_Oxis_Research™
A Division of OXIS Health Products, Inc.

BIOXYTECH® Nitrotyrosine-EIA
Enzyme Immunoassay for Nitrotyrosine
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Catalog Number 21055.

INTRODUCTION

The Analyte

Various pathways, including the formation of peroxynitrite, lead to a stable nitrotyrosine product in biological systems (1,2,3). Peroxynitrite formed by the reaction between two free radicals, nitric oxide and superoxide, reacts with the phenolic ring of tyrosine and forms nitrotyrosine. As a strong oxidant as well as nitrating agent, peroxynitrite mediates tyrosine nitration reactions with enzymes, for example, inactivation of a-antiproteinase (4) and endogenous antioxidants/enzymes such as catalase and Mn-SOD in many biological and cell culture systems (5,6,7). Thus nitrotyrosination is involved in the pathology of several inflammatory human diseases such as: chronic myocardial dysfunction, respiratory distress syndrome, inflammatory bowel disease, lung injuries, asthma, atherosclerotic plaques, rheumatoid arthritis, chronic renal failure, Lou-Gherig's disease (ALS or amyotrophic lateral sclerosis), septic shock, etc. (8,9,10,11,12). For example, nitrotyrosine in rheumatoid patients' serum levels range up to 1.2 μM, compared to a healthy control group, 0 μM, $p < 0.02$ (13). Demonstration of nitrotyrosine in biological samples (tissues, plasma and urine) therefore, infers the presence of peroxynitrite or related nitrogen-centered oxidants.

Principle of the Procedure

The BIOXYTECH® Nitrotyrosine-EIA is a "sandwich" ELISA. Antigen captured by a solid phase monoclonal antibody (nitrated keyhole limpet hemocyanin raised in mouse) is detected with a biotin-labeled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate then binds to the biotinylated antibody. A tetramethylbenzidine (TMB) substrate is added and the yellow product is measured at 450 nm.

REAGENTS

Materials Provided

• Dilution Buffer (10X)	Protein stabilized phosphate buffered saline, containing 2-chloroacetamide as preservative, 10x concentrated, 10 mL.
• Nitrotyrosine Standard	Lyophilized, 4.5 μM when reconstituted.
• Nitrotyrosine Antibody	Lyophilized (2 vials).
• Streptavidin Peroxidase	Lyophilized, containing 2-chloroacetamide as preservative.
• TMB Substrate	6 mL.
• Substrate Buffer	6 mL.
• Substrate Dilution Buffer	12 mL.
• Washing Buffer (40X)	Containing Tween-20, 40x concentrated, 20 mL.
• Stop Solution	Containing citric acid, 2.0 M, 22mL.
• Precoated Plate	Two microplates coated with nitrotyrosine antibody.

Materials Required But Not Provided

- Deionized water (DI H_2O)
- Test tubes and beakers
- Adjustable pipettes (50-500 μL) with disposable tips, a multi-channel pipette is helpful.
- Microplate reader for absorbance measurements 450 nm, preferably temperature-controlled to 25°C.

Warnings and Precautions

Use established laboratory precautions when handling or disposing any chemical contained in this product. Refer to the Material Safety Data Sheet for risk, hazard, and safety information. If any of the components come in contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.

Reagent Storage and Handling

DO NOT FREEZE. Store all components at 2-8°C until immediately before use. Only use the 96-well precoated plates supplied with the kit. All reconstituted solutions are good for 1 month at 2-8°C.

PROCEDURE

Reagent Preparation

All reagents should be brought to room temperature prior to use.

- **Dilution Buffer:** Mix one part Dilution Buffer (10X) with 9 parts DI H_2O (for example, mix the 10 mL supplied with 90 mL water for 100 mL of "*Working Dilution Buffer*").

- **Nitrotyosine Antibody:** Reconstitute a vial of Nitrotyrosine Antibody with 1 mL DI H_2O (one vial is provided for each of the two plates). Add 11 mL of the *Working Dilution Buffer* to the reconstituted antibody. This solution may be stored at 2-8°C for 1 month. For longer storage, immediately prepare aliquots and store at -70°C. Avoid freeze thaw cycles.

- **Streptavidin Peroxidase:** Reconstitute the vial of Streptavidin Peroxidase with 1 mL DI H_2O. Add 23 mL of the *Working Dilution Buffer* to the vial. This solution may be stored at 2-8°C for 1 month. For longer storage, immediately prepare aliquots and store at -70°C. Avoid freeze thaw cycles.

- **Washing Buffer:** This solution should be diluted 40 times with DI H_2O (for example, mix the 20 mL supplied with 780 mL of water for 800 mL of "*Working Wash Buffer*").

- **Stop Solution:** Ready to use.

- **TMP Substrate:** The TMP Substrate is prepared just prior to use, and therefore, its preparation is described as part of the Assay Procedure. Once prepared, it must be kept in the dark until use.

Standard Preparation

1. Reconstitute the contents of the Nitrotyrosine Standard with DI H_2O. The required volume is on the vial label. This solution is 4.5 µM.
2. Label 8 test tubes 1-8.
3. Add 300 µL of *Working Dilution Buffer* into tubes 1 through 7.
4. Add 500 µL of *Working Dilution Buffer* into tube 8. This is the zero standard.
5. Pipet 150 µL of reconstituted Nitrotyrosine Standard into tube1 and mix well.
6. Transfer 150 µL of solution from tube 1 into tube 2 and mix well.
7. Continue the serial dilution of 150 µL from each tube to the next through to tube 7. **Do not add anything to tube 8!**
8. These solutions may be stored at 2-8°C for 1 month. For longer storage, immediately prepare aliquots and store at -70°C. Avoid freeze thaw cycles.

Standard Tube Concentrations:

Tube	1	2	3	4	5	6	7	8
nM	1500	500	166.7	55.6	18.5	6.2	2.1	0

Sample Preparation

Samples should be diluted, if necessary, using *Working Dilution Buffer*. A sample volume of 100 µL is required for each replicate desired.

Plasma Guidelines:

1. Draw blood in EDTA or heparin tube.
2. Centrifuge whole blood within 2 or 3 hours of draw at 3000 x *g* for 10 minutes at 4°C.
3. Remove plasma supernatant. Plasma samples can be stores at 2-8°C for 24 hours.
4. For longer storage, sample should be stored at = -20°C. Avoid freeze/thaw cycles.

Assay Procedure

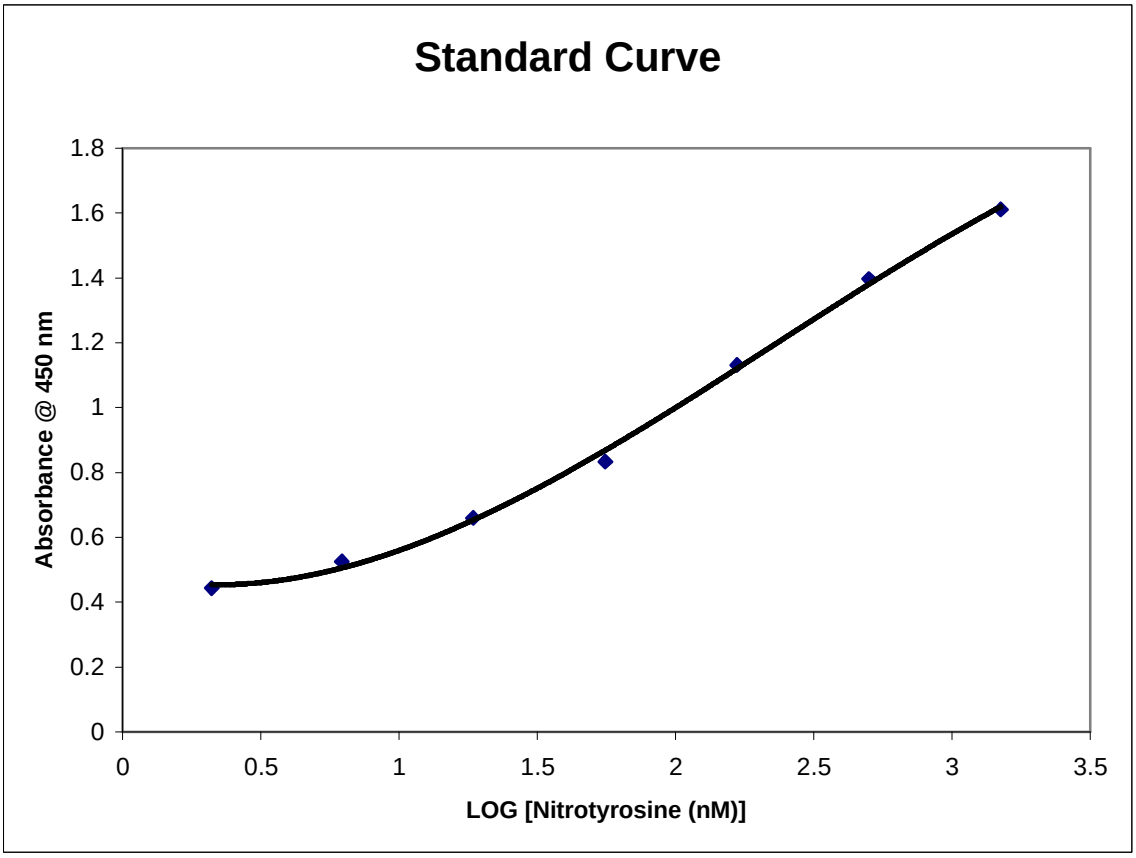
1. Add 100 µL of standard, sample or control to each well, as appropriate.
2. Cover the plate and incubate 1 hour at room temperature.
3. Wash wells 4 times with Wash Buffer. Empty the contents and blot dry on a lint-free paper towel.
4. Add 100 µL Nitrotyrosine Antibody.
5. Cover the plate and incubate 1 hour at room temperature.
6. Wash wells 4 times with Wash Buffer. Empty the contents and blot dry on a lint-free paper towel.
7. Add 100 µL Streptavidin Peroxidase.
8. Cover the plate and incubate 1 hour at room temperature.
9. Prepare TMB Substrate 15 minutes prior to the completion of the Streptavidin Peroxidase incubation:
   a. Mix 6 mL of TMB Substrate with 6 mL Substrate Buffer and 12 mL Substrate Dilution Buffer.
   b. If less than both plates are used at one time, prepare only the volume needed with a 1:1:2 ratio as above.
   c. Store in the **DARK** and use within 15 minutes of preparation.
10. Wash wells 4 times with Washing Buffer. Empty the contents and blot dry on a lint-free paper towel.
11. Add 100 µL prepared TMB Substrate.
12. Cover the plate and incubate at room temperature for 30 minutes in the **DARK.**
13. Add 100 µL Stop Solution and mix well.
14. Read the absorbance at 450 nm.

Calculations

1. The mean absorbance value of the “zero standard” should be less than 0.4.
2. Calculate the average absorbance values for each set of replicates for standards, samples and controls.
3. Plot a standard curve of absorbance values at 450 nm as a function of the logarithm of nitrotyrosine standard concentrations. A standard curve may be plotted graphically by point-to-point or using third-order polynomial regression (see below).
4. Read the concentration of samples from the curve and their corresponding absorbance values.

Sample Calculation

A plasma sample was collected in an EDTA tube. The plasma was diluted 10x with *Working Dilution Buffer* and assayed in triplicate. The average of the triplicate absorbance values at 450 nm (0.848, 0.827 and 0.824) for the sample is 0.833. An absorbance of 0.833 on the curve corresponds to 1.75 for the log of concentration. This is equivalent to 56.2 nM Nitrotyrosine (the inverse log of 1.75, $10^{1.75}$). The result is multiplied by the original dilution of the plasma (10x) to obtain the concentration in the original sample, 562 nM.



PERFORMANCE CHARACTERISTICS

Assay Precision

Intra Assay (%CV)	2.32
Inter Assay (%CV)	11.17

Sensitivity

The detection limit of the assay is 2 nM.

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