




## Instructions for Use


# Nor- / Metanephrine - 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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## **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 EDTA plasma. 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-/metanephrine 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 catecholamine is inversely proportional to the catecholamine concentration of the sample.

## **2. Precautions**

- For in vitro use only.
- Some reagents contain sodium azide as preservative. Avoid skin contact.
- 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.
- Where required reagents show the respective hazard symbol on the label.

## **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 prepared reagents refer to Preparation of Reagents. Do not use components beyond the expiration date shown on the kit labels. Do not mix various lots of any kit component within an individual assay.

## 4. Contents of the Kit

### Reagents for Sample Preparation:

- 4.1 **Acylation Reagent** **ACYL-REAG** 2 vials  
2.5 ml, lyophilised
- 4.2. **Acylation Buffer** **ACYL-BUFF** 1 vial  
4 ml, ready for use  
Tris-HCl-Puffer
- 4.3 **Standards 1 – 6** **CAL 1 - 6** 6 vials  
each 1.75 ml, lyophilized  
Concentrations variable, see q.c. certificate:
- | Standard                       | 1 | 2  | 3  | 4   | 5   | 6     |
|--------------------------------|---|----|----|-----|-----|-------|
| <b>Normetanephrine</b> (pg/ml) | 0 | 20 | 60 | 200 | 700 | 3,000 |
| <b>Metanephrine</b> (pg/ml)    | 0 | 20 | 60 | 200 | 700 | 3,000 |
- 4.4 **Control 1 & 2** **CON 1 & 2** 2 vials  
each 1.75 ml, lyophilized  
Concentrations: See q.c. certificate
- 4.5 **Precipitator 1** **PRECI 1** 1 vials  
6 ml, ready for use
- 4.6 **Precipitator 2** **PRECI 2** 1 vials  
6 ml, ready for use
- 4.7 **Precipitation Tubes** **PRECI-TUBE** 100 pcs.
- 4.8 **Reaction Plate** **ACYL-PLATE** 1 plate with 96 wells

### Reagents for the ELISA

- 4.9 **MT-Strips** 2 x 12 strips  
**STRIPS-MN** **STRIPS-NMN**  
8 wells each, break apart  
precoated with metanephrine (12 strips), colour-coded blue,  
and normetanephrine (12 strips), colour-coded yellow

4.10	<b>Metanephrine Antiserum</b> 3 ml, ready for use, from rabbit, blue coloured	<b>AS-MN</b>	1 vial
4.11	<b>Normetanephrine Antiserum</b> 5.5 ml, ready for use, from rabbit, yellow coloured	<b>AS-NMN</b>	1 vial
4.12	<b>Enzyme Conjugate</b> 21 ml, ready for use anti-rabbit IgG-POD conjugate	<b>CONJ</b>	1 vial
4.13	<b>Wash Buffer</b> 20 ml, concentrated dilute with dist. water to 500 ml.	<b>WASH</b>	2 vials
4.14	<b>Substrate</b> 21 ml TMB solution, ready for use	<b>SUB</b>	1 vial
4.15	<b>Stop Solution</b> 21 ml, ready for use contains 0.3 M sulphuric acid	<b>STOP</b>	1 vial
4.16	<b>Solvent</b> 6 ml, ready for use contains acetone (please note that Solvent reacts with many plastic materials including plastic trays; Solvent does not react with normal pipette tips and with glass devices)	<b>SOLVENT</b>	1 vial
4.17	<b>Adhesive Foil</b> ready for use	<b>FOIL</b>	2 pcs

Additional materials and equipment required but not provided:

- Pipettes (20, 25, 50, 100 µl and 400 µl)
- Horizontal shaker (400 – 500 rpm)
- Microplate washing device
- Centrifuge capable of at least 3,000 x g
- Vortex mixer
- Microplate photometer
- Distilled water

## 5. Sample Collection and Storage

EDTA samples are required for the assay. Do not use heparin or citrate plasma. Physical and psychical stress usually cause a high increase of the catecholamine concentration. Therefore, it is recommended to let the patient rest for 20 to 30 minutes after the venipuncture and before collecting the blood sample. Haemolytic and especially lipemic samples should not be used for the assay, because false low values will be obtained with such samples.

The plasma samples can be stored at 2 - 8 °C up to 6 hours. Samples can be stored at -20 °C for at least 6 months.

## 6. Sample Preparation

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

### 6.1. Preparation of Reagents

#### Standards and Controls

Dissolve standards and controls with 1.75 ml dist. water each and mix carefully. Reconstituted standards and controls can be kept frozen at -20 °C and may be thawed only once.

#### Acylation Reagent

Dissolve contents of one vial with 2.5 ml Solvent and mix on a shaker for 5 minutes. The Acylation Reagent has to be prepared just prior to use. If both vials are needed, pool the dissolved contents of the two vials.

Please note that Solvent reacts with many plastic materials including plastic trays. Solvent does not react with normal pipette tips and with glass devices.

#### Attention

Solvent is volatile and the dissolved Acylation Reagent evaporates quickly. Therefore, please do not use a tray with big surface together with a multichannel pipette for pipetting Acylation Reagent. Rather, use an Eppendorf multipipette, or similar device, fill the syringe directly from the vial (using a yellow tip) with dissolved Acylation Reagent and add well by well.

## 6.2. Precipitation

Allow all reagents to reach room temperature.

Duplicates are recommended. However, if sample volume is limited use singlet for precipitation and duplicates for subsequent acylation.

1. Pipette each 400 µl standards, controls and plasma samples into the Precipitation Tubes.
2. Pipette each 50 µl Precipitator 1 into all tubes.
3. Pipette each 50 µl Precipitator 2 into all tubes.
4. Close Precipitation Tubes carefully and mix thoroughly (vortex).
5. Incubate for 60 minutes at room temperature.
6. Centrifuge for 10 minutes with at least 3,000 x g.

Take 100 µl of the supernatant for the acylation.

## 6.3. Acylation

1. Pipette each 100 µl of the supernatant into the respective wells of the Reaction Plate for acylation
2. Pipette each 25 µl Acylation Buffer into all wells.
3. Dissolve Acylation Reagent as described above and mix for 5 minutes.
4. Pipette 20 µl prepared Acylation Reagent into all wells and continue with step 5. immediately.

### Attention

Solvent is volatile and the dissolved Acylation Reagent evaporates quickly. Therefore, please do not use a tray with big surface together with a multichannel pipette for pipetting Acylation Reagent. Rather, use an Eppendorf multipipette with a yellow tip (or similar device), fill the syringe directly from the vial with dissolved Acylation Reagent and add well by well.

5. Place on an orbital shaker at 400 to 500 rpm and incubate for 60 minutes at room temperature. Do not cover the wells or the plate, leave the plate open on the shaker.

Take 50 µl for the Metanephrine and 20 µl for the Normetanephrine ELISA.

## 7. Assay Procedure

### 7.1 Preparation of Reagents

#### Wash Buffer Concentrate

Prepare the Wash Buffer Concentrate by diluting one vial of concentrate with distilled water to a final volume of 500 ml.

#### 7.2.1

#### **Metanephrine ELISA**

Allow reagents and samples to reach room temperature. Duplicates are recommended.

1. Pipette each 50  $\mu$ l acylated Standards, Controls, and Patient Samples into the respective wells.
2. Pipette each 25  $\mu$ l Metanephrine Antiserum into all wells.
3. Cover with adhesive foil and incubate for at least 15 to 20 hours at 2 - 8  $^{\circ}$ C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 300  $\mu$ l prepared Wash Buffer. Repeat the washing procedure two more times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.
5. Pipette each 100  $\mu$ l Enzyme Conjugate into all wells.
6. Incubate for 30 minutes at room temperature on an orbital shaker with 400 – 500 rpm.
7. Washing: Repeat step 4.
8. Pipette each 100  $\mu$ l Substrate into all wells.
9. Incubate 25 to 35 minutes at room temperature on an orbital shaker with 400 – 500 rpm, preferably in the dark.
10. Pipette 100  $\mu$ l Stop Solution into all wells and shake the plate briefly.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

### 7.2.2

## **Normetanephrine ELISA**

Allow reagents and samples to reach room temperature. Duplicates are recommended.

1. Pipette each 20  $\mu$ l acylated Standards, Controls, and Patient Samples into the respective wells.
2. Pipette each 50  $\mu$ l Normetanephrine Antiserum into all wells.
3. Cover with adhesive foil and incubate for at least 15 to 20 hours at 2 - 8  $^{\circ}$ C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 300  $\mu$ l prepared Wash Buffer. Repeat the washing procedure two more times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.
5. Pipette each 100  $\mu$ l Enzyme Conjugate into all wells.
6. Incubate for 30 minutes at room temperature on an orbital shaker with 400 – 500 rpm.
7. Washing: Repeat step 4.
8. Pipette each 100  $\mu$ l Substrate into all wells.
9. Incubate 25 to 35 minutes at room temperature on an orbital shaker with 400 – 500 rpm, preferably in the dark.
10. Pipette 100  $\mu$ l Stop Solution into all wells and shake the plate briefly.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

## 8. Calculation of Results

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.

The concentration of the controls and samples can be read directly from this standard curve by using their average optical density.

## 9. Assay Characteristics

### 9.1 Reference Ranges

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

Plasma	Metanephrine	Normetanephrine
	< 140 pg/ml	< 200 pg/ml

### 9.2 Sensitivity

The lower limit of detection was determined by taking the 3fold standard deviation of the absorbance of the Zero Reference and reading the corresponding value from the standard curve.

Metanephrine	Normetanephrine
10 pg/ml	15 pg/ml

### 9.3 Specificity

The following cross reactivities were tested:

#### Metanephrine

Compound	Cross React. %
Metanephrine	100
Normetanephrine	0.102
3-Methoxytyramine	0.002
Adrenaline	0.019
Noradrenaline	< 0.001
Dopamine	< 0.001
L-Dopa	< 0.001
Tyramine	< 0.001
Tyrosine	< 0.001
Homovanillic acid	< 0.001
Vanillic mandelic acid	< 0.001

#### Normetanephrine

Compound	Cross React. %
Normetanephrine	100
Metanephrine	0.010
3-Methoxytyramine	0.024
Adrenaline	0.002
Noradrenaline	0.020
Dopamine	0.002
L-Dopa	< 0.001
Tyramine	< 0.001
Tyrosine	< 0.001
Homovanillic acid	< 0.001
Vanillic mandelic acid	< 0.001

## 9.4 Recovery

Plasma from a healthy donor was enriched with different amounts of Normetanephrine / Metanephrine and measured in the ELISA (concentrations in pg/ml).

### Metanephrine

added	measured	calculated	% recovery
0	57		
10	61	67	91
20	75	77	97
30	78	87	90
50	107	107	100
75	117	132	89
100	145	157	92
200	237	257	92
400	444	457	97
600	537	657	81
800	864	857	101
1000	964	1057	91
1500	1524	1557	98
2500	2578	2557	101

mean **94**

### Normetanephrine

added	measured	calculated	% recovery
0	77		
10	92	87	106
20	99	97	102
30	99	107	93
50	131	127	103
75	151	152	99
100	186	177	105
200	286	277	103
400	478	477	100
600	556	677	82
800	783	877	89
1000	875	1077	81
1500	1297	1577	82

mean **95**

## 9.5 Linearity

Linearity was measured by diluting spiked patient plasma with standard 1 (all concentrations in pg/ml).

### Metanephrine

<b>dilution</b>	<b>measured</b>	<b>recalculated</b>	<b>% recovery</b>
orig.	3250	3250	
3 + 2	1941	3235	100
2 + 3	1296	3240	100
1 + 3	667	2668	82
1 + 5	486	2916	90
1 + 9	331	3310	102
1 + 15	204	3264	100
1 + 25	131	3406	105
1 + 40	85	3486	107

**mean recovery**

**98**

### Normetanephrine

<b>dilution</b>	<b>measured</b>	<b>recalculated</b>	<b>% recovery</b>
orig.	2965	2965	
3 + 2	1577	2628	89
2 + 3	1059	2648	89
1 + 3	706	2824	95
1 + 5	464	2784	94
1 + 9	317	3170	107
1 + 15	223	3568	120
1 + 25	110	2860	96
1 + 40	70	2870	97

**mean recovery**

**98**

## 9.6 Reproducibility

Reproducibility was tested by measuring the intra-assay coefficients of variation.

### Metanephrine EIA

sample	n	mean	s.d.	c.v. (%)
1	40	52	4.9	9.4
2	40	270	17.4	6.5

### Normetanephrine EIA

sample	n	mean	s.d.	c.v. (%)
1	40	65	5.5	8.4
2	40	263	11.8	4.5

## 10. Literature

### 1. Plasma metanephrines: a novel and cost-effective test for pheochromocytoma.

Braz J Med Biol Res 2000 Oct;33(10):1157-69

Eisenhofer G, Walther M, Keiser HR, Lenders JW, Friberg P, Pacak K. Clinical Neurocardiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-1620, USA. [graeme@catecholamine.org](mailto:graeme@catecholamine.org)

### 2. Plasma metanephrines in the diagnosis of pheochromocytoma

Ann Intern Med 1995 Jul 15; 123 (2): p101-9

Lenders JW; Keiser HR; Goldstein DS; Willemsen JJ; Friberg P; Jacobs MC; Kloppenborg PW; Thien T; Eisenhofer G  
National Institutes of Health, Bethesda, Maryland, USA.

Further literature upon request.

## Pipetting Scheme Sample Preparation (Metanephrine and Normetanephrine)

### Precipitation

	Standards	Controls	Samples
Standard 1 – 6	400 µl		
Controls 1 & 2		400 µl	
Patient Plasma			400 µl
Precipator 1	50 µl	50 µl	50 µl
Precipator 2	50 µl	50 µl	50 µl

Mix thoroughly (vortex) and incubate for 60 minutes at room temperature

centrifuge for 10 minutes with at least 3,000 x g

take 100 µl of the supernatant for the acylation

### Acylation

	Standards & Controls	Samples
Standards & Controls	100 µl	
Samples		100 µl
Acylation Buffer	25 µl	25 µl

prepare Acylation Reagent: dissolve contents of one vial in 2.5 ml Solvent, mix thoroughly, and use immediately

Acylation Reagent	20 µl	20 µl
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Incubate for 60 minutes at room temperature on a shaker (400-500 rpm)  
do not cover wells or plate

Metanephrine	<b>50 µl</b> for the ELISA
Normetanephrine	<b>20 µl</b> for the ELISA

## Pipetting Scheme ELISA (Metanephrine and Normetanephrine)

Standards	Controls	Samples
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### Metanephrine

Standard 1 - 6	µl	50		
Controls 1 & 2	µl		50	
Patient Sample	µl			50
Met. Antiserum	µl	25	25	25

### Normetanephrine

Standard 1 - 6	µl	20		
Controls 1 & 2	µl		20	
Patient Sample	µl			20
Normet. Antiserum	µl	50	50	50

cover with adhesive foil and incubate for 15 - 20 hours at 2 - 8 °C

3 x washing

Enzyme Conjugate	µl	100	100	100
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incubate for 30 minutes at room temperature on an orbital shaker

3 x washing

Substrate	µl	100	100	100
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incubate for 25 to 35 minutes at room temperature on an orbital shaker

Stop Solution	µl	100	100	100
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read absorbance at 450 nm