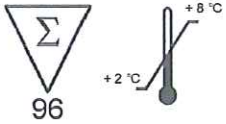
Manual

Nitrotyrosine ELISA

For the in vitro determination of nitrotyrosine in human EDTA-plasma, serum and stool

Valid from 16.01.2013

REF K 7824



IVD CE

1. Intended use

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) is intended for the quantitative determination of protein-bound nitrotyrosine in human EDTA-plasma, serum and stool. It is for *in vitro* 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 (MnSOD) 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.

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 7824MTP	PLATE	Microtiter plate, precoated	96 wells
K 7824WP	WASHBUF	ELISA wash concentrate, 10x	1 x 100 ml
K 7824AP	ASYBUF	Assay buffer, ready-to-use	1 x 50 ml
K 7824ST	STD	Standards, lyophilized	4 x 5 vials
K 7824KO1	CTRL	Control, lyophilized	4 vials
K 7824KO2	CTRL	Control, lyophilized	4 vials
K 7824K	CONJ	Conjugate (goat anti-nitrotyrosine, peroxidase-labeled)	1 x 200 µl
K 7824TMB	SUB	TMB substrate (Tetramethylbenzidine), ready-to-use	1 x 15 ml
K 7824AC	STOP	ELISA stop solution, ready-to-use	1 x 15 ml

4. Material required but not supplied

- Ultra pure water*
- Precision pipettors and disposable tips to deliver 10 - 1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge

- Vortex-Mixer
- Standard laboratory polypropylene reaction vessels (1.5 ml)
- Standard laboratory reaction vessel (15 ml)
- 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).

5. Preparation and storage of reagents

- Reagents with a volume less than 100 µl should be centrifuged before use to avoid loss of volume.
- The **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 re-dissolved at room temperature or at 37°C using a water bath before dilution of the buffer solutions. The **WASHBUF** (wash 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**.
- The lyophilized **STD** (standards) and **CTRL** (controls) are stable at **2-8°C** until the expiry date stated on the label. The **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 it to ensure complete reconstitution. **Reconstituted STD (standards) and CTRL (controls) are not stable and cannot be stored.**
- The **CONJ** (Conjugate, HRP-antibody) must be diluted **1:101** with diluted wash buffer. It should be freshly prepared for each run. The remainder of the diluted conjugate should be discarded. The **CONJ** (Conjugate, HRP-antibody) is stable at **2-8°C** until the expiry date stated on the label.
- Store **unused microtiter strips** sealed in the aluminium foil bag with desiccating agent at 2-8° C. Strips are stable until the expiry date stated on the label.
- 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. SAMPLE PREPARATION

EDTA-plasma or serum samples

Pipet **50 µl** of fresh EDTA-plasma or serum sample in a 1,5 ml reaction vial, add **200 µl ASYBUF** (assay buffer) and mix well (corresponds to **1:5 dilution**).

Stool samples

Extraction of the stool sample

Diluted wash buffer is used as a sample extraction buffer. We recommend the following sample preparation:

1a. Stool Sample Application System (SAS) (Cat. No.: K 6998SAS)

Stool sample tube – Instruction for use

Please note that the dilution factor of the final stool suspension depends on the used amount of stool sample and the volume of the buffer.

SAS with 0.75 ml buffer:

Applied amount of stool:	15 mg
Buffer volume:	0,75 ml
Dilution factor:	1:50

Please follow the instructions for the preparation of stool samples using the SAS as follows:

a) The raw stool sample has to be thawed. For remarkably inhomogeneous samples we recommend a mechanical homogenization using an applicator, inoculation loop or similar device.

b) **Fill the empty sample tube** with **0.75 ml** of ready-to-use extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.

c) Unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample. The lower part of the dipstick exhibits notches which need to be covered completely with stool after inserting it into the sample. Place the dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off leaving 15 mg of sample to be diluted. Screw tightly to close the tube.

d) Shake the tube well until no stool sample remains in the notches. Important: Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for app. 10 minutes improves the result.

e) After sample suspension, centrifuge the tube for 10 min at 3000xg.

1b. Sample preparation kit from Roche Diagnostics, Mannheim, Germany (Cat. No. 10 745 804 322)

Alternatively, other stool sample preparation kits (e.g. sample preparation kit from Roche Diagnostics, Mannheim, Germany) can be used. In the Roche sample preparation kit, 100 mg of stool sample are suspended in 5 ml of extraction buffer using a vibrator mixer (e.g. Vortex mixer). Centrifugation of the suspension is recommended.

The sample suspension (1a. or 1b.) is not stable.

Dilution I (1a. or 1b.) **1:50**

Dilution of samples

Stool samples

After centrifugation, the supernatant of the extraction (dilution step I) is diluted **1:20** with **diluted wash buffer**. For example:

30 µl supernatant (dilution I) + 570 µl diluted wash buffer **(dilution II)**

Final dilution 1 : 1000

For analysis, **100 µl** of **dilution II** is pipetted per well.

7. 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 microtiter plate coated with polyclonal anti-nitrotyrosine antibody. During the first incubation step, nitrated proteins are bound by the immobilized primary antibody. Then a peroxidase-conjugated polyclonal 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** (Standards), **CTRL** (controls) and **SAMPLE** (sample/s) in duplicate on a protocol sheet
3.	Take as many **microtiter strips** as **needed** out of the kit
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** (Standards), **CTRL** (controls) and **SAMPLE** (sample/s) in duplicate into respective well
6.	Cover plate or strips with foil tightly and **incubate for 2.5 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 1h** 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.

8. EVALUATION

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.

EDTA-plasma and serum samples

The obtained nitrotyrosine concentration must be multiplied by the dilution factor of **5**.

Stool samples

The obtained nitrotyrosine concentration must be multiplied by the dilution factor of **1000**.

Controls

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

9. LIMITATIONS

Samples with nitrotyrosine concentrations outside the standard curve range should be further diluted with assay buffer (EDTA-plasma or serum samples) or with diluted wash buffer (stool samples) in order to obtain readings within the standard curve, and re-assayed.

The corresponding dilution factor must be considered by the calculation of the nitrotyrosine concentration.

10. 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 sample are outside the acceptable limits.

11. PERFORMANCE CHARACTERISTICS

Reference range of nitrotyrosine

Serum

Serum samples of evidently healthy persons (n=78) were measured.

Min: **48 nM**

Max: **1533 nM**

Median: **207 nM**

For 95% of this collective (95 percentile) a nitrotyrosine concentration of 553 nM and less was obtained.

For 10% of this collective a nitrotyrosine concentration < LoB (Limit of Blank) was obtained.

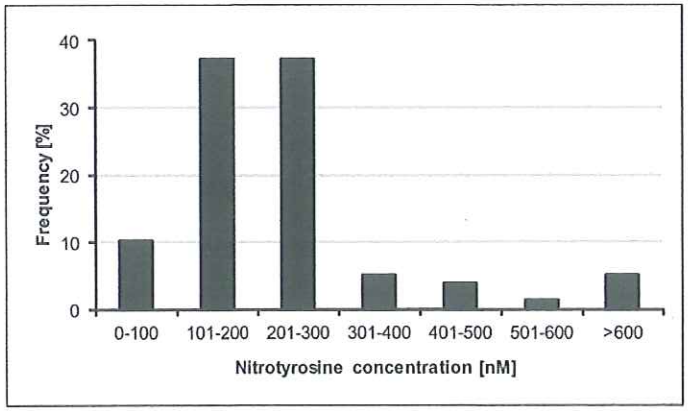


Fig. Reference range of nitrotyrosine

Precision and reproducibility

Intra-Assay

Each of two serum samples was measured 26 times by one technician within the same assay.

Intra-Assay (n=26)		
Sample	Nitrotyrosine [nM]	CV [%]
1	1832	2.7
2	543	9.6

Inter-Assay

Each of two serum sample was independently measured by six, respectively seven different technicians on three different days.

Inter-Assay (n=20)		
Sample	Nitrotyrosine [nM]	CV [%]
1	1876	4.7
2	596	11.0

Recovery

Two different serum samples were spiked with varying concentrations of the nitrotyrosine standard and measured.

Recovery n=2

Endogenous Nitrotyrosine in sample [nM]	Nitrotyrosine Spike [nM]	Nitrotyrosine expected [nM]	Nitrotyrosine measured [nM]
110	300	410	407
110	600	710	731
110	900	1010	1098
119	600	719	666
119	900	1019	975
119	1200	1319	1247

Blank and calculated detection limit

The calculated detection limit (LoB; Limit of Blank) was set as B_0 + 1.645*SD. Standard 1 (blank) was measured 168 times.

LoB = 20.7 nM

This LoB value was estimated based on the concentration of the calibration curve without considering the sample dilution factor. When considering the sample dilution factor, a **LoB** value of **104 nM** was obtained.

12. PRECAUTIONS

- For *in vitro* diagnostic 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 ProClin as bactericide. ProClin is 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. Furthermore, do not assemble cavities of different microplates for analyses, even if the microplates are of the same charge.
- 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.