Version 17-04/24

User's Manual

Nitrotyrosine ELISA Kit

REF **DEIA3981**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

Nitrotyrosine ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the competitive measurement of Free Nitrotyrosine protein in plasma, serum, cell lysates, urine, and other sample matrices. The ELISA utilizes an Nitrotyrosine-coated plate and an HRP-conjugated antibody for detection which allows for an assay range of 62.5 to 8000 nM Free Nitrotyrosine, with a sensitivity of 50 nM. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

General Description

Nitrotyrosine has been identified as a marker of inflammation and NO production. Nitrotyrosine is formed in presence of the active metabolite NO. Various pathways including the formation of peroxinitrite lead to nitrotyrosine production. Since nitrotyrosine is a stable end product of peroxynitrite oxidation, assessment of its plasma concentration may be useful as a marker of NO-dependent damage in vivo. Since NO_X is only an indicator for enhanced NO production, protein associated nitrotyrosine might be a more suitable marker for damage induced by reactive nitrogen intermediates derived from NO. Furthermore, most proteins have a longer half-life in the circulation than NO_X levels. The presence of nitrotyrosine has been detected in various inflammatory processes including atherosclerotic plaques, celiac disease, rheumatoid arthritis, chronic renal failure and septic shock. In normal plasma low, undetectable, levels of nitrotyrosine are present.

Nitrosylation of the amino acid tyrosine occurs both for free tyrosine and for protein bound tyrosine.

Principles of Testing

The microtiter wells are coated with capture Nitrosylated BSA. Free Nitrotyrosine protein in the sample competes with antigen coated on the microtiter plate for the antibody. After the addition of enzyme conjugate, TMB Substrate is used and the signal is measured by spectrophotometer. The absorption is inversely proportional to the Nitrotyrosine concentration in the sample.

Reagents And Materials Provided

1. Nitrosylated BSA Coated Plate: 1 x 96 tests
2. Anti-Nitrotyrosine:HRP Conjugated Detection Antibody: 1 x 75µl
3. Nitrotyrosine Antibody Diluent (Blue) : 1 x 13ml
4. Nitrotyrosine Standard: 1 x 110µl
5. Plate Cover: 2 units
6. Sample and Standard Diluent (Red) : 1 x 50ml
7. Stop Solution: 1 x 13ml
8. TMB Substrate: 1 x 13ml
9. Wash Buffer Concentrate (10×): 1 x 50ml

Materials Required But Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

1. Microplate reader capable of measuring absorbance at 450 nm
2. Double distilled water (ddH2O)
3. Pipettes and pipette tips, including multi-channel pipette
4. Assorted glassware for the preparation of reagents and buffer solutions
5. Tubes for the preparation of reagents and buffer solutions
6. Timer
7. Centrifuge capable of spinning at 2000×g
8. 0.2 µm filter
9. Log-log graph paper or computer and software for ELISA data analysis

Storage

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Specimen Collection And Preparation

General Sample Information

1. We recommend that you use fresh samples. We suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
2. All samples must be free of organic solvents prior to assay.
3. Samples that cannot be assayed immediately should be stored as indicated below.
4. 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.

Plasma/Serum

1. Storage: Collect plasma using established methods and store at - 80°C.
2. Dilution: Serum samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

Cell Lysates

1. Storage: Collect lysates using established methods and store at -80°C until use.

Urine

1. Storage: Fresh urine samples should be centrifuged at 2,000 × g for 10 minutes or filtered with a 0.2 µm filter before this assay, and stored at -20°C immediately after collection.
2. Dilution: Urine samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

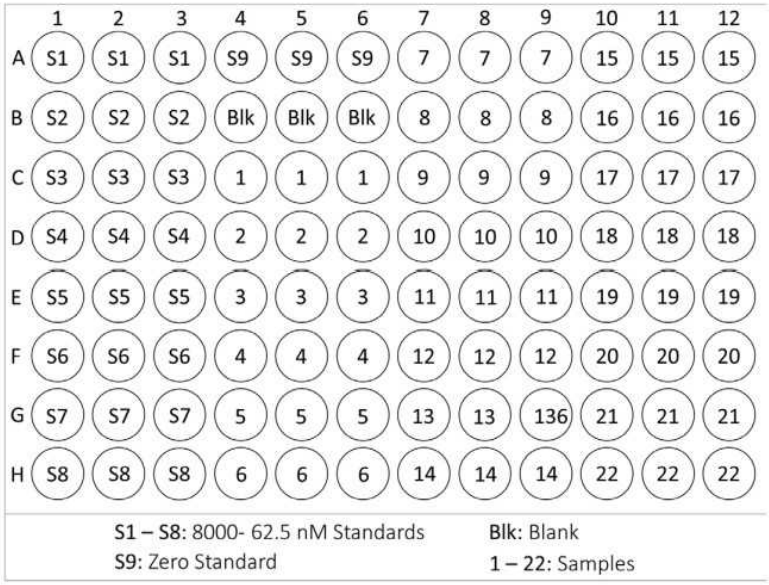
Plate Preparation

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents.

NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.

For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis. We suggest you record the contents of each well on the template sheet provided.



Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

1. **1× Wash Buffer:**

   Prepare 1× wash buffer by diluting 10× wash buffer in distilled or deionized water. For example, if preparing 500 mL of 1× wash buffer, dilute 50 mL of 10× wash buffer into 450 mL of distilled water. Mix well.

   NOTE: Store reconstituted 1× wash buffer at 4°C for up to one (1) month. Do not use 1× wash buffer if it becomes visibly contaminated during storage.

2. **Anti-Nitrotyrosine: HRP Conjugated Detection Antibody**

   Determine the amount of Antibody Preparation required. For every strip-well used (8-wells), prepare 0.5 mL of Antibody Preparation. Prepare Antibody Preparation by diluting the Anti-Nitrotyrosine:HRP Conjugated Detection Antibody Concentrate 1:100 with Nitrotyrosine Antibody Diluent. For example, if 6 mL of Antibody Preparation is required (one whole plate), dilute 60 µL of Antibody in 6 mL of Nitrotyrosine Antibody Diluent. Mix well prior to use.

3. **Nitrotyrosine Standard**

Note: Always prepare a fresh set of standards for every use. Diluted standard solution is unstable and must be prepared immediately prior use. Do not store for future use.

1) Centrifuge the Nitrotyrosine Standard (stock) vial before removing the cap. This process with assure that all of the standard is collected and available for use.
2) Label eight (8) polypropylene tubes, each with one of the following standard values: 8000 nM, 4000 nM, 2000 nM, 1000 nM, 500 nM, 250 nM 125 nM and 62.5 nM.
3) Add 500 µL of Sample and Standard Diluent to Tube #1.
4) Add 250 µL of Sample and Standard Diluent to Tube #2, 3, 4, 5, 6, 7 and 8.
5) Add 10 µL of the 408 µM Nitrotyrosine Standard to Tube #1. Mix well.
6) Transfer 250 µL from Tube #1 to Tube #2. Mix well.
7) Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix well, etc.) up to and including Tube #8.
8) Finally, add 250 µL Sample and Standard Diluent to another 1.5 mL polypropylene tube (Tube #9), which is the zero standard (0 ng/mL).

Standard #	Standard volume	Assay Buffer volume	Final volume	End Conc. (nmol)
Stock	N/A	N/A	N/A	408 µM
S1	10 µL of stock	500 µL	250 µL	8000
S2	250 µL of S1	250 µL	250 µL	4000
S3	250 µL of S2	250 µL	250 µL	2000
S4	250 µL of S3	250 µL	250 µL	1000
S5	250 µL of S4	250 µL	250 µL	500
S6	250 µL of S5	250 µL	250 µL	250
S7	250 µL of S6	250 µL	250 µL	125
S8	250 µL of S7	250 µL	500 µL	62.5
S9 (Blank)	N/A	250 µL	250 µL	0

Assay Procedure

Note:

Equilibrate all materials and prepared reagents to correct temperature prior to use.

We recommended to assay all standards, controls and samples in triplicate.

Prepare all reagents, working standards, and samples as directed in the previous sections.

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.

1. Addition of the Reagents

1) Add 50 µL (in triplicate) of each of the following to appropriate wells:
2) Prepared Nitrotyrosine Standard (Tube #1 through Tube #8) into wells labelled S1-S8
3) Zero Standard (Tube #9- Sample and Standard Diluent, which represents 0 nM into wells labelled S9.

4) Samples (previously prepared) into wells labelled 1-22.

5) Add 50 µL of the previously diluted Nitrotyrosine Antibody Preparation to each well, except the blank.

6) Add 50 µL of Standard and Sample Diluent and 50 µL of Antibody Diluent into wells labelled as the blank.

2. Incubate the Plate

1) Cover each plate with the plate cover and incubate 1 hour at room temperature (20- 25˚C).

3. Plate Washing

1) Carefully remove adhesive plate cover. Gently squeeze the long sides of the plate frame before washing to ensure all strips remain securely in the frame.

2) Empty plate contents. Use a multi-channel pipette to fill each well completely (300 µL) with 1× Wash buffer, then empty plate contents. Repeat procedure three additional times, for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

   NOTE: Follow the same procedure when using an automated plate washer as well. Take care to avoid microbial contamination of equipment. Automated plated washers can easily become contaminated thereby causing assay variability.

4. TMB Substrate Incubation and Reaction Stop

1) Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used.

2) Do NOT use a glass pipette to measure the TMB Substrate solution. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT.

3) Add 100 µL of TMB Substrate into each well.

4) Cover carefully with the second provided plate cover.

5) Allow the enzymatic color reaction to develop at room temperature (20-25°C) in the dark for 30 minutes. The substrate reaction yields a blue solution.

6) After 30 minutes, carefully remove the plate cover, and stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

5. Absorbance Measurement

NOTE: Evaluate the plate within 30 minutes of stopping the reaction.

1) Wipe underside of wells with a lint-free tissue.

2) Measure the absorbance on an ELISA plate reader set at 450 nm.

Calculation

1. Many plate readers come with data reduction software that plot data automatically

2. The following procedure is recommended for preparation of the data prior to graphical analysis.

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

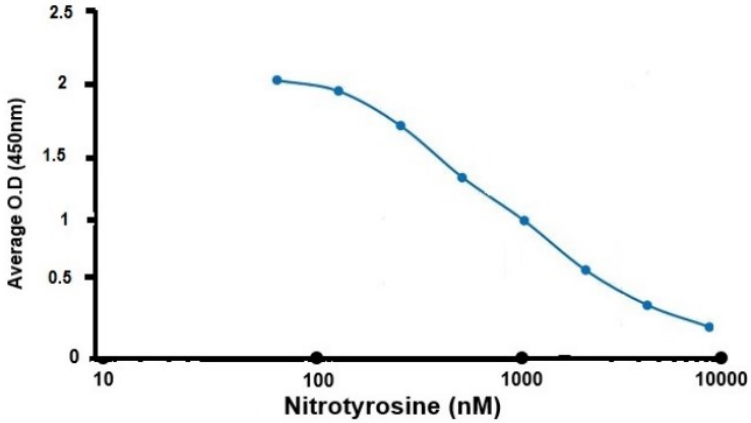
2) Plot Net OD versus Concentration of Nitrotyrosine for the standards. Sample concentrations may be calculated off of Net OD values using the desired curve fitting.

3) 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.

Typical Standard Curve

Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



Precision

Intra-Assay Precision: To determine Intra-Assay Precision, three samples of known concentration were assayed thirty times on one plate. The intra-assay coefficient of variation of the Nitrotyrosine ELISA has been determined to be <10%.

Inter-Assay Precision: To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The inter-assay coefficient of variation of the Nitrotyrosine ELISA has been determined to be <15%.

Detection Range

62.5 nM - 8000 nM

Sensitivity

The sensitivity of the Nitrotyrosine ELISA kit has been determined to be 50 nM pure nitrotyrosine.

Precautions

Please read these instructions carefully prior to beginning the assay.

1. All kit components have been formulated and quality control tested to function successfully as a kit.
2. We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
3. Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific

components.

4. Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
5. All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
6. All ELISA reagents must be at room temperature before use.
7. Vigorous plate washing is essential.
8. Use new disposable pipette tips for each transfer to avoid crosscontamination.
9. Use a new adhesive plate cover for each incubation step.
10. Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
11. Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
12. 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.
13. Include a standard curve each time the assay is performed.
14. Run both standards and samples in triplicate.
15. Buffers may crystallize over time. Warm crystallized buffer until the salt crystals return to solution. Ensure that your components return to RT before use in the assay.
16. This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
17. Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
18. Avoid foaming or bubbles when mixing or reconstituting components.
19. Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
20. Ensure plates are properly sealed or covered during incubation steps.
21. Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

22. Completely aspirate all solutions and buffers during wash steps.
23. When preparing your standards, it is critical to briefly spin down the vial first.
24. The Standard should be aliquoted into smaller portions; it should be used right away or else frozen for later use.
25. Be sure to discard the working standard dilutions after use – they do not store well.

Limitations

1. Assay kit intended for research use only. Not for use in diagnostic procedures.

2. Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
3. Troubleshooting:

Problem	Cause	Solution
Poor Standard Curve	Improper standard solution	Confirm dilutions are made correctly.
	Standard degraded	Store and handle standard as recommended.
	Curve doesn't fit scale	Try plotting using different scales
	Pipetting Error	Use calibrated pipettes and proper pipetting technique.
No Signal	Plate washings too vigorous	Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
	Wells dried out	Do not allow wells to dry out. Cover the plate for incubations.
High Background	Wells are insufficiently washed	Wash wells as per protocol
	Contaminated wash buffer	Prepare fresh wash buffer
	Waiting too long to read the plate after adding stop solution	Read plate immediately
Low sensitivity	Standard is degraded	Replace standard
	Mixing or substituting reagents from other kits	Avoid mixing components