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**Instructions for use**  
**Metanephrine Plasma ELISA** Fast Track

Enzyme Immunoassay for the determination of free Metanephrine in plasma.

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

**REF**

**IB89181**



## **1. Introduction**

### **1.1 Intended use and principle of the test**


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

Alternatively the assay can also be run automatically on an ELISA processor such as the Gemini instrument from Stratec Biomedical. The Gemini protocol is available upon request.

Metanephrine (Metadrenaline) 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 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-form. 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. pheochromocytoma) 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 research of pheochromocytoma and follow-up of pheochromocytoma subjects.

This assay is for research use only, not for use in diagnostic procedures.

## **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. Microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up.
- (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<sub>2</sub>SO<sub>4</sub>. 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

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

### 2.2.2 Drug interferences

Please refer to point "Sample collection and storage".

### 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

<b>BA D-0090</b>	<b>FOILS</b>	<b>Adhesive Foil</b> - Ready to use
Content:	Adhesive Foils in a resealable pouch	
Volume:	1 x 4 foils	
<b>BA E-0030</b>	<b>WASH-CONC 50x</b>	<b>Wash Buffer Concentrate</b> - Concentrated 50x
Content:	Buffer with a non-ionic detergent and physiological pH	
Volume:	1 x 20 ml/vial, light purple cap	
<b>BA E-0040</b>	<b>CONJUGATE</b>	<b>Enzyme Conjugate</b> - Ready to use
Content:	Goat anti-rabbit immunoglobulins conjugated with peroxidase	
Volume:	1 x 12 ml/vial, red cap	
<b>BA E-0055</b>	<b>SUBSTRATE</b>	<b>Substrate</b> - Ready to use
Content:	Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide	
Volume:	1 x 12 ml/vial, black cap	
<b>BA E-0080</b>	<b>STOP-SOLN</b>	<b>Stop Solution</b> - Ready to use
Content:	0.25 M sulfuric acid	
Volume:	1 x 12 ml/vial, light grey cap	

**BA E-0131** MI ADR MN **Metanephrine Microtiter Strips** - Ready to use  
 Content: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable blue pouch with desiccant

**BA E-8110** MN-AS **Metanephrine Antiserum** - Ready to use  
 Content: Rabbit anti- Metanephrine antibody, blue coloured  
 Volume: 1 x 6 ml/vial, blue cap

**Standards and Controls** - Ready to use

Cat. no.	Component	Colour/Cap	Concentration	Concentration	Volume/ Vial
			pg/ml	pmol/l	
			MN	MN	
<b>BA E-8301</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD A</span>	white	0	0	4 ml
<b>BA E-8302</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD B</span>	light yellow	36	183	4 ml
<b>BA E-8303</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD C</span>	orange	120	608	4 ml
<b>BA E-8304</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD D</span>	dark blue	360	1 825	4 ml
<b>BA E-8305</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD E</span>	light grey	1 200	6 084	4 ml
<b>BA E-8306</b>	<span style="border: 1px solid black; padding: 0 2px;">STANDARD F</span>	black	3 600	18 252	4 ml
<b>BA E-8351</b>	<span style="border: 1px solid black; padding: 0 2px;">CONTROL 1</span>	light green	Refer to QC-Report for expected value and acceptable range!		4 ml
<b>BA E-8352</b>	<span style="border: 1px solid black; padding: 0 2px;">CONTROL 2</span>	dark red			4 ml

Conversion: Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)

Content: Acidic buffer with non-mercury stabilizer, spiked with a defined quantity of Metanephrine

**BA E-8327** ADJUST-BUFF **Adjustment Buffer** - Ready to use  
 Content: Tris-Buffer  
 Volume: 1 x 10 ml/vial, yellow cap

**BA R-8313** ASSAY-BUFF **Assay Buffer** - Ready to use  
 Content: 25% organic solvent  
 Volume: 1 x 30 ml/vial, orange 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.

**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, dark 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 8 ml/vial, white cap

#### 4.2 Additional materials and 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)
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer

## 5. **Sample collection and storage**

Medications like Serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, MAO inhibitors, antihypertensive drugs and L-DOPA can influence Metanephrine and Normetanephrine level. Sympathomimetic agents, sport and smoking can also influence Metanephrine and Normetanephrine level.

Alcohol and caffeinated drinks should be avoided the day before and including the day of sample collection.

### **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.

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**

The ELISA can be run using an overnight incubation without shaking (results within approx. 24 hours) or alternatively as a fast version with shortened antiserum incubation times with shaking (results within approx. 6 hours).

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the Extraction Plate and microwell plate (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 antibodies, the enzyme conjugates, and the activity of the enzyme used are temperature dependent, 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 for the Enzyme Immunoassay is between 20 - 25 °C.

### 6.1 **Preparation of reagents**

#### **Wash Buffer**

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

Storage: 1 month at 2 - 8 °C

#### **Cleaning Buffer**

Dilute the 20 ml Cleaning Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 500 ml.

Storage: 1 month 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!

## 6.2 Preparation of samples

### Extraction

1.	Pipette <b>20 µl</b> of <b>standards</b> and <b>controls</b> into the respective wells of the <b>Extraction Plate</b> .
2.	Add <b>20 µl Standard A</b> to all wells containing <b>plasma samples</b> .
3.	Add <b>200 µl</b> of <b>Equalizing Reagent</b> to the wells with <b>standards and controls</b> .
4.	Pipette <b>200 µl</b> of <b>plasma samples</b> to the respective wells.
5.	Incubate plate <b>for 2 hours</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 600 rpm).
6.	Empty plate and blot dry by tapping the inverted plate on absorbent material.
7.	Pipette <b>250 µl</b> of <b>Assay Buffer</b> into all wells. Incubate the plate for <b>5 min</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 600 rpm). Empty plate and blot dry by tapping the inverted plate on absorbent material.
8.	Wash the plate <b>3 x</b> by adding <b>350 µl</b> of <b>Cleaning Buffer</b> , <b>discarding</b> the content and <b>blotting dry each time</b> by tapping the inverted plate on absorbent material.
9.	Pipette <b>100 µl</b> of <b>Elution Buffer</b> into all wells. <i>Please note: the colour changes caused by the elution buffer can vary between standards and samples.</i>
10.	Cover plate with adhesive foil. Incubate <b>15 min</b> at <b>RT</b> (20 - 25 °C) on a <b>shaker</b> (approx. 600 rpm). Remove the foil.  <b>Do not decant the supernatant thereafter!</b> The following volumes of the supernatant are needed for the subsequent ELISA: <b>Metanephrine 50 µl</b>

## 6.3 Metanephrine ELISA

1.	Pipette <b>25 µl</b> of <b>Adjustment Buffer</b> into all wells of the <b>Metanephrine Microtiter Strips</b> .
2.	Pipette <b>50 µl</b> of the extracted <b>standards, controls and samples</b> into the respective wells.
3.	Preparation of <b>Acylation Solution</b> : Pipette <b>80 µl Acylation Reagent Concentrate</b> (BA R-8312) to <b>3 ml water</b> (deionized, distilled, or ultra-pure) and mix thoroughly.
4.	Pipette <b>25 µl</b> of the freshly prepared <b>Acylation Solution</b> into all wells.
5.	Incubate for <b>15 min</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 600 rpm).
6.	Pipette <b>50 µl</b> of the <b>Metanephrine Antiserum</b> into all wells.
7.	Cover the plate with <b>Adhesive Foil</b> , shake for <b>1 min</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> and incubate for <b>15 - 20 h</b> (overnight) at <b>2 – 8 °C</b> without shaking. <i>Alternatively incubate for 2 h at RT (20 - 25 °C) on a shaker (approx. 600 rpm).</i>
8.	Remove the foil. Discard or aspirate the contents of the wells. Wash the plate <b>4 x</b> by adding <b>300 µl</b> of <b>Wash Buffer</b> , <b>discarding</b> the content and <b>blotting dry each time</b> by tapping the inverted plate on absorbent material.
9.	Pipette <b>100 µl</b> of the <b>Enzyme Conjugate</b> into all wells.
10.	Incubate for <b>30 min</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 600 rpm).
11.	Discard or aspirate the contents of the wells. Wash the plate <b>4 x</b> by adding <b>300 µl</b> of <b>Wash Buffer</b> , <b>discarding</b> the content and <b>blotting dry each time</b> by tapping the inverted plate on absorbent material.
12.	Pipette <b>100 µl</b> of the <b>Substrate</b> into all wells and incubate for <b>20 - 30 min</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 600 rpm). <b>Avoid exposure to direct sunlight!</b> 
13.	Add <b>100 µl</b> of the <b>Stop Solution</b> to all wells and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14.	<b>Read</b> the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to <b>450 nm</b> (if available a reference wavelength between 620 nm and 650 nm is recommended).

## 7. Calculation of results

<b>Measuring range (overnight ELISA)</b>	<b>Metanephrine</b>
	15.1 – 3 600 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 the included Equalizing Reagent and have to be re-assayed.

### Conversion

Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)

### Expected reference value

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

The expected reference values indicated below are based on method comparison studies to LC-MS/MS <sup>(1)</sup> with blood samples taken in the sitting position.

<b>Metanephrine</b>
< 65 pg/ml

For the interpretation of the results, a grey area has to be considered. This grey area does not depend on the methodology used and is reflected in a slight to mediate increase in Metanephrine and Normetanephrine up to 4 times the upper cut-off (Eisenhofer et al. 2003). Approx. 20 % of the tumors are found in this grey area, especially in the case of the Hereditary Syndrome, incidental tumors and in sporadic cases of Pheochromocytomas with a diameter less than 1 cm.

In case of a result in the grey area, it is recommended to collect a new sample together with an anamnesis concerning especially influences like the medication and age of the subject.

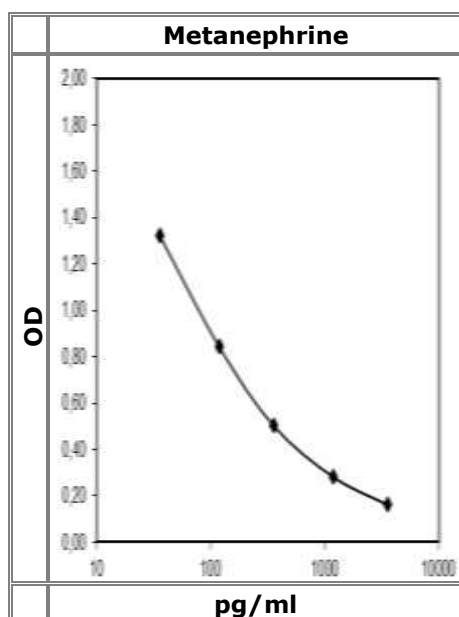
### 7.1 Quality control

It is recommended to use control samples according to national regulations. Use controls at both normal and pathological 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 (overnight ELISA)

Analytical Sensitivity	Metanephrine	
	LOD (pg/ml)	14.9
	LOQ (pg/ml)	15.1

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)
		Metanephrine
	Derivatized Metanephrine	100
	Derivatized Normetanephrine	0.05
	3-Methoxytyramin	< 0.01
	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
	Normetanephrine	< 0.01
	Acetaminophen	< 0.01

Precision							
Intra-Assay				Inter-Assay			
	Sample	Mean (pg/ml)	CV (%)		Sample	Mean (pg/ml)	CV (%)
Metanephrine	1	66.3	11.4	Metanephrine	1	67.8	17.6
	2	122	13.5		2	134	12.7
	3	308	10.6		3	319	11.0
	4	783	9.2		4	847	7.5

Linearity		Serial dilution up to	Mean (%)	Range (%)
	Metanephrine		1:64	107

Recovery		Mean (%)	Range (%)
	Metanephrine		88

Method Comparison: ELISA vs. LC-MS/MS <sup>(1)</sup>	Metanephrine	$y=0.91x + 1.8; r^2 = 0.96; n = 46$

## 9. References/Literature

- (1) De Jong et al. Plasma free metanephrine measurement using automated online solid phase extraction HPLC-Tandem mass spectrometry. Clin Chem, 53(9): 1684-1693 (2007)
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- (5) Manz et al. Development of enantioselective immunoassays for free plasma metanephrines. Ann.N.Y.Acad.Sci., 1018:582-587 (2004)
- (6) De Jong et al. Dietary Influences on Plasma and Urinary Metanephrines: Implications for Diagnosis of Catecholamine-Producing Tumors. J Clin Endocrinol Metab, 94(8):2841-2849 (2009)
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- (8) Eisenhofer et al. Biochemical Diagnosis of Pheochromocytoma: How to Distinguish True- from False-Positive Test Results. The Journal of Clinical Endocrinology & Metabolism, 88(6):2656-2666 (2003)















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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		Batch code		For in-vitro diagnostic use only!
	Consult instructions for use		Content		CE labelled
	Caution		Catalogue number		For research use only!