DIAGNOSTIKA GMBH

Instructions for Use

Adrenaline ELISA

Enzyme Immunoassay

for the Quantitative Determination of

Adrenaline

in Plasma and Urine

CE

IVD

REF EA604/96

Σ 12 x 8

+2 +8 °C 2 – 8 °C

DLD Gesellschaft für Diagnostika und medizinische Geräte mbH
Adlerhorst 15 • 22459 Hamburg • Germany • Tel: +49 40 5558710 • Fax: +49 40 55587111
Internet: http://www.dld-diagnostika.de • E-Mail: contact@dld-diagnostika.de

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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, ganglio-neuroma). An increased or decreased concentration of the catechol-amines can also be found with hypertension, degenerative cardiac diseases, schizophrenia and manic-depressive psychosis.

The assay kit provides materials for the quantitative measurement of adrenaline in plasma and urine. Adrenaline is extracted using a cis-diol-specific affinity gel and acylated to N-acyladrenaline and then converted enzymatically into N-acylmetanephrine.

The competitive Adrenaline - ELISA kit uses the microtitre plate format. Adrenaline is bound to the solid phase of the microtiter plate. Acylated adrenaline from the sample and solid phase bound adrenaline 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 adrenaline 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 adrenaline is inversely proportional to the adrenaline 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**	1 vial
4.3	**HCl** 21 ml, ready for use 0.025 M HCl colour coded yellow orange	**HCL**	1 vial

4.4 **Standards (1 - 7)** **CAL 1 - 7** 7 vials
Each 4 ml, ready for use

Concentrations:

Standard	1	2	3	4	5	6	7
Adrenaline (ng/ml)	0	0.5	1.5	5	15	50	250
Adrenaline (nmol/l)	0	2.7	8.2	27.3	81.9	273	1,365

For only determination of urine samples: Standard 2 is not required.

For only determination of plasma samples: Standard 7 is not required.

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 1.7 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

Reagents for ELISA:

4.11 **Adrenaline-Antiserum** **AS-AD** 1 vial
2.5 ml, ready for use, rabbit
colour coded blue

4.12 **MT-Strips** 12 strips **STRIPS-AD**
8 wells each, break apart, precoated with:
derivatized adrenaline (12 strips), colour coded blue

4.13 **POD Conjugate** **CONJ** 1 vial
Each 12 ml, ready for use,
Anti-rabbit IgG-POD conjugate/ peroxidase

4.14 **Wash Buffer** **WASH** 2 vials
20 ml, concentrate
Dilute content with dist. water to 500 ml total volume

4.15 **Substrate** **SUB** 1 vial
12 ml TMB solution, ready for use

4.16 **Stop Solution** **STOP** 1 vial
12 ml, ready for use
Contains 0.3 M sulphuric acid

4.17 **Adhesive Foil** **FOIL** 10 pieces
Ready for use

Additional materials and equipment required but not provided:

- Pipettes (10, 15, 50, 125, 300, 700 µl)
- Repeating dispenser for 10, 20, 50, 100, 150, 200, 250 µl und 1 ml
- Horizontal shaker
- Microplate washing device
- Microplate photometer
- Distilled water

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.
The plasma samples can be stored at 2 - 8 °C up to 6 hours. For a longer period (up to 1 week) the samples should be stored at -20 °C.

Urine

The total volume of urine excreted during a 24-hours period should be collected and mixed in a single bottle containing 10 - 15 ml of 6 M hydrochloric acid as preservative. Avoid exposure to direct sun light. Determine the total volume and take an aliquot for the measurement. For patients with suspected kidney disorders the creatinine concentration should be tested, too. Urine samples can be stored at -20 °C for at least 6 months.

6. Preparation of Reagents and Samples

6.1. Preparation of Reagents

Wash Buffer

Dilute the content 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.

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 1.7 ml distilled water.
Add 0.3 ml COENZYME and 0.7 ml ENZYME-BUFF (total volume: 2.7 ml) and mix thoroughly.

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 bottle of enzyme mix.

6.2. Preparation of Samples

Allow reagents and samples to reach room temperature.
Determinations in duplicates are recommended.
Each 20 µl of Standards, Control 1 & 2 and urine samples are extracted.
Each 300 µl of plasma samples are extracted.

1. Pipette each 20 µl Standard 1 - 7, 20 µl Control 1 & 2 and each 20 µl Urine Sample into the respective wells of the extraction plate. Add 250 µl of distilled water to these wells to correct for volume.

   Pipette each 300 µl Plasma Sample into the respective wells (no volume correction required).

2. Pipette each 50 µl Extraction Buffer into all wells.

3. Incubate 60 minutes at room temperature on an orbital shaker (400 - 600 r/min).

4. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.

5. Pipette each 1 ml Wash Buffer into all wells and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking).

6. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.

7. Pipette each 150 µl Acylation Buffer into all wells.

8. Pipette each 50 µl 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 the plate for 20 minutes at room temperature on an orbital shaker (400 - 600 r/min).

10. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.

11. Pipette each 1 ml Wash Buffer into all wells and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking).

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 200 µl HCl (0.025 M) into all wells.

15. Incubate the plate with adhesive foil for 20 minutes at room temperature on an orbital shaker (400 - 600 r/min).

    Caution: Do <u>not</u> decant the supernatant thereafter.

    Take each 100 µl of the supernatant for the adrenaline assay.

7. Test Procedure ELISA

Allow reagents to reach room temperature. Duplicates are recommended.

1. Pipette each 20 µl of freshly prepared Enzyme Mix into all wells (colour coded blue).
2. Pipette each 100 µl prepared Standards, Controls and Patient Samples into the respective wells (colour coded blue).
3. Incubate the plate with adhesive foil for 30 minutes at room temperature (20 – 25 ºC) on an orbital shaker (400 - 600 r/min).
4. Pipette each 20 µl Adrenaline-Antiserum (colour coded blue) into all wells.
5. Cover the plate with adhesive foil, shake for 10 seconds and incubate for 12 – 20 hours (overnight) at 2-8 °C.
6. Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl Wash Buffer. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 4 times.
7. Pipette each 100 µl POD-Conjugate into all wells.
8. Incubate for 30 minutes at room temperature on an orbital shaker (400 - 600 r/min).
9. Washing: Repeat wash step 6.
10. Pipette each 100 µl Substrate into all wells.
11. Incubate 25 to 35 minutes at room temperature (20 – 25 ºC) on an orbital shaker (400 - 600 r/min).
12. Pipette 100 µl Stop Solution into all wells.
13. 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.

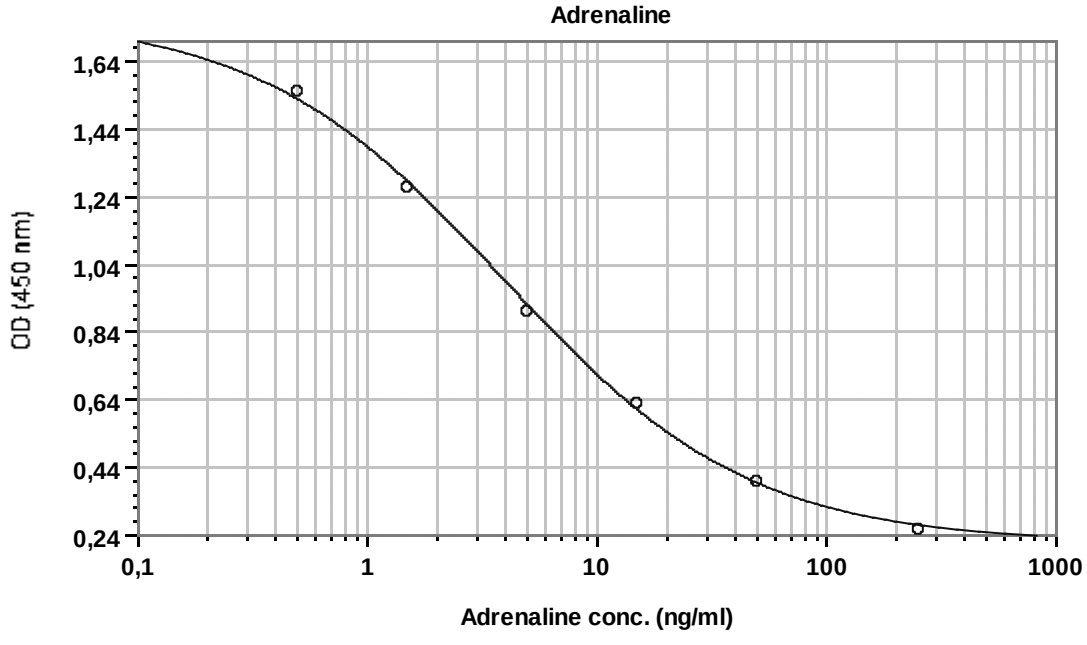
A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

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

The read concentrations of adrenaline in **plasma samples** have to be **divided by 15** due to the use of 300 µl plasma sample in relation to 20 µl standard.

Typical Example

Below is listed a typical example of a standard curve with the Adrenaline ELISA:



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
○	Std (Standards: Concentration vs MeanValue)	1,774	0,811	3,897	0,22	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.

	Adrenaline
Urine	< 20 µg/day
Plasma	< 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.

	Adrenaline
Sensitivity (Urine):	0.08 ng/ml
Sensitivity (Plasma):	5 pg/ml

9.3. Specificity (Cross Reactivity)

Structural related components were tested for possible interference with the antiserum against adrenaline used in the ELISA method.

Components	Cross Reactivity (%) Adrenaline-Ab
Adrenaline	100
Noradrenaline	0.030
Dopamine	< 0.01
Metanephrine	0.48
Normetanephrine	< 0.01
3-Methoxytyramine	< 0.01
L-Dopa	< 0.002
Tyramine	< 0.002
Tyrosine	< 0.002
Homovanillic acid	< 0.002
Vanillic mandelic acid	< 0.002

9.4. Recovery

Increasing amounts of adrenaline were added to an urine and to a plasma sample. 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 ng/ml

Urine					Plasma			
added	**measured**	**expected**	**% recovery**		**added**	**measured**	**expected**	**% recovery**
0.00	0.71				0.00	0.03		
1.46	2.6	2.2	**120**		0.07	0.09	0.10	**92**
2.38	3.9	3.1	**126**		0.12	0.13	0.15	**90**
3.70	4.7	4.4	**105**		0.19	0.18	0.22	**82**
5.36	7.2	6.1	**118**		0.29	0.33	0.32	**104**
9.80	12.7	10.5	**120**		0.48	0.54	0.51	**107**
19.2	19.4	19.9	**97**		0.71	0.69	0.74	**94**
32.7	40.2	33.4	**120**		1.22	1.25	1.24	**100**
61.4	59.5	62.1	**96**		1.92	2.13	1.95	**109**
		mean recovery:	**113**				mean recovery:	**97**

9.5. Linearity

The linearity of the ELISA method was investigated using different dilutions of an urine and a plasma sample.

Concentrations in ng/ml

Urine					Plasma			
dilution	**meas-ured**	**recalculated value**	**% Re-covery**		**dilution**	**meas-ured**	**recalculated value**	**% Re-covery**
Orig.	39.2				Orig.	2.11		
1+1	20.9	19.6	**107**		1+1	1.13	1.05	**107**
1+2	12.4	13.1	**95**		1+2	0.63	0.70	**90**
1+4	7.4	7.8	**94**		1+4	0.43	0.42	**101**
1+9	4.1	3.9	**104**		1+9	0.20	0.21	**97**
1+14	2.7	2.6	**102**		1+14	0.13	0.14	**95**
		mean linearity:	100				mean linearity:	98

9.6. Reproducibility

Intra-Assay

The reproducibility of the ELISA method was investigated by determing the intra-assay-coefficients of variation (cv) by repeated measurements of two samples with different concentrations.

Concentrations in ng/ml

sample	n	mean value	sd	cv (%)
1	40	9.7	0.88	**9.0**
2	40	14.3	1.11	**7.7**

Pipetting Scheme Sample Preparation

		Standards	Controls	Urine	Plasma
Standard 1 - 7	µl	20			
Control 1&2	µl		20		
Patient Urine	µl			20	
Patient Plasma	µl				300
Dist. Water	µl	250	250	250	
Extraction Buffer	µl	50	50	50	50

Incubate 60 minutes at RT (shake: 400 - 600 r/min)

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1	1

Incubate 5 minutes at RT (slow shaking)

Decant plate and remove residual liquid

Acylation Buffer	µl	150	150	150	150
Acyl. Reagent	µl	50	50	50	50

Immediately shake 20 minutes at RT (shake: 400 - 600 r/min)

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1	1

Incubate 5 minutes at RT (slow shaking)

Decant plate and remove residual liquid

Wash Buffer	ml	1	1	1	1

Incubate 5 minutes at RT (slow shaking)

Decant plate and remove residual liquid

HCl	µl	200	200	200	200

Incubate 20 minutes with adhesive foil at RT (shake: 400 - 600 r/min)

Caution: Do not decant the supernatant thereafter

For the ELISA take each 100 µl for Adrenaline

Pipetting Scheme - ELISA

		Standards	Controls	Samples
Adrenaline (blue)				
Enzyme mix (Fresh)	µl	20	20	20
Standard 1 – 7	µl	100		
Controls 1&2	µl		100	
Samples	µl			100
Cover with adhesive foil; shake 30 min at room temperature				
Adrenaline Antiserum	µl	20	20	20

Cover the plate with adhesive foil
Shake for 10 seconds
Incubate for 12 – 20 hours (overnight) at 2-8 °C

4 x washing

POD-Conjugate	µl	100	100	100

Incubate for 30 minutes at room temperature on an orbital shaker

4 x washing

Substrate	µl	100	100	100

Incubate 25 to 35 minutes at room temperature on an orbital shaker

Stop Solution	µl	100	100	100

Reading of absorbance at 450 nm