DLD DIAGNOSTIKA GMBH

Instructions for Use

BI-CAT® ELISA

Enzyme Immunoassay for the
Quantitative Determination of
Adrenaline and Noradrenaline in Plasma, Urine Cell Culture and other biological Samples

RUO

For Research Use Only
Not for Use in Diagnostic Procedures

REF EA613/192

2 x (12 x 8)

2 – 8 °C

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Symbols

RUO	For Research Use Only		
CONT	Content		Expiry Date
LOT	Lot Number		Store at
	Manufactured by		Sufficient for ... determinations
REF	Catalogue Number		Consult Instructions for Use

The symbols of the components of the kit are described in section 4 Contents of the Kit.

1 Introduction and Principle of the Test

The BI-CAT® ELISA kit contains reagents and materials for the quantitative determination of adrenaline and noradrenaline in plasma, urine, cell culture samples, tissue homogenates and other samples.

Catecholamine is the name of a group of aromatic amines (noradrenaline, adrenaline, dopamine, and their derivatives) which act as hormones and neurotransmitters, respectively. Adrenaline and noradrenaline are formed from dopamine. They act on the cardiac musculature and the metabolism (adrenaline) as well as on the peripheral circulation (noradrenaline) and help the body to cope with acute and chronic stress.

This assay is for research use only! Not for use in diagnostic procedures!

Adrenaline and noradrenaline are extracted using a cis-diol-specific affinity gel and acylated to N-acylnoradrenaline and N-acyladrenaline and then converted enzymatically into N-acylnormetanephrine and N-acylmetanephrine.

The competitive BI-CAT® ELISA kit uses the microtiter plate format. Adrenaline and noradrenaline, respectively, are bound to the solid phase of the microtiter plate. Acylated catecholamine from the sample and solid phase bound catecholamine 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 catecholamine is detected by peroxidase-conjugated anti-rabbit IgG. The substrate TMB / peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase catecholamine is inversely proportional to the catecholamine concentration of the sample.

2 Precautions

- For research use only. For professional use only!
- Before carrying out the test, the instructions for use, as included in the kit, should be read completely and the content understood.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy; however these materials should be handled as potentially infectious.
- Individual components of different lots and test kits should not be interchanged. The expiry dates and storage conditions stated on the packaging and the labels of the individual components must be observed.
- When handling the reagents, controls and samples, the current laboratory safety guidelines and good laboratory practice should be observed.
- Wear protective clothing, disposable gloves, and eye protection while performing the test.
- Some components of this kit are containing hazardous reagents. These components are marked with the adequate hazard label. Further information is in section 4 and in the corresponding MSDS.
- Avoid any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.
- Avoid contact with individual reagents, these can cause irritation and chemical burns.
- Dispose of waste according to state and local environmental protection regulations.

3 Storage and Stability

The kit is shipped at ambient temperature and is subsequently stable until the stated expiry date when stored between 2 - 8 °C. Once opened, the kit is stable until the expiry date.

The shelf life of the ready-to-use reagents is indicated on the respective bottle label. The shelf life and storage conditions of the prepared reagents is stated under 6.1.

Bring all reagents to room temperature before use and refrigerate immediately after use.

4 Contents of the Kit

4.1 Reagents for sample preparation

Extraction Plate — **EX-PLATE** — 2 Plates

48 wells each, coated with boronate affinity gel

Extraction-Buffer — **EX-BUFF** — 2 vials

6 ml each, ready for use

HCl — **HCL** — 1 vial

13 ml, ready for use, 0.025 M HCl

Standards (1 - 6) — **CAL 1** - **CAL 6** — 6 vials

4 ml each, ready for use,
concentrations:

CAL		1	2	3	4	5	6
Adrenaline	(ng/ml)	0	0.15	0.5	1.5	5	15
	(nmol/l)	0	0.82	2.7	8.2	27.3	81.9
Noradrenaline	(ng/ml)	0	1	3	10	30	100
	(nmol/l)	0	5.9	17.7	59.1	177	591

Controls 1 & 2 — **CON 1** & **CON 2** — 2 vials

4 ml each, ready for use,
concentrations: see QC certificate

Acylation Reagent — **ACYL-REAG** — 1 vial

6 ml, ready for use, Contains DMSO and DMF
(please note that solvent reacts with many plastic materials including plastic trays; It does not react with normal pipette tips and with glass devices)


Warning


Danger

Acylation Buffer — **ACYL-BUFF** — 1 vial

20 ml, ready for use

Enzyme — **ENZYME** — 3 vials

2 ml each, lyoph., Catechol-O-Methyltransferase

Coenzyme — **COENZYME** — 1 vial

1.75 ml, ready for use, S-Adenosyl-L-Methionine

Enzyme Buffer 2 ml, ready for use	**ENZYME-BUFF**  Warning	1 vial
Sample Stabilizer 20 ml, ready for use	**STABILIZER**  Warning	1 vial

4.2 Reagents for ELISA

Adrenaline Antiserum 4 ml, ready for use, rabbit, color coded blue	**AS-AD**  Warning	1 vial
Noradrenaline Antiserum 6 ml, ready for use, rabbit, color coded yellow	**AS-NAD**  Warning	1 vial
MT-Strips 8 wells each, break apart, precoated with adrenaline, color coded blue	**STRIPS-AD**	12 strips
MT-Strips 8 wells each, break apart, precoated with noradrenaline, color coded yellow	**STRIPS-NAD**	12 strips
Enyzme Conjugate 12 ml each, ready for use, Anti-rabbit IgG-Peroxidase	**CONJ**  Warning	2 vials
Wash Buffer 20 ml each, concentrate, Dilute content with dist. water to 1000 ml total volume	**WASH**	2 vials
Substrate 12 ml each, TMB solution, ready for use	**SUB**	2 vials

Stop Solution **STOP** 2 vials
12 ml each, ready for use,
contains 0.3 M sulphuric acid

Adhesive foil **FOIL** 10 pieces
Ready for use

Additional materials and equipment required but not provided:

- Pipettes for pipetting 20, 25, 90, 500, 1000 µl
- Repeating dispenser for 30, 50, 100, 150, 250 µl, 1000 µl
- Horizontal shaker
- Microplate washing device or multichannel pipette
- Microplate photometer (450 nm)
- Distilled water
- Paper towels, pipette tips, timer

5 Sample Collection and Storage

5.1 Plasma

EDTA plasma samples are required for the assay.

Haemolytic, icteric and especially lipemic samples should not be used for the assay, otherwise false low values will be obtained with such samples.

Immediately after collection, the plasma samples should be centrifuged (preferably at 2 - 8 °C) and frozen. The samples are stable up to 1 week at -20 °C. Storage at -80 °C should be preferred, if possible.

To improve the stability, each sample should be enriched with the Sample Stabilizer STABILIZER before freezing (add 20% of the sample volume), e.g.:

Sample volume	+ Stabilizer volume	= Total volume
20 µl	4 µl	24 µl
50 µl	10 µl	60 µl
100 µl	20 µl	120 µl
200 µl	40 µl	240 µl
300 µl	60 µl	360 µl
500 µl	100 µl	600 µl

Note: Multiply the determined adrenaline and noradrenaline concentration of the sample by the factor 1.2 (see section 8).

We recommend using EGTA/Gluthation (GSH) blood collection tubes. The addition of the Sample Stabilizer is then not necessary.

Before use, mix and centrifuge (10 minutes, 2,000 xg) stabilized plasma samples.

In general, mix and centrifuge (10 minutes, 2,000 xg) plasma samples after thawing.

5.2 Urine Samples

Spontaneous urine and collected urine can be used.
The urine samples must be acidified to stabilize them.

Spontaneous urine:
Acidify urine with hydrochloric acid, e.g. 15 ml urine + 100 µl 6 mol/l HCL.

Collected urine:

The total volume of urine excreted during a 24-hour period should be collected in a single bottle containing 10 – 15 ml of 6 N hydrochloric acid as preservative. Avoid exposure to direct sun light. Determine the total volume and take an aliquot for the measurement. With suspected kidney disorders, the creatinine concentration should be determined in addition.

Urine samples can be stored at -20 °C for at least 6 months.

Before use, mix and centrifuge (e.g. 3 minutes, 3,000 xg) urine.

Further, dilute urine samples 1:5 with dist. water.

5.3 Cell culture samples and various biological samples

The storage and stability of such samples depends on the type of sample and how they are collected. Therefore, only a general procedure for collection and storage can be outlined:

It is recommended to freeze the samples immediately after collection. The samples should be stable at -20 °C for up to 1 week. Storage at -80 °C should be preferred, if possible.

To improve the stability, each sample should be enriched with the Sample Stabilizer STABILIZER before freezing (10% of the sample volume), e.g.:

Sample volume	+ Stabilizer volume	= Total volume
20 µl	2 µl	22 µl
50 µl	5 µl	55 µl
100 µl	10 µl	110 µl
200 µl	20 µl	220 µl
300 µl	30 µl	330 µl
500 µl	50 µl	550 µl

Note: Multiply the determined adrenaline and noradrenaline concentration of the sample by the factor 1.1 (see section 8).

Acidified samples, which have a pH value of 5 or less must not be enriched with the Sample Stabilizer and must be frozen immediately after collection.

5.4 Tissue samples

Tissue samples can be homogenized in Sample Stabilizer STABILIZER diluted 1:20 (e.g. 19 ml dist. water + 1 ml Sample Stabilizer).

For all sample types, the following basic principles should be followed:

- Avoid excess of acid. This might exceed the buffer capacity of the extraction buffer EX-BUFF. After adding the extraction buffer (see 6.2) a pH value of 7 or above is mandatory, otherwise binding of adrenaline and noradrenaline to the boronate affinity gel will not take place. If the pH value is below 7 it is necessary to repeatedly add 50 µl of Extraction Buffer EX-BUFF until the pH value is at or above 7. The pH can be determined by pipetting 0.5 µl on pH indicator paper.
  Acidified samples, which have a pH value of 5 or less must not be enriched with the Sample Stabilizer and have to be frozen immediately after collection.
- Avoid substances in the samples with a cis-diol-structure (boric acid, sorbitol, mannitol, etc.). These substances reduce the recovery of extraction and lead to false low values.

6 Preparation of Reagents and Samples

6.1 Preparation of Reagents

Allow reagents to reach room temperature.

6.1.1 Wash Buffer

Dilute the content (20 ml, 50x) of the bottle WASH with distilled water to a total volume of 1000 ml, mix briefly.

Store the diluted wash buffer at 2 – 8 °C for a maximum period of 4 weeks. Should the kit be used in several runs, then prepare only the required amount of wash buffer for each run.

6.1.2 Enzyme Mix

NOTE: The enzyme mix must be prepared freshly prior to the assay (no longer than 20 – 30 minutes in advance). After use, discard the reagent.

Reconstitute the content of one vial ENZYME with 2 ml distilled water. Add 0.5 ml COENZYME and 0.5 ml ENZYME-BUFF (total volume: 3.0 ml) and mix thoroughly.

The two additional bottles of ENZYME allow a second and a third run of the test. If the whole kit is to be used in one run, two bottles of enzyme mix need to be prepared and pooled (in e.g. a 10 ml test tube).

All other reagents are ready for use.

6.2 Preparation of Samples

Preparation of the standards, controls and the samples is identical for adrenaline and noradrenaline and is therefore performed only once, in one extraction plate.
Allow reagents and samples to reach room temperature.
Determinations in duplicates are recommended.
Each 20 µl of Standards, Controls and Urine Samples (diluted 1:5) are extracted. Each 300 µl of plasma samples are extracted. Of other sample types 1 µl up to 300 µl can be extracted.

1. Pipette each 20 µl Standard 1 – 6 CAL 1 - CAL 6 , 20 µl Control 1 & 2 CON 1 & CON 2 and 20 µl Urine Sample (diluted 1:5 with dist. water) into the respective wells of the extraction plate EX-PLATE. Add 250 µl of distilled water to these wells to correct for volume.
   Pipette 300 µl Plasma Sample into the respective wells (no volume correction required).
   Pipette 1 µl up to 300 µl of other sample types into respective wells and correct volume of each well to 300 µl with distilled water, e.g. 100 µl sample + 200 µl dist. water.
2. Add 100 µl Extraction Buffer EX-BUFF into each well.
3. Incubate 60 minutes at room temperature on an orbital shaker at medium speed.
4. Decant the plate and remove residual liquid by firmly tapping the inverted plate on a paper towel.
5. Pipette 1 ml Wash Buffer (see 6.1.1) into each well and incubate for 5 minutes at room temperature on an orbital shaker at low speed.
6. Decant the plate and remove residual liquid by firmly tapping the inverted plate on a paper towel.
7. Pipette 150 µl Acylation Buffer ACYL-BUFF into each well.
8. Add 50 µl Acylation Reagent ACYL-REAG into each well and continue with next step, immediately.
   (please note that solvent reacts with many plastic materials including plastic trays; it does not react with normal pipette tips and with glass devices)

9. Incubate the plate for 20 minutes at room temperature on an orbital shaker at medium speed.
10. Decant the plate and remove residual liquid by firmly tapping the inverted plate on a paper towel.
11. Pipette 1 ml Wash Buffer (see 6.1.1) into each well and incubate for 5 minutes at room temperature on an orbital shaker at low speed.
12. Decant the plate and remove residual liquid by firmly tapping the inverted plate on a paper towel.
13. Repeat the wash steps 11. and 12 twice.
14. Pipette 100 µl HCl HCL into each well to elute adrenaline and noradrenaline.
15. Incubate the plate sealed with adhesive foil FOIL for 20 minutes at room temperature on an orbital shaker at medium speed.
    Caution: Do not decant the supernatant.
16. Add 50 µl of freshly prepared Enzyme Mix (s. 6.1.2) into each well. A color change to red occurs and indicates which wells have already been pipetted.
17. Incubate the plate sealed with adhesive foil FOIL for 30 minutes at room temperature on an orbital shaker at medium speed.
    Caution: Do not decant the supernatant.

    Take 90 µl of each supernatant for the Adrenaline ELISA and 25 µl each for the Noradrenaline ELISA.

7 Test Procedure ELISA

7.1 Adrenaline ELISA

1. Transfer each 90 µl prepared Standards, Controls and Samples into the STRIPS – AD wells (color coded blue). Thereby, slightly tilt EX-PLATE to facilitate pipetting the supernatants.
2. Add 30 µl Adrenaline-Antiserum AS-AD (color coded blue) into each well.
3. Cover the plate with adhesive foil FOIL, shake for 10 seconds and incubate for 15 - 20 hours (overnight) at 2 – 8 °C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl Wash Buffer (see 6.1.1). Remove residual liquid by firmly tapping the inverted plate on clean absorbent paper towel. Repeat the washing procedure 3 times.
5. Pipette 100 µl Enzyme Conjugate CONJ into each well.
6. Incubate for 30 minutes at room temperature on an orbital shaker at medium speed.
7. Washing: Repeat wash step 4.
8. Pipette 100 µl Substrate SUB into each well.
9. Shake plate for 10 seconds on orbital shaker, then place on table, cover with a box and incubate for 30 ± 5 minutes at room temperature (20 - 25 °C) without shaking.
10. Add 100 µl Stop Solution STOP into each well. Shake plate for 10 seconds on orbital shaker.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

7.2 Noradrenaline ELISA

1. Transfer each 25 µl prepared Standards, Controls and Samples into the STRIPS – NAD wells (color coded yellow). Thereby, slightly tilt EX-PLATE to facilitate pipetting the supernatants.
2. Add 50 µl Noradrenaline-Antiserum AS-NAD (color coded yellow) into each well.
3. Cover the plate with adhesive foil FOIL, shake for 10 seconds and incubate for 15 - 20 hours (overnight) at 2 – 8 °C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl Wash Buffer (see 6.1.1). Remove residual liquid by firmly tapping the inverted plate on clean absorbent paper towel. Repeat the washing procedure 3 times.
5. Pipette 100 µl Enzyme Conjugate CONJ into each well.
6. Incubate for 30 minutes at room temperature on an orbital shaker at medium speed.
7. Washing: Repeat wash step 4.
8. Pipette 100 µl Substrate SUB into each well.
9. Shake plate for 10 seconds on orbital shaker, then place on table, cover with a box and incubate for 30 ± 5 minutes at room temperature (20 - 25 °C) without shaking.
10. Add 100 µl Stop Solution STOP into each well. Shake plate for 10 seconds on orbital shaker.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

8 Calculation of Results

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.

A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

Correcting read concentrations:

Controls: The concentrations can be read off the standard curve directly without any further conversion.

Urine Samples: The read concentrations must be multiplied by 5, due to the 1:5 dilution of the samples.

Plasma Samples: The read concentrations must be divided by 15, due to the use of 300 µl plasma sample in relation to 20 µl per standard. Further, when 20% Sample Stabilizer (see 5.1) was added to the samples, the read concentration must be multiplied by 1.2.

Other Sample Types: As sample volumes of 1 µl up to 300 µl can be used, the read concentrations must be divided by a volume factor calculated as following

$$\text{Volume factor} = \frac{\text{Sample volume used for extraction [µl]}}{\text{20 µl (Volume of Standards)}}$$

Example:

200 µl Sample were used for extraction and the read concentration is 0.6 ng/ml.

Volume factor is 200 µl / 20 µl = 10

Concentration of sample is 0.6 ng/ml / 10 = 0.060 ng/ml = 60 pg/ml

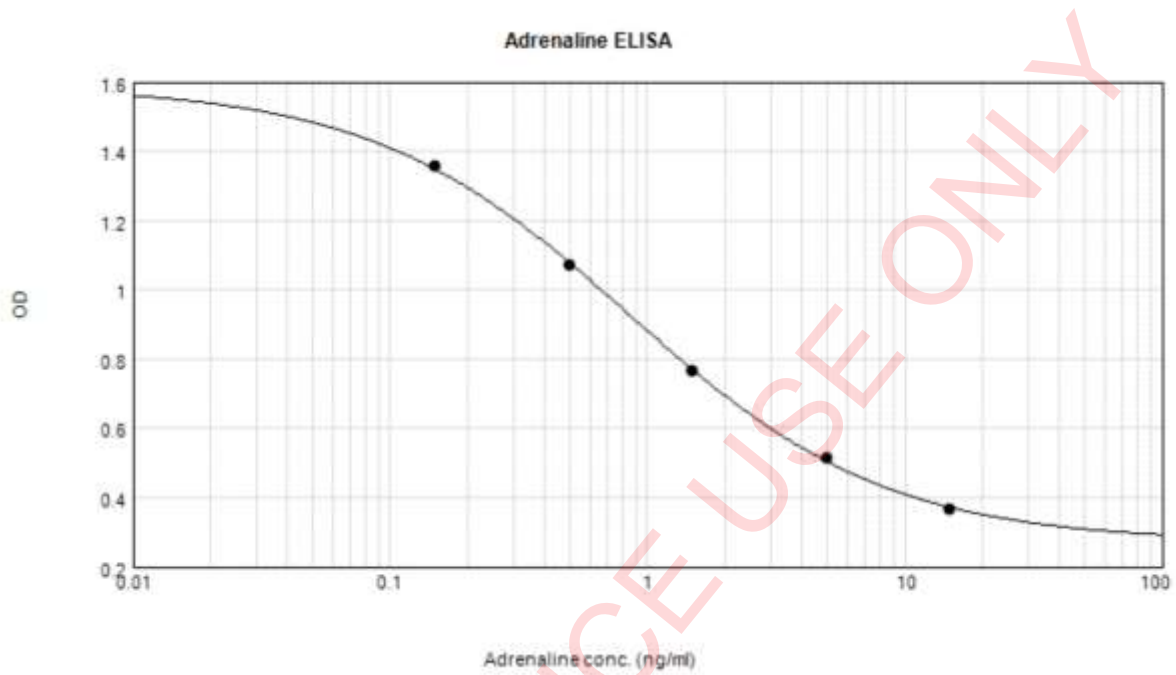
Further, when 10% Sample Stabilizer (see 5.3) was added to the samples, the concentration must be multiplied by 1.1.

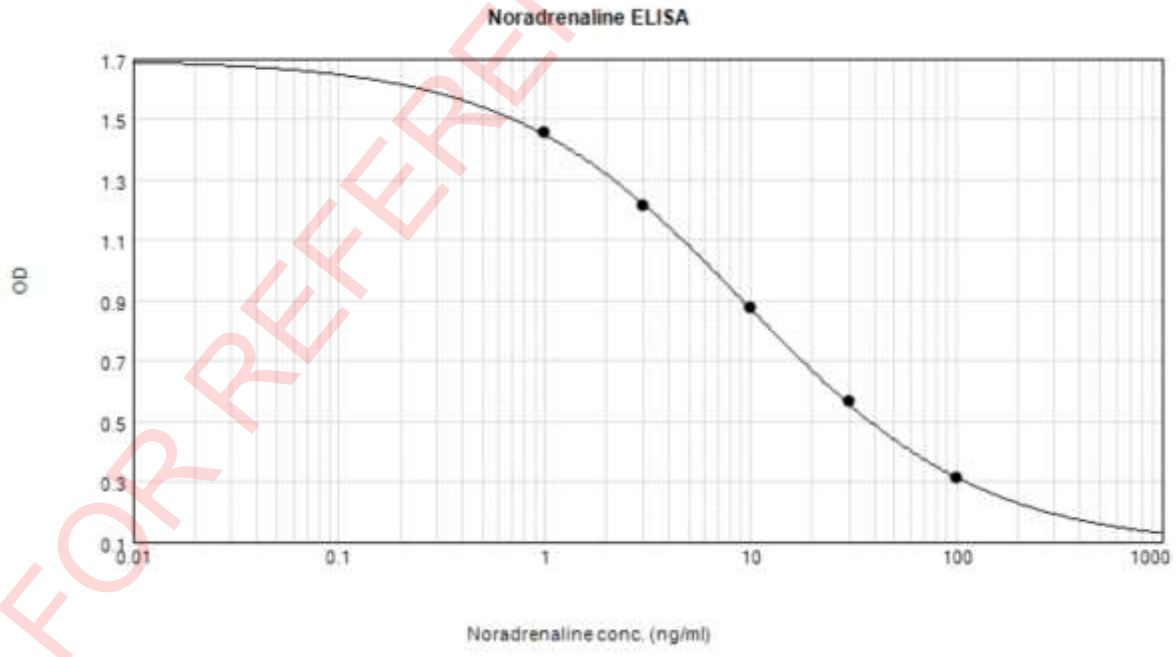
Adrenaline: 1 ng/ml = 5.46 nmol/l

Noradrenaline: 1 ng/ml = 5.91 nmol/l

Quality Control: Test results are valid only if the kit controls are within the ranges specified on the QC Certificate. Otherwise, the test should be repeated.

Typical Example (do not use for calculation of results):





9 Assay Characteristics

9.1 Assay Data

9.1.1 Sensitivity

Adrenaline

Matrix	LLOD	Calculation
Urine	0.27 ng/ml	ODCal1 – 2xSD
EDTA-Plasma	3.6 pg/ml	ODCal1 – 2xSD

Noradrenaline

Matrix	LLOD	Calculation
Urine	0.44 ng/ml	ODCal1 - 2xSD
EDTA-Plasma	5.9 pg/ml	ODCal1 - 2xSD

9.1.2 Specificity (Cross Reactivity)

Components	Cross Reactivity (%) Adrenaline-Ab	Cross Reactivity (%) Noradrenaline-Ab
Adrenaline	100	< 0.0052
Noradrenaline	0.085	100
Dopamine	0.00041	0.68
Metanephrine	0.0013	< 0.002
Normetanephrine	< 0.0001	0.0040
3-Methoxytyramine	< 0.0001	< 0.002
L-Dopa	< 0.0001	< 0.002
Tyramine	< 0.0001	< 0.002
Tyrosine	< 0.00005	< 0.0006
Homovanillic acid	< 0.00001	< 0.0002
Vanillic mandelic acid	< 0.00001	< 0.0002

9.1.3 Recovery after spiking

Adrenaline

Matrix	Range (ng/ml)	Mean (%)	Range (%)
Urine	2.1 – 25.8	105	102 – 110
EDTA-Plasma	0.008 – 0.237	92	87 – 98

Noradrenaline

Matrix	Range (ng/ml)	Mean (%)	Range (%)
Urine	15.8 – 92.3	103	95 – 109
EDTA-Plasma	0.19 – 2.10	101	89 – 111

9.1.4 Linearity

Adrenaline

Matrix	Range (ng/ml)	Max. dilution	Mean (%)	Range (%)
Urine	2.6 – 23.1	1:10 (dist. water)	103	93 - 112
EDTA-Plasma	0.04 – 0.245	1:7 (dist. water)	107	103 - 113

Noradrenaline

Matrix	Range (ng/ml)	Max. dilution	Mean (%)	Range (%)
Urine	6.4 – 105.6	1:15 (dist. water)	97	88 – 106
EDTA-Plasma	0.47 – 3.07	1:7 (dist. water)	100	89 - 107

9.1.5 Reproducibility

Adrenaline

Matrix	Range (ng/ml)	Intra-Assay-CV
Urine	3.9 – 14.3	8.5 – 6.4 %
EDTA-Plasma	0.044 – 0.148	11.3 – 7.1 %

Noradrenaline

Matrix	Range (ng/ml)	Intra-Assay-CV
Urine	27.5 – 74.3	11.0 – 4.7 %
EDTA-Plasma	0.247 – 1.009	13.1 – 8.0 %

9.1.6 Method Comparison

Adrenaline

Matrix	Method	Correlation
Urine	HPLC	Y = 1.12 x HPLC - 3.7; R = 0.982; N = 32

Noradrenaline

Matrix	Method	Correlation
Urine	HPLC	Y = 1.03 x HPLC + 4.9; R = 0.994; N = 32

9.2 Calibration

The calibration of the BI-CAT® ELISA is carried out by weighing the pure substance. The correctness of the method was determined by comparison of method (see 9.1.6).

9.3 Limitations of Method

Samples measured above the highest standard must be diluted with the appropriate medium as stated in 9.1.4 and re-assayed. The values of diluted samples must be multiplied by the appropriate dilution factor.

9.4 Interferences

Hemolytic, lipemic and icteric specimens should not be used in the BI-CAT® ELISA.

Do not use non-acidified urine collection.

10 Changes to declare

Version _11 (valid as of lot AN129): Changes are highlighted in grey.

Version _10 (valid as of lot A122): Hazard symbol was removed from POD Conjugate. Further changes are highlighted in grey.

Pipetting Scheme - Sample Preparation

Adrenaline and Noradrenaline in one plate

		Standards	Controls	Urine	Plasma	Other
EX-PLATE:						
CAL 1 – 6	µl	20				
CON 1 & 2	µl		20			
Urine (1:5 dil.)	µl			20		
Plasma	µl				300	
Other Types	µl					1 to 300
Dist. Water	µl	250	250	250		299 to 0
EX-BUFF	µl	100	100	100	100	100

Shake 60 minutes at RT
Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1	1	1

Incubate 5 minutes at RT (slow shaking)
Decant plate and remove residual liquid

ACYL-BUFF	µl	150	150	150	150	150
ACYL-REAG	µl	50	50	50	50	50

Immediately, shake 20 minutes at RT
Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1	1	1

Incubate 5 minutes at RT (slow shaking)
Decant plate and remove residual liquid
Repeat washing steps 2x
Decant plate and remove residual liquid

HCL	µl	100	100	100	100	100

Cover with FOIL; shake 20 minutes at RT

Enzyme Mix (fresh)	µl	50	50	50	50	50

Cover with FOIL; shake 30 minutes at RT

Caution: Do not decant the supernatant thereafter

Use 90 µl for the Adrenaline ELISA and 25 µl for the Noradrenaline ELISA

Pipetting Scheme – ELISA

Adrenaline and Noradrenaline in two separate plates

		Adrenaline (blue) STRIPS-AD			**Noradrenaline (yellow)** STRIPS-NAD		
		Acylated			Acylated		
		Stand.	Contr.	Samples	Stand.	Contr.	Samples
Transfer from EX-PLATE into STRIPS	µl	90	90	90	25	25	25
AS-AD (blue)	µl	30	30	30			
AS-NAD (yellow)	µl				50	50	50

Cover plates with FOIL
Shake for 10 seconds
Incubate for 15 – 20 hours (overnight) at 2 – 8 °C
Decant plate and remove residual liquid

Wash Buffer	µl	250	250	250	250	250	250

Decant plate and remove residual liquid
Repeat washing steps 3x
Decant plate and remove residual liquid

CONJ	µl	100	100	100	100	100	100

Shake 30 minutes at RT
Decant plate and remove residual liquid

Wash Buffer	µl	250	250	250	250	250	250

Decant plate and remove residual liquid
Repeat washing steps 3x
Decant plate and remove residual liquid

SUB	µl	100	100	100	100	100	100

Shake for 10 seconds
Incubate 30 ± 5 minutes at RT, covered with a box, without shaking

STOP	µl	100	100	100	100	100	100

Shake for 10 seconds
Read absorbance at 450 nm (ref. 570 – 650 nm)