



Instructions for Use

Nor- / Metanephrine - ELISA

Enzyme Immunoassay


for the Quantitative Determination of


Normetanephrine and Metanephrine

in Urine



REF EA614/192

 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 normetanephrine and metanephrine in urine. 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 nor-/metanephrines is inversely proportional to the nor-/metanephrines concentration of the sample.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative. Avoid skin contact.
- 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 Extraction:

- 4.1 **Acylation Reagent** **ACYL-REAG** 2 vials
2.5 ml, lyophilised
- 4.2. **Acylation Buffer** **ACYL-BUFF** 1 vial
3.5 ml, ready for use
Tris-HCl-Puffer
- 4.3 **Standards 1 – 6** **CAL 1 – 6** 6 vials
Each 1 ml, ready for use
Concentrations:

Standard	1	2	3	4	5	6
Metanephrine (ng/ml)	0	20	60	200	600	2,400
Normetanephrine (ng/ml)	0	30	100	300	1,000	4,000

- 4.4 **Control 1 & 2** **CON 1 & 2** 2 vials
Each 1 ml, ready for use
Concentrations: See q.c. certificate
- 4.5 **Hydrochloric Acid** **HCL** 1 vial
12 ml, ready for use
0.1 M HCl
- 4.6 **Hydrolysis Tubes** **HYDRO-TUBE** 1 x 100 pcs.
- 4.7 **Solvent** **SOLVENT** 1 vial
6 ml, ready for use
Contains DMF and DMSO. Please note that Solvent reacts with many plastic materials including plastic trays. Solvent does not react with normal pipette tips and with glass devices.

Reagents for the ELISA

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

4.9	Metanephrine Antiserum 5.5 ml, ready for use, from rabbit blue coloured	AS-MN	1 vial
4.10	Normetanephrine Antiserum 5.5 ml, ready for use, from rabbit yellow coloured	AS-NMN	1 vial
4.11	Enzyme Conjugate 21 ml, ready for use Anti-rabbit IgG-POD conjugate	CONJ	1 vial
4.12	Wash Buffer 20 ml, 25x concentrated Dilute with dist. water to 500 ml.	WASH	2 vials
4.13	Substrate 21 ml TMB solution, ready for use	SUB	1 vial
4.14	Stop Solution 21 ml, ready for use Contains 0.3M sulphuric acid, not corrosive	STOP	1 vial
4.15	Adhesive Foil	FOIL	2 pieces

Additional materials and equipment required but not provided:

- Pipettes (10, 20, 25, 50, 100 µl, 500 µl)
- Water bath or heat block (90°C)
- Horizontal shaker
- Microplate washing device
- Microplate photometer
- Distilled water

5. Sample Collection and Storage

Spontaneous urine can be used for this test as well as collected urine. In this case the total volume of urine excreted during a 24-hours period should be collected and mixed in a single bottle containing 10 - 15 ml of 6 M hydrochloric acid as preservative. Avoid exposure to direct sun light. Determine the total volume and take an aliquot for the measurement. For patients with suspected kidney disorders the creatinine concentration should be tested, too. Urine samples can be stored at -20 °C for at least 6 months.

6. Preparation of Reagents and Samples

6.1 Preparation of Reagents

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.

Wash Buffer

Dilute contents of one bottle with distilled water to 500 ml. The diluted Wash Buffer should be stored at 2 – 8 °C and is stable for four weeks.

All other reagents are ready for use.

6.2. Preparation of Samples

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

Allow all reagents to reach room temperature.

Hydrolysis

1. Pipette each **10 µl standards**, urine samples and controls into the corresponding hydrolysis tubes.
2. Pipette each **100 µl Hydrochloric Acid** (0.1 M) into all tubes.
3. Seal the tubes, mix thoroughly (vortex) and incubate for 30 min at 90 °C in a water bath or similar device.
4. Let the tubes cool down to room temperature.
Centrifuge shortly to pool dispersed drops, discard lids.

NOTE: For the measurement of the free normetanephrine and the free metanephrine only, leave out point 3 to 4.

Acylation

5. Pipette each **25 µl Acylation Buffer** into all tubes.
6. Pipette each **25 µl Acylation Reagent** into all tubes and continue with step 7. Immediately.
7. Mix thoroughly (vortex) and incubate for 15 minutes at room temperature.
8. Pipette each **0.5 ml dist. water** into all tubes and mix thoroughly (vortex).

7. Assay Procedure

7.1

Metanephrine ELISA

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

1. Pipette each **20 µl acylated Standards, Controls and patient samples** into the respective wells.
2. Pipette each **50 µl Metanephrine Antiserum** into all wells.
3. Incubate for 1 hour at room temperature on an orbital shaker.
4. Discard or aspirate the contents of the wells and wash thoroughly with each **300 µl Wash Buffer**. Repeat the washing procedure 4 times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.
5. Pipette each **100 µl Enzyme Conjugate** into all wells.
6. Incubate for 20 minutes at room temperature on an orbital shaker.
7. Washing: Repeat step 4.
8. Pipette each **100 µl Substrate** into all wells.
9. Incubate 15 to 25 minutes at room temperature on an orbital shaker, preferably in the dark.
10. Pipette **100 µl Stop Solution** into all wells.
11. Read the optical density at 450 nm within 10 minutes (reference wavelength between 570 and 650 nm) in a microplate photometer.

7.2

Normetanephrine ELISA

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

1. Pipette each **20 µl acylated Standards, Controls and patient samples** into the respective wells.
2. Pipette each **50 µl Normetanephrine Antiserum** into all wells.
3. Incubate for 1 hour at room temperature on an orbital shaker.
4. Discard or aspirate the contents of the wells and wash thoroughly with each **300 µl Wash Buffer**. Repeat the washing procedure 4 times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.
5. Pipette each **100 µl Enzyme Conjugate** into all wells.
6. Incubate for 20 minutes at room temperature on an orbital shaker.
7. Washing: Repeat step 4.
8. Pipette each **100 µl Substrate** into all wells.
9. Incubate 15 to 25 minutes at room temperature on an orbital shaker, preferably in the dark.
10. Pipette 100 µl **Stop Solution** into all wells.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

8. Calculation of the 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.

Metanephrine	Normetanephrine
< 350 µg/day	< 600 µg/day

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
4 ng/ml	10 ng/ml

9.3 Specificity

The following cross reactivities were tested:

Metanephrine

Compound	Cross React. %
Metanephrine	100
Normetanephrine	0.430
3-Methoxytyramine	<0.025
Adrenaline	0.875
Noradrenaline	< 0.025
Dopamine	< 0.025
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	0.775
3-Methoxytyramine	0.282
Adrenaline	<0.022
Noradrenaline	1.360
Dopamine	<0.022
L-Dopa	< 0.001
Tyramine	< 0.001
Tyrosine	< 0.001
Homovanillic acid	< 0.001
Vanillic mandelic acid	< 0.001

9.4 Recovery

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

Metanephrine

added	measured	calculated	% recovery
0	103		
10	111	113	98
18	128	121	106
30	154	133	116
50	175	153	114
69	177	172	103
112	218	215	101
146	195	249	78
208	289	311	93
266	319	369	86
357	517	460	112
459	653	562	116
555	762	658	116
769	1,079	872	124
952	1,332	1,055	126

mean 106

Normetanephrine

added	measured	calculated	% recovery
0	63		
14	64	77	83
27	83	90	92
56	139	119	117
94	190	157	121
129	188	192	98
209	343	272	126
272	346	335	103
391	529	454	117
500	623	563	111
357	472	420	112
459	631	522	121
555	650	618	105
769	887	832	107
952	1,185	1,015	117

mean 109

9.5 Linearity

Linearity was measured by diluting spiked patient urine with water (all concentrations in ng/ml).

Metanephrine

dilution	measured	recalculated	% recovery
orig.	2,134		
3 + 1	1,578	2,104	99
2 + 1	1,293	1,940	91
1 + 1	1,136	2,272	106
2 + 3	887	2,218	104
1 + 2	672	2,016	94
1 + 3	429	1,716	80
1 + 5	302	1,812	85
1 + 7	217	1,736	81
1 + 10	169	1,859	87
1 + 15	103	1,648	77
1 + 20	85	1,785	85

mean recovery

90

Normetanephrine

dilution	measured	recalculated	% recovery
orig.	1,243		
3 + 1	879	1,172	94
2 + 1	839	1,258	101
1 + 1	633	1,266	102
2 + 3	490	1,225	99
1 + 2	402	1,206	97
1 + 3	281	1,124	90
1 + 5	206	1,236	99
1 + 7	123	984	79
1 + 10	100	1,100	88
1 + 15	63	1,008	81
1 + 20	52	1,092	88

mean recovery

93

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	110	11.3	10.3
2	40	414	40.9	9.9

Normetanephrine EIA

sample	n	mean	s.d.	c.v. (%)
1	40	63	4.5	7.2
2	40	1,323	64	4.8

Pipetting Scheme Sample Preparation (Metanephrine and Normetanephrine)

Hydrolysis

	Standards	Controls	Samples
Standard 1 - 6	10 µl		
Controls 1&2		10 µl	
Patient Urine			10 µl
Hydrochloric Acid	100 µl	100 µl	100 µl

incubate for 30 minutes at 90 °C *
Let tubes cool down to room temperature
Centrifuge shortly
Discard lids

Acylation

Acyl Buffer	25 µl	25 µl	25 µl
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prepare Acylation Reagent

Acylation Reagent	25 µl	25 µl	25 µl
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vortex, incubate for 15 minutes at room temperature

Dist. water	500 µl	500 µl	500 µl
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vortex

pipette each 2 x **20 µl** for the ELISA

* leave out for determination of free normetanephrine and free metanephrine

Pipetting Scheme ELISA Metanephrine/ Normetanphrine

		Standards	Controls	Samples
Standard 1-6	µl	20		
Controls 1&2	µl		20	
Patient Sample	µl			20
specific Antiserum	µl	50	50	50

incubate for 1 hour at room temperature on an orbital shaker.

4 x washing

Enzym Conjugate	µl	100	100	100
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incubate for 20 minutes at room temperature on an orbital shaker

4 x washing

Substrate	µl	100	100	100
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incubate for 15 to 25 minutes at room temperature on an orbital shaker

Stop Solution	µl	100	100	100
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Reading of absorbance at 450 nm