DRG® Nitrotyrosin (Plasma) (EIA-5168)

USA: RUO

As of 29 Mar. 2010 rm (Vers. 1.0)

Please use only the valid version of the package insert provided with the kit.

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 *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 (SOD) reacts with superoxide ($O2•^-$) 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

Content	Kit Components	Quantity
PLATE	Microtiter plate, precoated	12 x 8 wells
WASHBUF	ELISA wash concentrate 10x	1 x 100 ml
CONJ	HRP-antibody (concentrate)	1 x 150 µl
STD	Standards (lyophilized)	4 x 6 x 500 µl
ASYBUF	Assay buffer	1x 50 ml
CTRL POS	Positive control	4 x 500 µl
CTRL NEG	Negative control	4 x 500 µl
SUB	TMB substrate (Tetramethylbenzidine), ready-to-use	1 x 15 ml
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
Bidistilled water (aqua bidest.)
Microtiter plate reader at 450 nm (reference wave length 620 or 690 nm)

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 aqua bidest. **1:10** before use (100 ml WASHBUF + 900 ml aqua bidest.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at 37°C using a water bath before dilution.
The **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 controls (**CTRL POS** and **CTRL NEG**) must be reconstituted with **500 µl** of **ASYBUF** (assay buffer) and used in the assay without any further dilution.

– The **CONJ** (HRP-antibody) must be diluted **1:100** with diluted wash buffer.
It should be freshly prepared for each run.
The remainder of the diluted conjugate should be discarded.

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- The **STD** (Standards) must be reconstituted with **500 µl ASYBUF** (assay buffer) and used directly in the assay. The concentration of the standards is given on the enclosed data sheet specification.
- 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

Pipet **50 µl** of fresh EDTA-plasma in a 1.5 ml reaction vial, add **200 µl ASYBUF** (assay buffer) and mix well (corresponds to 1:5 dilution).

7 ASSAY PROCEDURE

7.1 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.

7.2 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), **CTRL POS** (positive control), **CTRL NEG** (negative control) 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), **CTRL POS** (positive control), **CTRL NEG** (negative control) and **SAMPLE** (sample) 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** 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

*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 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.

Plasma samples

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

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

Plasma samples with nitrotyrosine concentrations **outside the standard curve** range should be diluted further with ASYBUF (assay buffer) to obtain readings within the standard curve and re-assayed.

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.

10.1 Expected values

EDTA-Plasma

The following results were obtained by the analysis of plasma samples of evidently healthy persons (n=44):

Mean value: 264,8 nM (lowest value - 47.3 nM; highest value - 1341.3 nM)

75 % of this collective (3rd quartile) had values under 307.9 nM.

It is recommended that each laboratory should determine its own normal range.

11 PERFORMANCE CHARACTERISTICS

11.1 Precision and reproducibility

EDTA-Plasma samples

Intra assay, n=7

Sample 1	[nM]
	1084.8
	1179.1
	1241.0
	978.4
	1043.5
	1031.7
	1249.9
Mean value	1115.5
SD	99.9
CV [%]	9.0

Inter assay. Sample 1; n=3

Day 1 [nM]	Day 2 [nM]	Day 3 [nM]	Mean value [nM]	SD	CV [%]
1147.8	927.0	1115.5	1063.4	97.4	9.16

11.2 Recovery

Two samples were spiked with different amounts of Nitrotyrosine standard and measured.

EDTA Plasma samples (n=2)

Sample [nM]	Nitrotyrosine Spike [nM]	Nitrotyrosine expected [nM]	Nitrotyrosine measured [nM]	Recovery [%]
751.7	1406	2157.7	1683.8	78.1
751.7	703	1454.7	1346.4	92.6
751.7	351	1102.7	1065.4	96.6
97.9	1406	1503.9	1279.9	85.1
97.9	703	800.9	644.9	80.5

11.3 Linearity

One sample was diluted with sample dilution buffer and analyzed. The results are shown in the table below:

EDTA Plasma samples (n= 1)

Dilution [%]	Expected [nM]	Measured [nM]
0	971.4	971.4
10	874.3	946.6
20	777.1	705.8
30	680.0	634.3
40	582.8	508.1
50	485.7	329.3
60	388.6	307.4

12 PRECAUTIONS

For *in vitro* 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

This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
All reagents in the kit package are for *in vitro* diagnostic 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. DRG 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 DRG together with a written complaint.

15 REFERENCES / LITERATURE

1. Gonsette RE (2008) Neurodegenaration in multiple sclerosis : The role of oxidative stress and excitotoxicity. J Neurol Sci. Vol 274. Issue 1-2. 48-53
2. Ischiropoulos H (2008) Protein tyrosine nitration – An update. Arch Biochem Biophys Oct 30
3. Peluffe G. Radi R (2007) Biochemistry of protein tyrosine nitration in cardiovascular pathology. Cardiovasc Res. Jul 15 : 75(2) :291-302
4. Souza JM et al. (2008) Protein tyrosine nitration- functional alteration or just a biomarker ? Free Radic Biol Med. Aug 15 ; 45 (4) :357-356

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SYMBOLS USED WITH DRG ASSAYS

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
RUO				
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilstrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..