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Manual

Nitrotyrosine ELISA

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

Valid from 2019-08-07

REF **K 7824**

Σ 96

+2 °C +8 °C

IVD

CE

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1. INTENDED USE

This Immundiagnostik AG assay is an enzyme immunoassay intended for the quantitative determination of protein-bound nitrotyrosine in EDTA plasma, serum and stool. For *in vitro* diagnostic 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 ($\bullet OO^-$) 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 (e. g. 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.	Label	Kit components	Quantity
K 7824	PLATE	Microtiter plate, precoated	12 x8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10 x	1 x 100 ml
K 7824	ASYBUF	Assay buffer, ready-to-use	1 x 25 ml
K 7824	STD	Standards, lyophilised (see specification for concentrations)	4 x 5 vials
K 7824	CTRL1	Control 1, lyophilised (see specification for range)	4 vials
K 7824	CTRL2	Control 2, lyophilised (see specification for range)	4 vials
K 7824	CONJ	Conjugate concentrate (goat anti-nitrotyrosine, peroxidase-labelled)	1 x 200 µl
K 0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultrapure water*
- Stool sample application system such as cat. no.: K 6998SAS
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- Multi-channel pipets or repeater pipets

- Centrifuge, 3000 *g*
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of ultrapure 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. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** 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.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF + 900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** is stable at **2–8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for 1 month**.
- The **lyophilised standards (STD)** and **controls (CTRL)** are stable at **2–8 °C** until the expiry date stated on the label. **Reconstitution** details are given in the **specification data sheet**. **Standards and controls** (reconstituted STD and CTRL) **are not stable and cannot be stored.**
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:101** in **wash buffer** (100 µl CONJ + 10 ml wash buffer). The **CONJ** is stable at **2–8 °C** until the expiry date stated on the label. **Conjugate** (1:101 diluted CONJ) **is not stable and cannot be stored.**
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample stability

Serum samples are stable for 4 days at -20 °C, 2–8 °C or room temperature. More than 3 freeze-thaw cycles should be avoided.

EDTA-plasma samples are stable for 4 days at -20 °C or for one day at 2–8 °C or room temperature. Repeated freezing and thawing should be avoided.

Preparation of samples

EDTA plasma or serum

Fresh EDTA plasma or serum samples must be diluted **1:5** before performing the assay, e. g.

50 µl sample + **200 µl** ASYBUF (assay buffer), mix well.

For testing in duplicates, pipette **2 x 100 µl** of each prepared sample per well.

Extraction of the stool samples

Wash buffer (1:10 diluted WASHBUF) is used as a sample extraction buffer. We recommend the following sample preparation:

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

Stool sample tube – Instructions for use

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

SAS with 0.75 ml sample extraction 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 particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.

b) Fill the **empty sample tube** with **0.75 ml** of **wash buffer** (1:10 diluted WASHBUF) before using it with the sample. Important: Allow the sample extraction buffer to reach room temperature.

c) Unscrew the tube (yellow part of cap) to open. Insert the yellow dipstick into the sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place 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 sample extraction buffer for ~ 10 minutes improves the result.

e) Allow sample to stand for ~10 minutes until sediment has settled. Floating material like shells of grains can be neglected.

f) Carefully unscrew the complete cap of the tube including the blue ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again.

Dilution I: **1:50**

Dilution of samples

Stool samples

The supernatant of the extraction (dilution I) is diluted **1:20 in wash buffer** (diluted WASHBUF). For example:

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

Final dilution: **1 : 1 000**

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

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of nitrotyrosine. It utilises 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 immobilised 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 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. concentration is generated, using the values obtained from the standards. Nitrotyrosine, present in the patient samples, is determined directly from this curve.

Test procedure

Bring all **reagents and samples to room temperature** (15–30 °C) and mix well.

Mark the positions of standards/samples/controls on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminium packaging at 2–8 °C. Strips are stable until the expiry date stated on the label.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	Wash the pre-coated microtiter plate **5 x** with **250 µl wash buffer**. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
2.	Add each **100 µl standards/controls/samples** into the respective wells.
3.	Cover plate or strips with foil tightly and incubate for **2.5 h** at room temperature (15 - 30 °C) **on a horizontal shaker***.
4.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
5.	Add **100 µl conjugate** (diluted CONJ) in each well.
6.	Cover plate or strips with foil tightly and incubate for **1 h** at room temperature (15 - 30 °C) **on a horizontal shaker***.
7.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
8.	Add **100 µl substrate** (SUB) in each well.
9.	Incubate for **10–20 min**** at room temperature (15–30 °C) **in the dark**.

10.	Add **100 µl stop solution** (STOP) and mix well.
11.	Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at **405 nm** against 620 nm as a reference.

* We recommend shaking the strips at 550 rpm with an orbit of 2 mm.

** The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

8. 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 standard must be specified with a value less than 1 (e. g. 0.001).

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 linear abscissa for the concentration.

The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the programme used, the duplicate values should be evaluated manually.

EDTA plasma and serum samples

The obtained results have to be multplied by the **dilution factor of 5** to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result by the dilution factor used.

Stool samples

The obtained results have to be multplied by the **dilution factor of 1 000** to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result by the dilution factor used.

9. LIMITATIONS

Samples with concentrations above the measurement range (see definition below) can be further diluted and re-assayed. Please consider this higher dilution when calculating the results.

Samples with concentrations lower than the measurement range (see definition below) cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:

highest concentration of the calibration curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:

LoB × sample dilution factor to be used

LoB see chapter "Performance Characteristics".

10. QUALITY CONTROL

Immundiagnostik AG recommends the use of external controls for internal quality control, if possible.

Control samples should be analysed 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.

Reference range

Based on Immundiagnostik AG studies of serum samples of apparently healthy persons (n = 78), the following values were estimated:

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.

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n=26

The repeatability was assessed with 3 serum samples under constant parameters (same operator, measurement system, day and kit lot).

Sample	Mean value [nM]	CV [%]
1	1831.48	2.7
2	851.66	5.7
3	546.95	8.7

Reproducibility (Inter-Assay); n=76

The reproducibility was assessed with 3 serum samples under varying parameters (different operators, measurement systems, days and kit lots).

Sample	Mean value [nM]	CV [%]
1	1879.29	5.8
2	790.69	10.3
3	591.22	11.8

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, nitrotyrosine spikes with known concentrations were added to 4 serum samples. The results below were obtained without consideration of the sample dilution factor.

Sample [nM]	Spike [nM]	Expected [nM]	Obtained [nM]	Recovery [%]
110.09	150	260.09	263.16	101.18
	300	410.09	407.24	99.31
	600	710.09	731.32	102.99
	900	1010.09	1097.54	108.66
	1200	1310.09	1441.92	110.06
118.74	150	268.74	234.53	87.27
	300	418.74	363.38	86.78
	600	718.74	665.83	92.64
	900	1018.74	974.45	95.65
	1200	1318.74	1247.08	94.57
76.05	150	226.05	178.30	78.88
	300	376.05	312.69	83.15
	600	676.05	654.27	96.78
	900	976.05	987.86	101.21
	1200	1276.05	1258.69	98.64
49.44	150	199.44	199.88	100.22
	300	349.44	345.61	98.91
	600	649.44	687.66	105.89
	900	949.44	1041.29	109.67
	1200	1249.44	1442.57	115.46

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A with a serial dilution of 3 different serum samples.

For nitrotyrosine in serum, EDTA-plasma and stool, the method has been demonstrated to be linear from 57.79 to 1500 nM based on the standard curve without considering possibly used sample dilution factors, showing a non-linear behaviour of less than ±20 % in this interval.

Sample	Dilution	Expected [nM]	Obtained [nM]	Recovery [%]
1	*1:10*	*2000.00*	*2000.00*	*100.00*
	1:11.88	*1684.21*	*1835.79*	*109.00*
	1:13.2	*1515.15*	*1537.88*	*101.50*
	1:15.83	1263.26	1181.15	93.50
	1:20	1000.00	945.00	94.50
	1:23.75	842.11	825.26	98.00
	1:40	500.00	507.50	101.50
	1:47.5	421.05	452.63	107.50
	1:80	250.00	281.25	112.50
	1:97.5	210.53	254.74	121.00
2	1:5	700.67	700.67	100.00
	1:10	350.33	355.86	101.58
	1:20	175.17	185.33	105.80
	1:40	87.58	103.33	117.98
3	1:5	231.18	231.18	100.00
	1:10	115.59	119.46	103.35
	1:20	57.79	59.87	103.60

Italic: extrapolated data above the range of the standard curve

Analytical sensitivity

The following value has been estimated based on the concentrations of the standards without considering possibly used sample dilution factors (n=168).

Limit of blank, LoB 20.08 nM

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic 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.

- Kit reagents contain sodium azide or ProClin as bactericides. Sodium azide and ProClin are toxic. Substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still should be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analysed with each run.
- Reagents should not be used beyond the expiration date stated 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.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to the enclosed manual.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- 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 incorrect use.

- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

15. REFERENCES

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Used symbols: