DIAGNOSTIKA GMBH

Instructions for Use

Nor-/Metanephrine in Plasma ELISA

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

CE IVD

Item No. EA612/192

Σ 2 x 96

+2 +8 °C 2 – 8 °C

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Symbols

IVD	In Vitro Diagnostic Medical Device	CE	EC Declaration of conformity
CONT	Content		Expiry Date
LOT	Lot Number		Store at
	Manufactured by	Σ	Sufficient for ... determinations
REF	Catalogue Number		Consult Instructions for Use

The symbols of the components of the kit are described in chapter 4 Contents of the Kit.

1 Introduction and Principle of the Test

Normetanephrine and metanephrine are physiologically formed from the catecholamines by the enzyme catechol-O-methyltransferase (COMT). Increased levels of normetanephrine and metanephrine can be found in patients with pheochromocytoma, ganglioneuroma and related tumors of neurogenic origin. However, the diagnosis should not be solely based on the elevated Nor-/ Metanephrine levels as measured in this ELISA.

The Nor-/Metanephrine ELISA Kit provides materials for the quantitative determination of derivatized metanephrine and normetanephrine in human EDTA-plasma. During sample preparation, proteins in the plasma are separated from the metanephrine and normetanephrine through addition of precipitation reagents. Then metanephrine and normetanephrine are quantitatively converted into their derivatives N-acyl-metanephrine and N-acyl-normetanephrine by the acylation reagent.

The Nor-/Metanephrine ELISA is a competitive enzyme immunoassay. Antigen bound to the solid phase and free antigen in solution compete for a defined number of antibody binding sites. When the system is in equilibrium, the unbound antigen-antibody complex is removed in a washing step and the correspondingly bound complex is detected using a peroxidase conjugate and determined via the conversion of tetramethylbenzidine (TMB). The TMB/POD reaction is stopped and monitored at 450 nm. The concentration of the antigen-antibody complex bound to the solid phase is inversely proportional to the concentration of antigen in the sample. The Nor-/Metanephrine ELISA is intended for manual processing.

2 Warnings and Precautions

- For in vitro diagnostic use only. For professional use only.
- Before carrying out the test, the valid instructions for use, as included in the kit, should be read completely and the content understood.
- 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.
- Individual components of different lots and test kits should not be interchanged. The expiry dates and storage conditions stated on the packaging and the labels of the individual components must be observed.
- When handling reagents, controls and samples follow good laboratory practice and safety guidelines.
- Wear lab coat, disposable gloves and protective glasses.

- Some components of this kit contain 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 any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.
- Avoid contact with reagents.
- Broken glass can cause injury. Be cautious with glass vials.
- 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

The kit is shipped at ambient temperature and is subsequently stable until the stated expiry date when stored between 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

Metanephrine-Microtiter Strips **STRIPS-MN** 12 strips
8 wells each, break-apart
Precoated with metanephrine, colour-coded blue

Normetanephrine-Microtiter Strips **STRIPS-NMN** 12 strips
8 wells each, break-apart
Precoated with normetanephrine, colour-coded yellow

Standards (1 - 6) **CAL 1 - 6** 7 vials
1.5 ml each, lyophilised, (see 6.1.1), concentrations: see QC certificate
2 x Standard 1 for dilution of high level samples

Control 1 & 2 **CON 1 & 2** 2 vials
1.5 ml each, lyophilised, (see 6.1.1), range: see QC certificate

Acylation Reagent **ACYL-REAG** 3 vials
2.5 ml, lyophilised, (see 6.1.2)

Acylation-Buffer **ACYL-BUFF** 1 vial
6 ml, ready for use
 Warning

Metanephrine-Antiserum **AS-MN** 1 vial
0.60 ml, concentrated, (see 6.1.3), colour-coded blue
Rabbit-anti-N-acyl-metanephrine
 Warning

Normetanephrine-Antiserum **AS-NMN** 1 vial
4 ml, ready for use, colour-coded yellow
Rabbit-anti-N-acyl-normetanephrine
 Warning

Enzyme Conjugate **CONJ** 2 vials
13 ml, ready for use, anti-rabbit IgG-POD conjugate
 Warning

Waschpuffer **WASH** 2 vials
20 ml, concentrated (50x), (see 6.1.4)

Substrate **SUB** 2 vials
13 ml TMB solution, ready for use

Stop Solution **STOP** 2 vials
13 ml, ready for use, contains 0.3 M sulphuric acid

Precipitation Tubes **PRECI-TUBE** 100 pieces
For precipitation

Precipitator 1 **PRECI 1** 1 vial
3.5 ml, ready for use, irritant
 Warning

Precipitator 2 **PRECI 2** 1 vial
3.5 ml, ready for use, irritant
 Danger

Solvent **SOLVENT** 1 vial
10 ml, ready for use, contains acetone,
irritant, highly flammable
 Warning
 Danger

Adhesive Foil **FOIL** 4 pieces

Ready for use

Additional materials and equipment required but not provided:

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

5 Specimen Collection and Storage

EDTA plasma should be used.

Medication, 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 or other stimulants 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

6.1.1 Standards and Controls

Dissolve standards CAL 1 – 6 and controls CON 1 & 2 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.

6.1.2 Acylation Reagent

Dissolve the content of one bottle ACYL-REAG with 2.5 ml Solvent SOLVENT and shake for minimum 15 minutes on a roll mixer or similar shaker. The Acylation Reagent must always be prepared immediately before use and is stable for at least 3 hours. The two additional bottles allow 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.

6.1.3 Metanephrine Antiserum

Dilute the concentrated Metanephrine Antiserum AS-MN 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.

6.1.4 Wash Buffer

Dilute the content (20 ml) of (50x) WASH with dist. water to a total volume of 1,000 ml, mix shortly.

The diluted wash buffer must be stored at 2 – 8 °C for a maximum period of 4 weeks. Should the kit be used in several runs, then prepare only the required amount of wash buffer for each run.

All other reagents are ready for use.

6.2 Preparation of Samples (Precipitation and Acylation)

Allow reagents 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 **200 µl** each of **dissolved Standards** CAL 1 – 6, **Controls** CON 1 & 2 (see 6.1.1) and **Samples** into the respective marked Precipitation Tubes PRECI-TUBE.
2. Pipette **25 µl Precipitator 1** PRECI 1 into each tube.
3. Pipette **25 µl Precipitator 2** PRECI 2 into each tube.
4. Mix tubes thoroughly (vortex mixer).
5. Centrifuge tubes for 15 minutes with at least 4,000 x g, preferably with a swing-out rotor.
   Attention: 4,000 x g is not identical to 4,000 x rpm (rounds per minute) and has to be adjusted for each centrifuge and rotor.
6. Pipette **50 µl Acylation Buffer** ACYL-BUFF into each tube and continue with the next step, 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 multipette (or similar device), fill the syringe directly from the vial with dissolved Acylation Reagent and pipette tube by tube.
   Pipette **40 µl dissolved Acylation Reagent** ACYL-REAG (see 6.1.2) into one tube and immediately vortex the tube softly at medium speed for 2 to 4 seconds and then continue 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, preferably with a swing-out rotor.

   Take 50 µl each for the Metanephrine and Normetanephrine ELISA.

7 Assay Procedure

7.1 Metanephrine ELISA

1. Pipette **50 µl each of acylated Standards, Controls and Samples** into the respective wells of the coated microtiter strips STRIPS-MN (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 **25 µl diluted Metanephrine Antiserum** AS-MN (see 6.1.3) into each well. Colour changes to blue.
4. Cover the plate with adhesive foil 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 **300 µl diluted Wash Buffer** WASH (see 6.1.4) into each well, 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 **100 µl Enzyme Conjugate** CONJ into each well.
7. Incubate for 30 minutes at room temperature on an orbital shaker (medium shaking rate).
8. Washing: Repeat step 5.
9. Pipette **100 µl Substrate** SUB into each well.
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** STOP into each well, mix for minimum of 10 seconds.
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 **50 µl each of acylated Standards, Controls and Samples** into the respective wells of the coated microtiter strips STRIPS-NMN (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 **25 µl Normetanephrine Antiserum** AS-NMN into each well. Colour changes to orange.
4. Cover the plate with adhesive foil 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 **300 µl diluted Wash Buffer** WASH (see 6.1.4) into each well, 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 **100 µl Enzyme Conjugate** CONJ into each well.
7. Incubate for 30 minutes at room temperature on an orbital shaker (medium shaking rate).
8. Washing: Repeat step 5.
9. Pipette **100 µl Substrate** SUB into each well.
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** STOP into each well, mix for minimum of 10 seconds.
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

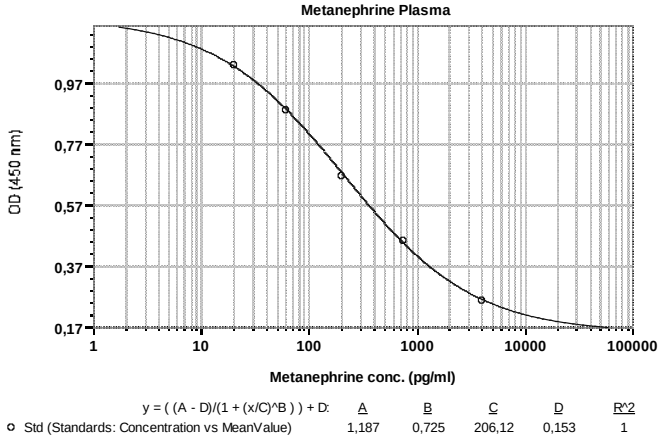
Concentrations of the standards: See QC certificate.

Conversion:

Metanephrine: 1 pg/ml = 5.07 pmol/l
Normetanephrine: 1 pg/ml = 5.46 pmol/l

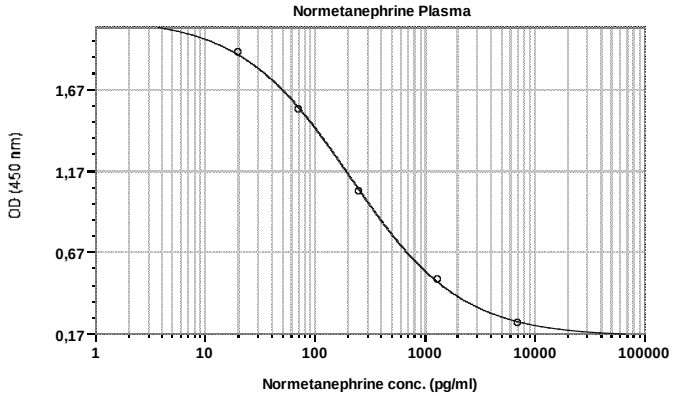
The concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline). The concentration of the controls and samples can be read directly from the standard curve in pg/ml.

Below are typical examples of standard curves:
Metanephrine Plasma ELISA



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
o	Std (Standards: Concentration vs MeanValue)	1,187	0,725	206,12	0,153	1

Normetanephrine Plasma ELISA



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
o	Std (Standards: Concentration vs MeanValue)	2,108	0,865	206,38	0,157	1

Quality control: All kit controls must be found within the acceptable ranges as printed on the QC 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 x LC/MS - 23; R = 0.991; N = 32
Normetanephrine	LC/MS	Y = 0.99 x 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 must be diluted with CAL 1 and reassayed.

In cases with moderate increases in normetanephrine, the clonidine suppression test can be useful in distinguishing between endogenous hypersecretion and false positive results.

9.10 Interfering Substances

Hemolytic, lipemic and icteric samples should not be used. Although common interfering substances have been evaluated in this test, other substances that have not been evaluated, such as medications or heterophilic antibodies as found in people who regularly come into contact with animals or animal products, can cause interference.

10 Changes to declare

Version _10: Changes/Additions are highlighted in grey.

Version _9: Changes/Additions are highlighted in grey. Valid from MNPE 149.

Version _8: Changes/Additions are highlighted in grey.

Version _7: Section 4: Change in volume provided. AS-MN to 0.6 ml and SOLVENT to 10 ml.

11 Literature

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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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- 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

For Metanephrine and Normetanephrine together in one PRECI-TUBE:

		Standards	Controls	Samples
PRECI-TUBEs:				
CAL 1 - 6	µl	200		
CON 1 & 2	µl		200	
EDTA-Plasma	µl			200
PRECI 1	µl	25	25	25
PRECI 2	µl	25	25	25

Vortex thoroughly
Centrifuge for 15 minutes at 4,000 x g

ACYL-BUFF	µl	50	50	50
ACYL-REAG	µl	40	40	40

Immediately, after pipetting ACYL-REAG into tube, vortex gently at medium speed for 2 to 4 seconds, before continuing with the next tube.
Take care not to disturb the pellet at the bottom of the tube.

Centrifuge for 15 minutes at 4,000 x g

Take 50 µl each for the Metanephrine and Normetanephrine ELISAs

Pipetting Scheme - ELISA

For Metanephrine and Normetanephrine in separate microtiterplates:

	Metanephrin (blue) STRIPS-MN			**Normetanephrin (yellow)** STRIPS-NMN		
	Acyl. Stand.	Acyl. Contr.	Acyl. Samples	Acyl. Stand.	Acyl. Contr.	Acyl. Samples
Transfer from PRECI-TUBEs into STRIPS	50	50	50	50	50	50

Shake for 1 hour at room temperature, do not cover plates

AS-MN	µl	25	25	25	-	-	-
AS-NMN	µl	-	-	-	25	25	25

Cover plate with FOIL
Shake for 2 hours at room temperature
4 x washing (approx. 300 µl WASH per well)

CONJ	µl	100	100	100	100	100	100

Shake for 30 minutes at room temperature
4 x washing (approx. 300 µl WASH per well)

SUB	µl	100	100	100	100	100	100

Shake for 30 ± 5 minutes at room temperature

STOP	µl	100	100	100	100	100	100

Mix for min. 10 seconds
Read absorbance at 450 nm