DIAGNOSTIKA GMBH

Instructions for Use

Nor- / Metanephrine in Urine ELISA

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

RUO

For Research Use Only
Not for Use in Diagnostic Procedures

REF	EA614/192
Σ	2 x (12 x 8)
	2 – 8 °C

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Symbols

RUO	For Research Use Only		
CONT	Content		Expiry Date
LOT	Lot Number		Store at
	Manufactured by		Sufficient for ... determinations
REF	Catalogue Number		Consult Instructions for Use

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

1 Introduction and Principle of the Test

Normetanephrine and metanephrine are physiologically formed from catecholamines by the enzyme catechol-O-methyltransferase (COMT).

The Nor- / Metanephrine ELISA kit provides materials for quantitative measurement of derivatized normetanephrine and metanephrine in human urine. During sample preparation conjugated normetanephrine and metanephrine are converted into free normetanephrine and metanephrine by hydrolysis. Normetanephrine and metanephrine are then quantitatively acylated into their derivates N-acyl-normetanephrine and N-acly-metanephrine.

The Nor-/ Metanephrine ELISA is a competitive enzyme immunoassay in the microtiter format. Solid-phase-bound antigen and the acylated antigen in solution compete for a defined number of antibody binding sites. When the system is in equilibrium, unbound antigen-antibody complex is removed in a washing step. The remaining solid-phase-bound complex is detected using peroxidase conjugate (POD) and its conversion of tetramethylbenzidine (TMB). The TMB/POD reaction is stopped and measured 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.

2 Precautions

- For research use only! For professional use only!
- Before carrying out the test, the instructions for use, as included in the kit, should be read completely and the content understood.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy; however 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 the reagents, controls and samples, the current laboratory safety guidelines and good laboratory practice should be observed.
- Wear protective clothing, disposable gloves, and eye protection while performing the test.
- Some components of this kit are containing hazardous reagents. These components are marked with the adequate hazard label. Further information is in section 4 and in the corresponding MSDS.
- Avoid contact with reagents. Avoid any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.

- Broken glass can cause injury. Be cautious with glass vials.
- Dispose of waste according to state and local environmental protection regulations.

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 the expiry date.

The shelf life of the ready-to-use reagents is indicated on the respective bottle label. The shelf life and storage conditions of the prepared reagents is stated under 6.1.

Bring all reagents to room temperature before use and refrigerate immediately after use.

4 Contents of the Kit

4.1 Reagents for sample preparation

Hydrolysis Tubes For hydrolysis	**HYDRO-TUBE**	98 pieces
Hydrochloric Acid 12 ml, ready for use, 0.1 M HCl	**HCL**	1 vial
Standards (1 - 6) 4 ml each, ready for use	**CAL 1** - **CAL 6**	6 vials
Control 1 & 2 4 ml each, ready for use, concentrations: see QC certificate	**CON 1** & **CON 2**	2 vials
Acylation Buffer 3.5 ml, ready for use, irritant	**ACYL-BUFF** Warning	1 vial
Acylation Reagent 1.75 ml, lyophilized, dissolve with Solvent	**ACYL-REAG**	3 vials

Solvent 7 ml, ready for use, contains DMSO	**SOLVENT**	1 vial

4.2 Reagents for ELISA

Metanephrine Antiserum 7 ml, ready for use, color coded blue, rabbit-anti-N-acyl-metanephrine	**AS-MN** Warning	1 vial
Normetanephrine Antiserum 7ml, ready for use, color coded yellow rabbit-anti-N-acyl-normetanephrine	**AS-NMN** Warning	1 vial
MT-Strips 8 wells each, break apart, coated with metanephrine, color coded blue	**STRIPS-MN**	12 strips
MT-Strips 8 wells each, break apart, coated with normetanephrine, color coded yellow	**STRIPS-NMN**	12 strips
Enyzme Conjugate 21 ml each, ready for use, Anti-rabbit IgG-Peroxidase	**CONJ** Warning	1 vial
Wash Buffer 20 ml each, 50x concentrate	**WASH**	2 vials
Substrate 21 ml, TMB solution, ready for use	**SUB**	1 vial
Stop Solution 21 ml, ready for use, contains 0.3 M sulphuric acid	**STOP**	1 vial
Adhesive foil Ready for use	**FOIL**	4 pieces

Additional materials and equipment required but not provided:

- Pipettes for pipetting 10, 20, 25, 50, 100 and 500 µl
- Multichannel pipette or Microplate washing device
- Eppendorf Multipette (or similar device)
- Distilled water
- Microplate photometer (450 nm)
- Orbital shaker
- Water bath or heat block
- Centrifuge
- Vortex mixer, Roll mixer
- Paper towels, pipette tips, timer

5 Sample Collection and Storage

5.1 Urine Samples

Spontaneous urine and collected urine can be used. Collected urine is recommended as it averages any fluctuations during a 24-hour period.

Collected urine:
The total volume of urine excreted during a 24-hour period should be collected in a single bottle containing 10 – 15 ml of 6 M hydrochloric acid (please, take caution) as preservative. Avoid exposure to direct sun light, store in the dark. Determine the total volume of urine collected, as it is required for calculating the µg/day value (see 8) and take an aliquot for the measurement.

With suspected kidney disorders, the creatinine concentration should be determined in addition.

Urine samples can be stored at -20 °C for at least 6 months. Avoid repeated freeze/thaw cycles.

Before use, mix and centrifuge.

6 Preparation of Reagents and Samples

6.1 Preparation of Reagents

Equilibrate reagents to room temperature.

6.1.1 Wash Buffer

Dilute the content (20 ml, 50x) of the bottle WASH with distilled water to a total volume of 1000 ml, mix briefly.

Store the diluted wash buffer 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.

6.1.2 Acylation Reagent

Before use, make sure that the SOLVENT equilibrates to room temperature (≥ 20 °C) and forms a homogenous, crystal-free solution.

Dissolve the content of one vial ACYL-REAG with 1.75 ml SOLVENT and shake for at least 5 minutes on a roll mixer or similar mixer. The Acylation Reagent must always be prepared immediately before performing the assay and is stable for approximately 3 hours.

The second and third vial allows a second and third run of the test, respectively. 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.

Discard Acylation Reagent after use.

All other reagents are ready for use.

6.2 Preparation of Samples (Hydrolysis and acylation)

Preparation of the standards, controls and the samples is identical for metanephrine and normetanephrine and is therefore performed only once.
Equilibrate reagents and samples to room temperature.
Determinations in duplicates are recommended.

1. Pipette **10 µl** of each **Standard** CAL 1 - CAL 6, **Control** CON 1 & CON 2 and **Urine Sample** into the respective hydrolysis tube HYDRO-TUBE (marked with a permanent marker).
2. Add **100 µl Hydrochloric Acid** HCL to each tube.
3. Seal the tubes, mix thoroughly (vortex) and incubate for 30 minutes at 90 to 100 °C in a water bath or heat block.
4. Let the tubes cool down to room temperature. Centrifuge briefly to collect any droplets on the tube walls and in the lid. Discard lids.
5. Add **25 µl Acylation Buffer** ACYL-BUFF to each tube.
6. Please note that the 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 multipette (or similar device), fill the syringe directly from the vial with dissolved Acylation Reagent and pipette tube by tube.
   Add **20 µl dissolved Acylation Reagent** ACYL-REAG (see 6.1.2) to each tube and immediately vortex for approx. 2 to 4 seconds at medium speed, then continue with the next tube. The color of the contents turns to red.
7. Incubate for 15 minutes at room temperature.
8. Add **500 µl distilled water** to each tube and mix thoroughly (vortex).

   Use 50 µl for the metanephrine ELISA and 20 µl the normetanephrine ELISA.

7 Test Procedure ELISA

7.1 Metanephrine ELISA

1. Transfer **50 µl of prepared Standards, Controls** and **Samples** from the Hydrolysis Tubes into the respective wells of the metanephrine strips STRIPS-MN wells (color coded blue).
2. Add **50 µl Metanephrine-Antiserum** AS-MN (color coded blue) to each well.
3. Cover the plate with adhesive foil FOIL and incubate for 1 hour at room temperature (20 – 25 °C) on an orbital shaker at medium speed.
4. Discard or aspirate the contents of the wells and wash with **300 µl Wash Buffer** WASH (see 6.1.1) per well. Discard or aspirate the contents of the wells and remove residual liquid by firmly tapping the inverted plate on clean absorbent paper towel. Repeat the washing procedure 3 times.
5. Pipette **100 µl Enzyme Conjugate** CONJ into each well.
6. Incubate for 20 min at room temperature (20 – 25 °C) on an orbital shaker at medium speed.
7. Washing: Repeat wash step 4.
8. Pipette **100 µl Substrate** SUB into each well.
9. Incubate for 20 ± 5 min at room temperature (20 - 25 °C) on an orbital shaker at medium speed.
10. Add **100 µl Stop Solution** STOP to each well. Shake plate for minimum 10 seconds on orbital shaker.
11. Within 15 minutes, read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

7.2 Normetanephrine ELISA

1. Transfer **20 µl of prepared Standards, Controls** and **Samples** from the Hydrolysis Tubes into the respective wells of the normetanephrine strips STRIPS-NMN wells (color coded yellow).
2. Add **50 µl Normetanephrine-Antiserum** AS-NMN (color coded yellow) to each well.
3. Cover the plate with adhesive foil FOIL and incubate for 1 hour at room temperature (20 – 25 °C) on an orbital shaker at medium speed.
4. Discard or aspirate the contents of the wells and wash with **300 µl Wash Buffer** WASH (see 6.1.1) per well. Discard or aspirate the contents of the wells and remove residual liquid by firmly tapping the inverted plate on clean absorbent paper towel. Repeat the washing procedure 3 times.
5. Pipette **100 µl Enzyme Conjugate** CONJ into each well.
6. Incubate for 20 min at room temperature (20 – 25 °C) on an orbital shaker at medium speed.
7. Washing: Repeat wash step 4.
8. Pipette **100 µl Substrate** SUB into each well.
9. Incubate for 20 ± 5 min at room temperature (20 - 25 °C) on an orbital shaker at medium speed.
10. Add **100 µl Stop Solution** STOP to each well. Shake plate for minimum 10 seconds on orbital shaker.
11. Within 15 minutes, read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

8 Calculation of Results

CAL:		1	2	3	4	5	6
Metanephrine	ng/ml	0	20	60	200	600	2,000
	nmol/l	0	101	304	1,014	3,042	10,140
Normetanephrine	ng/ml	0	30	100	300	1,000	3,000
	nmol/l	0	164	546	1,638	5,460	16,380

Metanephrine: 1 ng/ml = 5.07 nmol/l

Normetanephrine: 1 ng/ml = 5.46 nmol/l

The concentration of the standards in ng/ml (x-axis, logarithmic) are plotted against their corresponding optical density OD_{450nm} (y-axis, linear).

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

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

When using collected urine (24-hour periode):

The amount of analyte excreted per day [µg/day] is calculated as following:

Concentration of the sample [in ng/ml = µg/l] multipled by the volume of urine excreted per day [in l/day]

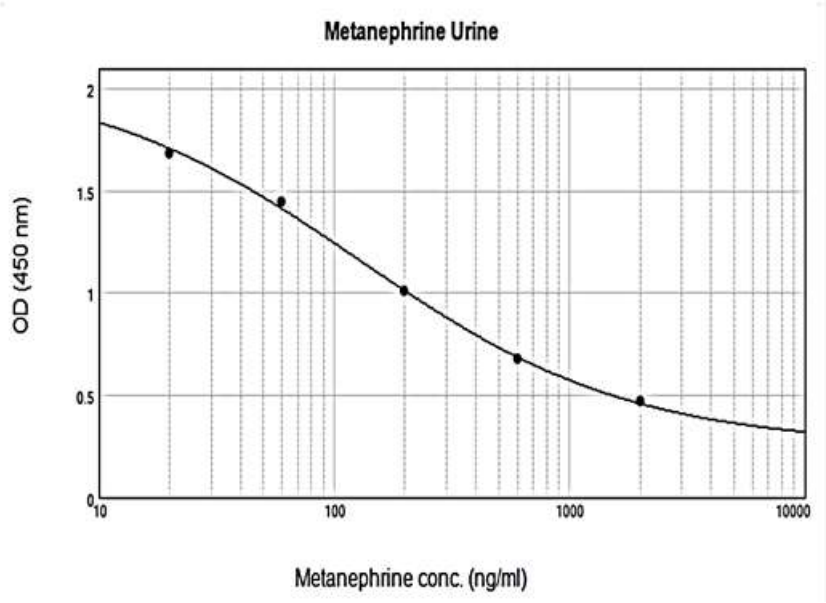
Example:

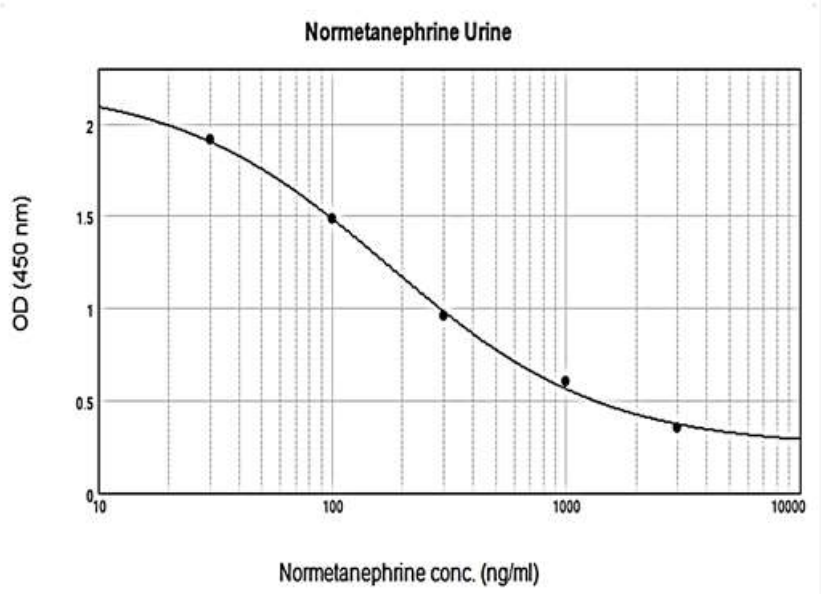
The concentration of the sample read from the curve is 365 ng/ml = 365 µg/l. The volume of urine collected during 24 hours is 1.2 l. Then the amount of analyte excreted during one day would be:

365 µg/l x 1.2 l/day = 438 µg/day

Quality Control: Test results are valid only if the kit controls are within the ranges specified on the QC Certificate. Otherwise, the test should be repeated.

Typical Example of standard curves (do not use for calculation of results):





9 Assay Characteristics

9.1 Analytical Sensitivity

The lower detection limit was determined by measuring the 2-fold standard deviation of the optical density (OD) of the zero standard and reading the corresponding concentration on the standard curve.

	LLOD	Calculation
Metanephrine	4 ng/ml	OD_{Cal1} – 2xSD
Normetanephrine	10 ng/ml	OD_{Cal1} – 2xSD

9.2 Analytical Specificity (Cross Reactivity)

The antibodies used in the assay are specific for the respective antigen. The following substances were tested for 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

9.3 Recovery after spiking

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

9.4 Linearity

	Range [ng/ml]	Max. dilution	Mean [%]	Range [%]
Metanephrine	85 - 2134	1 : 21 with CAL1	90	77 - 106
Normetanephrine	52 - 1243	1 : 21 with CAL1	93	79 - 102

9.5 Reproducibility

	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 %

9.6 Method Comparison

Matrix	Method	Correlation
Metanephrine	LC/MS	Y = 0.89 x LC/MS - 2; R^2 = 0.99; N = 32
Normetanephrine	LC/MS	Y = 1.01 x LC/MS - 20; R^2 = 0.98; N = 32

9.7 Calibration

The assay is calibrated by addition of defined stock solutions of metanephrine and normetanephrine.

9.8 Limitations of Method

Results are for research use only. Samples measured above the highest standard must be diluted with CAL 1 and re-assayed. The values of diluted samples must be multiplied by the appropriate dilution factor.

9.9 Interferences

Only use collected urine, which was acidified (see 5.1).

10 Changes to declare

Version _5 (valid as of lot MNUE110): Changes/additions are highlighted in grey.

Pipetting Scheme - Sample Preparation

Metanephrine and Normetanephrine in one tube

		Standards	Controls	Urine
HYDRO-TUBE:				
CAL 1 – 6	µl	10		
CON 1 & 2	µl		10	
Urine	µl			10
HCL	µl	100	100	100

Close tubes and vortex
Incubate for 30 minutes at 90 – 100 °C
Allow to cool to RT
Centrifuge briefly
Discard lid

ACYL-BUFF	µl	25	25	25
ACYL-REAG	µl	20	20	20

<u>Immediately,</u> mix for 2 – 4 seconds after adding to the tube and before proceeding to the next tube
Incubate for 15 minutes at RT

Dest. water	µl	500	500	500

Mix throughly

Use 50 µl for the Metanephrine ELISA and 20 µl for the Normetanephrine ELISA

Pipetting Scheme – ELISA

Metanephrine and Normetanephrine in two separate plates

		Metanephrine (blue) STRIPS-MN			**Normetanephrine (yellow)** STRIPS-NMN		
		Prepared			Prepared		
		Stand.	Contr.	Samples	Stand.	Contr.	Samples
Transfer from HYDRO-TUBE into STRIPS	µl	50	50	50	20	20	20
AS-MN (blue)	µl	50	50	50			
AS-NMN (yellow)	µl				50	50	50

Cover plates with FOIL
Incubate for 1 hour at RT with shaking
Decant plate and remove residual liquid

WASH	µl	300	300	300	300	300	300

Decant plate and remove residual liquid
Repeat washing steps 3x
Decant plate and remove residual liquid

CONJ	µl	100	100	100	100	100	100

Incubate for 20 minutes at RT with shaking
Decant plate and remove residual liquid

WASH	µl	300	300	300	300	300	300

Decant plate and remove residual liquid
Repeat washing steps 3x
Decant plate and remove residual liquid

SUB	µl	100	100	100	100	100	100

Incubate for 20 ± 5 minutes at RT with shaking

STOP	µl	100	100	100	100	100	100

Shake for minimum 10 seconds
Read absorbance at 450 nm (ref. 570 – 650 nm)