



Bi-Neph (Metanephrine & Normetanephrine) ELISA

For the quantitative determination of metanephrine and normetanephrine in urine.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	17-BINHU-E02-UFST
Size:	2 x 96 wells
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ALPCO Diagnostics

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1. Intended use and principle of the test

Enzyme Immunoassay for the quantitative determination of Metanephrine and Normetanephrine in urine. First Metanephrine (Metadrenaline) and Normetanephrine (Normetadrenaline) are 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 analytes 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.

During the sample preparation Metanephrine (Metadrenaline) is quantitatively acylated.

2. Advice on handling the test

2.1 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 normal range. In case of significant discrepancies between the pre-set assay characteristics of this test and the actual results please contact ALPCO for further instructions.

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

The results obtained with this test kit should not be taken as the sole reason for any therapeutic consequence but have to be correlated to other diagnostic tests and clinical observations.

2.2 Complaints

In case of complaints, please contact ALPCO with a written report containing all data as to how the test was conducted, the results received, and a copy of the original test printout.

2.3 General Notes

This test kit was produced according to the latest developments in technology and subjected to stringent internal and external quality control checks. Any alteration of the test kit or the test procedure as well as the usage of reagents from different sources may have a negative influence on the test results and are therefore not covered by any warranty. ALPCO is not liable for damages incurred in transit.

2.4 Disposal

Residual substances and/or all remaining chemicals, reagents and ready-to-use solutions are special refuse. Their disposal is subject to the local and federal laws and regulations. Inform the responsible authorities or disposal enterprises about the removal of these substances. The disposal of the kit must be made in accordance with local and federal regulations.

The appropriate safety data sheets of the individual products are available upon request. The safety data sheets correspond to the standard: ISO 11014-1.

2.5 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 procedure can affect the results. Do not use kit components beyond the expiration date. Avoid microbiological contamination of the reagents and of the deionized water.

2.6 Precautions

Follow the recommended 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 reagents are handled. When working with kit components or samples, always wear protective gloves and wash your hands thoroughly when exiting the lab. Avoid splashing and aerosolizing. Avoid any skin contact with reagents. Use protective clothing and disposable gloves. All steps must be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes.

Sodium azide could react with lead and copper plumbing and may form highly explosive metal azide. When disposing of waste in drains, rinse thoroughly with large volumes of water to prevent such formation.

All reagents of this test kit 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.

3. 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.

4.1 Contents of the kit

BA D-0023	REAC-TUBES	Reaction Tubes	2 x 50	ready for use
BA E-0030	WASH-CONC 50x	Wash Buffer Concentrate	1 x 20 mL	concentrate, dilute content with dist. water to a final volume of 1000 mL
BA E-0045	CONJUGATE	Enzyme Conjugate	2 x 12 mL	ready for use, anti-rabbit IgG conjugated with peroxidase
BA E-0055	SUBSTRATE	Substrate	2 x 12 mL	ready for use, containing a solution of tetramethylbenzidine (TMB)
BA E-0080	STOP-SOLN	Stop Solution	2 x 12 mL	ready for use, containing 0.25 M H ₂ SO ₄
BA E-0131	ARD MN	Adrenaline-Metanephrine Microtiter Strips	1 x 96 wells	12 strips, 8 wells each, break apart, pre-coated, blue coloured
BA E-0231	NAD NMN	Noradrenaline-Normetanephrine Microtiter Strips	1 x 96 wells	12 strips, 8 wells each, break apart, pre-coated, yellow coloured
BA E-8510	MN-AS	Normetanephrine Antiserum	1 x 12 mL	from rabbit, ready for use, yellow coloured, yellow screw cap
BA E-8410	MN-AS	Metanephrine Antiserum	1 x 12 mL	from rabbit, ready for use, blue coloured, blue screw cap
BA R-0012	ACYL-CONC	Acylation Concentrate	1 x 0.5 mL	Concentrate. Has to be diluted prior to use.
BA R-0075	ACYL-DILUENT	Acylation Diluent	1 x 4 mL	ready for use
BA R-8601	STANDARD A	Standard A	1 x 4 mL	ready for use
BA R-8602	STANDARD B	Standard B	1 x 4 mL	ready for use
BA R-8603	STANDARD C	Standard C	1 x 4 mL	ready for use
BA R-8604	STANDARD D	Standard D	1 x 4 mL	ready for use
BA R-8605	STANDARD E	Standard E	1 x 4 mL	ready for use
BA R-8606	STANDARD F	Standard F	1 x 4 mL	ready for use
BA R-8619	HCL	Hydrochloric Acid	1 x 30 mL	ready for use, contains 0.25 M HCl
BA R-8651	CONTROL 1	Control 1	1 x 4 mL	ready for use
BA R-8652	CONTROL 2	Control 2	1 x 4 mL	ready for use
BA R-8611	ACYL-BUFF	Acylation Buffer	1 x 30 mL	ready for use

4.2 Additional materials and equipment required but not provided with the kit

- Calibrated variable precision micropipettes (e.g. 10-100 µL / 100-1,000µL)
- 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

Note: 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

5. 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.

6. 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.

Note: The sample preparation (hydrolysis and acylation) is identical for both the Metanephrine and Normetanephrine assay and only has to be completed once.

6.1 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 2–8°C

Acylation Solution

Note: Before preparing the Acylation Solution make sure that the Acylation Diluent (BA R-0075) has reached room temperature ($\geq 20^{\circ}\text{C}$) and forms a homogenous, crystal-free solution.

Dilute the Acylation Concentrate (BA R-0012) 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

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

6.2 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 °C .
4.	Cool down the tubes to room temperature.
Note: If only measuring free Metanephrine and free Normetanephrine, skip steps 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 (20-25°C).
4.	Add 2.5 mL dist. water to all tubes.
Note: Take 25 μL of the acylated standards, controls and urine samples for the Metanephrine ELISA and Normetanephrine ELISA .	

6.3 Metanephrine ELISA

The usage of a shaker is not mandatory. The alternative protocol without a shaker is italicized and shaded grey.

1.	Pipette 25 µL of the acylated standards, controls and samples into the appropriate wells of the Metanephrine Microtiter Strips .
2.	Pipette 100 µL of the Metanephrine Antiserum into all wells.
3.	Incubate 30 min at RT (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: shake the Metanephrine Microtiter Strips shortly by hand and incubate for 1 hour at RT (20-25°C).</i>
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 (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: incubate for 15 min at RT (20-25°C).</i>
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 (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: incubate for 15 min ± 2 at RT (20-25°C).</i> Note: 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.

6.4 Normetanephrine ELISA

The usage of a shaker is not mandatory. The alternative protocol without a shaker is italicized and shaded 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 (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: shake the Normetanephrine Microtiter Strips shortly by hand and incubate for 1 hour at RT (20-25°C).</i>
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 (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: incubate for 15 min at RT (20-25°C).</i>
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 (20-25°C) on a shaker (approx. 600 rpm). <i>Without usage of a shaker: incubate for 15 min ± 2 at RT (20-25°C).</i> Note: 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.

7. Calculation of results

Standard	Concentration of the standards					
	A	B	C	D	E	F
Normetanephrine (ng/mL= μ g/L)	0	30	90	300	900	3 000
Normetanephrine (nmol/L)	0	164	491	1 638	4 914	16 380
Metanephrine (ng/mL= μ g/L)	0	20	60	200	600	2 000
Metanephrine (nmol/L)	0	101	304	1 014	3 042	10 140
Conversion:	Normetanephrine (ng/mL) \times 5.46 = Normetanephrine (nmol/L) Metanephrine (ng/mL) \times 5.07 = Metanephrine (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).

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

The amount of analyte excreted per day (μ g/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 μ 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:

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

7.1 Quality control

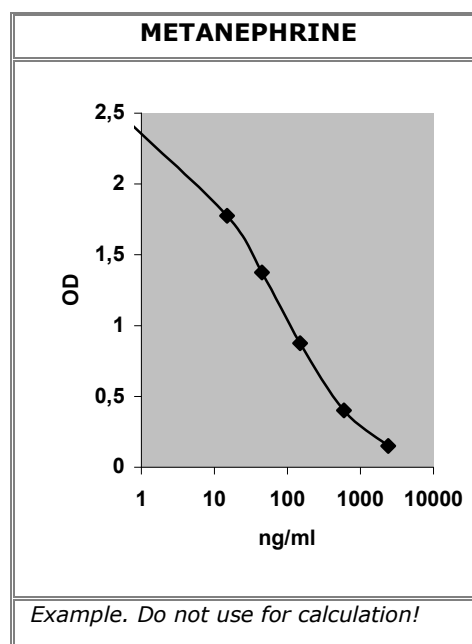
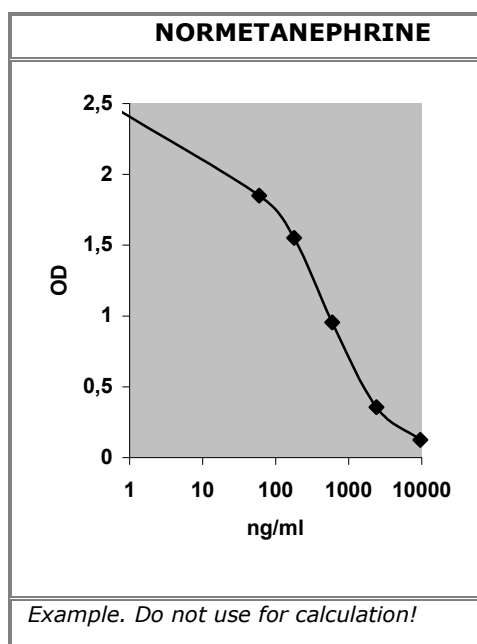
It is recommended to use control samples according to state and federal regulations. Use controls at both normal and pathological levels. The included controls or other commercial controls should fall within established confidence limits. The confidence limits of the kit controls are indicated on the QC Report.

7.2 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 20-25°C.

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

7.3 Typical calibration curves



8. Assay characteristics

Expected Reference Values		Metanephrine	Normetanephrine
	Urine	< 350 µg/day	< 600 µg/day

Analytical Sensitivity (Limit of Detection)		Metanephrine	Normetanephrine
	Urine	13 ng/mL	23 ng/mL

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)	
		Metanephrine	Normetanephrine
	Derivatized Metanephrine	100	0.11
	Derivatized Normetanephrine	0.15	100
	Derivatized 3-methoxytyramine	< 0.01	0.19
	Adrenaline	3.3	< 0.001
	Noradrenaline	< 0.001	0.64
	Dopamine	< 0.001	< 0.01
Vanillic mandelic acid, L-Dopa, Homovanillic acid, L-Tyrosin, Tyramin	< 0.001	< 0.001	

Precision							
Intra-Assay				Inter-Assay			
	Sample	Range (ng/mL)	CV (%)		Sample	Range (ng/mL)	CV (%)
Metanephrine	1	69 ± 8.6	12.6	Metanephrine	1	102 ± 15.4	15.1
	2	446 ± 23	5.2		2	448 ± 40	8.9
Normetanephrine	1	200 ± 34	17.2	Normetanephrine	1	191 ± 41	21.4
	2	857 ± 153	17.8		2	682 ± 131	19.3

Linearity			Range	Serial dilution up to	Mean (%)
	Metanephrine	Urine	40 – 1 600 ng/mL	1: 16	98
	Normetanephrine	Urine	40 – 5 200 ng/mL	1: 16	93

Recovery			Mean (%)	Range (%)	% Recovery after spiking
	Metanephrine	Urine	105	98 – 119	
	Normetanephrine	Urine	103	90 – 113	

Method Comparison versus HPLC*	Metanephrine	Urine	HPLC = 0.9 ELISA – 0.8	r = 0.99; n = 40
	Normetanephrine	Urine	HPLC = 0.9 ELISA + 0.6	r = 0.99; n = 40

* The concentrations were assessed using both the ELISA and the HPLC method (external QC samples from UK NEQAS). The correlation between ELISA and HPLC is excellent. Please take in mind, that the UK control values are the mean of about 40 different HPLC users, and contain always one pathological sample per sending.