



## Instructions for Use

# Nor-/ Metanephrine in Plasma ELISA

Enzyme Immunoassay  
for the Quantitative Determination of  
**Free**  
**Normetanephrine and Metanephrine**  
in Plasma



REF EA612/192



2 x 96



2 – 8 °C

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## Symbols


 In Vitro Diagnostic Medical Device

 CE labelled


 Content

 Use by


 Batch code

 Temperature limitation

 Manufacturer

 Sufficient for ... determinations

 Catalogue number

 Consult instructions for use

## 1. Introduction and Principle of the Test

Normetanephrine and metanephrine are physiologically formed from the catecholamines noradrenaline and adrenaline by the enzyme catechol-O-methyltransferase (COMT). Increased levels of normetanephrine and metanephrine can be found in patients suffering from pheochromocytoma, ganglioneuroma and other neurogenic tumors.

The assay kit provides materials for the quantitative measurement of free normetanephrine and metanephrine in human EDTA plasma. Normetanephrine and metanephrine are separated from plasma proteins through addition of precipitation reagents and then are quantitatively acylated to their N-acyl-derivates.

The competitive Nor-/ Metanephrine ELISA kit uses the microtitre plate format. Metanephrine and normetanephrine, respectively, are bound to the solid phase of the microtiter plate. Acylated nor-/metanephrines from the sample and solid phase bound nor-/metanephrines compete for a fixed number of antiserum binding sites. When the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase nor-/metanephrines is detected by anti-rabbit IgG/peroxidase. The substrate TMB/peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase nor-/metanephrines is inversely proportional to the nor-/metanephrines concentration of the sample.

## 2. Warnings and Precautions

- For in-vitro diagnostic use only. For professional use only.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious.
- Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
- By handling reagents, controls and samples follow good laboratory practice and safety guidelines.
- Wear lab coats, disposable gloves and protective glasses.
- Some components of this kit are containing hazardous reagents. These components are marked with the adequate hazard label. Further information: See 4. *Contents of the Kit* and the safety data sheet.
- Avoid contact with reagents. It may causes eye and skin irritations and chemical burns.


- Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled samples.





### 3. Storage and Stability

On arrival, store the kit at 2 - 8 °C. Once opened the kit is stable until its expiry date. For stability of the ready for use reagents: See vial labels. For stability and storage of prepared reagents refer to 6.1 *Preparation of Reagents*.

Allow all reagents to reach room temperature before use and refrigerate after use.

### 4. Contents of the Kit

4.1	<b>Microtiter Strips</b> 8 wells each, able to break off Precoated with metanephrine, colour-coded blue, or normetanephrine, colour-coded yellow	<b>STRIPS-MN</b>	<b>STRIPS-NMN</b>	2 x 12 strips
4.2	<b>Standards 1 – 6</b> Each 1.5 ml, lyophilised, concentrations: see q.c. certificate 2 x Standard 1 for dilution of high level samples	<b>CAL 1 – 6</b>		7 vials
4.3	<b>Control 1 &amp; 2</b> Each 1.5 ml, lyophilised, range: see q.c. certificate	<b>CON 1 &amp; 2</b>		2 vials
4.4	<b>Acylation Buffer</b> 6 ml, ready for use, irritant	<b>ACYL-BUFF</b>		1 vial
4.5	<b>Acylation Reagent</b> 2.5 ml, lyophilised, dissolve with Solvent	<b>ACYL-REAG</b>		3 vials
4.6	<b>Metanephrine Antiserum</b> 0.45 ml, concentrated, colour-coded blue Rabbit-anti-N-acyl-metanephrine	<b>AS-MN</b>		1 vial
4.7	<b>Normetanephrine Antiserum</b> 4 ml, ready for use, colour-coded yellow Rabbit-anti-N-acyl-normetanephrine	<b>AS-NMN</b>		1 vial
4.8	<b>Enzyme Conjugate</b> 13 ml, ready for use, anti-rabbit IgG-POD conjugate	<b>CONJ</b>		2 vials
4.9	<b>Wash Buffer</b> 20 ml, concentrated (50x)	<b>WASH</b>		2 vials

4.10 <b>Substrate</b> 13 ml TMB solution, ready for use	<b>SUB</b>		2 vials
4.11 <b>Stop Solution</b> 13 ml, ready for use, contains 0.3 M sulphuric acid	<b>STOP</b>		2 vials
4.12 <b>Precipitation Tubes</b> For Precipitation	<b>PRECI-TUBE</b>		100 pieces
4.13 <b>Precipitator 1</b> 3.5 ml, ready for use, irritant	<b>PRECI 1</b>		1 vial
4.14 <b>Precipitator 2</b> 3.5 ml, ready for use, irritant	<b>PRECI 2</b>		1 vial
4.15 <b>Solvent</b> 5.5 ml, ready for use, contains acetone, irritant, highly flammable	<b>SOLVENT</b>		 2 vials
4.16 <b>Adhesive Foil</b> Ready for use	<b>FOIL</b>		4 pieces

Additional materials and equipment required but not provided:

- Pipettes (25, 40, 50, 100 and 200 µl)
- Multichannel pipette or Microplate washing device
- Eppendorf Multipipette (or similar devices)
- Distilled water
- Microplate photometer (450 nm)
- Orbital shaker
- Centrifuge (4,000 x g)
- Vortex mixer

## 5. Specimen Collection and Storage

### Plasma

EDTA plasma should be used.

Drugs, alcohol and tobacco as well as stress influence the catecholamine release. This may lead to false positive results for metanephrine and normetanephrine.

If clinically acceptable, medication (i.e. L-Dopa, alpha-blocker, antidepressants, MAO inhibitor, etc.) should be stopped five days before blood collection.

Patient should adhere to an at least four hours fasting; no tea, coffee, alcohol, nicotine and no strong physical activity.

It is recommended to let the patient rest for 20 to 30 minutes after the venipuncture and before collecting the blood sample.

The samples can be stored up to 6 hours at 2 - 8 °C. For a longer storage the samples must be frozen at -20 °C and are stable for at least 12 months.

Repeated freezing and thawing should be avoided.

## 6. Preparation of Reagents and Samples

### 6.1 Preparation of Reagents

#### Standards and Controls

**CAL 1 – 6**

**CON 1 & 2**

Dissolve standards and controls with 1.5 ml dist. water each, vortex shortly and leave on a roll mixer or similar shaker for minimum 20 minutes. Handle with care in order to minimize foam formation.

The reconstituted standards and controls should be stored frozen at -20 °C and are stable until expiry date printed on vial label.

#### Acylation Reagent

**ACYL-REAG**

Dissolve the content of one bottle in 2.5 ml Solvent and shake for minimum 15 minutes on a roll mixer or similar shaker. The Acylation Reagent has always to be prepared immediately before use and is stable for at least 3 hours. The two additional bottles are allowing a second and a third run of the test. If the whole kit is to be used in one run it is recommended to pool the dissolved contents of two vials of Acylation Reagent. After use the reagent has to be discarded.

**Metanephrine Antiserum** **AS-MN**

Concentrate, has to be diluted 1 + 9 with dist. water before use. Diluted antiserum is stable for only one day. Therefore it is recommended to prepare the dilution freshly and only as much as necessary.

**Wash Buffer** **WASH**

Dilute the content with dist. water to a total volume of 1,000 ml, mix shortly. The diluted wash buffer has to be stored at 2 - 8 °C for a maximum period of 4 weeks. For longer storage the diluted wash buffer should be stored frozen at -20 °C and is stable until expiry date printed on vial label.

All other reagents are ready for use.

## 6.2 Preparation of Samples (Precipitation and Acylation)

Allow reagents and samples to reach room temperature.  
Determinations in duplicates are recommended.

The preparation of the standards, controls and plasma samples is identical for both metanephrine and normetanephrine and has to be done only once.

1. Pipette each **200 µl dissolved Standards, Controls and Samples** into the respective marked **Precipitation Tubes**.
2. Pipette each **25 µl Precipitator 1** into all tubes.
3. Pipette each **25 µl Precipitator 2** into all tubes.
4. Mix tubes strongly and thoroughly (vortex). Strongly vortex each tube.
5. Centrifuge tubes for 15 minutes with at least 4,000 x g, preferable with a swing-out rotor.  
Attention: 4,000 x g is not identical to 4,000 x rpm (round per minute) and has to be adjusted for each centrifuge and rotor.
6. Pipette each **50 µl Acylation Buffer** into all tubes and continue with step 7. immediately.
7. Please note that solvent reacts with many plastic materials including plastic trays; solvent does not react with normal pipette tips and with glass devices. Solvent is volatile and evaporates quickly. Therefore, please do not use a tray with big surface together with a multichannel pipette for pipetting dissolved Acylation Reagent. Rather, use an Eppendorf multipipette (or similar device), fill the syringe directly from the vial with dissolved Acylation Reagent and pipette tube by tube.

Pipette each **40 µl dissolved Acylation Reagent** into one tube and immediately softly vortex the tube at medium speed for 2 to 4 seconds and then start with the next tube. Take care not to disturb the pellet at the bottom of the tube. Colour changes to red.

8. Centrifuge tubes for 15 minutes with at least 4,000 x g, preferable with a swing-out rotor.

**Take each 50 µl for the Metanephrine and Normetanephrine ELISA.**



## 7. Assay Procedure

### 7.1

#### **Metanephrine ELISA**

1. Pipette each **50 µl acylated Standards, Controls and Samples** into the respective wells of the coated microtiter strips (blue).
2. Incubate for 1 hour at room temperature on an orbital shaker (medium shaking rate).  
Do not cover the wells or the plate; leave the plate open on the shaker.
3. Pipette each **25 µl Metanephrine Antiserum** into all wells. Colour changes to blue.
4. Cover the plate with adhesive foil and incubate for 2 hours at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
5. Discard or aspirate the contents of the wells, add each **300 µl diluted Wash Buffer**, again discard or aspirate the contents of the wells. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.  
*Alternatively use a microplate washing device.*
6. Pipette each **100 µl Enzyme Conjugate** into all wells.
7. Incubate for 30 minutes at room temperature on an orbital shaker (medium shaking rate).
8. Washing: Repeat step 5.
9. Pipette each **100 µl Substrate** into all wells.
10. Incubate for 30 ± 5 minutes at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
11. Pipette **100 µl Stop Solution** into all wells, shake shortly.
12. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

## 7.2

### **Normetanephrine ELISA**

1. Pipette each **50 µl acylated Standards, Controls and Samples** into the respective wells of the coated microtiter strips (yellow).
2. Incubate for 1 hour at room temperature on an orbital shaker (medium shaking rate).  
Do not cover the wells or the plate; leave the plate open on the shaker.
3. Pipette each **25 µl Normetanephrine Antiserum** into all wells. Colour changes to orange.
4. Cover the plate with adhesive foil and incubate for 2 hours at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
5. Discard or aspirate the contents of the wells, add each **300 µl diluted Wash Buffer**, again discard or aspirate the contents of the wells. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.  
*Alternatively use a microplate washing device.*
6. Pipette each **100 µl Enzyme Conjugate** into all wells.
7. Incubate for 30 minutes at room temperature on an orbital shaker (medium shaking rate).
8. Washing: Repeat step 5.
9. Pipette each **100 µl Substrate** into all wells.
10. Incubate for 30 ± 5 minutes at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
11. Pipette **100 µl Stop Solution** into all wells, shake shortly.
12. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

## 8. Calculation of Results

Concentrations of the standards: See q.c. certificate.

Conversion:

Metanephrine: 1 pg / ml = 5.07 pmol / l

Normetanephrine: 1 pg / ml = 5.46 pmol / l

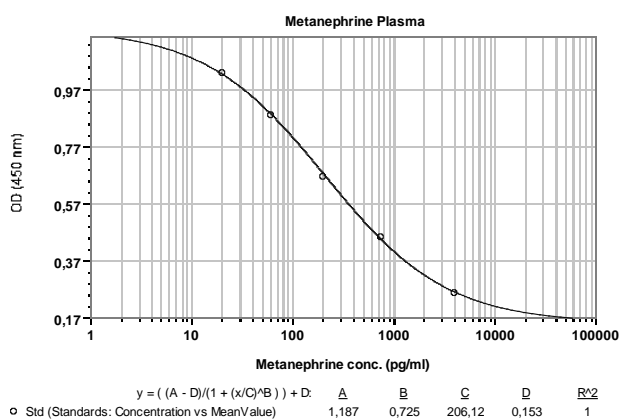
On a semilogarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Alternatively, the optical density of each standard and sample can be related to the optical density of the zero standard, expressed as the ratio OD/OD<sub>max</sub>, and then plotted on the y-axis.

A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

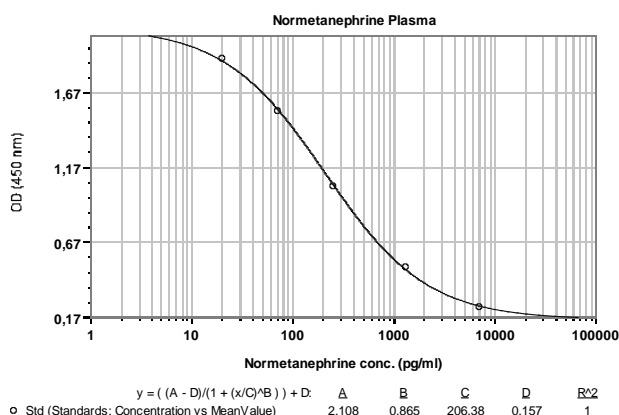
The concentration of the controls and samples can be read directly from the standard curve in pg / ml.

Below are listed typical examples of standard curves:

Metanephrine Plasma ELISA



Normetanephrine Plasma ELISA



### Quality control

All kit controls must be found within the acceptable ranges as printed on the q.c. certificate. If the criteria are not met, the run is not valid and should be repeated.

## 9. Assay Characteristics

### 9.1 Normal Range

The reference ranges given below should only be taken as a guideline. It is recommended that each laboratory should establish its own normal values.

Metanephrine	Normetanephrine
< 90 pg/ml	< 190 pg/ml

### 9.2 Sensitivity

	Lower detection limit	Calculation
Metanephrine	< 7 pg/ml	$OD_{Cal1} - 2xSD$
Normetanephrine	< 7 pg/ml	$OD_{Cal1} - 2xSD$

### 9.3 Specificity (Cross Reactivity)

Substance	Metanephrine (%)	Normetanephrine (%)
Metanephrine	100	0.015
Normetanephrine	0.130	100
3-Methoxytyramine	0.003	0.076
Adrenaline	0.039	0.0003
Noradrenaline	0.0008	0.0030
Tyramine	0.0005	0.0043
Dopamine	< 0.0001	0.0006
Homovanillic acid	< 0.0001	< 0.0001
Vanillic mandelic acid	< 0.0001	< 0.0001
L-Dopa	< 0.0001	< 0.0001
L-Tyrosine	< 0.0001	< 0.0001

### 9.4 Recovery

	Range (pg/ml)	Mean (%)	Range (%)
Metanephrine	20 - 900	94	82 - 117
Normetanephrine	34 - 1633	96	90 - 108

### 9.5 Linearity (Dilution with Standard 1)

	Range (pg/ml)	Highest Dilution	Mean (%)	Range (%)
Metanephrine	43 - 886	1 : 20	103	96 - 112
Normetanephrine	70 - 1613	1 : 20	93	86 - 105

### 9.6 Precision

	Range (pg/ml)	Intra-Assay-CV	Range (pg/ml)	Inter-Assay-CV
Metanephrine	157 - 403	7.9 - 7.8 %	118 - 276	8.8 - 8.6 %
Normetanephrine	193 - 757	8.4 - 4.1 %	246 - 551	9.3 - 9.2 %

### 9.7 Method Comparison

	Method	Correlation
Metanephrine	LC/MS	$Y = 1.04 \times LC/MS - 23$ ; $R = 0.991$ ; $N = 32$
Normetanephrine	LC/MS	$Y = 0.99 \times LC/MS - 8$ ; $R = 0.984$ ; $N = 32$

## 9.8 Calibration

The assay is calibrated by addition of defined stock solutions. The accuracy of the method was verified by comparing normal ranges (see 9.1) and other methods (see 9.7).

## 9.9 Limitations of Procedure

This assay is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated concerning the entire.

Samples showing concentrations above the highest standard have to be diluted with Standard 1 and reassayed.

## 9.10 Interfering Substances

Hemolytic, lipemic and icteric samples should not be used.

# 10. Literature

- Unger, N.; Pitt, C.; Petersenn, S.; et al. (2006):  
**Diagnostic value of various biochemical parameters for the diagnosis of pheochromocytoma in patients with adrenal mass**  
European Journal of Endocrinology 154 409–417
- Eisenhofer, G.; Walther, M.; Keiser, H.; et al. (2000):  
**Plasma metanephrines: a novel and costeffective test for pheochromocytoma**  
Brazilian Journal of Medical and Biological Research 33: 1157-1169
- Bravo, E. (2004):  
***Pheochromocytoma: Current Perspectives in the Pathogenesis, Diagnosis, and Management***  
Arq Bras Endocrinol Metab 48/5:746-750
- Candito, M.; Billaud, E.; Chauffert, M.; et al. (2002):  
**Biochemical diagnosis of pheochromocytoma and neuroblastomas**  
Ann Biol Clin (Paris). 2002 Jan-Feb;60(1):15-36.
- Lenders, J.; Pacak, K.; McClellan, M.; et al. (2002)::  
**Biochemical Diagnosis of Pheochromocytoma Which Test Is Best?**  
Jama, March 20, 2002-Vol 287, No. 11
- Eisenhofer, G.; Keiser, H.; Friberg, P.; et al. (1998):  
**Plasma Metanephrines Are Markers of Pheochromocytoma Produced by Catechol-O-Methyltransferase Within Tumors**  
Journal of Clinical Endocrinology and Metabolism Vol. 83, No. 6
- Lenders, J.; Keiser, H.; Goldstein, D.; et al. (1995):  
**Plasma Metanephrines in the Diagnosis of Pheochromocytoma**  
*Annals of Internal Medicine* • Volume 123 • Number 2

## Pipetting Scheme Sample Preparation (Metanephrine and Normetanephrine)

		Standard	Control	Plasma
<b>Standard 1 - 6</b>	μl	200		
<b>Control 1 &amp; 2</b>	μl		200	
<b>Plasma</b>	μl			200
<b>Precipitator 1</b>	μl	25	25	25
<b>Precipitator 2</b>	μl	25	25	25

Vortex strongly

Centrifuge for 15 minutes with at least 4,000 x g

<b>Acyl. Buffer</b>	μl	50	50	50
<b>Acyl. Reagent</b>	μl	40	40	40

Immediately vortex one tube softly at medium speed for 2 to 4 seconds, then start the addition of Acylation Reagent into the next tube and so on.

Take care not to disturb the pellet at the bottom of the tube.

Centrifuge for 15 minutes with at least 4,000 x g

**Take each 50 μl for the Metanephrine and Normetanephrine ELISA**

## Pipetting Scheme Metanephrine ELISA

	Standard	Control	Sample
<b>Acyl. Standard</b> $\mu\text{l}$	50		
<b>Acyl. Control</b> $\mu\text{l}$		50	
<b>Acyl. Sample</b> $\mu\text{l}$			50

Shake for 1 hour at room temperature, leave plate open

<b>Metanephrine Antiserum</b> $\mu\text{l}$	25	25	25
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Cover plate with adhesive foil

Shake for 2 hours at room temperature

4 x washing

<b>Enzyme conjugate</b> $\mu\text{l}$	100	100	100
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Shake for 30 minutes at room temperature

4 x washing

<b>Substrate</b> $\mu\text{l}$	100	100	100
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Shake for 30  $\pm$  5 minutes at room temperature

<b>Stop Solution</b> $\mu\text{l}$	100	100	100
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Reading of absorbance at 450 nm

## Pipetting Scheme Normetanephrine ELISA

	Standard	Control	Sample
<b>Acyl. Standard</b> $\mu\text{l}$	50		
<b>Acyl. Control</b> $\mu\text{l}$		50	
<b>Acyl. Sample</b> $\mu\text{l}$			50

Shake for 1 hour at room temperature, leave plate open

<b>Normetanephrine Antiserum</b> $\mu\text{l}$	25	25	25
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Cover plate with adhesive foil

Shake for 2 hours at room temperature

4 x washing

<b>Enzyme conjugate</b> $\mu\text{l}$	100	100	100
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Shake for 30 minutes at room temperature

4 x washing

<b>Substrate</b> $\mu\text{l}$	100	100	100
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Shake for  $30 \pm 5$  minutes at room temperature

<b>Stop Solution</b> $\mu\text{l}$	100	100	100
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Reading of absorbance at 450 nm