Nitrotyrosine ELISA Kit

Enzyme Immunoassay for the quantitative determination of Nitrotyrosine in plasma, serum, cell lysates, urine and other sample matrices.

Catalog number: ARG81132

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INTRODUCTION

Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide. Nitrotyrosine is identified as an indicator or marker of cell damage, inflammation as well as NO (nitric oxide) production. Nitrotyrosine is formed in the presence of the active metabolite NO. Generally in many disease states, oxidative stress increases the production of superoxide (O2−) and NO forming peroxynitrite (ONOO−) a destructive free radical oxidant. The production of ONOO− is capable of oxidizing several lipoproteins and of nitrating tyrosine residues in many proteins. It is difficult to determine the production of ONOO− so, usually nitrotyrosine in proteins are the detectable marker for indirectly detecting ONOO−. It is detected in large number of pathological conditions and is considered a marker of NO-dependent, reactive nitrogen species-induced nitrative stress. Nitrotyrosine is detected in biological fluids such as plasma, lung aspirants-BALF (Broncho alveolar lining fluid) and urine. Increased level of nitrotyrosine is detected in rheumatoid arthritis septic shock and coeliac disease. In all these studies nitrotyrosine was undetected in healthy subjects. Nitrotyrosine is also found in numerous other disease-affected tissues, such as the cornea in keratoconus. Peroxynitrite and/or nitrative stress may participate in the pathogenesis of diabetes. Nitrotyrosine, as a marker of reactive oxygen species, has also been linked to degeneration of dopamine neurons. Tyrosine is the precursor to dopamine, a neurotransmitter that's important for motivation, attention, learning, circadian rhythms, and other biological processes. [Provide from Wikipedia]

PRINCIPLE OF THE ASSAY

This assay employs the competitive enzyme immunoassay technique for the quantification of Free Nitrotyrosine in plasma, serum, cell lysates, urine, and other sample matrices. Nitrotyrosine has been pre-coated onto a microtiter plate. Nitrotyrosine in samples and the Nitrotyrosine coated on wells are competing for a limited amount of HRP-conjugated Nitrotyrosine monoclonal antibody. After incubation, the wells are washed with wash buffer to remove unbound material. The substrate solution (TMB substrate) is added and incubated, stopped with STOP solution, resulting in the development of a distinct color which absorbance is measured at 450 nm. The concentration of Nitrotyrosine in samples or standards is indirectly proportional to the color intensity or OD readings by the spectrometer.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C (except for standard solution which should be stored at -20°C). Use the kit before expiration date.

Component	Quantity	Storage information
Nitrosylated-BSA coated microplate	96 wells	4°C
Nitrotyrosine standard (408 µM)	1 vial (110 µl)	-20°C
Nitrotyrosine HRP-conjugated antibody	1 vial (75 µl)	4°C
Sample/Standard Diluent	50 ml (Ready to use)	4°C
Nitrotyrosine antibody Diluent	13 ml (Ready to use)	4°C
10X Wash Buffer Concentrate	50 ml	4°C
TMB substrate	13 ml (Ready to use)	4°C, keep in dark
STOP solution	13 ml (Ready to use)	4°C
Plate Cover	2	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Briefly spin down the all vials before use.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate solution. Do NOT return leftover TMB Substrate to bottle.
- Take care not to contaminate the TMB Substrate. Do not expose the TMB

solution to glass, foil or metal. If the solution is blue before use, do NOT use it.

- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Use a new adhesive plate cover for each incubation step.
- Always add the Antibody Preparation after the rest of the reagents, as this is a competitive assay.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in at least duplicates (triplicate is recommended).

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated. Prepare at least 180µL of your diluted sample to permit assay in triplicate (50µL/well).

Note:

- All samples must be free of organic solvents prior to assay.

- Samples that cannot be assayed immediately should be stored as indicated below.

- Please be advised that all suggested dilutions below are simply recommended as a starting point, and it may be necessary to adjust the dilution based on experimental results.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at r-80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples-80°C. Avoid repeated freeze-thaw cycles.

Serum/Plasma samples may be diluted 1:4 (v:v) in Sample/Standard Diluent as the starting dilution prior to testing (e.g. 40 µl of samples + 120 µl of Sample/Standard Diluent).

Urine – Urine samples should be centrifuged at 2,000 X g for 10 minutes, or filtered with 0.2 µm filter before assay. Assay immediately or aliquot and stored at -20°C or-80°C immediately after collection. Dilute urine samples 1:4 (v:v) in sample/standard diluent as starting dilution prior to testing (e.g. 40 µl of samples + 120 µl of Sample/Standard Diluent).

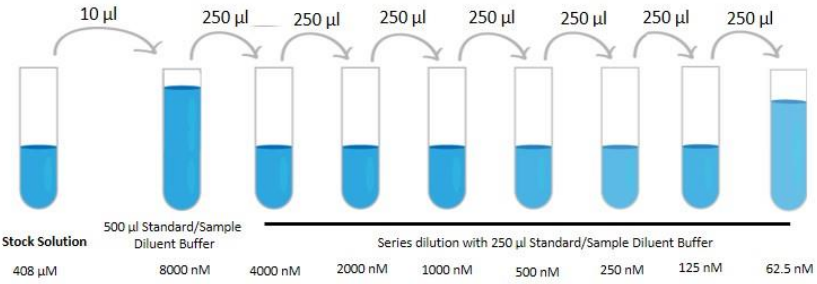
Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at 4°C. Collect the supernatants and assay immediately or aliquot and store samples -80°C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 50 ml of 10X Wash buffer into a total of 500 ml with distilled water. Store 1X Wash buffer at 2-8°C for up to 1 month. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.
- Nitrotyrosine antibody: Determine the amount of antibody required. For

every strip –well used (8 wells), prepare 0.5 ml of antibody solution. To prepare antibody working solution, dilute the HRP-conjugated Nitrotyrosine Antibody 1:100 with antibody diluent. For example, if 6 ml of antibody working solution is needed, dilute 60 µl of stock antibody into 6ml antibody diluent. Mix well prior to use.

- **Standards:** Centrifuge standard vial before removing the cap. Add 10 µl of concentrated standard solution (408 µM) into 500µl of Standard/Sample Diluent buffer and mix well to make an 8000 nM Standard S1. The Sample/Standard Diluent buffer serves as zero standard (S0, 0 nM), and the rest of the standard serial dilution can be diluted as according to the suggested concentration below: S1: 8000 nM, S2: 4000 nM, S3: 2000 nM, S4: 1000 nM, S5: 500 nM, S6: 250 nM, S7: 125 nM, S8: 62.5 nM. The Stock Standard should be aliquoted into smaller portions before use to ensure product integrity and store the aliquoted Stock Standard at -20°C. Avoid repeated freeze-thaw cycles.



ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use. Standards, samples and controls should be assayed in at least duplicates (triplicate is recommended).

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and store at 2-4°C. Be sure the packet is sealed with the desiccant inside.
2. Add 50 µl of standards (S0-S8) or samples in duplicate into appropriate wells.
3. Add 50 µl antibody working solution into each wells except for Blank wells.
4. Add 50 µl standard/sample diluent and 50 µl antibody diluent into another wells as Blank wells.
5. Cover plate with Plate Cover and incubate at room temperature (20-25°C) for 1 hour.
6. Aspirate each well and wash, repeating the process 3 times for a total 4 washes. Wash by filling each well with distilled water (300 µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
7. Add 100 µl TMB substrate into each well. Cover the plate with Plate Cover and incubate for 30 minutes at RT in dark. The substrate reaction yields a blue solution.
8. Remove the plate cover carefully. Add 100 µl STOP solution into each well.

The color of the solution should change from blue to yellow. Gently tap the plate to ensure thorough mixing.

9. Wipe underside of wells with a lint-free tissue. Read the OD with a microplate reader at 450 nm immediately or within 30 min after adding STOP solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and samples.

2. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD bound.

3. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

4. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

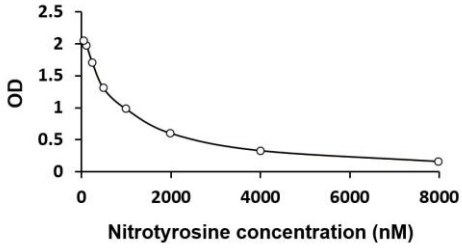
5. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

6. Samples that read at concentrations outside of the standard curve range will need to be re-analyzed using a different dilution. Make sure to multiply sample concentrations calculated off the curve by the dilution factor used during sample preparation to get starting sample concentration. (If samples generate

greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re- assayed at a lower sample dilution.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of Nitrotyrosine ranged from 62.5-800nM. The mean MDD was 50 nM.

Intra and inter-assay precision

% CV of Intra assay precision: <10%.

% CV of Inter assay precision: <15%.