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Instructions for use

Normetanephrine Plasma ELISA Fast Track

REF **BA E-8200R**

2°C – 8°C

Σ 96

RUO

For research use only – Not for use in diagnostic procedures

Table of contents

Related Products:

- 2-MET Plasma ELISA Fast Track
- Metanephrine Plasma ELISA Fast Track

1. Introduction

1.1 Intended use and principle of the test

Enzyme Immunoassay for the quantitative determination of free normetanephrine in plasma. The determination of normetanephrine helps in the detection of paragangliomas and pheochromocytomas.
Normetanephrine (normetadrenaline) is first extracted using an ion exchange matrix followed by an acylation process.
The subsequent competitive ELISA uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The acylated standards, controls and samples compete with the solid phase bound analytes for a fixed number of antibody binding sites. After the system is in equilibrium, free antigen and free antigen-antibody complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.
By means of a standard curve the normetanephrine concentrations in the samples are determined. Manual processing is recommended. The use of automatic laboratory equipment is the responsibility of the user. This product is not intended to clinical diagnoses.

1.2 Background

Metanephrine and normetanephrine are the metabolites of the catecholamines epinephrine and norepinephrine, respectively [2]. Cells derived from neuroendocrine tumors (e. g., pheochromocytoma and paraganglioma) are known to produce catecholamines, which are secreted episodically via vesicles into the blood stream [3, 4]. But beside this, a small portion of the catecholamines is metabolized inside the tumor cells to the corresponding catecholamines metabolites – namely metanephrine, normetanephrine (and 3-methoxytyramine in the case of dopamine) – which are secreted at low levels continuously into the blood stream [5, 6].

2. Procedural cautions, guidelines, warnings and limitations

2.1 Procedural cautions, guidelines and warnings

(1) This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and must be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
(2) The principles of Good Laboratory Practice (GLP) must be followed.
(3) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
(4) All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water. Avoid repeated freezing and thawing of reagents and specimens.
(5) The microplate contains snap-off strips. Unused wells must be stored at 2 – 8 °C in the sealed foil pouch with desiccant and used in the frame provided. Microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up.
(6) Duplicate determination of sample is highly recommended.
(7) Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials, and devices are prepared for use at the appropriate time.
(8) Incubation times do influence the results. All wells should be handled in the same order and time intervals.
(9) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
(10) A standard curve must be established for each run.
(11) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report provided with the kit.
(12) Do not mix kit components with different lot numbers within a test and do not use reagents beyond expiry date as shown on the kit labels.
(13) Avoid contact with Stop Solution containing 0.25 M H_2SO_4. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.
(14) TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water.

(15) For information about hazardous substances included in the kit please refer to Safety Data Sheet (SDS). The Safety Data Sheet for this product is made available directly on the website of the manufacturer or upon request.
(16) Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.
(17) In case of any severe damage to the test kit or components, the manufacturer has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components must not be used for a test run. They must be stored properly until the manufacturer decides what to do with them. If it is decided that they are no longer suitable for measurements, they must be disposed of in accordance with national regulations.
(18) Reagents of this kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by approved procedures. All reagents however, should be treated as potential biohazards in use and for disposal.

2.2 Limitations

Any inappropriate handling of samples or modification of this test might influence the results.
Commercially available synthetic normetanephrine is always a mixture of the D- and L-form. This has important implications if synthetic normetanephrine is used to enrich native samples. The antibody used in this kit has a specific D- and L-form recognition rate. Please contact the manufacturer for details in case synthetic normetanephrine was used to enrich native samples.

2.2.1 Interfering substances

Samples containing precipitates or fibrin strands might cause inaccurate results.
Hemolytic samples (up to 1 mg/ml hemoglobin), icteric samples (up to 0.25 mg/ml bilirubin) and lipemic samples (up to 17 mg/ml triglycerides) have no influence on the assay results.
If the concentrations cannot be estimated and there are doubts as to whether the above limit values for hemolytic, icteric or lipemic samples are complied with, the samples should not be used in the assay.

2.2.2 Drug interferences

Medications like antihypertensive agents, antidepressants, antipsychotics, sympathomimetics and L-DOPA can influence plasma metanephrines levels. Caffeinated beverages, nicotine, and mood-enhancing drugs can also affect plasma metanephrines levels. In addition, stress and physical strain should be avoided shortly before sampling.

2.2.3 High-Dose-Hook effect

No hook effect was observed in this test.

3. Storage and stability

Store kit and reagents at 2 – 8 °C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Once opened, the reagents are stable for 2 months when stored at 2 – 8 °C. Once the resealable pouch has been opened, care should be taken to close it tightly again including the desiccant.

4. Materials

4.1 Contents of the kit

BA D-0090	**FOILS**	**Adhesive Foil** – ready to use
Content:	Adhesive foils in a resealable pouch	
Volume:	1 x 4 foils	
BA E-0030	**WASH-CONC 50x**	**Wash Buffer Concentrate** – concentrated 50x
Content:	Buffer with a non-ionic detergent and physiological pH	
Volume:	1 x 20 ml/vial, purple cap	
BA E-0040	**CONJUGAT**	**Enzyme Conjugate** – ready to use
Content:	Goat anti-rabbit immunoglobulins conjugated with peroxidase	
Volume:	1 x 12 ml/vial, red cap	
Description:	Species is goat	
BA E-0055	**SUBSTRATE**	**Substrate** – ready to use
Content:	Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide	
Volume:	1 x 12 ml/vial, black cap	

BA E-0080	**STOP-SOLN**	**Stop Solution** – ready to use
Content:	0.25 M sulfuric acid	
Volume:	1 x 12 ml/vial, grey cap	
Hazards identification:		
	H290 May be corrosive to metals.	
BA E-0231	**Ш NAD NMN**	**Normetanephrine Microtiter Strips** – ready to use
Content:	1 x 96 well (12x8) antigen precoated microwell plate in a resealable yellow pouch with desiccant	
BA E-8210	**NMN-AS**	**Normetanephrine Antiserum** – ready to use
Content:	Rabbit anti-normetanephrine antibody in buffer with proteins and non-mercury preservative, yellow coloured	
Volume:	1 x 6 ml/vial, yellow cap	
Description:	Species of antibody is rabbit, species of protein in buffer is bovine	
BA E-8327	**ADJUST-BUFF**	**Adjustment Buffer** – ready to use
Content:	Tris-Buffer	
Volume:	1 x 10 ml/vial, yellow cap	
BA R-8312	**ACYL-CONC**	**Acylation Concentrate** – concentrated
Content:	Acylation reagent in DMSO	
Volume:	1 x 1.5 ml/vial, white cap	
Hazards identification:		
	H302 Harmful if swallowed. H319 Causes serious eye irritation. H335 May cause respiratory irritation.	
BA R-8313	**ASSAY-BUFF**	**Assay Buffer** – ready to use
Content:	25% organic solvent	
Volume:	1 x 30 ml/vial, orange cap	
BA R-8318	**EXTRACT-PLATE 96**	**Extraction Plate** – ready to use
Content:	1 x 96 well plate, precoated with ion-exchanger in a resealable pouch	
BA R-8325	**CLEAN-CONC 25X**	**Cleaning Concentrate** – concentrated 25x
Content:	Buffer with sodium acetate	
Volume:	1 x 20 ml/vial, brown cap	
BA R-8326	**ELUTION-BUFF**	**Elution Buffer** – ready to use
Content:	0.1 M sodium hydroxide, dark purple coloured	
Volume:	1 x 14 ml/vial, green cap	
BA R-8828	**EQUA-REAG**	**Equalizing Reagent** – ready to use
Content:	Human serum, negative for HIV I/II, HBsAg and HCV	
Volume:	1 x 14 ml/vial, white cap	
Description:	Species is human	

4.2 Calibration and Controls

Standards and Controls – ready to use

Cat. no.	Component	Colour/ Cap	Concentration [pg/ml] NMN	Concentration [pmol/l] NMN	Volume/ Vial
BA E-8301	**STANDARD A**	white	0	0	4 ml
BA E-8302	**STANDARD B**	yellow	72	393	4 ml
BA E-8303	**STANDARD C**	orange	240	1310	4 ml
BA E-8304	**STANDARD D**	blue	720	3931	4 ml
BA E-8305	**STANDARD E**	grey	2400	13104	4 ml
BA E-8306	**STANDARD F**	black	7200	39312	4 ml
BA E-8351	**CONTROL 1**	green	Refer to QC-Report for expected value and acceptable range!		4 ml
BA E-8352	**CONTROL 2**	red			4 ml

Conversion: normetanephrine [pg/ml] x 5.46 = normetanephrine [pmol/l]

Content: Acidic buffer with non-mercury stabilizer, spiked with a defined quantity of metanephrine and normetanephrine.

4.3 Additional materials required but not provided in the kit

- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)

4.4 Additional equipment required but not provided in the kit

- Calibrated precision pipettes to dispense volumes between 20 – 350 µl; 3 ml
- Microtiter plate washing device (manual, semi-automated or automated)
- ELISA reader capable of reading absorbance at 450 nm and if possible 620 – 650 nm
- Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Vortex mixer

5. Sample collection and storage

EDTA- or Heparin-Plasma

Whole blood should be collected into centrifuge tubes (Monovette or Vacuette) containing EDTA or heparin as anti-coagulant and centrifuged (according to manufacturer's instructions) immediately after collection. When in doubt, it is recommended that hemolytic, icteric, and lipemic samples not be used in the assay (see 2.2.1).

Storage: up to 3 days at 2 – 8 °C, for longer period (up to 6 months) at -20 °C.

Repeated freezing and thawing should be avoided.

6. Test procedure

Allow all reagents and samples to reach room temperature and mix thoroughly by gentle inversion before use. Number the Extraction Plate and microwell plates (Microtiter Strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up). Duplicate determinations are recommended.

The binding of the antisera and of the enzyme conjugate and the activity of the enzyme are temperature dependent. The higher the temperature, the higher the absorption values will be. Varying incubation times will have similar influences on the absorbance. The optimal temperature during the Enzyme Immunoassay is between 20 – 25 °C.

If the product is prepared in parts, unused wells in Reaction and Extraction Plates should be covered to avoid contamination. After preparation, the used wells must be labeled to prevent double use.

During the overnight incubation at 2 – 8 °C with the antiserum, the temperature should be uniform all over the ELISA plate to avoid any drift and edge-effect.

⚠ *The use of a microtiter plate shaker with the following specifications is mandatory: shaking amplitude 3 mm; approx. 600 rpm. Shaking with differing settings might influence the results.*

6.1 Preparation of reagents and further notes

Wash Buffer

Dilute the 20 ml Wash Buffer Concentrate **WASH-CONC** **50X** with water to a final volume of 1000 ml.

Storage: 2 months at 2 – 8 °C

Cleaning Buffer
Dilute the 20 ml Cleaning Concentrate **CLEAN-CONC** **25X** with water to a final volume of 500 ml.
Storage: 2 months at 2 – 8 °C

Acylation Solution

⚠ As the Acylation Solution is only **stable for a maximum of 3 minutes,** it should not be prepared before starting the assay. Therefore, its preparation is described in the protocol in chapter 6.3, step 3. Discard after use!

Normetanephrine Microtiter Strips
In rare cases residues of the blocking and stabilizing reagent can be seen in the wells as small, white dots or lines. These residues do not influence the quality of the product.

Extraction Plate
In rare cases residues of the cation exchanger can be seen in the wells as small, black dots or lines. These residues do not influence the quality of the product.

6.2 Preparation of samples – Extraction

The following extraction procedure can be run with 200 µl or 250 µl of plasma sample.
The procedure for 250 µl plasma is highlighted in grey and italicised and may be used in case higher supernatant volumes for pipetting to the subsequent ELISA are preferred.
The ELISA procedure itself is not affected by this alternative protocol.

1.	Pipette **20 µl** of **standards** and **controls** into the respective wells of the **EXTRACT-PLATE** **96**.
	Alternatively** pipette **25 µl** of **standards** and **controls.
2.	Add **20 µl** **STANDARD A** to all wells intended for the **plasma samples**.
	***Alternatively** add **25 µl** **STANDARD A**.*
3.	Add **200 µl** of **EQUA-REAG** to the wells with **standards and controls.**
	***Alternatively** add **250 µl** of **EQUA-REAG**.*
4.	Pipette **200 µl** of **plasma samples** to the respective wells.
	Alternatively** pipette **250 µl** of **plasma samples.
5.	Incubate plate **for 2 h** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
6.	Empty plate and blot dry by tapping the inverted plate on absorbent material.
7.	Pipette **250 µl** of **ASSAY-BUFF** into all wells. Incubate the plate for **5 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm). Empty plate and blot dry by tapping the inverted plate on absorbent material.
8.	Wash the plate **3 times** by adding **350 µl** of **Cleaning Buffer**, **discarding** the content and **blotting dry each time** by tapping the inverted plate on absorbent material.
9.	Pipette **100 µl** of **ELUTION-BUFF** into all wells.
	***Alternatively** pipette **125 µl** of **ELUTION-BUFF**.*
	Please note: the colour changes caused by the elution buffer can vary between standards and samples.
10.	Cover plate with **FOIL**. Incubate **15 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
⚠	Remove the **FOIL**.
	Do not decant the supernatant thereafter!
	The following volume of the supernatant is needed for the subsequent ELISA:
	Normetanephrine 25 µl

6.3 Normetanephrine ELISA

1.	Pipette **25 µl** of **ADJUST-BUFF** into all wells of the **Normetanephrine Microtiter Strips Ш NAD NMN**.
2.	Pipette **25 µl** of the extracted **standards, controls and samples** into the respective wells. *Please hold the Extraction Plate at a slight angle in order to facilitate this pipetting step.*
3.	Preparation of **Acylation Solution**: Pipette **80 µl** **ACYL-CONC** to **3 ml water** and mix thoroughly
4.	Pipette **25 µl** of the freshly prepared **Acylation Solution** into all wells.
5.	Incubate for **15 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
6.	Pipette **50 µl** of the **Normetanephrine Antiserum** **NMN-AS** into all wells.
7.	Cover the plate with **Adhesive Foil**, shake for **1 min** at **RT** (20 – 25 °C) on a **shaker** and incubate for **15 – 20 h** (overnight) at **2 – 8 °C** without shaking.
8.	Remove the foil. Discard or aspirate the contents of the wells. Wash the plate **4 times** by adding **300 µl** of **Wash Buffer, discarding** the content and **blotting dry each time** by tapping the inverted plate on absorbent material.
9.	Pipette **100 µl** of the **CONJUGATE** into all wells.
10.	Incubate for **30 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
11.	Discard or aspirate the contents of the wells. Wash the plate **4 times** by adding **300 µl** of **Wash Buffer, discarding** the content and **blotting dry each time** by tapping the inverted plate on absorbent material.
12.	Pipette **100 µl** of the **SUBSTRATE** into all wells and incubate for **20 – 30 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm). ***Avoid exposure to direct sunlight!***
13.	Add **100 µl** of the **STOP-SOLN** to all wells and shake the microtiter plate shortly.
14.	**Read** the absorbance of the solution in the wells within 10 min, using a microplate reader set to **450 nm** (if available a reference wavelength between 620 nm and 650 nm is recommended).

7. Calculation of results

Measuring range	Normetanephrine
	22.8 – 7200 pg/ml

The standard curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x-axis) using a concentration of 0.001 pg/ml for Standard A (this alignment is mandatory because of the logarithmic presentation of the data).
Use non-linear regression for curve fitting (e. g. 4-parameter, marquardt).

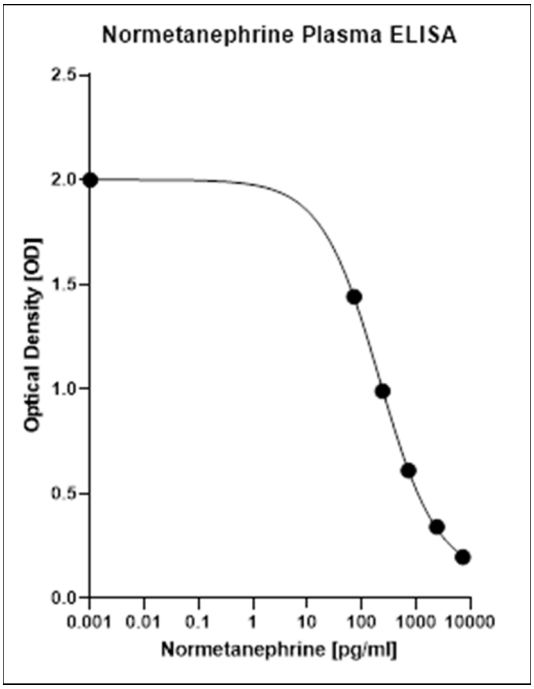
⚠ *This assay is a competitive assay. This means: the OD-values are decreasing with increasing concentrations of the analyte. OD-values found below the standard curve correspond to high concentrations of the analyte in the sample and have to be reported as being positive.*

The concentrations of the **samples and controls** can be read directly from the standard curve.
Samples found with concentrations higher than the highest standard (Standard F) should be diluted accordingly with the included Equalizing Reagent **EQUA-REAG** and have to be re-assayed.

Conversion:
normetanephrine [pg/ml] x 5.46 = normetanephrine [pmol/l]

7.1 Typical standard curve

⚠*Example, do not use for calculation!*



8. Controls

The confidence limits of the kit controls are indicated on the QC-Report.

9. Assay characteristics

9.1 Performance data

Analytical Sensitivity	
	Normetanephrine
Limit of Blank (LOB)	11.7 pg/ml
Limit of Detection (LOD)	17.9 pg/ml
Limit of Quantification (LOQ)	22.8 pg/ml

Analytical Specificity (Cross Reactivity)	
Substance	**Cross Reactivity [%]**
	Normetanephrine
Metanephrine	0.72
Normetanephrine	100
3-Methoxytyramin	6.5*
Adrenaline	< 0.01
Noradrenaline	< 0.01
Dopamin	< 0.01
Vanillic mandelic acid	< 0.01
Homovanillic acid	< 0.01
L-DOPA	< 0.01
L-Tyrosin	< 0.01
Tyramine	< 0.01
Acetaminophen	< 0.01

*Normetanephrine concentrations are not influenced by 3-methoxytyramine in case of normal 3-methoxytyramine concentrations. Only very high 3-methoxytyramine concentrations found in rare cases of exclusively dopamine secreting tumours can cause false positive results.

Precision							
Intra-Assay				**Inter-Assay**			
	Sample	Mean [pg/ml]	CV [%]		Sample	Mean [pg/ml]	CV [%]
Normetanephrine	1	149	9.5	Normetanephrine	1	156	10.6
	2	282	9.1		2	287	5.0
	3	734	8.2		3	769	5.1
	4	1956	10.5		4	1949	5.9

Lot-to-Lot			
	Sample	Mean ± SD [pg/ml]	CV [%]
Normetanephrine (n=6)	1	231 ± 29.9	13.0
	2	1688 ± 116	6.9

Recovery			
	Range [pg/ml]	Mean [%]	Range [%]
Normetanephrine	77.4 – 7285	109	105 – 114

Linearity			
	Serial dilution up to	Mean [%]	Range [%]
Normetanephrine	1:64	98	92 – 102

Method Comparison: ELISA vs. LC-MS/MS [14]	
Normetanephrine	$y = 0.93x + 13$; $r^2 = 0.99$; n = 48

9.2 Metrological Traceability

The values assigned to the standards and controls of the Normetanephrine Plasma ELISA Fast Track are traceable to SI Units by calibrated weighing with quality-controlled analyte.

Standards and Controls	
	Uncertainty [%]
Normetanephrine	2.0

2-MET Plasma ELISA Fast Track	
	Expanded Uncertainty [%] k = 2*
Normetanephrine	10.8

*This defines an interval about the measured result that will include the true value with a probability of 95%.

10. References/Literature

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For updated literature or any other information please contact your local supplier.

11. Changes

Version	Release Date	Chapter	Change
20.0-r	2022-03-25	All	- The alternative version, 2 h at RT incubation with antiserum, was removed - Sample stability (chapter 5) changed - LOB and Lot to Lot were added to the assay characteristics (chapter 9.1) - Metrological traceability was added (chapter 9.2) - References/Literature was updated (chapter 10)

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number		Distributor
	Date of manufacture			RUO	For research use only!