



Instructions for Use

Noradrenaline / Dopamine High Sensitive ELISA

(high sensitivity and small sample volume)

Enzyme Immunoassay


for the Quantitative Determination of


Noradrenaline / Dopamine

For research use only

REF EA631/192

 2 x 96

 2 – 8 °C

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Contents

1. Introduction and Principle of the Test	Page	3
2. Precautions	Page	4
3. Storage and Stability	Page	4
4. Contents of the Kit	Page	4
5. Sample Collection and Storage	Page	7
6. Preparation of Reagents and Samples	Page	9
7. Test Procedure ELISA	Page	12
Noradrenaline ELISA	Page	12
Dopamine ELISA	Page	13
8. Calculation of Results	Page	14
9. Assay Characteristics	Page	16
Pipetting Scheme Sample Preparation	Page	19
Pipetting Scheme ELISA	Page	20

1. Introduction and Principle of the Test

Catecholamine is the name of a group of aromatic amines (noradrenaline, adrenaline, dopamine, and their derivatives) which act as hormones and neurotransmitter, 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.

An increased production of catecholamines can be found with tumours of the chromaffine system (pheochromocytoma, neuroblastoma, ganglioneuroma). An increased or decreased concentration of the catecholamines can also be found with hypertension, degenerative cardiac diseases, schizophrenia and manic-depressive psychosis.

The assay kit provides materials for the quantitative measurement of noradrenaline and dopamine in low concentrated samples and for small sample volumes. Noradrenaline and dopamine are extracted using a cis-diol-specific affinity gel and acylated to N-acylnoradrenaline and N-acyldopamine and then converted enzymatically into N-acylnormetanephrine and N-acyl-3-methoxytyramine.

The competitive Noradrenaline / Dopamine - Sensitive - ELISA kit uses the microtitre plate format. Noradrenaline and dopamine, 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 anti-rabbit IgG / peroxidase. 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 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.
- Some components of this kit are containing hazardous reagents. These components are marked with the adequate hazard 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 Sample Preparation:

4.1	Extraction Plate 48 wells coated with boronate affinity gel	EX-PLATE	2 plates
4.2.	Extraction-Buffer 6 ml, ready for use colour coded purple	EX-BUFF	2 vials
4.3	HCl 21 ml, ready for use 0.025 M HCl colour coded yellow orange	HCL	1 vial

4.4 **Standards (A - F)** **CAL A - F** 6 vials
 Each 4 ml, ready for use

Concentrations:

Standard	A	B	C	D	E	F
Noradrenaline (ng/ml)	0	0.15	0.5	1.5	5	25
Noradrenaline (nmol/l)	0	0.89	3.0	8.9	29.6	148
Dopamine (ng/ml)	0	0.3	1.25	5	20	100
Dopamine (nmol/l)	0	2.0	8.2	32.7	131	653

4.5 **Control 1 & 2** **CON 1 & 2** 2 vials
 Each 4 ml, ready for use
 Concentrations: see q.c. certificate

4.6 **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).

4.7 **Acylation Buffer** **ACYL-BUFF** 1 vial
 20 ml, ready for use
 colour coded purple

4.8 **Enzyme** **ENZYME** 3 vials
 each 2 ml, lyophilized
 Catechol-O-methyltransferase

4.9 **Coenzyme** **COENZYME** 1 vial
 1 ml, ready for use
 S-adenosyl-L-methionine

4.10 **Enzyme Buffer** **ENZYME-BUFF** 1 vial
 3.5 ml, ready for use

4.11 **Enzyme Plate** **ENZYME-PLATE** 1 piece
 96 wells, ready for use

4.12 **Sample Stabilizer** **STABILIZER** 1 vial
 20 ml, ready for use

Reagents for ELISA:

4.13	Noradrenaline-Antiserum 2.5 ml, ready for use, rabbit colour coded yellow	AS-NAD	1 vial
4.14	Dopamine-Antiserum 2.5 ml, ready for use, rabbit colour coded green	AS-DOP	1 vial
4.15	MT-Strips 8 wells each, break apart, precoated with: noradrenaline (12 strips), colour coded yellow dopamine (12 strips), colour coded green	STRIPS-NAD STRIPS-DOP	2 x 12 strips
4.16	POD Conjugate Each 12 ml, ready for use, Anti-rabbit IgG-POD conjugate / peroxidase	CONJ	2 vials
4.17	Wash Buffer 20 ml, concentrate Dilute content with dist. water to 500 ml total volume	WASH	3 vials
4.18	Substrate 12 ml TMB solution, ready for use	SUB	2 vials
4.19	Stop Solution 12 ml, ready for use Contains 0.3 M sulphuric acid	STOP	2 vials
4.20	Adhesive Foil Ready for use	FOIL	10 pieces

Additional materials and equipment required but not provided:

- Pipettes (20, 50, 100, 150, 175, 280 µl)
- Repeating dispenser for 20, 25, 50, 100, 150 and 1 ml
- Orbital shaker
- Multichannel pipette or Microplate washing device
- Microplate photometer
- Distilled water
- Heating cabinet with 37 °C (optional)

5. Sample Collection and Storage

Plasma

EDTA plasma samples are required for the assay. Physical and psychical stress usually causes a high increase of the catecholamine concentration. Therefore, it is recommended to let the patient rest for 20 to 30 minutes after the venipuncture and before collecting the blood sample.

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

Immediately after collection the plasma samples should be centrifuged (preferable at 2 - 8 °C) and freezed. The samples are stable up to 1 week at -20 °C.

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

Sample volume	Stabilizer volume
20 µl	4 µl
50 µl	10 µl
100 µl	20 µl
200 µl	40 µl
300 µl	60 µl
500 µl	100 µl

Urine, Cell culture samples and various biological samples:

The stability of such samples depends on the sample type and the way of collection. Therefore, a general procedure for collection and storage is not possible. However, it is recommended to freeze the samples immediately after collection. The samples should be stable at -20 °C for up to 1 week.

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

Sample volume	Stabilizer volume
20 µl	2 µl
50 µl	5 µl
100 µl	10 µl
200 µl	20 µl
300 µl	30 µl
500 µl	50 µl

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

Tissue samples

Tissue samples can be homogenized in 0.01 N HCl in the presence of 0.15 mM EDTA and 4 mM sodium metabisulfite.

The following basic principles should be followed:

- Avoid excess of acid. This might exceed the buffer capacity of the extraction buffer. After adding the extraction buffer a pH value of 7 or above is mandatory. If the pH value is below 7 it is necessary to repeatedly add 50 µl of Extraction Buffer until the pH value is at or above 7.
Acidified samples, which have a pH value of 5 or less must not enriched with the Sample Stabilizer.
- Avoid substances in the samples with a cis-diol-structure (boric acid, Sorbitol, mannitol, etc.). These substances reduce the recovery of extraction.

6. Preparation of Reagents and Samples

6.1. Preparation of Reagents

6.1.1 Wash Buffer

Dilute the contents of the bottle with distilled water to a total volume of 500 ml.

For further use the diluted wash buffer must be stored at 2 - 8 °C for a maximum period of 4 weeks.

6.1.2 Enzyme Mix

NOTE: The enzyme mix has to be prepared freshly prior to the assay (not longer than 10 - 15 minutes in advance). After use the reagent has to be discarded.

Reconstitute the content of one vial labelled **ENZYME** with 2 ml distilled water.

Add 0.3 ml **COENZYME** and 0.7 ml **ENZYME-BUFF** , mix thoroughly (total volume: 3 ml).

The two additional bottles of **ENZYME** are allowing a second and a third run of the test. If the whole kit is to be used in one run it is sufficient to prepare one vial of enzyme mix.

All other reagents are ready for use.

6.2 Preparation of Samples

The preparation of the standards, the controls and the samples is identical for noradrenaline and dopamine and has to be done only once.

Allow reagents to reach room temperature.

Determinations in duplicates are recommended.

Each 20 μ l of Standards and Control 1 & 2 are extracted.

Each 1 μ l - 300 μ l of samples are extracted (alternatively: > 300 μ l up to 500 μ l).

1. Pipette each 20 μ l Standard A - F, 20 μ l Control 1 & 2 and each 1 μ l - 300 μ l Sample into the respective wells of the extraction plate.
Correction for volume:
Pipette 280 μ l of distilled water into the wells of the standards and controls (final volume: 300 μ l).
Pipette as much distilled water into the wells of the samples to obtain a final volume of 300 μ l, e.g. 100 μ l sample + 200 μ l distilled water.
For sample volumes above 300 μ l up to 500 μ l: fill up all wells to a final volume of 500 μ l.
Within a run the final volume has to be the same in all wells (300 μ l or 500 μ l).
2. Pipette each 100 μ l Extraction Buffer into all wells.
3. Cover the plate with adhesive foil and incubate for 60 minutes at room temperature on an orbital shaker (high shaking rate).
4. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
5. Pipette each 1 ml prepared Wash Buffer into all wells and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking rate).
6. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.

7. Pipette each 150 μ l Acylation Buffer into all wells.
8. Pipette each 50 μ l Acylation Reagent into all wells and continue with step 9. 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 for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
10. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
11. Pipette each 1 ml prepared Wash Buffer into all wells and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking rate).
12. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
13. Repeat the wash steps 11. and 12.
14. Pipette each 175 μ l HCl (0.025 M) for elution into all wells.
15. Cover the plate with adhesive foil and incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
Caution: Do not decant the supernatant thereafter.
16. Transfer 150 μ l from the extraction plate into the respective wells of the enzyme plate.
17. Pipette each 25 μ l of freshly prepared Enzyme Mix (s. 6.1.2) into all wells of the enzyme plate. Colour changes to red.
18. Cover the plate with adhesive foil and incubate for 1 minute at room temperature on an orbital shaker (medium shaking rate).
19. Incubate the plate for 90 minutes at 37°C without shaking.
(Alternatively: 120 minutes at room temperature (20 - 25°C) on an orbital shaker at medium shaking rate).
Caution: Do not decant the supernatant thereafter.

Transfer each 50 μ l of the supernatant for the noradrenaline assay and 100 μ l for the dopamine assay.

7. Test Procedure ELISA

The ELISA plates for Noradrenaline and Dopamine are different and must not be interchanged.

7.1 Noradrenaline ELISA

1. Pipette each 50 µl prepared Standards, Controls and Samples into the respective wells (colour coded yellow).
2. Pipette each 20 µl Noradrenaline-Antiserum (colour coded yellow) into all wells.
3. Cover the plate with adhesive foil, shake briefly and incubate for 15 – 20 hours (overnight) at 2 - 6 °C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl prepared Wash Buffer. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.
5. Pipette each 100 µl POD-Conjugate into all wells.
6. Incubate for 60 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Washing: Repeat wash step 4.
8. Pipette each 100 µl Substrate into all wells.
9. Incubate for 35 to 45 minutes at room temperature (20 - 25°C) on an orbital shaker (medium shaking rate). Avoid exposure to direct sun light.
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 within 15 minutes.

7.2 Dopamine ELISA

1. Pipette each 100 μ l prepared Standards, Controls and Samples into the respective wells (colour coded green).
2. Pipette each 20 μ l Dopamine-Antiserum (colour coded green) into all wells).
3. Cover the plate with adhesive foil, shake briefly and incubate for 15 – 20 hours (overnight) at 2 - 6 °C.
4. Discard or aspirate the contents of the wells and wash thoroughly with each 250 μ l prepared Wash Buffer. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.
5. Pipette each 100 μ l POD-Conjugate into all wells.
6. Incubate for 60 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Washing: Repeat wash step 4.
8. Pipette each 100 μ l Substrate into all wells.
9. Incubate for 35 to 45 minutes at room temperature (20 - 25°C) on an orbital shaker (medium shaking rate). Avoid exposure to direct sun light.
10. Pipette 100 μ l Stop Solution into all wells.
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

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.

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

The concentration of the controls can be read off the standard curve directly without any further conversion.

The read concentrations of the samples have to be divided by a correction factor due to the use of 1 μ l - 300 μ l sample volume in relation to 20 μ l standard.

$$\text{Correction factor} = \frac{\text{Sample volume for extraction } (\mu\text{l})}{20 \mu\text{l (Standard volume)}}$$

Example:

300 μ l sample was extracted and the concentration read off from the standard curve is 0.6 ng/ml.

Correction factor = $300 \mu\text{l} / 20 \mu\text{l} = 15$

Concentration of the sample = $0.6 \text{ ng/ml} / 15 = 0.040 \text{ ng/ml} = 40 \text{ pg/ml}$

Conversion to pmol/l:

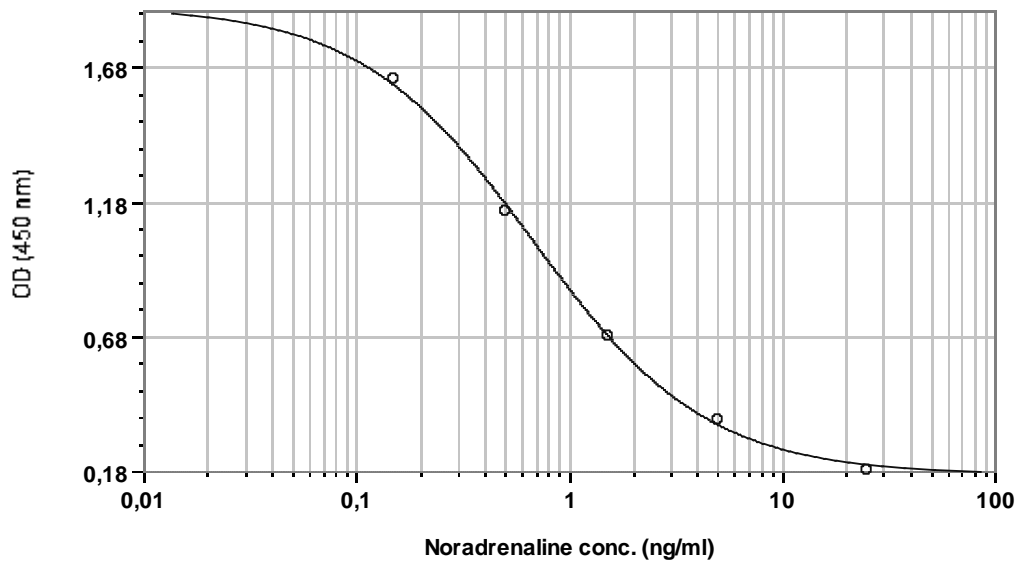
Noradrenaline: $1 \text{ pg} / \text{ml} = 5,91 \text{ pmol} / \text{l}$

Dopamine: $1 \text{ pg} / \text{ml} = 6,53 \text{ pmol} / \text{l}$

Typical Examples

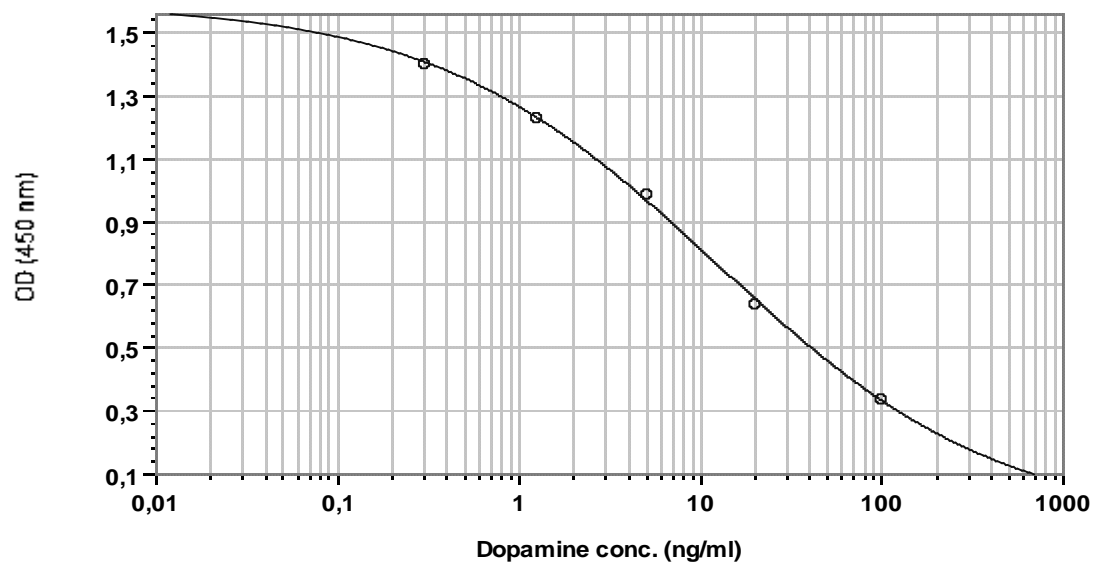
Below typical examples of standard curves with the Noradrenaline / Dopamine – Sensitive - ELISA are shown:

Noradrenaline ELISA



$y = ((A - D) / (1 + (x/C)^B)) + D$: A B C D R²
 ○ Std (Standards: Concentration vs MeanValue) 1,91 1,064 0,666 0,17 0,999

Dopamine ELISA



$y = ((A - D) / (1 + (x/C)^B)) + D$: A B C D R²
 ○ Std (Standards: Concentration vs MeanValue) 1,594 0,557 12,159 -0,059 0,999

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.

	Noradrenaline	Dopamine
Plasma	< 600 pg/ml	< 100 pg/ml

9.2 Sensitivity

The lower limit of detection was determined by taking the 2fold standard deviation of the absorbance of the Zero Reference and reading the corresponding value from the standard curve. The sensitivity depends on the sample volume and can be calculated with the corresponding correction factor (see 8. Calculation of Results)

	Noradrenaline	Dopamine
Sensitivity:	<u>36 pg/ml (213 pmol/l)</u> Correction factor	<u>73 pg/ml (477 pmol/l)</u> Correction factor
Example for 300 µl Sample (Correction factor 15):	$\frac{36 \text{ pg/ml}}{15} = 2.4 \text{ pg/ml}$ (14 pmol/l)	$\frac{73 \text{ pg/ml}}{15} = 4.9 \text{ pg/ml}$ (32 pmol/l)

9.3 Specificity (Cross Reactivity)

Structural related components were tested for possible interference with the antisera against noradrenaline and dopamine used in the ELISA method.

Substance	Cross Reactivity (%) Noradrenaline-Ab	Cross Reactivity (%) Dopamine-Ab
Noradrenaline	100	0.23
Dopamine	0.092	100
Adrenaline	< 0.012	< 0.020
Metanephrine	< 0.012	< 0.020
Normetanephrine	0.16	< 0.020
3-Methoxytyramine	< 0.012	0.28
L-Dopa	< 0.005	< 0.01
Tyramine	< 0.005	0.011
Tyrosine	< 0.005	< 0.01
Homovanillic acid	< 0.005	< 0.01
Vanilmandelic acid	< 0.005	< 0.01

9.4 Recovery

Increasing amounts of noradrenaline and dopamine were added to an EDTA plasma sample and to a cell culture medium (RPMI 1640). Each spiked sample was assayed. The analytical recovery was estimated at different concentrations by using the theoretically expected and the actually measured values.

Concentrations in pg/ml

Noradrenaline

EDTA-Plasma				cell culture medium			
added	measured	expected	% recovery	added	measured	expected	% recovery
0,0	10.5			0.0	4.1		
8,8	16.5	19.3	86	8.8	14.5	13.0	112
22,4	30.3	32.9	92	22.4	25.9	26.5	98
34,4	44.1	44.8	98	34.4	37.7	38.5	98
67,2	69.8	77.6	90	67.2	71.6	71.3	100
88,2	93.6	98.7	95	88.2	93.1	92.4	101
151,5	145.2	162.0	90	151.5	149.3	155.6	96
223,9	218.3	234.3	93	223.9	220.7	228.0	97
294,1	255.7	304.6	84	294.1	307.8	298.3	103
757,6	696.2	768.0	91	757.6	672.5	761.7	88
		mean:	91			mean:	99

Dopamine

EDTA-Plasma				cell culture medium			
added	measured	expected	% recovery	added	measured	expected	% recovery
0.0	57.7			0.0	54.9		
56.0	117.9	113.7	104	56.0	77.9	110.9	70
114.5	164.7	172.2	96	114.5	181.8	169.4	107
151.5	177.9	209.2	85	151.5	200.1	206.4	97
223.9	284.9	281.6	101	223.9	274.8	278.8	99
307.7	295.7	365.4	81	307.7	273.3	362.6	75
606.1	491.5	663.8	74	606.1	548.4	661.0	83
895.5	965.5	953.3	101	895.5	1090.2	950.5	115
1176.5	884.5	1234.2	72	1176.5	1098.2	1231.4	89
1538.5	1218.5	1596.2	76	1538.5	1459.1	1593.4	92
		mean:	88			mean:	92

9.5 Reproducibility

Intra-Assay

The reproducibility of the ELISA method was investigated by determining the intra-assay-coefficients of variation (cv) by repeated measurements for EDTA-Plasma and cell culture medium (RPMI 1640) with different concentrations.

Concentrations in pg/ml

Noradrenaline

sample	n	mean value	sd	cv (%)
EDTA-Plasma	16	192	12.0	6.3
Cell culture medium	24	296	14.9	5.0

Dopamine

sample	n	mean value	sd	cv (%)
EDTA-Plasma	16	260	28.4	11.0
Cell culture medium	24	557	54.0	9.7

Pipetting Scheme Sample Preparation (Noradrenaline and Dopamine)

		Standards	Controls	Samples
Standard A - F	μl	20		
Control 1 & 2	μl		20	
Sample	μl			1 - 300
Dist. Water	μl	280	280	Fill up to 300
Extraction Buffer	μl	100	100	100

Cover the plate with adhesive foil; Shake for 60 minutes at room temperature

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1
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Shake for 5 minutes at room temperature (slow shaking rate)

Decant plate and remove residual liquid

Acylation Buffer	μl	150	150	150
Acylation Reagent	μl	50	50	50

Immediately: Shake for 20 minutes at room temperature

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1
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Shake for 5 minutes at room temperature (slow shaking rate)

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1
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Shake for 5 minutes at room temperature (slow shaking rate)

Decant plate and remove residual liquid

HCl	μl	175	175	175
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Cover the plate with adhesive foil; Shake for 20 min at room temperature

Caution: Do not decant the supernatant thereafter

Transfer to Enzyme Plate	μl	150	150	150
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Enzyme Mix (fresh)	μl	25	25	25
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Cover the plate with adhesive foil; Shake for 1 minute at room temperature

Incubate for 90 minutes at 37°C

Caution: Do not decant the supernatant thereafter

For the ELISA take each 50 μl for Noradrenaline
 100 μl for Dopamine

Pipetting Scheme ELISA (Noradrenaline and Dopamine)

		Standards	Controls	Samples
Noradrenaline (yellow)				
Standard A - F	μl	50		
Controls 1 & 2	μl		50	
Samples	μl			50
Noradrenaline Antiserum	μl	20	20	20

Dopamine (green)				
Standard A - F	μl	100		
Controls 1 & 2	μl		100	
Samples	μl			100
Dopamine Antiserum	μl	20	20	20

Cover the plates with adhesive foil and shake briefly
Incubate for 15 – 20 hours (overnight) at 2 - 6 °C

4 x washing

POD-Conjugate	μl	100	100	100
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Shake for 60 minutes at room temperature

4 x washing

Substrate	μl	100	100	100
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Shake for 35 – 45 minutes at room temperature

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