

Nitrotyrosine (NT) ELISA Kit

Catalog No.: abx150362

Size: 96T

Range: 3.7 ng/ml - 300 ng/ml

Sensitivity: < 1.35 ng/ml

Storage: Store standard, detection reagent A, detection reagent B and the 96-well plate at -20°C, and the rest of the kit components at 4°C.

Application: For quantitative detection of NT in Serum, Plasma and other biological fluids.

Introduction: Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxyntirite 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 peroxyntirite (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. Peroxyntirite and/or nitrative stress may participate in the pathogenesis of diabetes

Principle of the Assay

This kit is based on a competitive binding enzyme-linked immuno-sorbent assay technology. An antibody specific to NT is pre-coated onto the 96 well plate. A competitive inhibition reaction is launched between biotin labeled NT and unlabeled NT with the pre-coated antibody specific to NT. After washing away the unbound conjugates, avidin conjugated to Horseradish Peroxidase is added to each microplate well and incubated. After TMB substrate solution is added only wells that contain NT will produce a blue color product that changes into yellow after adding acidic stop solution. The intensity of the color yellow is inverse proportional to the NT amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450nm in a microplate reader, and then the concentration of NT can be calculated.

Kit components

1. One pre-coated 96 well plate
2. Standard: 2 tubes
3. Standard Diluent Buffer: 20 ml
4. Wash Buffer (30X): 20 ml. Dilution: 1:30
5. Diluent A: 12 ml
6. Diluent B: 12 ml
7. Stop solution: 6 ml
8. TMB substrate : 9 ml
9. Detection Reagent A (100X): 120 µl
10. Detection Reagent B (100X): 120 µl
11. Plate sealer: 4

Material Required But Not Provided

1. 37°C incubator
2. Microplate reader (wavelength: 450nm)
3. Precision pipette and disposable pipette tips
4. Automated plate washer
5. ELISA shaker
6. 1.5ml tubes
7. Plate cover
8. Absorbent filter papers
9. 100 ml and 1 L volume graduated cylinders

Protocol

A. Preparation of sample and reagents

1. Sample

Isolate the test samples soon after collecting, analyze immediately or store at 4°C for up to 5 days. Otherwise, store at -20°C for up to one month or -80°C for up to two months to avoid loss of bioactivity. Avoid multiple freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum at room temperature (~2 hr) or overnight at 4°C. Centrifuge at approximately 1000 × g for 20 min. Analyze the serum immediately or aliquot and store at -20°C or -80°C.
- **Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or aliquot and store at -20°C. Avoid hemolysis and high cholesterol samples.
- **Other biological fluids:** Centrifuge at approximately 1000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.

Note:

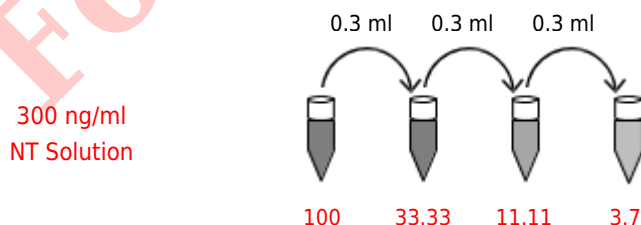
- » Please bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used.
- » Samples must be diluted so that the expected concentration falls within the kit's range. Sample should be diluted in 0.01 mol/L PBS (PH=7.0-7.2).
- » If the sample are not indicated in the manual's applications, a preliminary experiment to determine the validity of the kit will be necessary.
- » Fresh sample or recently obtained samples are recommended to prevent protein degradation and denaturalization that may lead to erroneous results.

2. Wash buffer

Dilute the concentrated Wash buffer 30-fold (1/30) with distilled water (i.e. add 20 ml of concentrated wash buffer into 580 ml of distilled water).

3. Standard

Bring samples and all kit components to room temperature. Prepare the Standard with 0.4ml of Standard Diluent buffer (kept for 10 min at room temperature) to make the 300 ng/ml Standard Solution (use within one hour), which serves as the highest standard. Mix gently and avoid foaming or bubbles. Label 4 tubes with 100 ng/ml, 33.33 ng/ml, 11.11 ng/ml, 3.7 ng/ml. Aliquot 0.6 ml of the Standard diluent buffer into each tube. Add 0.3 ml of 300 ng/ml standard solution into the 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube, mix thoroughly, and so on.



4. Detection Reagent A and B Preparation

Centrifuge Detection Reagent A and B briefly before use. Detection Reagent A and B should be diluted 100-fold with the Diluent A and B respectively and mixed thoroughly. They are sticky solutions therefore pipette with a slow, smooth action to reduce volume errors. The solution should be prepared no more than 2 hours prior to the experiment. The Reconstituted Standard, Detection Reagent A and Detection Reagent B can only be used once.

B. Assay Procedure

Equilibrate the kit components and samples to room temperature before use. It is recommended to plot a standard curve for each test.

1. Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate or triplicate.
2. Add 50 µl of the diluted standards into the standard wells. Aliquot 50 µl Standard Diluent Buffer to the control (zero) well.
3. Add 50 µl of appropriately diluted sample into the test sample wells. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.
4. Immediately aliquot 50 µl of Detection Reagent A working solution (if it appears cloudy mix gently until the solution is uniform) to each well. Shake the plate gently to mix thoroughly (a microplate shaker is recommended).
5. Seal the plate with a cover and incubate for 1 h at 37°C.
6. Discard the solution and wash the plate 3 times with wash buffer. Each time let the wash buffer sit for 1-2 min. Do not let the wells dry completely at any time.

Manual Washing: Discard the solution without touching the side walls. Tap the plate on absorbent filter papers or other absorbent material. Fill each well completely with Wash buffer and vortex mildly on ELISA shaker for 2 min. Discard the contents and tap the plate on absorbent filter papers or other absorbent material. Repeat this procedure three times.

Automated Washing: Discard the solution and wash the plate three times overfilling the wells with Wash buffer. After the final wash invert the plate and tap on absorbent filter papers or other absorbent material. It is recommended that the washer be set for a soaking time of 1 min.

7. Aliquot 100 µl of Detection Reagent B working solution into each well, seal and incubate at 37°C for 30 min.
8. Repeat the aspiration/wash process 5 times as explained in step 6.
9. Aliquot 90 µl of TMB Substrate into each well. Seal the plate with a cover and incubate at 37°C for 10-20 min. Avoid exposure to light. The incubation time is for reference use only, the optimal time should be determined by end user. Do not exceed 30 min.
10. Add 50 µl of Stop solution into each well. The color should change to yellow. Gently tap the plate to ensure thorough mixing.
11. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance at 450nm immediately.

This assay is competitive, therefore there is an inverse correlation between [target] concentration in the sample and the absorbance measured. Create a graph with the log of the standard concentration (y-axis) and absorbance measured (x-axis). Apply a best fit trendline through the standard points. Use this graph calculate sample concentrations based on their OD values. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Note: If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

C. Precautions

1. Before using the kit, centrifuge the tubes briefly to bring down the contents trapped in the lid.
2. Wash buffer may crystallize and separate. If this happens warm to room temperature and mix gently until the crystals are completely dissolved.
3. Avoid foaming or bubbles when mixing or reconstituting components. Prepare the standards within 15 min of starting the experiment.
4. It is recommended measuring each standard and sample in duplicate or triplicate.
5. Do NOT let the wells uncovered for extended periods between incubation. Once reagents are added to the wells DO NOT let the strips dry at any time during the assay. This can inactivate the biological material on the plate. Incubation time and temperature must be controlled.
6. Ensure plates are properly sealed or covered during incubation steps.
7. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
8. Do not reuse pipette tips and tubes to avoid cross contamination.
9. Do not use expired components or components from a different kit.
10. The TMB Substrate is light sensitive and should be protected from direct sunlight and UV sources. Store the TMB substrate in the dark and to avoid edge effect of plate incubation for temperature differences it is recommended to equilibrate the TMB substrate for 30 min at room temperature. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.

D. Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, medium and high levels of NT were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, medium and high levels of NT were tested on 3 different plates, 8 replicates in each plate.

$$CV (\%) = \frac{SD}{\text{mean}} \times 100$$

Intra-Assay: CV < 10%

Inter-Assay: CV < 12%