Nitrotyrosine ELISA Kit

Catalog# NBP2-59717-96Tests(96-Well Kit)

Catalog# NBP2-59717-5x96Tests(5 x 96-Well Kit)

Colorimetric detection of Nitrotyrosine

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GENERAL INFORMATION

Materials Supplied

	Item	96 wells Quantity/Size
	Nitrosylated BSA Coated Plate	1 Plate
	Nitrotyrosine Standard	1 vial/110µl
	Nitrotyrosine HRP Conjugated Monoclonal Antibody	1 vial/75µl
	Sample and Standard Diluent (Red)	1 vial/50mL
	Nitrotyrosine Antibody Diluent (Blue)	1 vial/13mL
	Wash Buffer Concentrate (10X)	1 vial/ 50mL
	TMB Substrate	1 vial/13mL
	Stop Solution	1 vial/13mL
	Plate Cover	2

If any of the items listed above are damaged or missing, please contact our Customer Service department at 303-730-1950. We cannot accept any returns without prior authorization.

WARNING: Not for human or animal disease diagnosis or therapeutic drug use.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Novus Biologicals StressXpress® ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

Novus Biologicals suggests running all ELISA kits in triplicate, but replication must be defined by the user.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 303-730-1950

Fax: 303-294-9025

E-Mail: technical@novusbio.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if the components are stored as directed and used before the expiration date indicated on the outside of the box.

All reagents are stable as supplied at 4°C. For optimum storage, the Standard should be aliquotted into smaller portions and then stored appropriately. Avoid repeated freeze/thaw cycles (10 µL of Standard can prepare a triplicate standard curve.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and a repeat pipettor.
3. Deionized or distilled water
4. Materials used for **Sample Preparation** (see page 8).

Assay Precautions

- All ELISA reagents must be at room temperature before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- 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.
- Individual components may contain preservatives. Wear gloves while preforming the assay. Please follow proper disposal procedures.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in triplicate.
- 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.

INTRODUCTION

Background

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.

About This Assay

Novus Biologicals Nitrotyrosine ELISA is a competitive assay that can be used for the quantification of Free Nitrotyrosine 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 8000nM Free Nitrotyrosine, with a sensitivity of 50nM. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

Assay Overview

1. Prepare standard and samples in the Sample and Standard Diluent.
2. Add 50 µL of prepared standards and samples in triplicate to appropriate wells.
3. Add 50 µL of the diluted antibody preparation to the appropriate wells.
4. Cover plate with Plate Cover and incubate at room temperature (20-25°C) for 1 hour.
5. Wash plate 4 times with 1X Wash Buffer.
6. Add 100 µL of TMB Substrate to each well.
7. Cover plate and develop the plate in the dark at room temperature for 30 minutes.
8. Add 100 µL of Stop Solution to each well.
9. Measure absorbance on a plate reader at 450 nm.
10. Plot the standard curve and calculate sample concentrations.

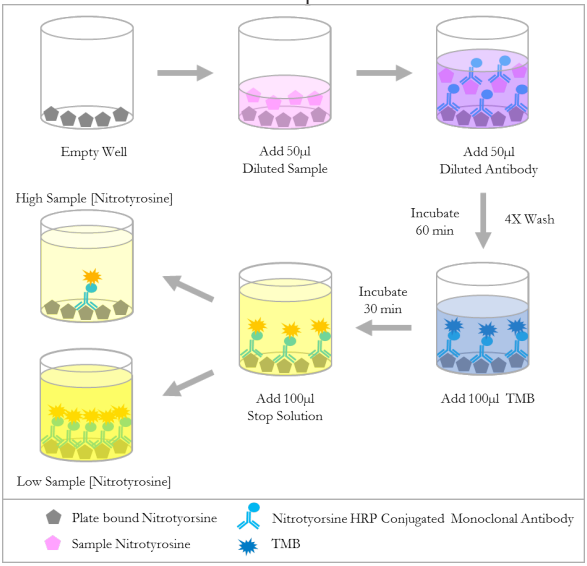


Figure 1. Schematic of the Nitrotyrosine *StressXpress*® Competitive ELISA

PRE-ASSAY PREPARATION

Sample Preparation

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

NOTE: Prepare at least 180µL of your diluted sample to permit assay in triplicate (approximately 50µL/ well).

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- 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

Storage: Collect plasma using established methods and store at -80°C.

Dilution: Serum samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

Cell Lysates

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

Urine

Storage: Fresh urine samples should be centrifuged at 2,000 x g for 10 minutes or filtered with a 0.2µm filter before this assay, and stored at -20°C immediately after collection.

Dilution: Urine samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

Reagent Handling/ Preparation

Standard Preparation (S1-S8)

NOTE: The Standard should be aliquotted into smaller portions before use to ensure product integrity. Avoid freeze/thaw cycles. (10 µL of Standard can prepare a triplicate standard curve).

1. Centrifuge the Nitrotyrosine Standard 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. Similarily, 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 1.5mL polypropylene tube (Tube #9), which is the zero standard (0 ng/mL).

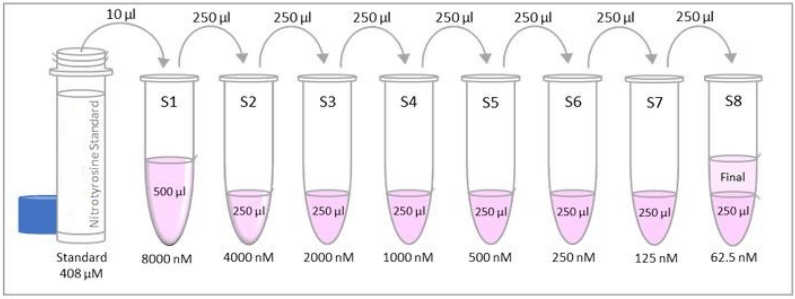


Figure 2. Preparation of the Nitrotyrosine standards

1X Wash Buffer Preparation

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

Store reconstituted 1X Wash Buffer at 2-8ºC for up to one (1) month. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

Nitrotyrosine: HRP Conjugate Monoclonal Antibody Preparation

1. Determine the amount of Antibody Preparation required. For every strip-well used (8-wells), prepare 0.5 mL of Antibody Preparation.
2. Prepare Antibody Preparation by diluting the Nitrotyrosine: HRP Conjugate 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.

Plate Set Up

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 2-4°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 in Figure 3, 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 (see page 20).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S9	S9	S9	7	7	7	15	15	15
B	S2	S2	S2	Blk	Blk	Blk	8	8	8	16	16	16
C	S3	S3	S3	1	1	1	9	9	9	17	17	17
D	S4	S4	S4	2	2	2	10	10	10	18	18	18
E	S5	S5	S5	3	3	3	11	11	11	19	19	19
F	S6	S6	S6	4	4	4	12	12	12	20	20	20
G	S7	S7	S7	5	5	5	13	13	136	21	21	21
H	S8	S8	S8	6	6	6	14	14	14	22	22	22

S1 – S8: 8000- 62.5 nM Standards
S9: Zero Standard
Blk: Blank
1 – 22: Samples

Figure 3. Sample plate format

Performing the Assay

Assay Hints

- Use different tips to pipette the buffer, standard, sample, and antibody.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.
- 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.

Well	Standard OR Sample Preparation	Standard and Sample Diluent	Antibody Preparation	Antibody Diluent	Total Volume Per Well
Standard (S1-S8)	50 µL	Included in Standard Prep	50 µL	Included in Ab. Prep	100 µL
Zero Standard (S9)	-	50 µL	50 µL	Included in Ab. Prep	100 µL
Blank	-	50 µL	-	50 µL	100 µL
Samples (1-22)	50 µL	Included in Sample Prep	50 µL	Included in Ab. Prep	100 µL

Table 1: Pipetting Summary

Addition of the Reagents

1. Add 50 µl (in triplicate) of each of the following to appropriate wells:

- Prepared Nitrotyrosine Standard (Tube #1 through Tube #8) into wells labelled S1-S8
- Zero Standard (Tube #9- Sample and Standard Diluent, which represents 0 nM into wells labelled S9.
- Samples (previously prepared- See **Sample Preparation**, page 8) into wells labelled 1-22.

2. Add 50 µl of the previously diluted Nitrotyrosine Antibody Preparation to each well, except the blank.

3. Add 50 µl of Standard and Sample Diluent and 50 µl of Antibody Diluent into wells labelled as the blank.

Incubate the Plate

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

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 1X 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.

TMB Substrate Incubation and Reaction Stop

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

1. Add 100 µL of TMB Substrate into each well.
2. Cover carefully with the second provided plate cover.
3. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. The substrate reaction yields a blue solution.
4. 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.

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.

Data Analysis Methods

This kit can be analyzed using any of the following methods:

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

B. 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.

Performance Characteristics

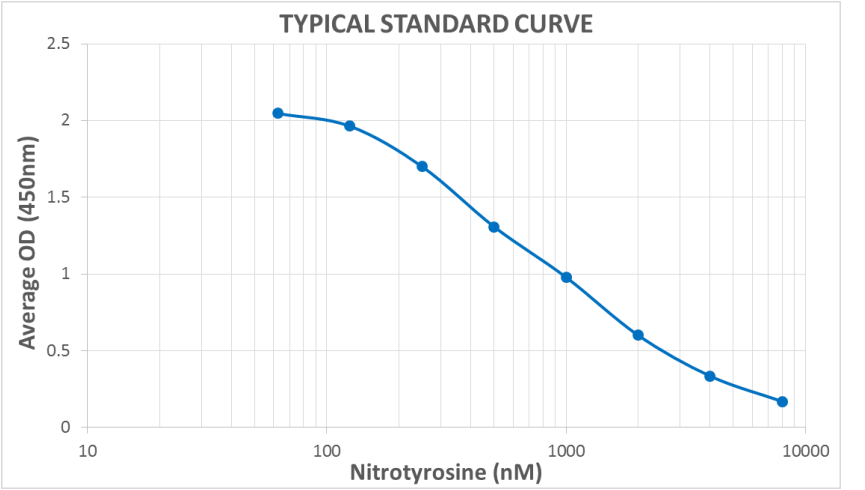


Figure 4. Typical standard curve
NOTE: This typical standard curve was generated using the Nitrotyrosine ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay Range: 62.5-8000 nM

Sensitivity:

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

Precision:

1. Intra-Assay Precision (Within Run 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%.

2. Inter-Assay Precision (Between Run 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%.

Assay Limitations:

- If samples generate greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re- assayed at a lower sample dilution.
- The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.
- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers

Troubleshooting

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

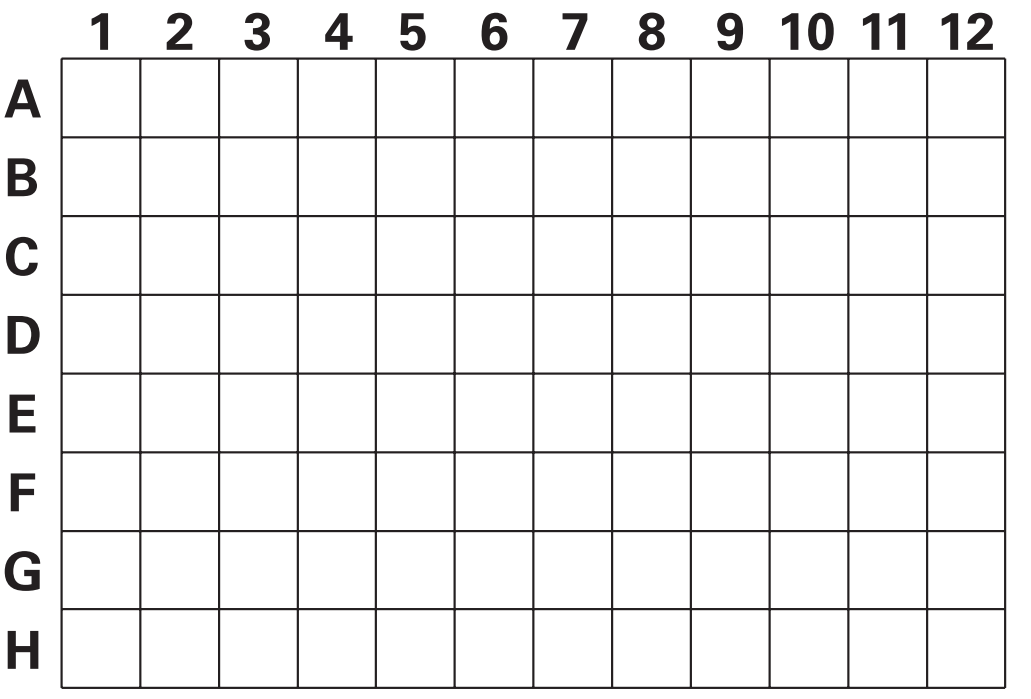
Warranty and Limitation of Remedy

Novus Biologicals makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Novus Biologicals **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Novus Biologicals will carry out its delivery obligations with due care and skill. Thus, in no event will Novus Biologicals have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Novus Biologicals is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Novus Biologicals, its directors or its employees.

Buyer's **exclusive remedy** and Novus Biologicals sole liability hereunder shall be limited to a refund of the purchase price, or at Novus Biologicals option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Novus Biologicals within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
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