human Nitric oxide Standard (640 nmol/ml) - 0.5 ml
4. Streptavidin:HRP Conjugate - 6 ml
5. (20X) Wash Buffer - 25 ml
6. Standard Diluent - 3 ml
7. TMB Substrate - 12 ml
8. Stop Solution - 12 ml
9. Instruction Manual

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Semi-log graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Storage Information:

1. All reagents should be stored at 2°C to 8°C.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 30 minutes prior before use, bring all components to room temperature (18-25°C). Store all the components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. The kit cannot test samples which contain NaN_3 , because NaN_3 inhibits HRP activity.
2. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
3. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

4. **Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 20-min at the 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
5. **Urine-** Collect urine in a sterile container, centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
6. **Cell Culture Supernatant-** Collect sample in a sterile container. Centrifuge for 20-mins at 2000-3000 rpm. Remove the supernatant carefully. When examining the components within the cell, dilute cell suspension with PBS (pH 7.2-7.4), if cell concentration is greater than 1 million/ml. Damage the cells by repeated freeze-thaw cycles to release intracellular components. Centrifuge for 20-min at 2000-3000 rpm. If precipitation appears, centrifuge again.
7. **Tissue Samples-** Rinse tissues in PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (pH7.4) with a glass homogenizer on ice. Thaw at 2-8°C or freeze at -20°C. Centrifuge at 2000-3000 RPM for approximately 20 minutes and collect the supernatant carefully.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Bring all reagents to Room Temperature prior to use.
2. To make 1X Wash Solution, add 25 ml of 20X Wash Buffer in 475 ml of DI water.

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Human Nitric oxide High Dose Hook Effect is due to excess of antibody for very high concentrations of Human Nitric oxide present in the sample.
3. Avoid assay of Samples containing Sodium Azide (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount Human Nitric oxide.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

- 1) Bring all reagents to room temperature prior to use. It is strongly recommended that all Standards and Samples should be run in duplicates or triplicates. A standard curve is required for each assay.
- 2) Standards Dilution: Prepare the standards as per the table given below using the provided standard Concentration and standard diluent.

Standard Concentration	Standard No	Dilution Particulars
640 nmol/ml	Standard, concentrated	Original Standard provided in the Kit
320 nmol/ml	Standard No.5	120 ul Original Standard + 120 ul Standard Diluent
160 nmol/ml	Standard No.4	120 ul Standard No.5 + 120 ul Standard Diluent
80 nmol/ml	Standard No.3	120 ul Standard No.4 + 120 ul Standard Diluent
40 nmol/ml	Standard No.2	120 ul Standard No.3 + 120 ul Standard Diluent
20 nmol/ml	Standard No.1	120 ul Standard No.2 + 120 ul Standard Diluent

** refer accompanying sheet with the Standard, concentrated in the kit*

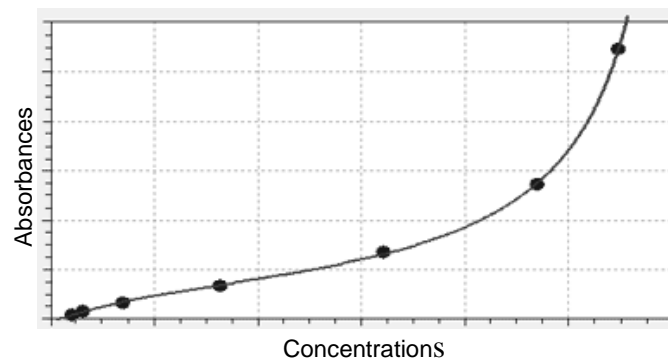
- 3) The quantity of the plates depends on the quantities of samples and standards to be tested. It is suggested to remove the number of strips required for the assay.
- 4) Pipette **50 ul of Standards** and **40 ul Samples** into the respective wells as mentioned in the work list.
Note do not add the sample, Biotin Conjugate and Streptavidin-HRP to the blank well.
- 5) Pipette **10 ul of Biotinylated Human Nitric oxide Antibody** into each sample well.
Do not pipette into the blank and standards wells. The standards offered in the kit are pre-offered as a complex of the standard and the biotin antibody for ease-of-use.
- 6) Pipette **50 ul of Streptavidin:HRP Conjugate** into each sample and standards well.
Do not pipette into the Blank well.

- 7) Cover the plate and incubate for 1 hour at 37°C in the incubator.
- 8) Aspirate and wash plate 4 times with **1X Wash Buffer** and blot residual buffer by firmly tapping the plate on an absorbent paper. Wipe off any liquid from the bottom of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
- 9) Add **100 ul TMB Substrate** in all the wells. Gently mix.
- 10) Incubate for 10 min at 37°C in dark.
- 11) Pipette **100 ul of Stop Solution**. Wells should turn from blue to yellow in color.
- 12) Read the absorbance at 450 nm within 15 minutes after adding the Stop Solution. Blanking the zero standard for net absorbance.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit, 4PL or a polynomial regression to the 2nd order is best recommended for automated results.

Typical Graph**Precautions:**

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Sensitivity:**Limit Of Quantification:**

It is defined as the lowest detectable concentration that can be determined with an acceptable repeatability and the LOQ was found to be **17 nmol/ml**.

Specificity:

The antibodies used in the kit for capture and detection are specific for human Nitric oxide.

Assay Range:

20 – 320 nmol/ml

Precision:

Intra-Assay: CV<10%

Inter-Assay: CV<12%

Dilutional Linearity:

The Linearity of the kit was assayed by testing samples spiked with 640 nmol/ml concentration of Human Nitric oxide and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8
serum (n=5)	81-111%	86-116%	87-117%
EDTA plasma (n=5)	82-112%	85-115%	88-118%
heparin plasma (n=5)	83-113%	84-114%	89-119%

Note: The kit has not been validated for concentrations and dilutional linearity / recovery beyond the concentration of 640 nmol/ml. In case your samples have expected concentrations beyond this range, you may validate the same using the Standard Diluent provided in the kit. However, we do not warrant for linearity beyond the range indicated above.

LIMITED WARRANTY

KinesisDx does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by KinesisDx, or against damages resulting from such non-KinesisDx made products or components. KinesisDx passes on to customer the warranty it received (if any) from the maker thereof of such non-Krishgen made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by KinesisDx.

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ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 5 Samples	50 ul	40 ul
3	Pipette Human NO Biotin Detection Antibody		10 ul
4	Pipette Streptavidin :HRP Conjugate	50 ul	50 ul
5	Incubate 60 minutes (37°C)		
6	1X Wash Buffer Decant, 4 x 300 ul		
7	Add TMB Substrate 100 ul		
8	Incubate in the dark 10 minutes (37°C)		
9	Pipette Stop Solution 100 ul		
10	Measure 450 within 15 mins		

Troubleshooting:

Problem	Possible cause	Investigation/Actions
High Absorbances	<ol style="list-style-type: none"> 1. Cross-contamination from other specimens 2. Insufficient or inefficient washing or reading 3. Wavelength of filter not correct. 4. High assay background. 5. Contaminated TMB 6. Incubation time too long or incubation temperature too high. 7. Incorrect dilution of serum 	<ul style="list-style-type: none"> > Repeat assay taking care when washing and pipetting. > Check washer efficiency > Check that the wavelength is 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. > Repeat assay and include a well that contains only sample diluent or sample absorbent (i.e. a blank well). > Check that TMB is colorless or faint blue. > Check incubation time and temperature. > Check incubator is at the correct temperature. > Repeat assay, ensuring correct serum dilution is used.
Low Absorbances	<ol style="list-style-type: none"> 1. Incubation time too short or incubation temperature too low. 2. Incorrect dilution or pipetting of sera 3. Incorrect filter wavelength. 4. Contaminated Conjugate solution. 5. Kit has expired. 6. Air blank reading high. 7. Incorrect storage of kit. 8. Kit reagents not equilibrated at room temperature 9. Incorrect reagents used. 10. Over washing of plate (e.g. inclusion of a long soak step). 	<ul style="list-style-type: none"> > Ensure time and temperature of assay incubation are correct. > Check incubator is set at the correct temperature. > Repeat assay ensuring correct dilutions and volumes are used. > Ensure controls are sufficiently mixed. > Check the wavelength is set at 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650nm. > Dispense conjugate directly from the bottle using clean pipette tip; avoid transferring Conjugate to another container if possible. > Do not return unused Conjugate to bottle. > Ensure all pipettes and probes used to dispense the Conjugates are clean and free from serum, detergent and bleach. > Check expiration date of kit and do not use if expired. Investigate causes of high background absorbance. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue/purple. > Allow sufficient time for reagents to equilibrate to room temperature prior to assay. > Check the reagents used match those listed on the specification sheet. > Repeat assay using recommended wash procedure.
Poor Duplicates	<ol style="list-style-type: none"> 1. Poor mixing of samples. 2. Poor pipette precision 3. Addition of reagents at inconstant timing intervals; reagent addition takes too long, air bubbles when adding reagents. 4. Inefficient washing - Wash buffer left in wells, inconsistent washing, inadequate washing. 5. Reader not calibrated or warmed up prior to plate reading. 6. Optical pathway not clean 7. Spillage of liquid from wells 8. Serum samples exhibit microbial growth, haemolysis or lipaemia. 9. Uneven well volumes due to evaporation. 	<ul style="list-style-type: none"> > Mix reagents gently and equilibrate to room temperature. > Calibration may need to be checked. > Check pipetting technique-change pipette tip for each sample and ensure excess liquid is wiped from the outside of the tip. > Use consistent timing when adding reagents. > Ensure all dilutions are made before commencing addition to plate. > Improve pipetting technique and skill. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Check reader precision > Check reader manual to ascertain warm up time of instrument. > Gently wipe bottom of plate. > Check reader light source and detector are clean. > Repeat assay, taking care not to knock the plate or splash liquid > It is not recommended to use serum samples exhibiting microbial growth, haemolysis or lipaemia. > Cover plate with a lid or plate sealer (not provided).
All wells yellow	<ol style="list-style-type: none"> 1. Contaminated TMB. 2. Contaminated reagents (e.g. Conjugate, Wash buffer). 3. Incorrect dilution of serum. 4. Incorrect storage of kit. 5. Inefficient washing- Wash buffer left in wells, inconsistent washing, inadequate washing. 6. If Conjugate reconstitute is required – Conjugate reconstituted incorrectly. 	<ul style="list-style-type: none"> > Check TMB is colorless or faint blue. > Check reagents for turbidity. > Repeat assay, ensuring correct serum dilution is used. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Repeat assay ensuring Conjugate is reconstituted according to assay method.

All wells negative

1. Test not performed correctly – correct reagents not added or not added in the correct sequence.
 - > Check procedure and check for unused reagents.
 - > Ensure that Stop Solution was not added before Conjugate or TMB.
 - > Ensure that serum was diluted in correct Sample diluent; e.g. do not use Sample Absorbent for an IgG ELISA.
2. Contaminated Conjugate solution.
 - > Dispense Conjugate directly from the bottle using a clean pipette tip; avoid transferring Conjugate to another container if possible.
 - > Do not return unused Conjugate to bottle.
 - > Ensure all pipettes and probes used to dispense the Conjugate are clean and free from serum, detergent and bleach.
 - > Repeat assay using recommended wash procedure.
3. Over- washing of plate (e.g. inclusion of a long soak step).
4. Incorrect storage of kit.
 - > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple.
5. Wash Buffer made up with Stop Solution instead of Wash Buffer Concentrate
 - > Ensure Wash Buffer is made up correctly.