



Revised 22 Dec. 2011 rm (Vers. 7.1)

RUO in the USA*This kit is intended for Research Use Only.**Not for use in diagnostic procedures.**Please use only the valid version of the package insert provided with the kit.***Intended use and principle of the test**

Enzyme Immunoassay for measurement of Normetanephrine in urine

During the sample preparation Normetanephrine (Normetadrenaline) is quantitatively acylated.

The subsequent competitive ELISA kit uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The acylated standards, controls and samples and the solid phase bound analyte compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

Quantification of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

Advice on handling the test***Reliability of the test results***

In order to assure a reliable evaluation of the test results it must be conducted according to the instructions included and in accordance with current rules and guidelines (GLP, RILIBÄK, etc.). Special attention must be paid to control checks for precision and correctness during the test; the results of these control checks have to be within the norm range. In case of significant discrepancies between the pre-set assay characteristics of this test and the actual results please contact the manufacturer of the test kit for further instructions.

It is recommended that each laboratory establishes its own reference intervals. The values reported in this test instruction are only indicative.

Complaints

In case of complaints please submit to the manufacturer a written report containing all data as to how the test was conducted, the results received and a copy of the original test printout. Please contact the manufacturer to obtain a reclamation form and return it completely filled in to the manufacturer.

Disposal

Residual substances and/or all remaining chemicals, reagents and ready for use solutions, are special refuse. The disposal is subject to the laws and regulations of the federation and the countries. About the removal of special refuse the responsible authorities or refuse disposal enterprises inform. The disposal of the kit must be made according to the national official regulations. Legal basis for the disposal of special refuse is the cycle economic- and waste law. The appropriate safety data sheets of the individual products are available upon request. The safety data sheets correspond to the standard: ISO 11014-1.

Interference

Do not mix reagents and solutions from different lots. Consider different transport and storage conditions. Inappropriate handling of test samples or deviations from the test regulation can the results affect. Use no kit components beyond the

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expiration date. Avoid microbiological contamination of the reagents and the washing water. Consider incubation periods and wash references.

Precautions

Observe the incubation periods and washing instructions. Never pipette by mouth and avoid contact of reagents and specimens with skin. No smoking, eating or drinking in areas where samples or kit test tubes are handled. When working with kit components or samples, always wear protective gloves and wash your hand thoroughly as soon as you have finished the work. Avoid spraying of any kind. Avoid any skin contact with reagents. Use protective clothing and disposable gloves.

All steps have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes. Sodium azide could react with lead and copper tubes and may form highly explosive metal azide. When clearing up, rinse thoroughly with large volumes of water to prevent such formation.

All reagents of this testkit which contain human or animal serum or plasma have been tested and confirmed negative for HIV I/II, HbsAg and HCV by FDA approved procedures.

All reagents, however, should be treated as potential biohazards in use and for disposal.

Storage and stability

Store the reagents at 2 - 8 °C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Do not mix various lots of any kit component within an individual assay.

Contents of the kit

REAC-TUBES	Reaction Tubes	2 x 50	ready for use
WASH-CONC 50x	Wash Buffer Concentrate	1 x 20 mL	concentrate, dilute content with dist. water to a final volume of 1000 mL
CONJUGATE	Enzyme Conjugate	1 x 12 mL	ready for use, anti-rabbit IgG conjugated with peroxidase
SUBSTRATE	Substrate	1 x 12 mL	ready for use, containing a solution of tetramethylbenzidine (TMB)
STOP-SOLN	Stop Solution	1 x 12 mL	ready for use, containing 0.25 M H ₂ SO ₄
96 NAD NMN	Noradrenaline-Normetanephrine Microtiter Strips	1 x 96 wells	12 strips, 8 wells each, break apart, pre-coated, yellow coloured
NMN-AS	Normetanephrine Antiserum	1 x 12 mL	from rabbit, ready for use, yellow coloured, yellow screw cap
ACYL-CONC	Acylation Concentrate	1 x 0.5 mL	Concentrate. Has to be diluted prior to use.
ACYL-DILUENT	Acylation Diluent	1 x 4 mL	ready for use
STANDARD A	Standard A	1 x 4 mL	ready for use

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STANDARD B	Standard B	1 x 4 mL	ready for use
STANDARD C	Standard C	1 x 4 mL	ready for use
STANDARD D	Standard D	1 x 4 mL	ready for use
STANDARD E	Standard E	1 x 4 mL	ready for use
STANDARD F	Standard F	1 x 4 mL	ready for use
HCL	Hydrochloric Acid	1 x 30 mL	ready for use, contains 0.25 M HCl
CONTROL 1	Control 1	1 x 4 mL	ready for use
CONTROL 2	Control 2	1 x 4 mL	ready for use
ACYL-BUFF	Acylation Buffer	1 x 30 mL	ready for use

Additional materials and equipment required but not provided in the kit

- Calibrated variable precision micropipettes (e.g. 10-100 µL / 100-1.000 µL)
- Microtiter plate washing device
- ELISA reader capable of reading absorbance at 450 nm and 620 or 650 nm
- Centrifuge capable of at least 3.000 x g
- Absorbent material (paper towel)
- Distilled water, Vortex mixer
- Temperature controlled water bath (90 °C) or similar heating device

■ The assay can be performed with or without shaking. If a shaker is used, it should have the following characteristics: shaking amplitude 3mm; approx. 600 rpm

Sample collection and storage

Spontaneous or 24-hour urine, collected in a bottle containing 10-15 mL of 6 M HCl, should be used.

Determine the total volume of urine excreted during a period of 24 h for calculation of the results.

Storage: for longer periods (up to 6 months) at -20 °C.

Repeated freezing and thawing should be avoided.

Avoid exposure to direct sunlight.

Test procedure

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the Reaction Tubes accordingly. Duplicate determinations are recommended.

Preparation of reagents**Wash Buffer**

Dilute the 20 mL Wash Buffer Concentrate with distilled water to a final volume of 1000 mL.

Storage: up to 6 months 2 °C - 8 °C

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Acylation Solution

Before preparing the Acylation Solution make sure that the Acylation Diluent has reached room temperature ($\geq 20\text{ }^{\circ}\text{C}$) and forms a homogenous, crystal-free solution.

Dilute the Acylation Concentrate 1 + 60 with Acylation-Diluent in a glass or polypropylene-vial.

Acylation Concentrate	10 μL	20 μL	25 μL	50 μL
Acylation-Diluent	600 μL	1.2 mL	1.5 mL	3 mL

The Acylation Solution has to be prepared freshly prior to the assay (not longer than 60 minutes in advance). Discard after use!

Sample preparation and acylation

Hydrolysis

1. Pipette **25 μL** of standards, **25 μL** of controls, and **25 μL** of urine samples into the respective **Reaction Tubes**.
2. Add **250 μL Hydrochloric Acid** to all tubes.
3. Mix thoroughly (vortex) and hydrolyze for **30 min.** at **90 $^{\circ}\text{C}$** .
4. Cool down the tubes to room temperature.

For the measurement of the free Normetanephrine only, leave away step 3 and 4.

Acylation

1. Pipette **250 μL** of **Acylation Buffer** into all tubes.
2. Add **25 μL** of **Acylation Solution** to all tubes.
3. Mix thoroughly (vortex) and acylate for **15 minutes** at **RT** (20 $^{\circ}\text{C}$ - 25 $^{\circ}\text{C}$).
4. Add **2.5 mL dist. water** to all tubes.

Take **25 μL** of the acylated standards, controls and urine samples for the Normetanephrine ELISA.

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Normetanephrine ELISA

The usage of a shaker is not mandatory. The alternative protocol without shaker is highlighted in italic and shaded in grey.

1. Pipette **25 µL** of the **acylated standards, controls and samples** into the appropriate wells of the **Normetanephrine Microtiter Strips**.
 2. Pipette **100 µL** of the **Normetanephrine Antiserum** into all wells.
 3. Incubate **30 min at RT** (20 °C - 25 °C) on a shaker (approx. 600 rpm).
Without usage of a shaker: shake Normetanephrine Microtiter Strips shortly by hand and incubate for 1 hour at RT (20 °C - 25 °C).
 4. Discard or aspirate the contents of the wells and **wash** each well **3 times** thoroughly with **300 µL Washbuffer**. Blot dry by tapping the inverted plate on absorbent material.
 5. Pipette **100 µL** of the **Enzyme Conjugate** into all wells.
 6. Incubate for **15 min at RT** (20 °C - 25 °C) on a shaker (approx. 600 rpm).
Without usage of a shaker: incubate for 15 min at RT (20 °C - 25 °C).
 7. Discard or aspirate the contents of the wells and **wash** each well **3 times** thoroughly with **300 µL Washbuffer**. Blot dry by tapping the inverted plate on absorbent material.
 8. Pipette **100 µL** of the **Substrate** into all wells.
 9. Incubate for **15 ± 2 min at RT** (20 °C - 25 °C) on a shaker (approx. 600 rpm).
Without usage of a shaker: incubate for 15 min ± 2 at RT (20 °C - 25 °C).
- Avoid exposure to direct sun light!*
10. Add **100 µL** of the **Stop Solution** to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.
 11. **Read** the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to **450 nm** and a reference wavelength between 620 nm and 650 nm.

Calculation of results

Standard	Concentration of the standards					
	A	B	C	D	E	F
Normetanephrine (ng/mL = µg/L)	0	30	90	300	900	3 000
Normetanephrine (nmol/L)	0	164	491	1 638	4 914	16 380
Conversion:	Normetanephrine (ng/mL) x 5.46 = Normetanephrine (nmol/L)					

The calibration curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x-axis). Use a non-linear regression for curve fitting (e.g. spline, 4- parameter, akima).

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The concentrations of the samples and controls can be read directly from the standard curve.

The amount of analyte excreted per day ($\mu\text{g}/\text{day}$) is calculated according to:

$$\text{concentration of the sample (in } \mu\text{g/L)} \times \text{volume of urine excreted per day (in L/day)}$$

Example

The concentration of the sample read from the curve is $125 \mu\text{g/L}$. The amount of urine collected during 24 hours is 1.3 L. Then the amount of analyte excreted during one day would be:


$$125 \mu\text{g/L} \times 1.3 \text{ L/day} = 162.5 \mu\text{g/day}$$

Quality control

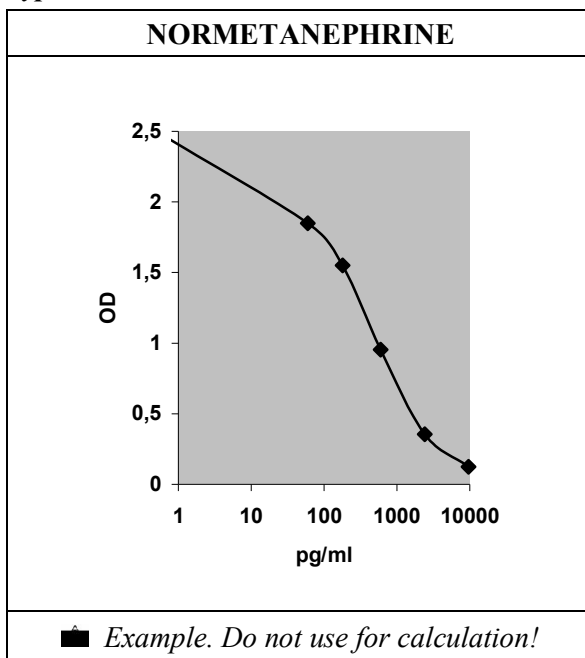
It is recommended to use control samples according to state and federal regulations.

Calibration

The binding of the antisera and the enzyme conjugates and the activity of the enzyme used are temperature dependent, and the extinction values may vary if a thermostat is not used. The higher the temperature, the higher the extinction values will be. The extinction values also depend on the incubation times. The optimal temperature during the Enzyme Immunoassay is between $20 \text{ }^\circ\text{C}$ - $25 \text{ }^\circ\text{C}$.

 In case of overflow, read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 405 nm

Typical calibration curve



Rev. 12/15/2011 cc