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Instructions for use

Nephrines (Plasma) RIA

^{125}I – Radioimmunoassay for the determination of free Metanephrine and free Normetanephrine in plasma.

For research use only, not for use in diagnostic procedures.

REF **IB88525**

Σ 2 x 100

+2 +8 °C

400 kBq

1. Introduction

1.1 Intended use and principle of the test

^{125}I – Radioimmunoassay for the determination of free Metanephrine and free Normetanephrine in plasma. For research use only, not for use in diagnostic procedures.

First, the plasma proteins are removed by precipitation. Then the metanephrine (metadrenaline) and normetanephrine (normetadrenaline) are acylated.
The assay procedure follows the basic principle of radioimmunoassay, involving competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of ^{125}I-labelled antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the antibody bound radioactivity is precipitated with a second antibody in the presence of polyethylene glycol. The precipitate is counted in a gamma counter. Determination of unknown samples is achieved by comparing their activity with a reference curve prepared with known standards.

⚠ *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, i.e. the L-portion, will be detected by use of this kit, these samples will be underestimated. Therefore only native samples should be used.*

1.2 Background

Metanephrine and normetanephrine are the metabolites of the catecholamines epinephrine and norepinephrine, respectively. Cells derived from neuroendocrine tumors (e.g. pheochromocytoma) 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.
Recent studies and publications have shown that the determination of these plasma free metanephrine and plasma free normetanephrine is the most accurate biochemical marker for the study of pheochromocytoma and follow-up of pheochromocytoma subjects.

2. Procedural cautions, guidelines, warnings and limitations

2.1 Precautions, 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) This assay was validated for a certain type of sample as indicated in *Intended Use* (please refer to Chapter 1). Any off-label use of this kit is in the responsibility of the user and the manufacturer cannot be held liable.
(3) 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.
(4) The principles of Good Laboratory Practice (GLP) have to be followed.
(5) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
(6) All kit reagents - with the exception of Precipitating Reagent – and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
(7) For the dilution or reconstitution purposes use deionized, distilled, or ultra-pure water.
(8) The radioactive material (^{125}Iodine, half life 60 days, emitting ionizing X-radiation with 28 keV and G-radiation with 35.5 keV) may be received, acquired, possessed and used only by physicians, laboratories or hospitals. In compliance with regulations, a copy of the customer's current radioisotope license must be on file with the supplier. Orders cannot be shipped until the license is received by the supplier (Radiation Protection Act of June 30, 1989).
(9) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
(10) 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.
(11) Incubation times do influence the results. All tubes should be handled in the same order and time intervals.
(12) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.

(13) A standard curve must be established for each run.
(14) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
(15) 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.
(16) 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 available directly on the website of the manufacturer or upon request.
(17) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.
(18) The results obtained with this test kit are for research use only, not for use in diagnostic procedures.
(19) Kit reagents must be regarded as hazardous waste and disposed according to national regulations.

2.2 Limitations

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

2.2.1 Interfering substances

Plasma

Samples containing precipitates or fibrin strands or which are haemolytic or lipemic might cause inaccurate results.

2.2.2 Drug interferences

There are no known substances (drugs) which ingestion interferes with the measurement of (nor-)metanephrine level in the sample.

2.2.3 High-Dose-Hook effect

No hook effect was observed in this test.

3. Storage and stability

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.

4. Materials

4.1 Content of the kit

BA D-0023 REAC-TUBES **Reaction Tubes** - Ready to use
Content: Reaction Tubes in a resealable pouch
Volume: 2 x 50 tubes

BA R-0030 PREC-REAG **Precipitating Reagent -** Ready to use
Content: Goat anti-rabbit serum in PEG phosphate buffer
Volume: 2 x 55 ml/vial, yellow cap

BA R-0028 EQUA-REAG **Equalizing Reagent** - Lyophilized
Content: Human serum, negative for HIV I/II, HBsAg and HCV
Volume: 2 vials, dark green cap

BA R-0120 ^{125}I ADR MN **^{125}I – Metanephrine** - Ready to use
Content: ^{125}I labeled Metanephrine, red coloured
Volume: 1 x 5.5 ml/vial, blue cap
Hazards identification:

Radioactive, activity < 200 kBq

BA R-0220 ^{125}I NAD NMN **^{125}I – Normetanephrine** - Ready to use
Content: ^{125}I labeled Normetanephrine, red coloured
Volume: 1 x 5.5 ml/vial, yellow cap
Hazards identification:

Radioactive, activity < 200 kBq

BA R-8110 AS MN **Metanephrine Antiserum** - Ready to use

Content: Rabbit anti- metanephrine antibody, blue coloured

Volume: 1 x 5.25 ml/vial, blue cap

BA R-8210 AS NMN **Normetanephrine Antiserum** - Ready to use

Content: Rabbit anti- normetanephrine antibody, yellow coloured

Volume: 1 x 5.25 ml/vial, yellow cap

Standards and **Controls** - Ready to use

Cat. no.	Component	Colour/Cap	Concentration pg/ml		Concentration pmol/l		Volume/ Vial
			MN	NMN	MN	NMN	
BA R-8301	STANDARD A	white	0	0	0	0	12 ml
BA R-8302	STANDARD B	light yellow	36	48	183	262	4 ml
BA R-8303	STANDARD C	orange	120	160	608	874	4 ml
BA R-8304	STANDARD D	dark blue	360	480	1 825	2 621	4 ml
BA R-8305	STANDARD E	light grey	1 200	1 600	6 084	8 736	4 ml
BA R-8306	STANDARD F	black	3 600	4 800	18 252	26 208	4 ml
BA R-8351	CONTROL 1	light green	Refer to QC report for expected value and acceptable range!				4 ml
BA R-8352	CONTROL 2	dark red					4 ml

Conversion: Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)
Normetanephrine (pg/ml) x 5.46 = Normetanephrine (pmol/l)

Content: Buffer with stabilizer and a precipitating reagent spiked with a defined quantity of metanephrine and normetanephrine

Hazards identification:

H302 Harmful if swallowed.

BA R-0050 ADJUST-BUFF **Adjustment Buffer** - Ready to use

Content: TRIS buffer

Volume: 3 x 4 ml/vial, green cap

BA R-8312 ACYL-CONC **Acylation Concentrate** – Concentrated

Content: Acylation reagent in DMSO

Volume: 1 x 1.5 ml/vial, dark grey cap

Hazards identification:

H302 Harmful if swallowed.

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

- Calibrated precision pipettes to dispense volumes between 25 - 500 µl; 3 ml; 10 ml
- Conical tubes and suitable rack
- Centrifuge capable of at least 3 000 x g
- Suitable device for aspirating or decanting the tubes
- For the alternative protocol with short incubation times, a shaker is needed (amplitude 3 mm; approx. 600 rpm)
- Gamma Counter
- Vortex mixer
- Water (deionized, distilled, or ultra-pure)

5. Sample collection and storage

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

6. Test procedure

Allow all reagents – with the exception of Precipitating Reagent - to reach room temperature and mix thoroughly by gentle inversion before use. Number the assay tubes accordingly. Duplicates are recommended.

⚠ *Pipetted liquids should not adhere to the wall of the RIA tubes. If necessary please centrifuge the tubes for 1 minute at 500 x g to spin down adhering liquids.*

6.1 Preparation of reagents

Equalizing Reagent
The Equalizing Reagent has to be reconstituted with 10 ml water (deionized, distilled, or ultra-pure).
Reconstituted Equalizing Reagent which is not used immediately has to be frozen for max 1 month at -20 °C (in aliquotes) and may be thawed only once.

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 the protocol in chapter 6.3, step 4 and chapter 6.4, step 4.
Discard after use!

6.2 Preparation of samples

⚠ *The precipitation procedure is the same for Metanephrine and Normetanephrine and has to be done only once.*

Precipitation

1.	Pipette **100 µl** of **standards** and **controls** and **500 µl** of **plasma samples** into the respective **Reaction Tubes.**
2.	Add **500 µl Equalizing Reagent** to all tubes containing **standards** and **controls**.
3.	Add **100 µl Standard A** to all tubes containing **plasma samples**.
4.	Mix the **Reaction Tubes** thoroughly (vortex) and centrifuge for **15 min** at **3 000 x g**.
⚠	Take **100 µl** of the clear supernatant for the **Metanephrine RIA** and **25 µl** of the clear supernatant for the **Normetanephrine RIA**.

6.3 Metanephrine RIA

⚠ *The use of conical tubes for the RIA is highly recommended!*

1.	Pipette **100 µl** of **water** (deionized, distilled, or ultra-pure) into the **tubes** for the **NSB**.
2.	Pipette **100 µl** of the **clear supernatants** of **standards, controls** and **samples** into the respective **tubes**.
3.	Pipette **50 µl** of **Adjustment Buffer** into **all tubes *(except totals)***.
4.	Preparation of **Acylation Solution**: Pipette **80 µl Acylation Reagent Concentrate** (BA R-8312) to **3 ml water** (deionized, distilled, or ultra-pure) and mix thoroughly.
5.	Pipette **25 µl** of the freshly prepared **Acylation Solution** into **all tubes *(except totals)***.
6.	Mix thoroughly (vortex) and incubate for **15 min** at **RT** (20 - 25 °C).
7.	Pipette **50 µl** of **Metanephrine Antiserum** into **all tubes *(except totals and NSB)***; mix thoroughly (vortex).
8.	Incubate for **1 h** at **RT** (20 - 25 °C).
9.	Pipette **50 µl** of the **^{125}I Metanephrine** into **all tubes** and mix thoroughly (vortex).
10.	Cover tubes. Incubate for **15 - 20 h** (overnight) at **2 - 8 °C**. ***Alternatively** incubate for **2 h** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).*
11.	Mix the chilled (2 - 8 °C) **Precipitating Reagent** thoroughly, pipette each **500 µl** into **all tubes *(except totals)***, and mix on a vortex.
12.	Incubate for **15 min** at **2 - 8 °C**.
13. ⚠	Centrifuge for **15 min** at **3 000 x g**, if possible in a refrigerated centrifuge. ***Continue without any delay with step 14.***
14.	**Decant** or aspirate the **supernatant <u>carefully</u> *(except totals)***. Blot the tubes dry and leave them upside down for 2 minutes.
15.	**Count** all tubes for **1 min** in a gamma counter.

6.4 Normetanephrine RIA

⚠ *The use of conical tubes for the RIA is highly recommended!*

1.	Pipette **25 µl** of **water** (deionized, distilled, or ultra-pure) into the **tubes** for the **NSB**.
2.	Pipette **25 µl** of the **clear supernatants** of **standards, controls** and **samples** into the respective **tubes**.
3.	Pipette **50 µl** of **Adjustment Buffer** into **all tubes *(except totals)***.
4.	Preparation of **Acylation Solution**: Pipette **80 µl Acylation Reagent Concentrate** (BA R-8312) to **3 ml water** (deionized, distilled, or ultra-pure) and mix thoroughly.
5.	Pipette **25 µl** of the freshly prepared **Acylation Solution** into **all tubes *(except totals)***.
6.	Mix thoroughly (vortex) and incubate for **15 min** at **RT** (20 - 25 °C).
7.	Pipette **50 µl** of **Normetanephrine Antiserum** into **all tubes *(except totals and NSB)***; mix thoroughly (vortex).
8.	Incubate for **1 h** at **RT** (20 - 25 °C).
9.	Pipette **50 µl** of the **^{125}I Normetanephrine** into **all tubes** and mix thoroughly (vortex).
10.	Cover tubes. Incubate for **15 - 20 h** (overnight) at **2 - 8 °C**. ***Alternatively** incubate for **2 h at RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).*
11.	Mix the chilled (2 - 8 °C) **Precipitating Reagent** thoroughly, pipette each **500 µl** into **all tubes *(except totals)***, and mix on a vortex.
12.	Incubate for **15 min** at **2 - 8 °C**.
13. ⚠	Centrifuge for **15 min** at **3 000 x g**, if possible in a refrigerated centrifuge. ***Continue without any delay with step 14.***
14.	**Decant** or aspirate the **supernatant <u>carefully</u> *(except totals)***. Blot the tubes dry and leave them upside down for 2 minutes.
15.	**Count** all tubes for **1 min** in a gamma counter.

7. Calculation of results

Measuring range	Metanephrine	Normetanephrine
	19 – 3 600 pg/ml	24 – 4 800 pg/ml

Subtract the mean cpm of the non-specific binding NSB from the mean cpm of standards, controls and samples.
The standard curve, from which the concentrations in the samples can be taken, is obtained by using the percentage of (B-NSB)/ (B_0-NSB) measured for the standards (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis).
Use non-linear regression for curve fitting (e.g. spline, 4- parameter, akima).

⚠ *This assay is a competitive assay. This means: the counts are decreasing with increasing concentrations of the analyte. Counts 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 Equalizing Reagent (BA R-0028) and have to be re-assayed.

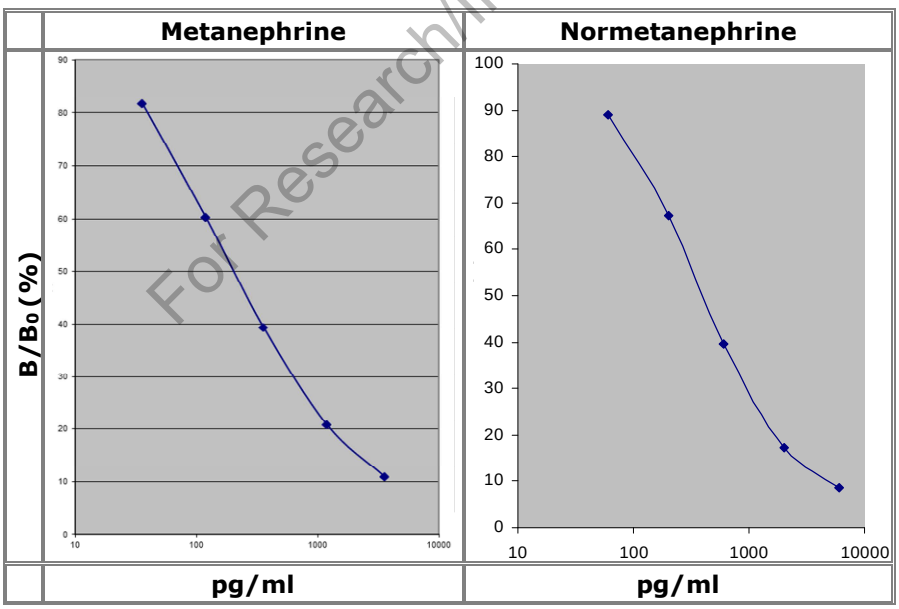
Conversion
Metanephrine (pg/ml) x 5.07 = Metanephrine (pmol/l)
Normetanephrine (pg/ml) x 5.46 = Normetanephrine (pmol/l)

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

Metanephrine	Normetanephrine
< 90 pg/ml	< 180 pg/ml

7.1 Quality control
It is recommended to use control samples according to national regulations. Use controls at both normal and elevated levels. The kit, or other commercially available, controls should fall within established confidence limits. The confidence limits of the kit controls are printed on the QC-Report.

7.2 Typical standard curves
⚠ *Examples, do not use for calculation!*



8. Assay characteristics

Analytical Sensitivity (Limit of Detection)		Metanephrine	Normetanephrine
	Plasma	19 pg/ml	24 pg/ml

	Substance	Cross Reactivity (%)	
		Metanephrine	Normetanephrine
Analytical Specificity (Cross Reactivity)	Derivatized Metanephrine	100	0.08
	Derivatized Normetanephrine	1.39	100
	3-Methoxytyramine	0.18	1.74
	Adrenaline	1.75	< 0.01
	Noradrenaline	0.03	< 0.01
	Dopamine	< 0.01	< 0.01
	Vanillic mandelic acid	< 0.01	< 0.01
	Homovanillic acid	< 0.01	< 0.01
	L-DOPA	< 0.01	< 0.01
	L-Tyrosin	< 0.01	< 0.01
	Tyramine	< 0.01	< 0.01

Precision							
Intra-Assay				**Inter-Assay**			
	Sample	Range (pg/ml)	CV (%)		Sample	Range (pg/ml)	CV (%)
Metanephrine	1	29.8 ± 2.9	9.8	Metanephrine	1	31.8 ± 5.3	17
	2	57.9 ± 6.4	11		2	67.8 ± 9.8	14
	3	523 ± 70	13		3	541 ± 70.4	13
Normetanephrine	1	234 ± 24	10	Normetanephrine	1	240 ± 20	8.3
	2	488 ± 43	8.7		2	518 ± 29	5.6
	3	1180 ± 93	7.9		3	1144 ± 71	6.2

Linearity			Range (pg/ml)	Serial dilution up to	Mean (%)
	Metanephrine	Plasma	25 - 2100	1: 65	91
	Normetanephrine	Plasma	40 - 6000	1: 129	100

Recovery			Mean (%)	Range (%)	% Recovery after spiking
	Metanephrine	Plasma	92	82 - 110	
	Normetanephrine	Plasma	107	95 - 119	

Method Comparison versus LC-MS/MS				
	Metanephrine	Plasma	LC-MS/MS = x – 13.2	r = 0.99; n = 50
	Normetanephrine	Plasma	LC-MS/MS = 1.2x – 29.6	r = 0.99; n = 50

9. References/Literature

(1) Niculescu et al. Plasma free metanephrine and normetanephrine levels are increased in Patients with chronic kidney disease. Endocr Pract, 20(2):139-44 (2014).

(2) Senn et al. Diagnostic Value of Biochemical Parameters in the Differential Diagnosis of an Adrenal Mass. Ann. N.Y. Acad. Sci, 1073:348-357 (2006)

(3) Manz et al. Development of enantioselective immunoassays for free plasma metanephrines. Ann.N.Y.Acad.Sci., 1018:582-587 (2004)

For orders, please contact:
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⚠ ***For updated literature or any other information please contact your local supplier.***

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code	I V D	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content	CE	CE labelled
	Caution	REF	Catalogue number	RUO	For research use only!