



Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSTM ***Nitrotyrosine ELISA***

Product NWK-NTR01
For Research Use Only



Simple ELISA kit for quantification of nitrotyrosine in biological samples.



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Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Notes:

Procedure Checklist

- Create an assay template
- Equilibrate reagents to room temperature.
- Set-up required number of strips in frame supplied.
- Prepare reagents
 - Reconstitute lyophilized components
 - Dilute Reagent Vials 1, 2, 4 and 5 as required.
- Perform a 1/3 serial dilution of the 4500 nM standard
- Prepare samples, making any necessary dilutions.
- Add 100 μ L standard, sample or control to each replicate well according to assay template.
- Apply Adhesive Cover and Incubate 1 Hour at room temperature.
- Wash wells 4 times with 200 μ L Working Wash Buffer
- Add 100 μ L Diluted Tracer to each well.
- Apply Adhesive Cover and Incubate 1 Hour at room temperature.
- Wash wells 4 times with 200 μ L Working Wash Buffer
- Add 100 μ L of diluted Streptavidin-Peroxidase Conjugate to each well.
- Apply Adhesive Cover and Incubate 1 Hour at room temperature.
- Wash wells 4 times with 200 μ L Working Wash Buffer
- Add 100 μ L TMB Substrate Solution to each well.
- Apply Adhesive Cover and incubate 20-30 minutes at room temperature in the dark.
- Stop the reaction by adding 100 μ L Stop Solution.
- Measure absorbance at 450 nm
- Analyze data using standard curve plot.

Introduction:

Nitrate (NO_3) and nitrite (NO_2) production are often used as markers of nitric oxide (NO) production. These analytes by themselves fail to address the eventual fate of NO or the possible adverse effects associated with its excess production and reaction with free radical species *in vivo*. Active NO metabolites can react with superoxide to form peroxynitrite (ONOO \cdot) a powerful oxidant and nitrating agent. Subsequent reaction of ONOO \cdot with proteins results in nitrotyrosine (NT) formation. As a stable end product of ONOO \cdot mediated oxidation/nitration, NT can be used as a surrogate index of NO dependent damage *in vivo* and has been associated with multiple disease states.

Intended Use:

The NWLSSTM Nitrotyrosine ELISA assay is intended for the quantitative measurement of nitrosylated protein adducts in biological samples. The NWLSSTM product allows quantification of oxidative/nitrosative stress and peroxynitrite formation on proteins in biological systems.

Test Principle:

The NWLSSTM Nitrotyrosine ELISA test is a simple "sandwich" ELISA using a plate bound capture antibody (anti nitrated KLH) to nitrotyrosine and a biotinylated secondary tracer antibody. Addition of streptavidin-peroxidase followed by tetramethylbenzidine (TMB) facilitates color development directly proportional to the nitrotyrosine present in the sample. The reaction is stopped using a citric acid solution and the assay is read on a plate reader at **450 nm**.

Specifications:

Format::	2 X 96 well ELISA	
Number of tests:	Triplicate =	48
	Duplicate =	80
Specificity:	Nitrosylated protein adducts	
Sensitivity:	2 nM	
Range:	2 nM—1500 nM	

Kit Contents:

Vial 1:	40X Concentrated Wash Buffer containing Tween-20: 20 mL
Vial 2:	10X Concentrated Dilution Buffer. Protein stabilized phosphate buffered saline containing 2-chloroacetamide preservative: 10 mL
Vial 3:	Lyophilized Nitrotyrosine Standard. Concentration is lot specific and indicated on label.
Vials 4A & 4B	Lyophilized Biotinylated Anti-nitrotyrosine in protein stabilized buffer with 2-chloroacetamide preservative.
Vial 5:	Lyophilized concentrated Streptavidin-peroxidase conjugate with 2-chloroacetamide preservative.
Vial 6:	TMB Substrate: 22 mL
Vial 7:	Stop Solution containing citric acid: 22 mL
Plates:	96 well microplates (12 X 8 well strips) pre-coated with anti-nitrotyrosine antibody: 2 each
Frames:	Microwell strip frames: 2 each
Covers:	Adhesive plate covers: 4 each

Required Materials Not Provided:

Adjustable micropipettes with disposable tips (50-1000 μ L). Multi-channel pipettes are useful and help to reduce intra-sample variability.

Serological pipettes.

Deionized water.

Polypropylene tubes

Automatic plate washer or other aspiration devices are optional.

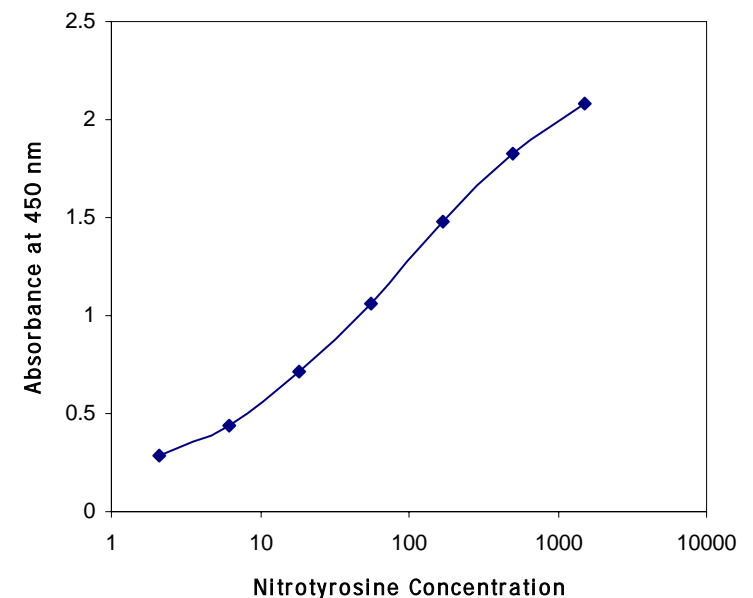
Required Instrumentation:

Plate reader with **450 nm** capability.

Data Analysis:

1. Plot the mean absorbance at 450 nm for each standard replicate versus the nitrotyrosine concentrations setting x-axis to log scale. We recommend using a 4-parameter curve fit. This can typically be done using the software provided with most modern plate readers. An example curve is shown below.

Absorbance vs. Nitrotyrosine Concentration



2. Unknown nitrotyrosine concentrations are determined by comparing their absorbance measurements at 450 with those of the standard curve.

Assay Protocol:

Allow approximately 4.5 hours for procedure.

1. Add 100 μ L standard, sample or control to each well according to the assay template created earlier.
2. Cover the wells with adhesive tape and incubate for 1 hour at room temperature (18-25°C).
3. Carefully remove the adhesive cover. Empty the plate by inverting and shaking contents over sink. Keep inverted and tap dry on a thick layer of tissues or paper towels. Wells may also be aspirated using a multi-channel pipette or plate washer. In either case, wash the plate 4 times with 200 μ L Working Wash Buffer waiting 20 seconds before aspirating.
5. Add 100 μ L of Diluted Tracer to each well keeping the same sequence as used in step 1. Avoid touching the side or top of the wells.
6. Cover the tray with adhesive tape and incubate for 1 hour at room temperature.
7. Repeat washing procedure as above.
8. Add 100 μ L of Diluted Streptavidin Peroxidase Conjugate to each well keeping the same sequence as used earlier.
9. Cover the tray with adhesive tape and incubate for 1 hour at room temperature.
10. Repeat washing procedure as above.
11. Add 100 μ L of TMB substrate solution (Vial 6) to each well, keeping the same sequence as used in earlier steps.
12. Cover the tray with adhesive tape and incubate in the dark for 20-30 minutes at room temperature.
13. Stop the reaction by adding 100 μ L stop solution (Vial 7) taking care to use the same sequence and timing as previous steps.
14. Measure the absorbance at 450 nm.

The mean absorbance for the zero buffer only control should be < 0.4.

Warnings, Limitations, Precautions:

Do not add sodium azide as preservative to any component since its presence will inactivate the peroxidase conjugate.

Components containing 2-chloroacetamide and citric acid may be hazardous if in direct contact with skin, eyes, etc. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact occurs, rinse the site immediately with water.

Storage Instructions:

Upon receipt, store this product at 2-8 °C...**DO NOT FREEZE.** Lyophilized components are stable for 1 month after reconstitution if stored at 2-8°C.

All reagents should be brought to room temperature (18-25°C) prior to use and stored at 2-8°C immediately after use.

Prolonged exposure of kit components to light should be avoided.

Coated microwell strips may be used until the product expiration date as long as they are returned to pouch and stored dry at 2-8°C.

Assay Preparation

1. Determine the number of wells required to assay standards, samples and controls for the appropriate replicate. It is recommended that testing be performed in duplicate or triplicate if possible.
2. Create an assay template showing positioning of standards, controls and samples.
3. Bring all samples and reagents to room temperature before use.
4. To avoid condensation, do not open foil pouches containing the microtiter strips until after they have reached room temperature. Next remove the required number of strips and place in the frame supplied.

Note: The bottom of some wells may be slightly white due to the preservation treatment. This does not influence assay performance.

Return unused wells to the storage bag with desiccant, seal and store at 2-8°C.

Reagent Preparation:**1. Vial 1, 40X Wash Buffer:**

For each 96 well plate to be assayed, dilute 10 mL of 40X Wash Buffer with 390 mL de-ionized water and/or for each 8 well strip to be assayed dilute 833 μ L of 40X Wash Buffer with 32.5 mL deionized water. Label as **Working Wash Buffer**

2. Vial 2, 10X Dilution Buffer:

For each 96 well plate to be assayed dilute 5 mL of 10X Dilution Buffer with 45 mL de-ionized water and/or for each 8 well strip to be assayed dilute 417 μ L of 10X Dilution Buffer with 3.75 mL deionized water. Label as **Working Dilution Buffer**

Note: Lyophilized components are under vacuum. In order to avoid product loss, allow pressure to equalize slowly before opening fully.

3. Vial 3: Lyophilized Standard:

Reconstitute the standard with deionized water according to the directions on the vial label to create a 4500 nM stock solution.

4: Vial 4A & 4B, Lyophilized, Biotinylated Anti-nitrotyrosine Tracer:

Reconstitute 1 vial with 1 mL deionized water per 96 well plate assayed. Next dilute the 1 mL reconstituted Tracer with 11 mL Working Dilution Buffer and/or for each 8 well strip to be assayed dilute 83 μ L reconstituted Tracer with 917 μ L Working Dilution Buffer.

5. Vial 5, Lyophilized, Streptavidin-Peroxidase (SAP) Conjugate:

Reconstitute the vial with 1 mL deionized water. For each 96 well plate to be assayed dilute 0.5 mL reconstituted SAP Conjugate with 11.5 mL Working Dilution Buffer and/or for each 8 well strip to be assayed, dilute 42 μ L reconstituted SAP Conjugate with 958 μ L Working Dilution Buffer.

6. Vial 6, TMB Substrate: and Vial 7, Stop Solution are supplied ready to use.

Standard Curve Preparation:

1. Label microtubes 1-8 and add 350 μ L Working Dilution Buffer to each.

2. Transfer 175 μ L of 4500 nM standard to tube 1, mix well and continue 1/3 serial dilutions across tubes 2-7 creating standards of 1500 - 2.1 nM. Leave tube 8 as a buffer only zero control.

Std Tube #:	1	2	3	4	5	6	7	8
Conc. (nM):	1500	500	166.7	55.6	18.5	6.2	2.1	zero control

Standards may be stored for 1 month at 2-8°C or longer at -80°C.

Sample Handling/Preparation

1. Before performing the assay, all samples should be brought to room temperature.

2. Samples should be diluted if necessary with Working Dilution Buffer and mixed gently taking care to avoid foaming.

Note that 100 μ L sample is required per replicate well.

Plasma or Serum samples:

1. Blood samples should be stored on ice prior to separation.

2. For plasma, EDTA anticoagulant is recommended.

3. Samples frozen long term at -80°C are suitable for assay however sample storage at -20°C may adversely affect recovery of the NT analyte.

4. Avoid multiple freeze thaw cycles. In the case of frozen samples, use thawed sample within 24 hours.

5. All samples should be handled according to standard guidelines for preventing transmission of blood borne pathogens.

6. It is recommended that plasma samples be diluted 10X prior to assay.

Tissue samples:

Testing of tissue homogenates should be possible with this assay but has not yet been validated. Please contact NWLSS for additional information.