Nitrotyrosine AccuSignal™ ELISA Kit

Nitrotyrosine AccuSignal™ competitive ELISA (Enzyme-Linked Immunosorbent Assay) Kit is *an in vitro* enzyme-linked immunosorbent assay designed for the quantification of free nitrotyrosine in plasma, serum, cell lysates, urine and other sample matrices. The ELISA utilizes a nitrotyrosine-coated plate and an HRP-conjugated antibody for detection which allows for an assay range of 62.5 to 8000 nM free nitrotyrosine, with a sensitivity of 50 nM. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

I. KIT COMPONENTS

Nitrotyrosine AccuSignal™ ELISA Kit			
Component	Catalog Number	Description	Size
Nitrosylated BSA Coated Plate	KOA0888A	96-well plate pre-coated with nitrosylated BSA	1
Target Protein Standard	KOA0888B	Nitrotyrosine standard	1 vial (110 µl)
Conjugated Monoclonal Antibody	KOA0888D	Nitrotyrosine HRP-conjugated monoclonal antibody	1 vial (75 µl)
Sample and Standard Diluent (Red)	KOD0102	Sample and standard diluent	1 vial (50 ml)
Antibody Diluent (Blue)	KOD0103	Nitrotyrosine antibody diluent	1 vial (13 ml)
Wash Buffer Concentrate	KOD0105	Wash buffer concentrate (10X)	1 vial (50 ml)
TMB Substrate	KOC0102	TMB color developing reagent	1 vial (13 ml)
Stop Solution	KOC0103	Stop solution	1 vial (13 ml)
Plate Cover	KOC0104	Adhesive cover	2

II. INTRODUCTION

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 concentration in plasma may be useful as a marker of NO-dependent damage *in vivo*. Since NOX is only an indicator for enhanced NO production, protein associated nitrotyrosine might be a more suitable marker for damage induced by reactive nitrogen intermediates. Furthermore, most proteins have a longer half-life in the circulation than NOX 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.

III. STORAGE AND STABILITY CONDITIONS

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

IV. REQUIRED EQUIPMENT AND REAGENTS NOT INCLUDED

- Microplate reader capable of measuring absorbance at 450 nm
- Automated plate washer
- Adjustable pipettes, repeat pipettor and pipette tips
- Clean tubes and Eppendorf tubes
- Distilled or deionized water

V. GENERAL CONSIDERATIONS

Please read the following instructions before starting the experiment.

- All ELISA reagents must be at room temperature before use.
- Vigorous plate washing is essential.
- Before using the Kit, briefly spin down the vials.
- Do not let 96-well plate to dry, this will inactivate active components on plate.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Use 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.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- 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.

VI. ELISA PROTOCOL

A. 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 180 µL of your diluted sample to permit assay in triplicate (approximately 50 µL/ well).

- 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.

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: Dilute urine samples 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing. For example: 45 µL of sample into 135 µL of Sample and Standard Diluent.

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.

B. Standard Preparation

NOTE: The Standard should be aliquoted 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 490 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. Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, 250 µL from Tube # 3 to Tube #4, 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 µM).

Standard #	Volume to Dilute (µL)	Diluent (µL)	Total Volume (µL)	Concentration (nM)
1	10 (standard 408µM)	490	500 µL	8000
2	250	250	500 µL	4000
3	250	250	500 µL	2000
4	250	250	500 µL	1000
5	250	250	500 µL	500
6	250	250	500 µL	250
7	250	250	500 µL	125
8	250	250	500 µL	62.5
9	0	250	250	0

C. Reagent Preparation

1X Wash Buffer Preparation

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

Determine the amount of Antibody Preparation required. For every strip-well used (8-wells), prepare 0.5 ml of Antibody Preparation.

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.

D. Assay Procedure

The 96-well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate 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 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.

	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

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.

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) 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.
4. Cover each plate with the plate cover and incubate 1 hour at room temperature (20- 25˚C).
5. 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.
6. 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.
7. Add 100 µL of TMB Substrate into each well.
   Note: 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.
8. Cover carefully with the second provided plate cover.
9. 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.

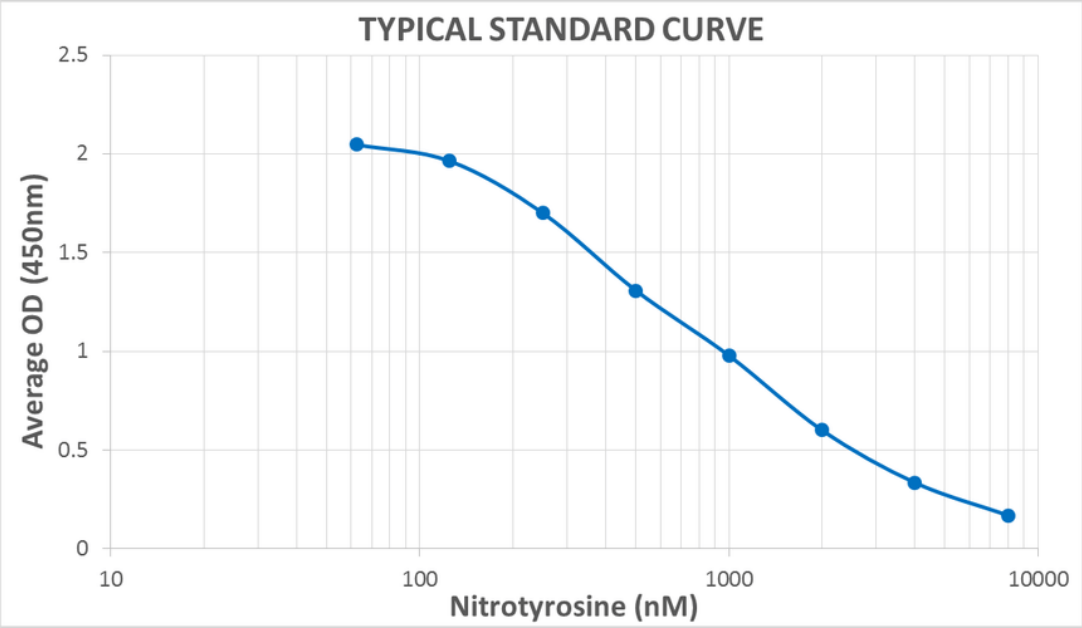
10. After 30 minutes, carefully remove the plate cover, and stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.
11. Wipe underside of wells with a lint-free tissue.
12. Measure the absorbance on an ELISA plate reader set at 450 nm.
    Note: Evaluate the plate within 30 minutes of stopping the reaction.

Calculations

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 8-OHdG 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.

VII. NITROTYROSINE ACCUSIGNAL™ ELISA KIT STANDARD CURVE



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 nM-8000 nM

Sensitivity	50 nM pure nitrotyrosine

VIII. INTRA AND INTER ASSAY PRECISION

Intra-Assay 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%.

Inter-Assay 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.

VIII. ASSAY SUMMARY

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

IX. 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

X. RELATED PRODUCTS

Component	Catalog #	Size
10x TBS pH 7.5	MB-012	1000 ml
10x TTBS pH 7.5	MB-013	1000 ml
10X PBS pH 7.2	MB-008	1000 ml
10x PBST pH 7.2	MB-075-1000	1000 ml