Normetanephrine ELISA Kit

Catalog Number KA1892

96 assays

Version: 03

Intended for research use only

www.abnova.com

Table of Contents

Introduction

Intended Use

Enzyme Immunoassay for the quantitative determination of free Normetanephrine in plasma.

Background

Metanephrine and Normetanephrine are the metabolites of the catecholamines Epinephrine and Norepinephrine, respectively. Cells derived from neuroendocrine tumors (e.g. pheochromcytoma) are known to produce catecholamines which are secreted episodically via vesicles into the blood stream. But beside this, a small portion of the catecholamines is metabolized inside the cells to the corresponding catecholamines metabolites – namely Metanephrine, Normetanephrine and 3-Methoxytyramine – which are secreted at low levels continuously into the blood stream.

Principle of the Assay

Normetanephrine (Normetadrenaline) is first extracted using an ion exchange matrix followed by an acylation process.
The subsequent competitive ELISA 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 antibody binding sites. After the system is in equilibrium, free antigen and free antigen-antibody 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 standards.
Note: The antibodies used in this test kit only recognise the biologically relevant L-forms of Metanephrines. Commercially available synthetic Normetanephrine or Metanephrine is always a mixture of the D- and L-forms. The ratio between both forms differs widely from lot to lot. This has important implications if synthetic Metanephrines are used to enrich native samples. As only about 50% of the synthetic Metanephrines - the L-portion - will be detected by use of this kit, spiked samples will be underestimated. Therefore native samples containing solely the L-form should be used.

General Information

Materials Supplied

List of component

Component	Description	Amount
Adhesive Foil	Ready to use. Adhesive foils in a resealable pouch.	4 slices
Wash Buffer Concentrate	Concentrated 50x. Buffer with a non-ionic detergent and physiological pH, light purple cap.	20 mL
Enzyme Conjugate	Ready to use, goat anti-rabbit immunoglobulins conjugated with peroxidase, red cap.	12 mL
Substrate	Ready to use, chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide, black cap.	12 mL
Stop Solution	Ready to use, 0.25 M sulfuric acid, light grey cap. Hazards identification:  H290 May be corrosive to metals.	12 mL
Normetanephrine Microtiter Strips	Ready to use, antigen precoated microwell plate in a resealable yellow pouch with desiccant.	96 (12x8) wells
Normetanephrine Antiserum	Ready to use, anti-normetanephrine rabbit antibody, yellow coloured, yellow cap.	6 mL
Adjustment Buffer	Ready to use. TRIS buffer, yellow cap.	10 mL
Assay Buffer	Ready to use, 25% organic solvent, orange cap.	30 mL
Acylation Concentrate	Concentrated, Acylation reagent in DMSO, dark gray cap. Hazards identification:  H302 Harmful if swallowed.	1.5 mL
Extraction Plate	Ready to use, precoated with ion-exchanger in a resealable pouch.	96 well plate
Cleaning Concentrate	Concentrated 25x. Buffer with sodium acetate, brown cap.	20 mL
Elution Buffer	Ready to use, 0.1 M Sodium hydroxide, dark purple colour, dark green cap	14 mL
Equalizing-Reagent	Ready to use, Human serum, negative for HIV I/II, HBsAg and HCV, white cap.	14 mL

Standards and Controls - Ready to use

Component	Colour/Cap	Concentration (pg/mL)	Concentration (pmol/L)	Amount
Standard A	white	0	0	4 mL
Standard B	light yellow	72	393	4 mL
Standard C	orange	240	1310	4 mL
Standard D	dark blue	720	3931	4 mL
Standard E	light grey	2400	13104	4 mL
Standard F	black	7200	39312	4 mL
Control 1	light green	Refer to QC report for expected value and acceptable range.		4 mL
Control 2	dark red	Refer to QC report for expected value and acceptable range.		4 mL

Conversion: Normetanephrine (pg/mL) x 5.46 = Normetanephrine (pmol/L)

Content: Acidic buffer with non-mercury stabilizer, spiked with defined quantity of normetanephrine.

Storage Instruction

Store the unopened reagents at 2-8°C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Once opened the reagents are stable for 1 month when stored at 2-8°C. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again.

Materials Required but Not Supplied

- ✓ Calibrated precision pipettes to dispense volumes between 20-350 µL; 3 mL
- ✓ Microtiter plate washing device (manual, semi-automated or automated)
- ✓ ELISA reader capable of reading absorbance at 450 nm and if possible 620-650 nm
- ✓ Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- ✓ Absorbent material (paper towel)
- ✓ Water (deionized, distilled, or ultra-pure)
- ✓ Vortex mixer

Precautions for Use

- Procedural cautions, guidelines and warnings

1. This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Reagents of this kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.

3. The principles of Good Laboratory Practice (GLP) have to be followed.
4. In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
7. The microplate contains snap-off strips. Unused wells must be stored at 2°C to 8°C in the sealed foil pouch with desiccant and used in the frame provided. Microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up.
8. Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
9. Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
10. Incubation times do influence the results. All wells should be handled in the same order and time intervals.
11. To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
12. A standard curve must be established for each run.
13. The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
14. Do not mix kit components with different lot numbers within a test and do not use reagents beyond expiry date as shown on the kit labels.
15. Avoid contact with Stop Solution containing 0.25 M H_2SO_4. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.
16. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them.
17. For information on hazardous substances included in the kit please refer to Material Safety Data Sheet (MSDS). The Material Safety Data Sheet for this product is made available directly on the website of the manufacturer or upon request.
18. Kit reagents must be regarded as hazardous waste and disposed according to national regulations.

- Limitations

  Any inappropriate handling of samples or modification of this test might influence the results.
- Interfering substance

  Samples containing precipitates or fibrin stands might cause inaccurate results.

  Hemolytic samples (up to 1 mg/mL hemoglobin), icteric samples (up to 25 mg/dL bilirubin) and lipemic samples (up to 1700 mg/dL triglycerides) have no influence on the assay results.
- Drug interferences

  Please refer to point “Sample preparation”.
- High-Dose-Hook effect: No hook effect was observed in this test.

Assay Protocol

Reagent Preparation

- ✓ Wash Buffer

  Dilute the 20 mL Wash Buffer Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 1000 mL.

  Storage: 1 month at 2-8°C.

- ✓ Cleaning Buffer

  Dilute the 20 mL Cleaning Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 500 mL.

  Storage: 1 month at 2-8°C.

- ✓ Acylation Solution

  As the Acylation Solution is only stable for a maximum of 3 minutes, it should not be prepared before starting the assay. Therefore its preparation is described in Assay Procedure, step 3.

  Discard after use!

- ✓ Normetanephrine Microtiter Strips

  In rare cases residues of the blocking and stabilizing reagent can be seen in the wells as small, white dots or lines. These residues do not influence the quality of the product.

- ✓ Extraction Plate

  In rare cases residues of the cation exchanger can be seen in the wells as small, black dots or lines. These residues do not influence the quality of the product.

Sample Preparation

- ✓ Sample collection and storage

  Medications like Serotonin-noradrenaline reuptake inhibitors, tryicyclic antidepressants, MAO inhibitors, antihypertensive drugs and L-DOPA can influence Metanephrine and Normetanephrine level. People who are taking such medication should consult with their doctor before specimen collection.

  Sympathomimetic agents, sport and smoking can also influence Metanephrine and Normetanephrine level. Alcohol and caffeinated drinks should be avoided the day before and including the day of sample collection.

- EDTA-or Heparin-Plasma

  Whole blood should be collected into centrifuge tubes (Monovette™ or Vacuette™) containing EDTA or heparin as anti-coagulant and centrifuged (according to manufacturer's instructions) immediately after collection.

  Haemolytic, icteric and lipemic samples should not be used for the assay.

  Storage: up to 6 hours at 2 - 8°C, for longer period (up to 6 month) at -20°C.

  Repeated freezing and thawing should be avoided.

Assay Procedure

The ELISA can be run using an overnight incubation without shaking (results within approx. 24 hours) or alternatively as a fast version with shortened antiserum incubation times with shaking (results within approx. 6 hours).

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the Extraction Plate and microwell plate (microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up).

Duplicate determinations are recommended.

The binding of the antibodies, the enzyme conjugates, and the activity of the enzyme used are temperature dependent, and the absorption values may vary if a thermostat is not used. The higher the temperature, the higher the absorption values will be. The absorption 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.

- Preparation of samples

  Extraction

1. Pipette 20 µL of standards, controls into the respective wells of the Extraction Plate.
2. Add 20 µL of Standard A to all wells containing plasma samples.
3. Add 200 µL of Equalizing Reagent to the wells with standards and controls.
4. Pipette 200 µL of plasma samples to the respective wells.
5. Incubate plate for 2 hours at RT (20 -25°C) on a shaker (approx. 600 rpm).
6. Empty plate and blot dry by tapping the inverted plate on absorbent material.
7. Pipette 250 µL of Assay Buffer into all wells. Incubate the plate for 5 min at RT (20 -25°C) on a shaker (approx. 600 rpm). Empty plate and blot dry by tapping the inverted plate on absorbent material.
8. Wash the plate 3 x by adding 350 µL of Cleaning Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
9. Pipette 100 µL of Elution Buffer into all wells.

   Please note: the colour changes caused by the elution buffer can vary between standards and samples.
10. Cover plate with adhesive foil. Incubate 15 min at RT (20 - 25°C) on a shaker (approx. 600 rpm).

    Remove the foil.

    Note: Do not decant the supernatant thereafter!

    The following volumes of the supernatant are needed for the subsequent ELISA:

    Normetanephrine 25 µL

- Normetanephrine ELISA

1. Pipette 25 µL of the Adjustment Buffer into all wells of the Normetanephrine Microtiter Strips.
2. Pipette 25 µL of the extracted standards, controls and samples into the respective wells.

3. Preparation of Acylation Solution:
   Pipette 80 µL Acylation Reagent Concentrate to 3 mL water (deionized, distilled, or ultra-pure) and mix thoroughly.
4. Pipette 25 µL of the freshly prepared Acylation Solution into all wells..
5. Incubate for 15 min at RT (20 -25°C) on a shaker (approx. 600 rpm).
6. Pipette 50 µL of the Normetanephrine Antiserum into all wells.
7. Cover the plate with Adhesive Foil, shake for 1 min at RT (20 -25°C) on a shaker and incubate for 15-20 h (overnight) at 2-8°C without shaking.
   Alternatively incubate for 2 h at RT (20 - 25°C) on a shaker (approx. 600 rpm).
8. Remove the foil. Discard or aspirate the contents of the wells. Wash the plate 4 x by adding 300 µL of Wash Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
9. Pipette 100 µL of the Enzyme Conjugate into all wells.
10. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).
11. Discard or aspirate the contents of the wells. Wash the plate 4 x by adding 300 µL of Wash Buffer, discarding the content and blotting dry each time by tapping the inverted plate on absorbent material.
12. Pipette 100 µL of the Substrate into all wells and incubate for 20-30 min at RT (20 -25°C) on a shaker (approx. 600 rpm). Avoid exposure to direct sunlight!
13. Add 100 µL of the Stop Solution to all wells and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm (if available a reference wavelength between 620 nm and 650 nm is recommended).

Data Analysis

Calculation of Results

Measuring range (overnight ELISA)	Normetanephrine
	22.8 to 7200 pg/mL

The standard 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).

Note: This assay is a competitive assay. This means: the OD-values are decreasing with increasing concentrations of the analyte. OD-values found below the standard curve correspond to high concentrations of the analyte in the sample and have to be reported as being positive.

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

Samples found with concentrations higher than the highest standard (Standard F) should be diluted accordingly with the included Equalizing Reagent and have to be re-assayed.

- Conversion

  Normetanephrine (pg/mL) x 5.46 = Normetanephrine (pmol/L)

- Expected reference values

  It is strongly recommended that each laboratory should determine its own reference values.

  The expected reference values indicated below are based on method comparison studies to LC-MS/MS [1] with blood samples taken in the sitting position.

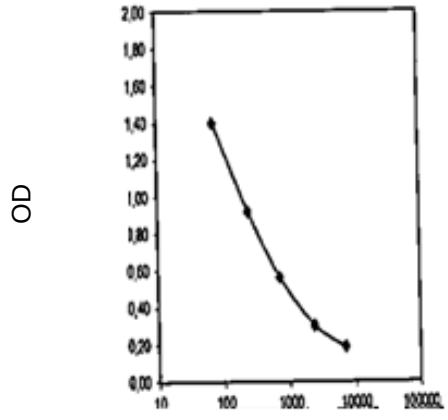
  Normetanephrine: < 196 pg/mL

- Quality Control

  The confidence limits of the kit controls are indicated in the QC-Report.

- Typical standard curve

  Example, do not use for calculation!



Performance Characteristics

- Analytical Sensitivity

	Normetanephrine
LOD (pg/mL)	17.9
LOQ (pg/mL)	22.8

- Analytical Specificity (Cross Reactivity)

Substance	Cross Reactivity (%)
	Normetanephrine
Derivatized Metanephrine	0.72
Derivatized Normetanephrine	100
3-Methoxytyramin	6.5*
Adrenaline	< 0.01
Noradrenaline	< 0.01
Dopamin	< 0.01
Vanillic mandelic acid	< 0.01
Homovanillic acid	< 0.01
L-DOPA	< 0.01
L-Tyrosin	< 0.01
Tyramine	< 0.01
Normetanephrine	< 0.01
Acetaminophen	< 0.01

*****Normetanephrine concentrations are not influenced by 3-Methoxytyramine in case of normal 3-Methoxytyramine concentrations. Only very high 3-Methoxytyramin concentrations found in rare cases of exclusively dopamine secreting tumours can cause false positive results.

- Precision

Intra-Assay			
	Sample	Mean (pg/mL)	CV (%)
Normetanephrine	1	149	9.5
	2	282	9.1
	3	734	8.2
	4	1956	10.5

Intra-Assay			
	Sample	Mean (pg/mL)	CV (%)
Normetanephrine	1	156	10.6
	2	287	5.0
	3	769	5.1
	4	1949	5.9

- Linearity

	Serial dilution up to	Mean (%)	Range (%)
Normetanephrine	1:64	98	92-102

- Recovery

	Mean (%)	Range (%)
Normetanephrine	109	105 – 114

- Method Comparison ELISA vs. LC-MS/MS[1]

Normetanephrine	y=0.93x +13; r^2=0.99; n=48

Resources

References

1. De Jong et al. Plasma free metanephrine measurement using automated online solid phase extraction HPLC-Tandem mass spectrometry. Clin Chem, 53(9): 1684-1693 (2007).
2. Eisenhofer et al. Laboratory evaluation of pheochromocytoma and paraganglioma. Clin Chem, 60:1486-1499 (2014).
3. Eisenhofer et al. Plasma metadrenalines: Do they provide useful information about sympatho-adrenal function and catecholamine metabolism? Clin Sci (Lond),88:533-542 (1995).
4. Berkel et al. Diagnosis of endocrine disease: Biochemical diagnosis of pheochromocytoma and paraganglioma. Eur J Endocrinol, 170: R109-R119.
5. Manz et al. Development of enantioselective immunoassays for free plasma metanephrines. Ann.N.Y.Acad.Sci., 1018:582-587 (2004).
6. De Jong et al. Dietary Influences on Plasma and Urinary Metanephrines: Implications for Diagnosis of Catecholamine-Producing Tumors. J Clin Endocrinol Metab, 94(8):2841-2849 (2009).
7. Deutschbein et al. Influences of Various Confounding Variable and Storage Conditions on Metanephrine and Normetanephrine Levels in Plasma. Clin Endocrinol, 72(2):153-160 (2010).
8. Eisenhofer et al. Biochemical Diagnosis of Pheochromocytoma: How to Distinguish True- from False-Positive Test Results. The Journal of Clinical Endocrinology & Metabolism, 88(6):2656-2666 (2003).

Plate Layout

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H