



## Instructions for Use


# Nor-/ Metanephrine in Urine ELISA

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



**REF** EA 614/192

 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 normetanephrine and metanephrine in human urine. Normetanephrine and metanephrine 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.
- 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 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 ml, ready for use	<b>CAL 1 – 6</b>		6 vials
4.3	<b>Control 1 &amp; 2</b> Each 1 ml, ready for use, range: See q.c. certificate	<b>CON 1 &amp; 2</b>		2 vials
4.4.	<b>Acylation Buffer</b> 3.5 ml, ready for use, irritant	<b>ACYL-BUFF</b>		1 vial
4.5	<b>Acylation Reagent</b> 1.75 ml, lyophilised, dissolve with Solvent	<b>ACYL-REAG</b>		3 vials
4.6	<b>Metanephrine Antiserum</b> 5.5 ml, ready for use, colour-coded blue Rabbit-anti-N-acyl-metanephrine	<b>AS-MN</b>		1 vial
4.7	<b>Normetanephrine Antiserum</b> 5.5 ml, ready for use, colour-coded yellow Rabbit-anti-N-acyl-normetanephrine	<b>AS-NMN</b>		1 vial
4.8	<b>Enzyme Conjugate</b> 21 ml, ready for use, anti-rabbit IgG-POD conjugate	<b>CONJ</b>		1 vial
4.9	<b>Wash Buffer</b> 20 ml, concentrated (50x)	<b>WASH</b>		2 vials
4.10	<b>Substrate</b> 21 ml TMB solution, ready for use	<b>SUB</b>		1 vial

4.11	<b>Stop Solution</b> 21 ml, ready for use, contains 0.3 M sulphuric acid	<b>STOP</b>	1 vial
4.12	<b>Hydrolysis Tubes</b> For hydrolysis	<b>HYDRO-TUBE</b>	100 pieces
4.13	<b>Hydrochloric Acid</b> 12 ml, ready for use, contains 0.1 M HCl	<b>HCL</b>	1 vial
4.14	<b>Solvent</b> 6 ml, ready for use, contains DMF and DMSO, toxic	<b>SOLVENT</b>	 1 vial
4.15	<b>Adhesive Foil</b> Ready for use	<b>FOIL</b>	4 pieces

Additional materials and equipment required but not provided:

- Pipettes (10, 20, 25, 50, 100, 500 µl)
- Multichannel pipette or Microplate washing device
- Eppendorf Multipipette (or similar devices)
- Distilled water
- Microplate photometer (450 nm)
- Orbital shaker
- Water bath or heat block
- Centrifuge
- Vortex mixer

## 5. Specimen Collection and Storage

Spontaneous urine can be used for this test as well as collected urine. In this case the total volume of urine excreted during a 24-hours period should be collected and mixed in a single bottle containing 10 - 15 ml of 6 M hydrochloric acid as preservative. Avoid exposure to direct sun light. Determine the total volume and take an aliquot for the measurement. For patients with suspected kidney disorders the creatinine concentration should be tested, too. Acidified urine samples can be stored at -20 °C for at least 6 months.

Repeated freezing and thawing should be avoided.

**Mix and centrifuge urine samples before use in the assay.**

## 6. Preparation of Reagents and Samples

### 6.1 Preparation of Reagents

#### Acylation Reagent

**ACYL-REAG**

Dissolve the content of one bottle in 1.75 ml Solvent and shake for minimum 5 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.

#### 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 (Hydrolysis and Acylation)

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

Allow all reagents to reach room temperature. Duplicates are recommended.

1. Pipette each **10 µl Standards, Controls and Urine Samples** into the corresponding waterproof marked hydrolysis tubes.
2. Pipette each **100 µl Hydrochloric Acid** into all tubes.
3. Seal the tubes, mix thoroughly (vortex) and incubate for 30 min at 90 to 100 °C in a water bath or heat block.
4. Let the tubes cool down to room temperature.  
Centrifuge shortly to pool dispersed drops, discard lids.

**NOTE: For the measurement of the free normetanephrine and the free metanephrine only, leave out incubation at 90 °C (point 3 to 4).**

5. Pipette each **25 µl Acylation Buffer** into all tubes, shake shortly.
6. Please note that Solvent reacts with many plastic materials including plastic trays; Solvent does not react with normal pipette tips and with glass devices. Please 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 **20 µl dissolved Acylation Reagent** into all tubes and continue with step 7. **immediately**.

7. Mix thoroughly (vortex) and incubate for 15 minutes at room temperature.
8. Pipette each **500 µl distilled water** into all tubes and mix thoroughly (vortex).

**Take 50 µl for the Metanephrine and 20 µl for the 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. Pipette each **50 µl Metanephrine Antiserum** into all wells. Colour changes to blue.
3. Cover the plate with adhesive foil and incubate for 1 hour at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
4. 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.*
5. Pipette each **100 µl Enzyme Conjugate** into all wells.
6. Incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Washing: Repeat step 4.
8. Pipette each **100 µl Substrate** into all wells.
9. Incubate for 20 ± 5 minutes at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
10. Pipette **100 µl Stop Solution** into all wells, shake shortly.
11. Read the optical density at 450 nm within 15 minutes (reference wavelength between 570 and 650 nm) in a microplate photometer.

## 7.2

### **Normetanephrine ELISA**

1. Pipette each **20 µl acylated Standards, Controls and Samples** into the respective wells of the coated microtiter strips (yellow).
2. Pipette each **50 µl Normetanephrine Antiserum** into all wells. Colour changes to yellow.
3. Cover the plate with adhesive foil and incubate for 1 hour at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
4. 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.*
5. Pipette each **100 µl Enzyme Conjugate** into all wells.
6. Incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Washing: Repeat step 4.
8. Pipette each **100 µl Substrate** into all wells.
9. Incubate for 20 ± 5 minutes at room temperature (20 - 25 °C) on an orbital shaker (medium shaking rate).
10. Pipette **100 µl Stop Solution** into all wells, shake shortly.
11. Read the optical density at 450 nm within 15 minutes (reference wavelength between 570 and 650 nm) in a microplate photometer.

## 8. Calculation of the Results

Standard:		1	2	3	4	5	6
<b>Metanephrine</b>	ng / ml	0	20	60	250	800	4,000
	nmol / l	0	101	304	1,268	4,056	20,280
<b>Normetanephrine</b>	ng / ml	0	30	100	300	1,000	4,000
	nmol / l	0	164	546	1,638	5,460	21,840

Conversion:

Metanephrine: 1 ng / ml = 5.07 nmol / l

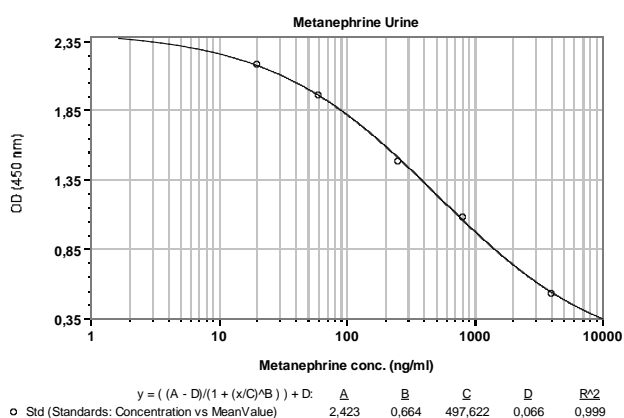
Normetanephrine: 1 ng / ml = 5.46 nmol / l

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  $OD/OD_{max}$ , and then plotted on the y-axis.

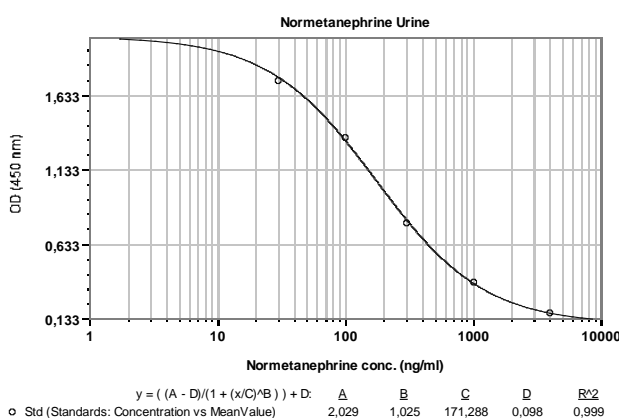
A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

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

Below are listed typical examples of standard curves:



Metanephrine Urine ELISA



Normetanephrine Urine 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
< 350 µg/day	< 600 µg/day

### 9.2 Sensitivity

	Lower detection limit	Calculation
Metanephrine	4 ng/ml	OD <sub>Cal1</sub> - 2xSD
Normetanephrine	10 ng/ml	OD <sub>Cal1</sub> - 2xSD

### 9.3 Specificity (Cross Reactivity)

Substance	Metanephrine (%)	Normetanephrine (%)
Metanephrine	100	0.775
Normetanephrine	0.430	100
3-Methoxytyramine	< 0.025	0.282
Adrenaline	0.875	< 0.022
Noradrenaline	< 0.025	1.360
Tyramine	0.001	< 0.001
Dopamine	< 0.025	< 0.022
Homovanillic acid	< 0.001	< 0.001
Vanillic mandelic acid	< 0.001	< 0.001
L-Dopa	< 0.001	< 0.001
L-Tyrosine	< 0.001	< 0.001

### 9.4 Recovery

	Range (ng/ml)	Mean (%)	Range (%)
Metanephrine	10 - 952	107	86 - 124
Normetanephrine	14 - 952	110	92 - 121

### 9.5 Linearity (Dilution with Standard 1)

	Range (ng/ml)	Highest Dilution	Mean (%)	Range (%)
Metanephrine	85 - 2134	1 : 21	90	77 - 106
Normetanephrine	52 - 1243	1 : 21	93	79 - 102

### 9.6 Precision

	Range (ng/ml)	Intra-Assay-CV	Range (ng/ml)	Inter-Assay-CV
Metanephrine	110 - 414	10.3 - 9.9 %	208 - 1964	10.1 - 14.8 %
Normetanephrine	63 - 1323	7.2 - 4.8 %	120 - 2963	5.3 - 10.1 %

### 9.7 Method Comparison

	Method	Correlation
Metanephrine	HPLC	Y = 0.95 x HPLC - 1; R = 0.99; N = 23
Normetanephrine	HPLC	Y = 0.85 x HPLC + 28; R = 1.00; N = 23

## 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 Interferenzen

Do not use collected urine samples which are not acidified.

# 10. Literature

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**Diagnostic value of various biochemical parameters for the diagnosis of pheochromocytoma in patients with adrenal mass**  
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- Bravo, E. (2004):  
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Arq Bras Endocrinol Metab 48/5:746-750
- Candito, M.; Billaud, E.; Chauffert, M.; et al. (2002):  
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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
- Heron, E.; Chatellier, G.; Billaud, E.; et al. (1996):  
**The Urinary Metanephrine-to-Creatinine Ratio for the Diagnosis of Pheochromocytoma**  
*Ann Intern Med.* 125 : 300-303

## Pipetting Scheme Sample Preparation (Metanephrine and Normetanephrine)

	Standard	Control	Urine
<b>Standard 1 - 6</b> $\mu\text{l}$	10		
<b>Control 1 &amp; 2</b> $\mu\text{l}$		10	
<b>Urine</b> $\mu\text{l}$			10
<b>Hydrochloric Acid</b> $\mu\text{l}$	100	100	100

Vortex

Incubate for 30 minutes at 90 – 100 °C \*  
Let tubes cool down to room temperature  
Centrifuge shortly, discard lids

<b>Acyl. Buffer</b> $\mu\text{l}$	25	25	25
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Shake shortly

<b>Acyl. Reagent</b> $\mu\text{l}$	20	20	20
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Vortex

Incubate for 15 minutes at room temperature

<b>Dest. Wasser</b> $\mu\text{l}$	500	500	500
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Vortex

Pipette **50  $\mu\text{l}$**  Metanephrine for the ELISA

Pipette **20  $\mu\text{l}$**  Normetanephrine for the ELISA

\* leave out for determination of free normetanephrine and free metanephrine

## Pipetting Scheme Metanephrine ELISA

		Standard	Control	Sample
<b>Acyl. Standard</b>	μl	50		
<b>Acyl. Control</b>	μl		50	
<b>Acyl. Sample</b>	μl			50
<b>Metanephrine Antiserum</b>	μl	50	50	50

Cover plate with adhesive foil

Shake for 1 hour at room temperature

4 x Washing

<b>Enzyme conjugate</b>	μl	100	100	100
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Shake for 20 minutes at room temperature

4 x Washing

<b>Substrate</b>	μl	100	100	100
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Shake for 20 ± 5 minutes at room temperature

<b>Stop Solution</b>	μl	100	100	100
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Reading of absorbance at 450 nm

## Pipetting Scheme Normetanephrine ELISA

	Standard	Control	Sample
<b>Acyl. Standard</b> $\mu\text{l}$	20		
<b>Acyl. Control</b> $\mu\text{l}$		20	
<b>Acyl. Sample</b> $\mu\text{l}$			20
<b>Normetanephrine Antiserum</b> $\mu\text{l}$	50	50	50

Cover plate with adhesive foil

Shake for 1 hour at room temperature

4 x Washing

<b>Enzyme conjugate</b> $\mu\text{l}$	100	100	100
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Shake for 20 minutes at room temperature

4 x Washing

<b>Substrate</b> $\mu\text{l}$	100	100	100
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Shake for  $20 \pm 5$  minutes at room temperature

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