Manufactured for Immuno-Biological Laboratories Inc.
(IBL-America)
8201 Central Avenue, NE, Suite P
Minneapolis, MN 55432
Tel: 763-780-2955
Toll Free: 1-888-523-1246

Instructions for use

Normetanephrine (Plasma) ELISA

Enzyme Immunoassay for the determination of free Normetanephrine in plasma.

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

REF **IB89182**

Σ 96

+2 +8 °C

1. Introduction

1.1 Intended use and principle of the test

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

First, the plasma proteins are removed by precipitation. After this 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 analytes 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 standards.

⚠ *The antibodies used in this test kit only recognise the biologically relevant L-forms of Metanephrines. Commercially available synthetic Normetanephrine or Metanephrine is always a mixture of the D- and L-forms. The ratio between both forms differs widely from lot to lot. This has important implications if synthetic Metanephrines are used to enrich native samples. As only about 50% of the synthetic Metanephrines - the L-portion - will be detected by use of this kit, spiked samples will be underestimated. Therefore native samples containing solely the L-form should be used.*

1.2 Background

Metanephrine and Normetanephrine are the metabolites of the catecholamines Epinephrine and Norepinephrine, respectively. Cells derived from neuroendocrine tumors (e.g. pheochromcytoma) are known to produce catecholamines which are secreted episodically via vesicles into the blood stream. But beside this a small portion of the catecholamines is metabolized inside the cells to the corresponding catecholamines metabolites – namely Metanephrine, Normetanephrine and 3-Methoxytyramine – which are secreted at low levels continuously into the blood stream.
Recent studies and publications have shown that the determination of these plasma free Metanephrine and plasma free Normetanephrine is the most accurate biochemical marker for the study of pheochromocytoma and follow-up of pheochromocytoma subjects.

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) 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.
(4) The principles of Good Laboratory Practice (GLP) have to be followed.
(5) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
(6) 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.
(7) For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
(8) 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.
(9) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
(10) 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.
(11) Incubation times do influence the results. All wells should be handled in the same order and time intervals.
(12) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
(13) A standard curve must be established for each run.
(14) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.

(15) 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.
(16) 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.
(17) 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.
(18) 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.
(19) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.
(20) The results obtained with this test kit are for research use only, not for use in diagnostic procedures.
(21) 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

Serum/Plasma

Samples containing precipitates or fibrin strands or which are haemolytic or lipemic might cause inaccurate results.

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 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, light purple cap

BA E-0040 CONJUGATE **Enzyme Conjugate** - Ready to use
Content: Goat anti-rabbit immunoglobulins conjugated with peroxidase.
Volume: 1 x 12 ml/vial, red cap

Standards and **Controls** - Ready to use

Cat. no.	Component	Colour/Cap	Concentration pg/ml NMN	Concentration pmol/l NMN	Volume/ Vial
BA R-8301	STANDARD A	white	0	0	12 ml
BA R-8302	STANDARD B	light yellow	48	262	4 ml
BA R-8303	STANDARD C	orange	160	874	4 ml
BA R-8304	STANDARD D	dark blue	480	2 621	4 ml
BA R-8305	STANDARD E	light grey	1 600	8 736	4 ml
BA R-8306	STANDARD F	black	4 800	26 208	4 ml
BA R-8351	CONTROL 1	light green	Refer to QC-Report for expected value and acceptable range!		4 ml
BA R-8352	CONTROL 2	dark red			4 ml

Conversion: Normetanephrine (pg/ml) x 5.46 = Normetanephrine (pmol/l)

Content: Buffer with stabilizer and a precipitating reagent spiked with a defined quantity of Normetanephrine

Hazards identification:

H302 Harmful if swallowed.

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-8210 NMN-AS **Normetanephrine Antiserum** - Ready to use

Content: Anti- Normetanephrine rabbit antibody, yellow coloured.

Volume: 1 x 6 ml/vial, yellow cap

BA E-8313 ASSAY-BUFF **Assay Buffer** - Ready to use

Content: TRIS-buffer containing proteins and a non-mercury preservative.

Volume: 1 x 12 ml/vial, orange cap

Hazards identification: H317 May cause an allergic skin reaction.

BA R-0028 EQUA-REAG **Equalizing Reagent** - Lyophilized

Content: Human serum, negative for HIV I/II, HBsAg and HCV

Volume: 2 vials, dark green cap

BA R-8312 ACYL-CONC **Acylation Concentrate** – Concentrated

Content: Acylation reagent in DMSO

Volume: 1 x 1.5 ml/vial, dark grey cap

Hazards identification:

H302 Harmful if swallowed.

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

- Calibrated precision pipettes to dispense volumes between 25 - 500 µl; 3 ml; 10 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
- Centrifuge capable of at least 3.000 x g
- microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer

5. Sample collection and storage

EDTA-, Citrate- or Heparin-Plasma
Whole blood should be collected into centrifuge tubes (Monovette™ or Vacuette™) containing EDTA, heparin or citrate as anti-coagulant and centrifuged (according to manufacturer's instructions) immediately after collection.
Haemolytic and lipemic samples should not be used for the assay.
Storage: up to 6 hours at 2 - 8 °C, for longer period (up to 6 month) at -20 °C.
Repeated freezing and thawing should be avoided.

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

Equalizing Reagent
Reconstitute the Equalizing Reagent with 10 ml water (deionized, distilled, or ultra-pure).
Reconstituted Equalizing Reagent which is not used immediately has to be stored in aliquots for max. 1 month at -20 °C and may be thawed only once.

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!

6.2 Precipitation

1.	Pipette **100 µl** of **standards**, **100 µl** of **controls,** and **500 µl** of **plasma samples** into the respective **Reaction Tubes.**
2.	Add **500 µl Equalizing Reagent** *(refer to 6.1)* to all tubes containing standards and controls.
3.	Add **100 µl Standard A** to all tubes containing plasma samples.
4.	Mix **Reaction Tubes** thoroughly (vortex) and centrifuge for **15 minutes** at **3,000 x g**.
⚠	Take **25 µl** of the clear supernatant for the Normetanephrine ELISA.

6.3 Normetanephrine ELISA

1.	Pipette **50 µl** of **Assay Buffer** into the appropriate wells of the **Normetanephrine Microtiter Strips**.
2.	Pipette **25 µl** of the **clear supernatant** from the **standards, controls and samples** into the wells.
3.	Preparation of **Acylation Solution**: Pipette **80 µl Acylation Reagent Concentrate** (BA R-8312) to **3 ml water** (deionized, distilled, or ultra-pure) 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** 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 content of the wells. Wash the plate **4 x** 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 **Enzyme 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 content of the wells and wash the plate **4 x** 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 Solution** to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14.	**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	Normetanephrine
	23 – 4 800 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). 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.

Samples found with concentrations higher than the highest standard (Standard F) should be diluted accordingly with Equalizing Reagent (BA R-0028) and have to be re-assayed.

Conversion
Normetanephrine (pg/ml) x 5.46 = Normetanephrine (pmol/l)

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

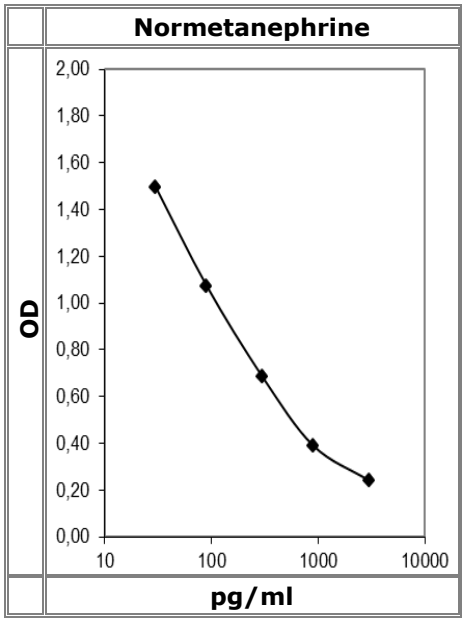
Normetanephrine
< 180 pg/ml

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 in the QC-Report.

7.2 Typical standard curve

⚠ *Example, do not use for calculation!*



8. Assay characteristics

Analytical Sensitivity (Limit of Detection)		**Normetanephrine**
	Plasma	23 pg/ml

	Substance	**Cross Reactivity (%)**
		Normetanephrine
Analytical Specificity (Cross Reactivity)	Derivatized Normetanephrine	100
	Derivatized Metanephrine	0.08
	3-Methoxytyramin.HCl	1.74
	Adrenaline	< 0.001
	Noradrenaline	< 0.001
	Dopamin.HCl	< 0.001
	VMS	< 0.001
	HMVS	< 0.001
	L-DOPA	< 0.001
	L-Tyrosin	< 0.001
	Tyramine.HCl	< 0.001
	Normetanephrine	< 0.001
	Acetaminophen	< 0.001

Precision							
Intra-Assay				**Inter-Assay**			
	Sample	Range (pg/ml)	CV (%)		Sample	Range (pg/ml)	CV (%)
Normetanephrine	1	167 ± 12	7.3	Normetanephrine	1	161 ± 18	11
	2	373 ± 29	7.8		2	370 ± 19	5.1
	3	832 ± 60	7.2		3	844 ± 51	6.0

			Range	Serial dilution up to	Mean (%)
Linearity	Normetanephrine	Plasma	58 – 5800	1: 129	109

			Mean (%)	Range (%)	% Recovery after spiking
Recovery	Normetanephrine	Plasma	92	80 – 108	

Method Comparison ELISA vs. LC-MS/MS	Normetanephrine	Plasma	LC-MS/MS = 1.2x + 10.5	r = 0.99; n = 59

9. References/Literature

(1) Jeyaraman et al. The role of urinary fractionated metanephrines in the diagnosis of phaeochromocytoma. The Indian Journal of Medical Research, 137(2):316-323 (2013)

(2) Unger et al. Plasma and Urinary Metanephrines Determined by an Enzyme Immunoassay, but not Serum Chromogranin A for the Diagnosis of Pheochromocytoma in Patients with Adrenal Mass. Exp Clin Endocrinol Diabetes, 120:494-500 (2012)

(3) Stefanescu et al. Salivary free catecholamines metabolites as possible biochemical markers in pheochromocytoma diagnosis. Acta Endocrinologica (Buc), VII (4): 431-442 (2011)

For orders, please contact:

Immuno-Biological Laboratories, Inc. (IBL-America)
8201 Central Ave NE, Suite P
Minneapolis, MN 55432
Email: info@ibl-america.com
Web: www.ibl-america.com
Phone: (888) 523-1246
Fax: (763) 780-2988

⚠ ***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	I V D	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content	CE	CE labelled
	Caution	REF	Catalogue number	RUO	For research use only!