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Instructions for use

Nephrines (Plasma) ELISA

Enzyme Immunoassay for the determination of free
Metanephrine and free Normetanephrine in plasma.

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

REF

IB89183



2 x 96



1. Introduction

1.1 Intended use and principle of the test

Enzyme Immunoassay for the determination of free Metanephrine and free Normetanephrine in plasma. For research use only, not for use in diagnostic procedures.

Metanephrine (Metadrenaline) and Normetanephrine (Normetadrenaline) are 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 compete with the solid phase bound analytes 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.

Determination of unknown samples is achieved by comparing their absorbance 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 - 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.

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.

2. Procedural cautions, guidelines, warnings and limitations

2.1 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) 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 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 dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
- (8) 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.
- (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 wells 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) Avoid contact with Stop Solution containing 0.25 M H₂SO₄. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.
- (17) 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.
- (18) 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.
- (19) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.
- (20) The results obtained with this test kit are for research use only, not for use in diagnostic procedures.
- (21) 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

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

2.2.2 Drug interferences

Please refer to point "Sample collection and storage".

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. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again.

4. Materials

4.1 Content of the kit

BA D-0090	FOILS	Adhesive Foil - Ready to use
Content:	Adhesive Foils in a resealable pouch	
Volume:	2 x 4 foils	
BA E-0030	WASH-CONC 50x	Wash Buffer Concentrate - Concentrated 50x
Content:	Buffer with a non-ionic detergent and physiological pH	
Volume:	2 x 20 ml/vial, light purple cap	
BA E-0040	CONJUGATE	Enzyme Conjugate - Ready to use
Content:	Goat anti-rabbit immunoglobulins conjugated with peroxidase	
Volume:	2 x 12 ml/vial, red cap	
BA E-0055	SUBSTRATE	Substrate - Ready to use
Content:	Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide	
Volume:	2 x 12 ml/vial, black cap	
BA E-0080	STOP-SOLN	Stop Solution - Ready to use
Content:	0.25 M sulfuric acid	
Volume:	2 x 12 ml/vial, light grey cap	
BA E-0131	ADR MN	Metanephrine Microtiter Strips - Ready to use
Content:	1 x 96 well (12x8) antigen precoated microwell plate in a resealable blue pouch with desiccant	
BA E-0231	NAD MN	Normetanephrine Microtiter Strips - Ready to use
Content:	1 x 96 well (12x8) antigen precoated microwell plate in a resealable yellow pouch with desiccant	

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

Content: Rabbit anti- Metanephrine antibody, blue coloured

Volume: 1 x 6 ml/vial, blue cap

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

Content: Rabbit anti- Normetanephrine antibody, yellow coloured

Volume: 1 x 6 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	4 ml
BA R-8302	STANDARD B	light yellow	36	72	183	393	4 ml
BA R-8303	STANDARD C	orange	120	240	608	1 310	4 ml
BA R-8304	STANDARD D	dark blue	360	720	1 825	3 931	4 ml
BA R-8305	STANDARD E	light grey	1 200	2 400	6 084	13 104	4 ml
BA R-8306	STANDARD F	black	3 600	7 200	18 252	39 312	4 ml
BA R-8351	CONTROL 1	light green	Refer to QC-Report for expected value				4 ml
BA R-8352	CONTROL 2	dark red	and acceptable range!				4 ml

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

Normetanephrine (pg/ml) x 5.46 = Normetanephrine (pmol/l)

Content: Acidic buffer with non-mercury stabilizer, spiked with a defined quantity of Metanephrine and Normetanephrine

BA E-8327 ADJUST-BUFF **Adjustment Buffer** - Ready to use

Content: Tris-Buffer

Volume: 1 x 10 ml/vial, yellow cap

BA E-8313 ASSAY-BUFF **Assay Buffer** - Ready to use

Content: 25% organic solvent

Volume: 1 x 20ml/vial, orange 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.

BA R-8318 EXTRACT-PLATE 96 **Extraction Plate** - Ready to use

Content: 1 x 96 well plate, precoated with ion-exchanger in a resealable pouch

BA R-8325 CLEAN-CONC 25x **Cleaning Concentrate** - Concentrated 25x

Content: Buffer with sodium acetate

Volume: 1 x 20 ml/vial, brown cap

BA R-8326 ELUTION-BUFF **Elution Buffer** - Ready to use

Content: 0.1 M Sodium hydroxide, dark purple coloured

Volume: 1 x 14 ml/vial, dark green cap

BA R-8828 EQUA-REAG **Equalizing-Reagent** - Ready to use

Content: Human serum, negative for HIV I/II, HBsAg and HCV

Volume: 1 x 14 ml/vial, white cap

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

- Calibrated precision pipettes to dispense volumes between 20 - 350 µl; 3 ml; 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
- Centrifuge capable of at least 3.000 x g
- Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer

5. Sample collection and storage

Medications like Serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, MAO inhibitors, antihypertensive drugs and L-DOPA can influence Metanephrine and Normetanephrine levels. Sympathomimetic agents, sport and smoking can also influence Metanephrine and Normetanephrine levels.

Alcohol and caffeinated drinks should be avoided the day before and including the day of sample collection.

EDTA-, Citrate- or Heparin-Plasma

Whole blood should be collected into centrifuge tubes (Monovette™ or Vacuette™) containing EDTA or heparin as an 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

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 plates (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, 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.

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

6.1 Preparation of reagents

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 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 the protocol in chapter 6.3, step 3 and chapter 6.4, step 3.

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.

Extraction

The following extraction procedure can be run with 200 µl or 250 µl of plasma sample.

The procedure for 250 µl plasma is highlighted in grey and italicized and may be used in case higher supernatant volumes for pipetting to the subsequent ELISA are preferred.

The ELISA procedure itself is not affected by this alternative protocol.

1.	Pipette 20 µl of standards and controls into the respective wells of the Extraction Plate . <i>Alternatively pipette 25 µl of standards and controls into the respective wells of the Extraction Plate.</i>		
2.	Add 20 µl Standard A to all wells containing plasma samples . <i>Alternatively add 25 µl Standard A to all wells containing plasma samples.</i>		
3.	Add 200 µl of Equalizing Reagent to the wells with standards and controls . <i>Alternatively add 250 µl of Equalizing Reagent to the wells with standards and controls.</i>		
4.	Pipette 200 µl of plasma samples to the respective wells. <i>Alternatively pipette 250 µl of plasma samples to the respective wells.</i>		
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. <i>Alternatively pipette 125 µl of Elution Buffer into all wells.</i> <i>Please note: the colour changes caused by the elution buffer can vary between standards and samples.</i>		
10.	Cover plate with adhesive foil. Incubate 15 min at RT (20 - 25 °C) on a shaker (approx. 600 rpm). Remove the foil.		
	Do not decant the supernatant thereafter!		
	The following volumes of the supernatant are needed for the subsequent ELISA:		
	<table border="1"><tr><td>Metanephrine 50 µl</td><td>Normetanephrine 25 µl</td></tr></table>	Metanephrine 50 µl	Normetanephrine 25 µl
Metanephrine 50 µl	Normetanephrine 25 µl		

6.3 Metanephrine ELISA

1. Pipette 25 µl of Adjustment Buffer into all wells of the Metanephrine Microtiter Strips .
2. Pipette 50 µl of the extracted standards, controls and samples into the respective wells. <i>Please hold the Extraction Plate at a slight angle in order to facilitate this pipetting step.</i>
3. 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.
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 Metanephrine 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 content 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 content 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 each well 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).

6.4 Normetanephrine ELISA

1. Pipette 25 µl of 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. <i>Please hold the Extraction Plate at a slight angle in order to facilitate this pipetting step.</i>
3. 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.
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 content 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 content 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 each well 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).

7. Calculation of results

Measuring range	Metanephrine	Normetanephrine
		15.1 – 3 600 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).



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 Equalizing Reagent (BA R-8828) 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.

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.

Metanephrine	Normetanephrine
< 65 pg/ml	< 196 pg/ml

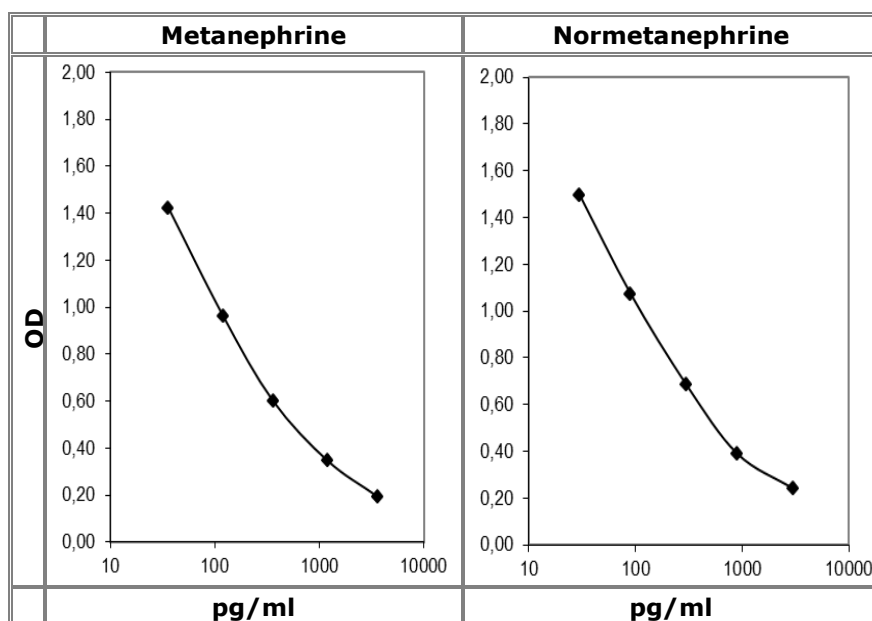
7.1 Quality control

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

7.2 Typical standard curves



Examples, do not use for calculation!



8. Assay characteristics (overnight ELISA)

Analytical Sensitivity		Metanephrine	Normetanephrine
	LOD (pg/ml)	14.9	17.9
	LOQ (pg/ml)	15.1	22.8

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)	
		Metanephrine	Normetanephrine
	Derivatized Metanephrine	100	0.72
	Derivatized Normetanephrine	0.05	100
	3-Methoxytyramin	< 0.01	6.5*
	Adrenaline	< 0.01	< 0.01
	Noradrenaline	< 0.01	< 0.01
	Dopamin	< 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
	Normetanephrine	< 0.01	< 0.01
	Acetaminophen	< 0.01	< 0.01

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

Precision							
Intra-Assay				Inter-Assay			
	Sample	Mean (pg/ml)	CV (%)		Sample	Mean (pg/ml)	CV (%)
Metanephrine	1	66.3	11.4	Metanephrine	1	67.8	17.6
	2	122	13.5		2	134	12.7
	3	308	10.6		3	319	11.0
	4	783	9.2		4	847	7.5
Normetanephrine	1	149	9.5	Normetanephrine	1	156	10.6
	2	282	9.1		2	287	5.0
	3	734	8.2		3	769	5.1
	4	1 956	10.5		4	1 949	5.9

Linearity	Serial Dilution up to		Mean (%)	Range (%)
	Metanephrine	1:64	107	101 - 124
	Normetanephrine	1:64	98	92 - 102

Recovery	Mean (%)		Range (%)
	Metanephrine	88	80 - 99
	Normetanephrine	109	105 - 114

Method Comparison: ELISA vs. LC-MS/MS(1)	Metanephrine	$y=0.91x + 1.8; r^2 = 0.96; n = 46$
	Normetanephrine	$y=0.93x + 13; r^2 = 0.99; n = 48$

9. References/Literature













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- (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)

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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		Batch code		For in-vitro diagnostic use only!
	Consult instructions for use		Content		CE labelled
	Caution		Catalogue number		For research use only!