
OxisResearch™

A Division of OXIS Health Products, Inc.

BIOXYTECH® Nitrotyrosine-EIA

Enzyme Immunoassay for Nitrotyrosine

For Research Use Only. Not For Use in Diagnostic Procedures.

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 α -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 μ M, compared to a healthy control group, 0 μ M, $p < 0.02$ (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 μ M when reconstituted.
- Nitrotyrosine Antibody Lyophilized (2 vials).
- Streptavidin Peroxidase Lyophilized, containing 2-chloroacetamide as preservative.
- TMB Substrate 22 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 H₂O)
- Test tubes and beakers
- Adjustable pipettes (50-500 μ L) 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.

Do not under any circumstances add sodium azide as preservative to any of the components since this will inactivate the peroxidase used in the assay.

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 H₂O (for example, mix the 10 mL supplied with 90 mL water for 100 mL of "*Working Dilution Buffer*").
- **Nitrotyrosine Antibody:** Reconstitute a vial of Nitrotyrosine Antibody with 1 mL DI H₂O (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 H₂O. 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 H₂O (for example, mix the 20 mL supplied with 780 mL of water for 800 mL of "*Working Wash Buffer*").
- **Stop Solution:** Ready to use.
- **TMB Substrate:** Ready to use.

Standard Preparation

1. Reconstitute the contents of the Nitrotyrosine Standard with DI H₂O. 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 tube 1 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 and serum samples must be diluted at least 10x before use.

Plasma Guidelines:

1. Draw blood in EDTA or heparin tube. Best results if EDTA plasma is used.
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 stored at 2-8°C for 24 hours.
4. For longer storage, sample should be stored at $\leq -20^\circ\text{C}$. Avoid freeze/thaw cycles.

Assay Procedure

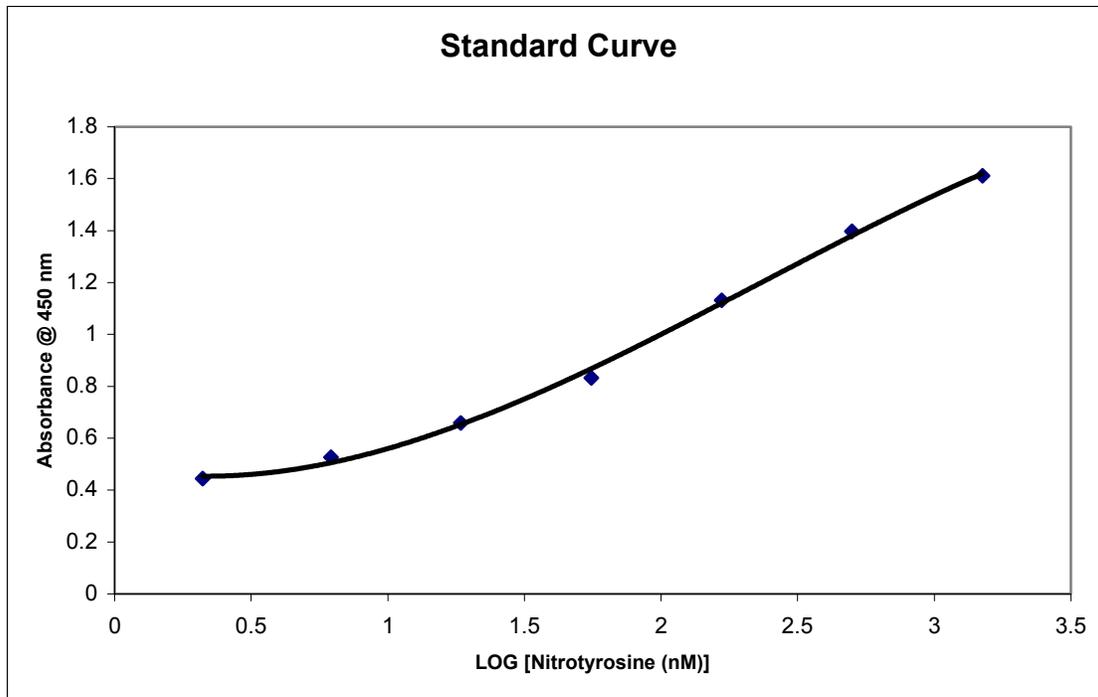
1. Add 100 μL of standard, sample or control to each well, as appropriate.
2. Cover the plate and incubate 1 hour at room temperature.
3. Wash wells 4 times with Wash Buffer. Empty the contents and blot dry on a lint-free paper towel.
4. Add 100 μL Nitrotyrosine Antibody.
5. Cover the plate and incubate 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. Wash wells 4 times with Washing Buffer. Empty the contents and blot dry on a lint-free paper towel.
10. Add 100 μL TMB Substrate.
11. Cover the plate and incubate at room temperature for 30 minutes in the **DARK**.
12. Add 100 μL Stop Solution and mix well.
13. 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.

REFERENCES

1. van der Vliet A, et al. (2000) "Nitric oxide: a proinflammatory mediator in lung disease?" *Rep. Res.* **1**: 67-72.
2. Wu W, et al. (1999) "Eosinophil peroxidase nitrates protein tyrosyl residues" *J. Biol. Chem.* **274(36)**: 25933-44.
3. van der Vliet A, et al. (1999) "Reactive nitrogen species and tyrosine nitration in the respiratory tract: Epiphenomena or a pathobiological mechanism of disease" *Am. J. Respir. Cri. Care. Med.* **160**: 1-9.
4. Whiteman M., et al. (1996) "Protection against peroxynitrite-dependent tyrosine nitration and α -antiproteinase inactivation by oxidized and reduced lipoic acid" *FEBS Lett.* **379**: 74-6.
5. Keng T., et al. (2000) "Peroxynitrite formation and decreased catalase activity in autoimmune MRL-lpr/lpr mice" *Mol. Med.* **6(9)**: 779-92.
6. MacMillan-Crow LA, et al. (1996) "Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts" *Proc. Natl. Acad. Sci. USA* **93**: 11853-8.
7. Monezen SL, Augustus O (2001) "EPR detection of glutathionyl and protein- tyrosyl radicals during the interaction of peroxynitrite with macrophages (J774)" *J. Biol. Chem.* **276(43)**: 37679-84.

8. Kooy NW, et al. (1997) "Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite" *Crit. Care Med.* **25(5)**: 812-9.
9. Beal MF, et al. (1997) "Increased 3-nitrotyrosine in both sporadic and familial amyotrophic sclerosis" *Ann. Neurol.* **42**: 646-54.
10. Sittipunt C, et al. (2001) "Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome" *Am. J. Res. Crit. Care Med.* **163**: 503-510.
11. Singer II, et al. (1996) "Expression of inducible nitric oxide and nitrotyrosine in colonic epithelium in inflammatory bowel disease" *Gastroenterology* **111**: 8771-85.
12. Chang L-Y, Crapo JM, (2002) "Inhibition of airway inflammation and hyper-reactivity by an antioxidant mimetic" *Free Radic. Biol. Med.* **33(3)**: 379-85.
13. Kaur H, Halliwell B, (1994) "Evidence for nitric-oxide-mediated oxidative damage in chronic inflammation: nitrotyrosine in serum and synovial fluid from rheumatoid patients" *FEBS Lett.* **350**: 9-12.

LIMITED WARRANTY

OXIS Health Products, Inc. warrants that, at the time of shipment, this product is free from defects in materials and workmanship. OXIS Health Products, Inc. makes no warranty, expressed or implied, including, but not limited to, the warranties of fitness for a particular use and merchantability which extends beyond the description of the product on the face hereof, except that this product will meet our specifications at the time of shipment.

OXIS Health Products, Inc. must be notified of any breach of this warranty within 14 days of receipt of this product. No claim shall be honored if OXIS Health Products, Inc. is not notified within this time period, or if the product has been stored in any way other than described in this product insert. The sole and exclusive remedy of the buyer for any liability based upon this warranty is limited to the replacement of this product, or refund of the invoice price of the goods. OXIS Health Products, Inc. shall not be liable otherwise or for incidental or consequential damages, including but not limited to the costs of handling.

TECHNICAL SUPPORT

An OXIS Health Products, Inc. Technical Support Representative can be reached by telephone at (800) 547-3686, (503) 283-3911, or by e-mail techsupport@oxis.com Monday through Friday 8:00 AM to 5:00 PM Pacific Time.

OxisResearch™
6040 N. Cutter Circle, Suite 317
Portland, OR 97217-3935 U.S.A.
503-283-3911 or 800-547-3686 Fax: 503-283-4058
Last revision January 2005

Made in the U.S.A.
BIOXYTECH® is a registered trademark of OXIS International, Inc.
Portland, OR 97217-3935
Copyright© 2002 OXIS Health Products, Inc. All rights reserved