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Instructions for use

Normetanephrine (Urine) ELISA

Enzyme Immunoassay for the determination of Normetanephrine in urine.

For research use only, not for use in diagnostic procedures.

REF

IB89185



1. Introduction

1.1 Intended use and principle of the test

Enzyme Immunoassay for the determination of Normetanephrine in urine. For research use only, not for use in diagnostic procedures.

During the sample preparation Normetanephrine (Normetadrenaline) is acylated.

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 and the solid phase bound analyte compete 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.

Determination of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

1.2 Background

Metanephrine and Normetanephrine are the metabolites of the catecholamines Epinephrine and Norepinephrine, respectively. They are metabolized to Vanillylmandelic acid or excreted with the urine.

Subjects with pheochromocytoma or other tumors derived from neuroendocrine cells show elevated urinary levels of total Metanephrines.

As catecholamine secretion from neuroendocrine cells might show high variations, urine samples collected over a period of 24 hours are used to average these fluctuations.

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 has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
- (2) This assay was validated for a certain type of sample as indicated in *Intended Use* (please refer to Chapter 1). Any off-label use of this kit is in the responsibility of the user and the manufacturer cannot be held liable.
- (3) The principles of Good Laboratory Practice (GLP) have to be followed.
- (4) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
- (5) All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- (6) For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
- (7) The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch with desiccant and used in the frame provided.
- (8) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
- (9) Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
- (10) Incubation times do influence the results. All wells should be handled in the same order and time intervals.
- (11) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
- (12) A standard curve must be established for each run.
- (13) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
- (14) 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.
- (15) Avoid contact with Stop Solution containing 0.25 M H₂SO₄. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.
- (16) 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. Wash contaminated objects before reusing them.
- (17) For information on hazardous substances included in the kit please refer to Material Safety Data Sheet (MSDS). The Material Safety Data Sheet for this product is made available directly on the website of the manufacturer or upon request.
- (18) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.
- (19) The results obtained with this test kit are for research use only, not for use in diagnostic procedures.
- (20) Kit reagents must be regarded as hazardous waste and disposed according to national regulations.

2.2 Limitations

Any inappropriate handling of samples or modification of this test might influence the results.

2.2.1 Interfering substances

24-hour urine

Please note the *sample collection and storage!* If the percentage of the final concentration of acid is too high, the buffer capacity of the Acylation Buffer is insufficient. As a consequence Normetanephrine will not be acylated adequately.

2.2.2 Drug interferences

There are no known substances (drugs) which ingestion interferes with the measurement of Normetanephrine level in the sample.

2.2.3 High-Dose-Hook effect

No hook effect was observed in this test.

3. Storage and stability

Store the unopened 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 1 month when stored at 2 - 8 °C. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again.

4. Materials

4.1 Content of the kit

BA D-0023	REAC-TUBES	Reaction Tubes - Ready to use
Content:	Reaction Tubes in a resealable pouch	
Volume:	2 x 50 tubes	
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, light purple cap	
BA E-0045	CONJUGATE	Enzyme Conjugate - Ready to use
Content:	Goat anti-rabbit immunoglobulins conjugated with peroxidase	
Volume:	1 x 12 ml/vial, red cap	
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, light grey cap	
BA E-0231	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-8510	NMN-AS	Normetanephrine Antiserum - Ready to use
Content:	Rabbit anti-Normetanephrine antibody, yellow coloured	
Volume:	1 x 12 ml/vial, yellow cap	

Standards and Controls - Ready to use

Cat. no.	Component	Colour/Cap	Concentration	Concentration	Volume/ Vial
			ng/ml (= µg/l)	nmol/l	
			NMN	NMN	
BA R-8601	STANDARD A	white	0	0	4 ml
BA R-8602	STANDARD B	light yellow	30	164	4 ml
BA R-8603	STANDARD C	orange	90	491	4 ml
BA R-8604	STANDARD D	dark blue	300	1 638	4 ml
BA R-8605	STANDARD E	light grey	900	4 914	4 ml
BA R-8606	STANDARD F	black	3 000	16 380	4 ml
BA R-8651	CONTROL 1	light green	Refer to QC-Report for expected value and acceptable range!		4 ml
BA R-8652	CONTROL 2	dark red			4 ml

Conversion: Normetanephrine (ng/ml) x 5.46 = Normetanephrine (nmol/l)

Content: Acidic buffer with non-mercury preservatives, spiked with defined quantity of Normetanephrine

BA R-0012 ACYL-CONC Acylation Concentrate - Concentrated

Content: Concentrated acylation reagent

Volume: 1 x 0.5 ml/vial

Hazards identification:



H 314 Causes severe skin burns and eye damage.

BA R-0075 ACYL-DILUENT Acylation Diluent - Ready to use

Content: Dimethylsulfoxide

Volume: 1 x 4 ml/vial, dark grey cap

BA R-8611 ACYL-BUFF Acylation Buffer - Ready to use

Content: TRIS buffer

Volume: 1 x 30 ml/vial, white cap

BA R-8619 HCL Hydrochloric Acid - Ready to use

Content: 0.25 M hydrochloric acid, yellow coloured

Volume: 1 x 30 ml/vial, dark green cap

4.2 Additional materials and equipment required but not provided in the kit

- Calibrated precision pipettes to dispense volumes between 10 – 600 µl; 1.2 – 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
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer
- Temperature controlled water bath (90 °C) or similar heating device



The assay can be performed with or without shaking. If a microtiter plate shaker is used, it should have the following characteristics: shaking amplitude 3 mm; approx. 600 rpm

5. Sample collection and storage

Spontaneous or 24-hour urine, collected in a bottle containing 10 - 15 ml of 6 M HCl, should be used. Determine the total volume of urine excreted during a period of 24 h for calculation of the results.

Storage: for longer periods (up to 6 months) at -20 °C.

Repeated freezing and thawing should be avoided.

Avoid exposure to direct sunlight.

6. Test procedure

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the Reaction Tubes accordingly. Duplicate determinations are recommended.

The binding of the antibodies and the enzyme conjugates and the activity of the enzyme used are temperature dependent, and the absorption values may vary if a thermostat is not used. The higher the temperature, the higher the absorption values will be. The absorption values also depend on the incubation times. The optimal temperature during the Enzyme Immunoassay is between 20 – 25 °C.



In case of overflow, read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 405 nm.

6.1 Preparation of reagents

Wash Buffer

Dilute the 20 ml Wash Buffer Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 1000 ml.

Storage: 1 month at 2 – 8 °C

Acylation Solution



Before preparing the Acylation Solution make sure that the Acylation Diluent (BA R-0075) has reached room temperature ($\geq 20^{\circ}\text{C}$) and forms a homogenous, crystal-free solution.


Dilute the Acylation Concentrate (BA R-0012) 1 + 60 with Acylation-Diluent in a glass or polypropylene-vial.

Acylation Concentrate	10 μl	20 μl	25 μl	50 μl
Acylation-Diluent	600 μl	1.2 ml	1.5 ml	3 ml


 *The Acylation Solution has to be prepared freshly prior to the assay (not longer than 60 minutes in advance). Discard after use!*

6.2 Sample preparation and acylation

Hydrolysis


1. Pipette 25 μl of standards, controls, and urine samples into the respective Reaction Tubes .
2. Add 250 μl Hydrochloric Acid to all tubes.
3. Mix thoroughly (vortex) and hydrolyze for 30 min at 90 °C .
4. Cool down the tubes to room temperature.
 For the measurement of the free Normetanephrine only, leave away step 3 and 4.

Acylation

1. Pipette 250 μl of Acylation Buffer into all tubes.
2. Add 25 μl of Acylation Solution (<i>refer to 6.1</i>) to all tubes.
3. Mix thoroughly (vortex) and acylate for 15 min at RT (20 – 25 °C).
4. Add 2.5 ml water (deionized, distilled, or ultra-pure) to all tubes.
 Take 25 μl of the acylated standards, controls and urine samples for the Normetanephrine ELISA .

6.3 Normetanephine ELISA


The usage of a shaker is not mandatory. The alternative protocol without shaker is highlighted in italic and shaded in grey.

1.	Pipette 25 µl of the acylated standards, controls and samples into the appropriate wells of the Normetanephine Microtiter Strips .
2.	Pipette 100 µl of the Normetanephine Antiserum into all wells.
3.	Incubate 30 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: shake Normetanephine Microtiter Strips shortly by hand and incubate for 1 h at RT (20 – 25 °C).</i>
4.	Discard or aspirate the content of the wells. Wash the plate 3 x by adding 300 µl of Wash Buffer , discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
5.	Pipette 100 µl of the Enzyme Conjugate into all wells.
6.	Incubate for 15 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: incubate for 15 min at RT (20 – 25 °C).</i>
7.	Discard or aspirate the content of the wells. Wash the plate 3 x by adding 300 µl of Wash Buffer , discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
8.	Pipette 100 µl of the Substrate into all wells.
9.	Incubate for 15 ± 2 min at RT (20 – 25 °C) on a shaker (approx. 600 rpm).  <i>Without usage of a shaker: incubate for 15 min ± 2 at RT (20 – 25 °C).</i> Avoid exposure to direct sunlight!
10.	Add 100 µl of the Stop Solution to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.
11.	Read the absorbance of the solution in the wells within 10 minutes, 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	Normetanephine
	23 – 3 000 ng/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). Use a non-linear regression for curve fitting (e.g. spline, 4- parameter, akima).

 *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.

The amount of analyte excreted per day (µg/day) is calculated according to:

$$\text{concentration of the sample (in } \mu\text{g/l)} \times \text{volume of urine excreted per day (in l/day)}$$

Example

The concentration of the sample read from the curve is 125 µg/l. The amount of urine collected during 24 hours is 1.3 l. Then the amount of analyte excreted during one day would be:

$$125 \mu\text{g/l} \times 1.3 \text{ l/day} = 162.5 \mu\text{g/day}$$

Conversion

$$\text{Normetanephine (ng/ml)} \times 5.46 = \text{Normetanephine (nmol/l)}$$

Expected reference value

It is strongly recommended that each laboratory should determine its own reference value.

	Normetanephine
24-hour urine	< 600 µg/day

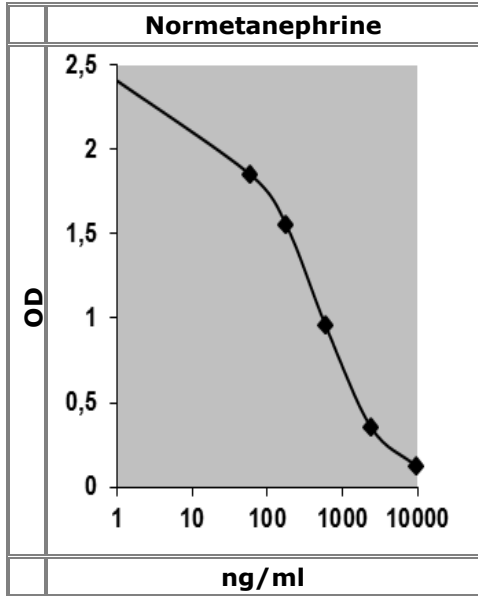
7.1 Quality control

It is recommended to use control samples according to national regulations. Use controls at both normal and elevated levels. The kit or other commercial controls should fall within established confidence limits. The confidence limits of the kit controls are indicated on the QC-Report.

7.2 Typical standard curve



Example, do not use for calculation!



8. Assay characteristics

Analytical Sensitivity (Limit of Detection)		Normetanephrine
	Urine	23 ng/ml

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)
		Normetanephrine
	Derivatized Metanephrine	0.11
	Derivatized Normetanephrine	100
	Derivatized 3-methoxytyramine	0.19
	Adrenaline	< 0.001
	Noradrenaline	0.64
	Dopamine	< 0.01
	Vanillic mandelic acid, L-Dopa, Homovanillic acid, L-Tyrosin, Tyramin	< 0.001

Precision							
Intra-Assay				Inter-Assay			
	Sample	Range (ng/ml)	CV (%)		Sample	Range (ng/ml)	CV (%)
Normetanephrine	1	200 ± 34	17.2	Normetanephrine	1	191 ± 41	21.4
	2	857 ± 153	17.8		2	682 ± 131	19.3

Linearity			Range	Serial dilution up to	Mean (%)
	Normetanephrine	Urine	40 – 5 200 ng/ml	1:16	93

Recovery			Mean (%)	Range (%)	% Recovery after spiking
	Normetanephrine	Urine	103	90 – 113	

Method Comparison versus HPLC*	Normetanephrine	Urine	HPLC = 0.9 ELISA + 0.6	r = 0.99; n = 40
*The concentrations were assessed using both the ELISA and the HPLC method (external QC samples from UK NEQAS). The correlation between ELISA and HPLC is excellent. Please take in mind, that the UK control values are the mean of about 40 different HPLC users, and contain always one pathological sample per sending.				

9. References/Literature







- (1) Parrott et al. Urinary corticosterone and normetanephrine levels after voluntary wheel and forced treadmill running in the db/db mouse. *Journal of Diabetes Mellitus*, 1(4):71-78 (2011)
- (2) Petramala et al. Multiple Catecholamine-Secreting Paragangliomas: Diagnosis after Hemorrhagic Stroke in a Young Woman. *Endocrine Practice*, 14(3):340-346 (2008)
- (3) Sato et al. Central control of bone remodeling by neuromedin U. *Nature Medicine*, 13:1234-1240 (2007)

For orders, please contact:

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

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code	IVD	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content	CE	CE labelled
	Caution	REF	Catalogue number	RUO	For research use only!