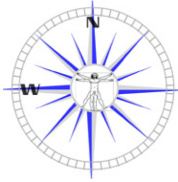


Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™
Nitrotyrosine Assay
(Competitive ELISA)

Product NWK-NTR02-01
For Research Use Only

This competitive ELISA is optimized for detection of mono-nitrated proteins and is expected to be more useful in detecting lower level nitrosylation than our original nitrotyrosine kit NWK-NTR01.

Nwkntr0201rev062014

Table of Contents

Section	Page
Introduction	3
Intended Use	3
Test Principle	3
Specifications	3
Kit Contents	4
Required Materials Not Provided	4
Required Instrumentation	4
Warnings, Precautions, Limitations	5
Storage Instructions	5
Assay Preparation	5
Reagent Preparation	6
Standard Curve Preparation	6
Sample Handling/Preparation	7
Assay Protocol	8
Data Analysis	9
Procedure Checklist	10
Statement of Limited Warranty	11
Notes	11

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 ($ONOO^-$) a powerful oxidant and nitrating agent. Subsequent reaction of $ONOO^-$ with proteins results in nitrotyrosine (NT) formation. As a stable end product of $ONOO^-$ 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:

This NWLSS™ Nitrotyrosine assay is intended for the quantitative measurement of nitrosylated protein adducts in biological samples. This competitive ELISA is optimized for detection of mono-nitrated proteins and is expected to be more useful in detecting lower level nitrosylation than our original nitrotyrosine kit NWK-NTR01.

Test Principle:

This NWLSS™ Nitrotyrosine Assay features a "competitive" ELISA format in which standards and samples are first incubated with a biotynylated Tracer Antibody in a separate U-bottom microplate. After incubation, the standards and samples are transferred to a microplate precoated with nitrated-HSA and allowed to incubate for an additional hour. During this step unbound Biotinylated Tracer Antibody binds to stationary phase NO-HSA. A subsequent wash removes excess Tracer and Tracer/protein complex. Addition of streptavidin-peroxidase followed by tetramethylbenzidine (TMB) facilitates color development inversely proportional to the nitrotyrosine present in the sample. The reaction is stopped using an oxalic acid solution and the assay is read on a plate reader at 450 nm.

Specifications:

Format::	1 X 96 well ELISA	
Number of tests:	Triplicate =	24
	Duplicate =	40
Specificity:	Nitrosylated protein adducts	
Sensitivity:	50 nM	
Range:	50 nM—1600 nM	

Kit Contents:

20X Concentrated Wash Buffer:	60 mL
10X Concentrated Dilution Buffer:	15 mL
Nitrotyrosine Standard (lyophilized) Contains 2-chloroacetamide as preservative. Concentration is lot specific and indicated on label.	2 Vials
100X Biotinylated Anti-Nitrotyrosine Tracer (lyophilized): Contains 2-chloroacetamide as preservative.	2 Vials
100X Streptavidin-peroxidase Reagent:	250 uL
TMB Substrate:	11 mL
Stop Solution (Containing 2% Oxalic Acid)	22 mL
12 X 8 Flat bottom microplate strips precoated w/NO-HSA	1 each
96 Well U Bottom microplate.	1 each

Required Materials Not Provided:

Adjustable micropipettes with disposable tips (50-1000 µL). 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.

Warnings, Limitations, Precautions:

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

Components containing 2-chloroacetamide and oxalic 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.

100X SAP Conjugate is stable up to expiration date when stored at 2-8°C . Working SAP Conjugate must be used the same day it is diluted. It is not stable when stored in diluted form.

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.

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

<u>Reagent Preparation:</u>

20X Wash Buffer:
For a full 96 wells, dilute 60 mL of 20X Wash Buffer with 1140 mL deionized water and/or for each 8 well strip to be assayed dilute 5 mL of 20X Wash Buffer with 95 mL deionized water. Label as **Working Wash Buffer.**

10X Dilution Buffer:
For a full 96 well plate dilute 15 mL of 10X Dilution Buffer with 135 mL deionized water and/or for each 8 well strip to be assayed dilute 1.25 mL of 10X Dilution Buffer with 11.25 mL deionized water. Label as **Working Dilution Buffer**

Note: Lyophilized components are under vacuum. Allow pressure to equalize slowly before opening fully.

Lyophilized Standard:
Reconstitute the standard with deionized water according to the directions on the vial label to create a 3200 nM stock solution.

Lyophilized, 100X Biotinylated Anti-Nitrotyrosine Tracer:
For each 96 wells to be assayed, reconstitute 1 vial Tracer with 250 uL deionized water. Next, dilute the 250 uL reconstituted Tracer with 24.75 mL *Working Dilution Buffer* and/or for each 8 well strip to be assayed dilute 21 µL reconstituted Tracer with 2.062 mL *Working Dilution Buffer.* Label as **Working Tracer.**

100X Streptavidin-Peroxidase (SAP) Reagent:
Spin down this vial prior to use. For a full 96 wells mix 250 uL of 100X SAP Conjugate with 24.75 mL *Working Dilution Buffer*. If less than a full plate will be assayed prepare the required volume by mixing 1 part 100X SAP Reagent with 99 parts Working Dilution Buffer. Label as **Diluted SAP Conjugate** and use the same day.

TMB Substrate: and Stop Solution are supplied ready to use.

<u>Standard Curve Preparation:</u>

1. Label microtubes 1-7 and add 400 µL Working Dilution Buffer to each.

2. Transfer 400 µL of 3200 nM standard to tube 1, mix well and continue 1/2 serial dilutions across tubes 2-6 creating standards of 1600 - 50 nM. Leave tube 8 as a buffer only zero control.

Std Tube # :	1	2	3	4	5	6	7
Conc. (nM):	1600	800	400	200	100	50	zero

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 125 μL 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 –80ºC are suitable for assay however sample storage at –20ºC 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 and serum samples be diluted 10X prior to assay.

Tissue samples:

Tissue samples can be homogenized in PBS, pH 7.3. Homogenates should be kept as concentrated as possible then tested at 1/2, 1/5, 1/10 etc. to determine the best dilution for the specific tissue type.

Assay Protocol:

Allow approximately 4.5 hours for procedure.

1. In the U-bottom plate provided add 125 μL of each standard, sample or control to each replicate well according to the assay template created earlier.

2. Add 125 μL **Working Tracer** to each well and mix gently.

3. Cover the wells using adhesive tape or plastic wrap and incubate for 1 hour at room temperature (18-25ºC).

4. Prepare the NO-HSA coated plate for use by washing 3 times with 200 μL **Working Wash Buffer** waiting 20 seconds before aspirating. After the final wash, empty the plate by inverting and shaking contents over sink. Keep inverted and tap dry on a thick layer of tissues or paper towels.

5. Transfer 100 μL of **Standards** and **Samples** mixed with **Working Tracer** to each of the flat bottom NO_HSA coated wells according to the assay template. Avoid touching the side or top of the wells.

6. Cover the wells and incubate for 1 hour at room temperature.

7. Repeat washing procedure as above in step 4.

8. Add 100 μL of **Diluted SAP Conjugate** to each well keeping the same sequence as used earlier.

9. Cover the wells and incubate for 1 hour at room temperature.

10. Repeat washing procedure as above in step 7.

11. Add 100 μL of TMB Substrate to each well, keeping the same sequence as used in earlier steps.

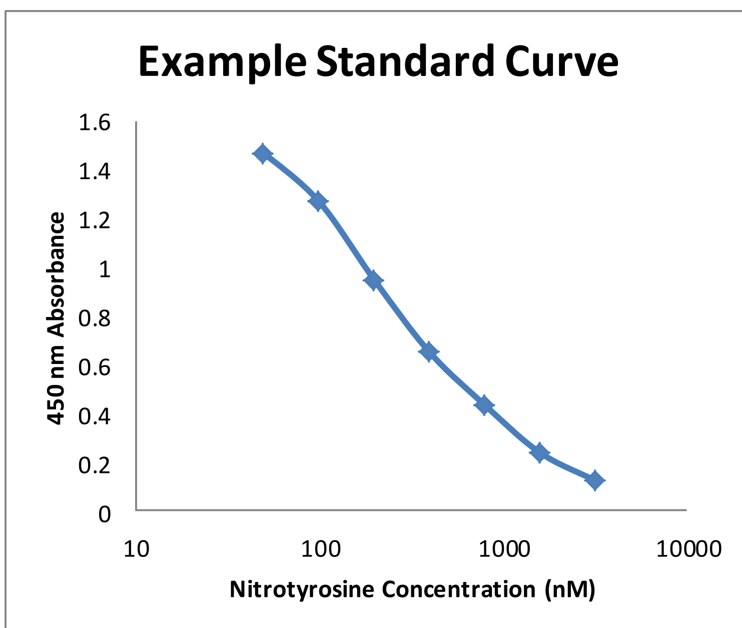
12. Cover the wells and incubate in the dark for 20-30 minutes at room temperature. Color development should be monitored to avoid over-development of high standards. Reaction can be stopped sooner if necessary.

13. Stop the reaction by adding 100 μL stop solution taking care to use the same sequence and timing as in previous steps.

14. Measure the absorbance at 450 nm.

The mean absorbance for the zero buffer only control should be > 1.7

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.



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

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 Reagents as required.

__ Perform a 1/2 serial dilution of the 3200 nM standard.

__ Prepare samples, making any necessary dilutions.

__ Add 125 µL standard, sample or control to each replicate well in the U-Bottom plate according to assay template.

__ Add 125 µL Working Tracer Reagent to each replicate well in the U-Bottom plate according to assay template.

__ Cover and Incubate 1 Hour at room temperature.

__ Prepare NO-HSA coated flat bottom plate for use by washing wells 3 times with 200 µL Working Wash Buffer.

__ Transfer 100 µL of Standard and Sample mixed with Diluted Tracer to each well of the pre-washed NO-HSAcoated coated plate.

__ Cover and Incubate 1 Hour at room temperature.

__ Wash wells 3 times with 200 µL Working Wash Buffer

__ Add 100 µL of diluted Streptavidin-Peroxidase Conjugate to each well.

__ Cover and Incubate 1 Hour at room temperature.

__ Wash wells 4 times with 200 µL Working Wash Buffer

__ Add 100 µL TMB Substrate Solution to each well.

__ Cover and incubate 20-30 minutes at room temperature in the dark.

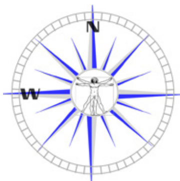
__ Stop the reaction by adding 100 µL Stop Solution.

__ Measure absorbance at 450 nm

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:



Northwest

Life Science Specialties, LLC

5131 NE 94th Avenue, Suite 201
Vancouver, WA 98662
Phone 360-449-3091 or Toll Free: 888-449-3091
Fax 360-449-3092
E-mail: sales@nwlifescience.com

nwkntr0201rev062014