Creative Diagnostics®

Version 13-05/24

User's Manual

Nitrotyrosine ELISA kit

REF **DIA-XY182**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Nitrotyrosine ELISA kit is to be used for the in vitro quantitative determination of nitrotyrosine in plasma and other biological samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures. The analysis should be performed by trained laboratory professionals.

General Description

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.

Principles of Testing

The Nitrotyrosine ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3½ hours.

Reagents And Materials Provided

Kit component	Cat. #	Quantity	Color code
Wash buffer 40x	WB01	1 vial (30 ml)	Colorless
Dilution buffer 10x	DB81	1 vial (15 ml)	Green
Standard		2 vials, lyophilized	White
Tracer, biotinylated		1 vial, 1 ml lyophilized	White
Streptavidin-peroxidase	CON03	1 tube, 0.25ml in solution	Brown
TMB substrate	TMB050/TMB100	1 vial (11 ml)	Brown
Stop solution	STOP110	1 vial (22 ml)	Red
12 Microtiter strips, pre-coated		1 plate	
Certificate of Analysis		1	
Manual		1	
Data collection sheet		2	

Materials Required But Not Supplied

Calibrated micropipettes and disposable tips.

Distilled or de-ionized water.

Plate washer: automatic or manual.

Polypropylene tubes.

Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.

Adhesive covers can be ordered separately. Please contact your local distributor.

Centrifuge for 1 ml tubes.

Storage

1. Upon receipt, store individual components at 2 - 8°C. Do not freeze.
2. Do not use components beyond the expiration date printed on the kit label.
3. The standard, tracer in lyophilized form and streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
4. The exact amount of the standard is indicated on the label of the vial and the Certificate of Analysis .
5. The standard is single use. After reconstitution the standard cannot be stored.
6. Once reconstituted the tracer are stable for 1 month if stored at 2 - 8°C.
7. The streptavidin-peroxidase can only be stored in concentrated solution and is not stable when stored diluted.
8. Upon receipt, foil pouch around the plate should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
9. Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality guaranteed for 1 month if stored at 2 - 8°C.

Specimen Collection And Preparation

Plasma

Keep freshly collected blood on ice. Within 20 minutes after blood sampling, separate plasma by centrifugation: 1500xg at 4°C for 15 min.

Transfer the plasma to a fresh polypropylene tube.

Most reliable results are obtained if EDTA plasma is used.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of nitrotyrosine. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of nitrotyrosine activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Plasma samples

Nitrotyrosine can be measured accurately if plasma samples are diluted at least 10x with supplied dilution buffer in polypropylene tubes.

Note that most reliable results are obtained with EDTA plasma.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline.

The recovery of nitrotyrosine from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of nitrotyrosine.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

Guideline for dilution of samples

Please see the table below for recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of dilution buffer required
1.	10x	Not necessary	25 µl (sample)	225 µl
2.	20x	Not necessary	15 µl (sample)	285 µl
3.	50x	Not necessary	10 µl (sample)	490 µl
4.	100x	Not necessary	10 µl (sample)	990 µl
5.	500x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	490 µl
6.	1000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

Reagent Preparation

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare the desired volume of wash buffer by diluting 1 part of the 40x wash buffer with 39 parts of distilled or de-ionized water.

Dilution buffer

Prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water. Concentrated dilution buffer may contain crystals. Where the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37°C. Do not shake the solution.

Standard solution

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 1. Prepare each Nitrotyrosin standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure 1*. The standard cannot be stored for repeated use.

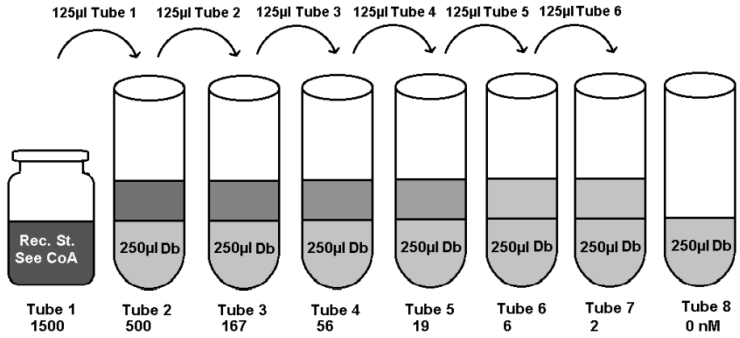

Figure 1

*) CoA: Certificate of Analysis, St: Standard, Db: Dilution buffer

Tracer solution

The tracer is reconstituted by pipetting 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. Where less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of dilution buffer.

Assay Procedure

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.
2. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
3. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
4. Incubate the strips or plate for 1 hour at room temperature.
5. Wash the plates 4 times with wash buffer using a plate washer or as follows*:

a. Carefully remove the cover, avoid splashing.

b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.

c. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate as described in 5b.

d. Repeat the washing procedure 5b/5c three times.

e. Empty the plate and gently tap on thick layer of tissues.

6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
7. Cover the tray and incubate the tray for 1 hour at room temperature.

8. Repeat the wash procedure described in step 5.
9. Add 100 µl of diluted streptavidin-peroxidase substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
10. Cover the tray and incubate the tray for 1 hour at room temperature.
11. Repeat the wash procedure described in step 5.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
13. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the micro well strips to direct sunlight. Covering the plate with aluminum foil is recommended.
14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
15. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

*) In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure. Make sure the plate washer is used as specified for the manual method.

TECHNICAL HINTS

1. User should be trained and familiar with ELISA assays and test procedure.
2. If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
3. Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
4. Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard range prepared on the same plate.
5. Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
6. Each time the kit is used, fresh dilutions of standard, sample, tracer, streptavidinperoxidase and buffers should be made.
7. Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
8. To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
9. The waste disposal should be performed according to your laboratory regulations.

Quality Control

The Certificate of Analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ. This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested

in the Creative Diagnosticsimmunoassay, the possibility of interference cannot be excluded. For optimal performance of this kit, it is advised to work according to good laboratory practice.

Interpretation Of Results

1. Calculate the mean absorbance for each set of duplicate standards, control and samples.
2. If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested. 3.The mean absorbance of the zero standard should be less than 0.3.
4. Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
5. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
6. Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

Detection Range

2-1500 nM

Detection Limit

2 nM

Specificity

The ELISA detects nitrotyrosine containing proteins. Since the assay detects a modified amino acid the assay is useful for proteins of all species.

Precautions

1. For research use only, not for diagnostic or therapeutic use.
2. This kit should only be used by qualified laboratory staff.
3. Do not under any circumstances add sodium azide as preservative to any of the components.
4. Do not use kit components beyond the expiration date.
5. Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by manufacturer. 6.The assay has been optimized for the indicated standard range. Do not change the standard range.
7. Open vials carefully: vials are under vacuum.
8. It is advised to spin down streptavidin-peroxidase tubes before use.
9. Do not ingest any of the kit components.

10. Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
11. The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
12. The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advise.
13. Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
14. Do not reuse micro wells or pour reagents back into their bottles once dispensed.
15. Handle all biological samples as potentially hazardous and capable of transmitting diseases.
16. Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
17. Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.