Preliminary

Nitrotyrosine ELISA

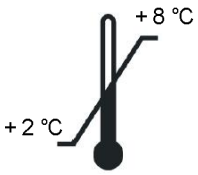
For the in vitro determination of Nitrotyrosine in human EDTA-plasma

For research use only

Please note that this manual is not final, and it may be changed in future.

Valid from 27.01.2011

K 7829



1. INTENDED USE

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) is intended for the quantitative determination of Nitrotyrosine in human EDTA plasma. It is for research use only.

2. INTRODUCTION

Nitrotyrosine is the nitrated form of the amino acid tyrosine. The accumulation of protein bound nitrotyrosine is associated with cardiovascular diseases that are based on inflammatory processes (e.g., atherosclerosis, myocardial infarction, diabetic vasculopathy, hypertension, or coronary heart diseases). A growing number of studies have also associated the accumulation of nitrotyrosine with neurological diseases (Alzheimer´s disease, Parkinson´s disease, multiple sclerosis, stroke). With treatment of some of the associated diseases the levels of nitrated tyrosines have been shown to decrease, so nitrotyrosine has been stated to be a marker of nitrosative stress.

During inflammatory processes, large amounts of nitric oxide (•NO) are locally released from L-arginine. This reaction is catalyzed by the enzyme NO-synthase (NOS). Other causes for the increased •NO production are exposure to chemicals or heavy metals, drugs, nicotine, or physical and psychological stress, as well as extraordinary physical strain with increased oxygen consumption.

In high concentrations, •NO that is not trapped by mitochondrial superoxide dismutase (SOD) reacts with superoxide ($O_2^{\bullet}$) to form peroxynitrite ($ONOO^-$). Peroxynitrite is implicated as a key oxidant species in several pathologies and is known to be cytotoxic (nitrosative stress).

Peroxinitrite is highly reactive and shows a high affinity to aromatic amino acids, e.g., to the phenolic ring of tyrosine. The nitration of tyrosine in general is a natural process within the post-translational protein modification.

Nitrotyrosine is a stable product and might be seen as a correlate of peroxynitrite production, and its accumulation in cells and tissues is a marker of oxidative stress and nitrosative stress, respectively (Ischiropoulos 2008).

Indications

- Cardiovascular diseases
- Neurological diseases
- Thyroid disturbances
- Blockade of biochemical pathways
- Mitochondriopathy

Consequences of nitrosative stress

- Modification of lipids and proteins (for example structural proteins in mitochondria)
- Inhibition of respiratory chain enzymes in the mitochondria
- Glutamate overload
- Disturbances in ion channels
- Calcium overload
- Initiation of apoptosis processes

3. MATERIAL SUPPLIED

Cat. No	Content	Kit Components	Quantity
K 7829MTP	PLATE	Microtiter plate, precoated	96 wells
K 7829WP	WASHBUF	ELISA wash concentrate 10x	1 x 100 ml
K 7829PV	SAMPLEBUF	Sample dilution buffer, ready-to-use	1 x 100 ml
K 7829K	CONJ	Conjugate (goat anti-human-serum proteins, peroxidase-labeled)	1 x 200 µl
K 7829ST	STD	Standards, lyophilized	6 vials
K 7829KO1	CTRL1	Control, 1lyophilized	1 vial
K 7829KO2	CTRL2	Control 2, lyophilized	1 vial
K 7829TMB	SUB	TMB substrate (Tetramethylbenzidine), ready-to-use	1 x 15 ml
K 7829AC	STOP	ELISA stop solution, ready-to-use	1 x 15 ml

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Standard laboratory polypropylene reaction vessels (1.5 ml)
- Standard laboratory reaction vessel (15 ml)
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- Vortex mixer
- Ultra Pure Water*
- Microtiter plate reader at 450 nm

*Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25°C (≤18.2 MΩ cm).

6. PREPARATION AND STORAGE OF REAGENTS

- To run assay more than once, ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. The kit can be used up to 4 times within the expiry date stated on the label.
- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.
- The **ELISA WASHBUF** (wash buffer concentrate) should be diluted with ultra pure water **1:10** before use (100 ml WASHBUF + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or at 37°C using a water bath before dilution. The **WASHBUF** (buffer concentrate) is stable at **2-8°C** until the expiry date stated on the label. Diluted **buffer solution** can be stored in a closed flask at **2-8°C for one month**.
- **STD** (Standards) and **CTRL** (controls) must be reconstituted with **500 µl** of ultra pure water. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards and controls are not stable**.
- The **CONJ** (conjugate, POD-antibody) must be diluted **1:100 in SAMPLEBUF** (sample dilution buffer) (100 µl CONJ + 10 ml SAMPLEBUF). The **CONJ** (conjugate, POD-antibody) is stable at 2-8 °C until the expiry date stated on the label. **Diluted conjugate is not stable and cannot be stored.**
- All other test reagents are ready to use. The test reagents are stable until the expiry date (see label of test package) when stored at **2-8°C**.

6. PRECAUTIONS

- For research use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Stop solution is composed of sulfuric acid, which is a strong acids acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Reagents should not be used beyond the expiration date shown on kit label.

7. SAMPLE PREPARATION

Pipette **15 µl** of fresh sample in a 1,5 ml reaction vial, add **885 µl of SAMPLEBUF** (sample dilution buffer) and mix well (corresponds to **1:60 dilution**).

8. ASSAY PROCEDURE

Principle of the test

The assay utilizes the sandwich technique with two polyclonal antibodies against nitrated proteins.

Standards, controls and diluted samples which are assayed for nitrotyrosine are added into the wells of a micro plate coated with polyclonal rabbit anti-nitrotyrosine antibody. During the first incubation step, nitrated proteins are bound by the immobilized primary antibody. Then a peroxidase-conjugated polyclonal rabbit anti- nitrotyrosine antibody is added into each microtiter well and a sandwich of

primary antibody - nitrated protein peroxidase-conjugate

is formed. Tetramethylbenzidine (TMB) is used as peroxidase substrate. Finally, an acidic stop solution is added to terminate the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of nitrotyrosine. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. standard concentration is generated, using the values obtained from the standard.

Test procedure

1.	Prior to use in the assay allow all **reagents and samples** to come to **room temperature** (18-26 °C) and mix well
2.	Mark the **positions of STD** (Standard), **CTRL1** (control 1), **CTRL2** (control 2) and **SAMPLE** (sample) on a protocol sheet
3.	**Take microtiter strips** out of the kit. Store unused strips covered at 2-8° C. Strips are stable until the expiry date stated on the label
4.	Wash each well **5 times by dispensing 250 µl of diluted wash buffer** into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
5.	Add **2 x 100 µL of the prepared STD** (Standard), **CTRL1** (control 1), **CTRL2** (control 2) and **SAMPLE** (sample) in duplicate into respective well
6.	Cover plate or strips with foil tightly and **incubate for 1 h** at room temperature (18 - 26°C) on the horizontal shaker
7.	Discard the contents of each well. Wash **5 times by dispensing 250 µL** of diluted wash buffer into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
8.	Pipette **100 µL of diluted CONJ** (HRP-antibody) into each well
9.	Cover plate or strips with foil tightly and **incubate for 1 h** at room temperature (18 - 26°C) on the horizontal shaker.
10.	Discard the contents of each well. Wash **5 times by dispensing 250 µL** of diluted wash buffer into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper

11. Add **100 µL of SUB** (TMB substrate) into each well
12. **Incubate for 10-20 min** at room temperature (18-26°C) in the dark.
13. Add **100 µL of STOP** (stop solution) into each well, mix thoroughly in a microtiter plate reader
14. Determine **absorption** immediately with an ELISA reader at **450 nm**. If the highest extinction of the standards **(STD)** is above the range of the photometer, absorption must be measured immediately at **405 nm** and the obtained results used for evaluation. If possible, the extinctions from each measurement should be compared with extinctions obtained at a reference wavelength, e. g. 595 nm, 620 nm, 630 nm, 650 nm and 690 nm can be used.

*The intensity of the colour change is temperature sensitive. We recommend to observe the colour change and to stop the reaction upon good differentiation.

9. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using the "4-Parameter-algorithm".

1. 4-Parameter-algorithm

   It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.01).

2. Point-to-point-calculation

   We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline-algorithm

   We recommend a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.01).

   The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Serum and plasma samples

The obtained nitrotyrosine concentration must be multiplied by a **factor** of **60**.

Controls

The nitrotyrosine concentration can be read directly from the calibration curve. The concentration range is given on the enclosed data sheet specification.

10. LIMITATIONS

Serum and plasma samples with nitrotyrosine concentrations **outside the standard curve** range should be diluted further with **SAMPLEBUF** (sample dilution buffer) to obtain readings within the standard curve and re-assayed.

11. QUALITY CONTROL

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control samples are outside the acceptable limits.

12. PRECAUTIONS

- For research use only.
- The quality control guidelines should be followed.
- Human material used in the kit components was tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Reagents of the kit package contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. The substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulphuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water.

13. TECHNICAL HINTS

- Do not mix different lot numbers of any kit component.
- Reagents should not be used beyond the expiration date shown on the kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- The assay should always be performed according the enclosed manual.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- All reagents in the kit package are for research use only.
- The guidelines for medical laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG together with a written complaint.

15. REFERENCES

Gonsette RE (2008) Neurodegenaration in multiple sclerosis : The role of oxidative stress and excitotoxicity. J Neurol Sci, Vol 274, Issue 1-2, 48-53

Ischiropoulos H (2008) Protein tyrosine nitration An update. Arch Biochem Biophys Oct 30

Peluffe G, Radi R (2007) Biochemistry of protein tyrosine nitration in cardiovascular pathology. Cardiovasc Res. Jul 15 : 75(2) :291-302

Souza JM et al. (2008) Protein tyrosine nitration- functional alteration or just a biomarker ? Free Radic Biol Med. Aug 15 ; 45 (4) :357-356

Used symbols:

	Temperature limitation	REF	Catalogue Number
IVD	In Vitro Diagnostic Medical Device		Contains sufficient for <n> tests
	Manufacturer		Use by
LOT	Lot number	RUO	For research use only