DIAGNOSTIKA GMBH

Instructions for Use

Adrenaline High Sensitive ELISA

(High sensitive and small sample volume)

Enzyme Immunoassay for the
Quantitative Determination of
Adrenaline

RUO

For Research Use Only
Not for use in diagnostic procedures

REF	EA632/96
Σ	12 x 8
	2 – 8 °C

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Symbols

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

Hazard Pictograms

 Danger

 Warning

1 Introduction and Principle of the Test

The Adrenaline High Sensitive ELISA provides materials for the quantitative measurement of adrenaline in low concentrated samples and for small sample volumes. Adrenaline is extracted using a cis-diol-specific boronate affinity gel and acylated to N-acyladrenaline and then converted enzymatically into N-acylmeta-nephrine.

The competitive Adrenaline High Sensitive ELISA kit uses the microtiter plate format with Adrenaline 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 conjugate. The peroxidase-induced conversion of the TMB substrate 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. Not for use in diagnostic procedures.
- Some reagents contain sodium azide as preservative. Some components of this kit contain hazardous reagents. These components are marked with the adequate hazard label. Refer to Safety Data Sheet. Avoid skin and eye contact. Wear protection, such as lab coat, appropriate gloves and eye protection.
- Discard waste according to state and local authorities.
- 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.

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

4.1 Reagents for Sample Preparation:

Extraction Plate 48 wells coated with boronate affinity gel	**EX-PLATE**	2 plates
Extraction Buffer 6 ml, ready for use	**EX-BUFF**	2 vials
HCl 21 ml, ready for use 0.025 M HCl	**HCL**	1 vial

Standards (A - F) **CAL A - F** 6 vials

4 ml each, ready for use

Concentrations:

Standard	A	B	C	D	E	F
Adrenaline (ng/ml)	0	0.15	0.5	1.5	5	25
Adrenaline (nmol/l)	0	0.82	2.7	8.2	27.3	137

Control 1 & 2 **CON 1 & 2** 2 vials

4 ml each, ready for use

Concentrations: see q.c. 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).

Danger

Warning

Acylation Buffer **ACYL-BUFF** 1 vial

20 ml, ready for use

Enzyme **ENZYME** 3 vials

2 ml each, lyophilized

Catechol-O-methyltransferase

Coenzyme **COENZYME** 1 vial

1 ml, ready for use

S-adenosyl-L-methionine

Enzyme Buffer **ENZYME-BUFF** 1 vial

3.5 ml, ready for use

Warning

Enzyme Plate **ENZYME-PLATE** 1 piece

96 wells, ready for use

Sample Stabilizer **STABILIZER** 1 vial

20 ml, ready for use

Warning

Reagents for ELISA

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

MT-STRIPS **STRIPS-AD** 12 strips
8 wells each, break apart, precoated with adrenaline (12 strips)

POD Conjugate **CONJ** 1 vial
12 ml each, ready for use,
Anti-rabbit IgG peroxidase conjugate  Warning

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

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

Stop Solution **STOP** 1 vial
12 ml, 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, 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 ºC (optional)

5 Sample Collection and Storage

5.1 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, otherwise false low values will be obtained with such samples.

Immediately after collection the plasma samples should be centrifuged (preferable at 2 - 8 °C) and frozen. 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 (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 achieved result of the sample by the factor 1.2.

5.2 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 (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 achieved result of the sample by the factor 1.1.

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.

5.3 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. 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 be 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 [WASH] 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] allow 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

Allow reagents to equilibrate to room temperature. Determinations in duplicates are recommended. Each 20 µl of Standards and Control 1 & 2 are extracted. Each 1 µl - 300 µl of samples are extracted (alternatively: > 300 µl up to 500 µl).

1. Pipette 20 µl of each Standard A – F [CAL A - F], 20 µl of each Control 1 & 2 [CON 1 & 2] and 1 µl - 300 µl of each Sample into the respective wells of the extraction plate [EX-PLATE].
   Within a run the final volume has to be the same in all wells: 300 µl or 500 µl.
   Correction of volume:
   Pipette 280 µl of distilled water into the wells of the standards and controls (final volume: 300 µl). Pipette as much distilled water into the wells of the samples to obtain a final volume of 300 µl, e.g. 100 µl sample + 200 µl distilled water.
   For sample volumes > 300 µl up to 500 µl: fill up all wells of the standards, controls and eventually samples to 500 µl with distilled water.
2. Pipette 100 µl Extraction Buffer [EX-BUFF] into each well.
3. Cover the plate with adhesive foil [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 1 ml prepared Wash Buffer [WASH] into each well 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 150 µl Acylation Buffer [ACYL-BUFF] into each well.
8. Add 50 µl Acylation Reagent [ACYL-REAG] into each well 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 or 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 1 ml prepared Wash Buffer [WASH] into each well 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 125 µl HCl [HCl] (0.025 M) for elution into each well.
15. Cover the plate with adhesive foil [FOIL] and incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
    Caution: Do not decant the supernatant.
16. Transfer 100 µl from each well of the extraction plate [EX-PLATE] into the respective wells of the enzyme plate [ENZYME-PLATE].
    (do not discard extraction plate, seal and keep for second or third assay run)
17. Add 20 µl of freshly prepared Enzyme Mix (s. 6.1) into each well. 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.

7 Test Procedure ELISA

1. Transfer 100 µl each of prepared Standards, Controls and Samples from the enzyme plate [ENZYME-PLATE] into the respective wells of the MT-Strips [STRIPS-AD].
   (do not discard enzyme plate, seal and keep for second or third assay run)
2. Add 20 µl Adrenaline-Antiserum [AS-AD] (colour coded blue) into each well.
3. Cover the plate with adhesive foil [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 250 µl prepared Wash Buffer [WASH] per well. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.
5. Pipette 100 µl POD-Conjugate [CONJ] into each well.
6. Incubate for 60 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Wash according to step 4.
8. Pipette 100 µl Substrate [SUB] into each well.
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. Add 100 µl Stop Solution [STOP] into each well.
11. Read the optical density at 450 nm (reference wavelength between 570 nm 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 µl - 300 µl sample volume in relation to 20 µl standard.

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

Example:

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

Correction factor = 300 µl / 20 µl = 15

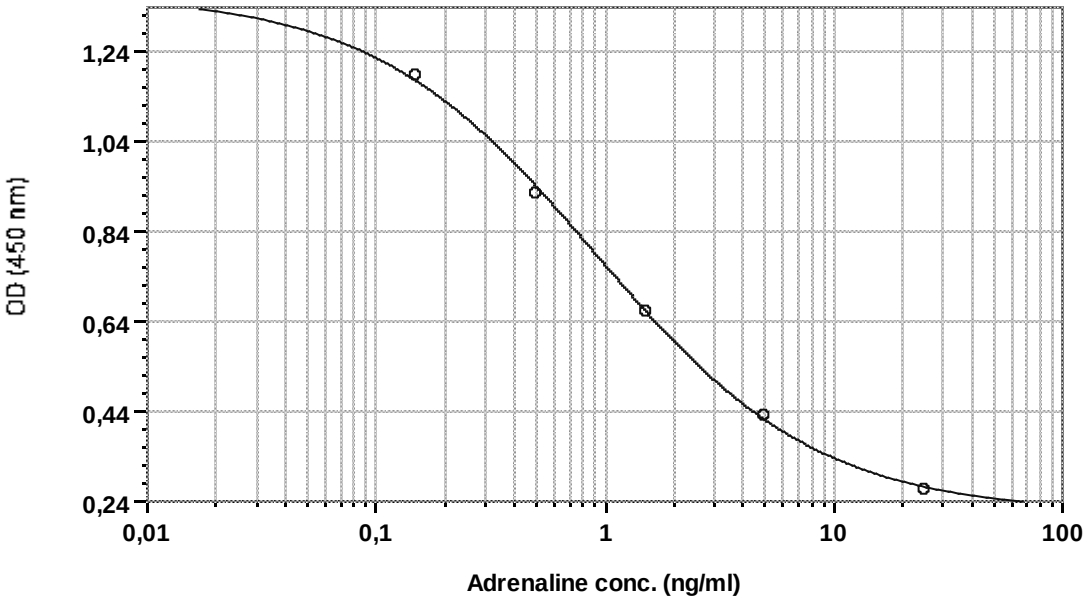
Concentration of the sample = 0.6 ng/ml / 15 = 0.040 ng/ml = 40 pg/ml

Conversion into pmol/l:

Adrenaline: 1 pg/ml = 5.46 pmol/l

Should the samples have been enriched with Sample Stabilizer, multiply the result with the appropriate dilution factor indicated in section 5.

Below a typical example of a standard curve with the Adrenaline High Sensitive ELISA is shown:



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
○	Std (Standards: Concentration vs MeanValue)	1,368	0,893	0,903	0,216	0,999

9 Assay Characteristics

9.1 Sensitivity

The lower limit of detection was determined by taking the 2-fold 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)

	Adrenaline
Sensitivity:	$\frac{\text{24 pg/ml (131 pmol/l)}}{\text{Correction factor}}$
Example for 300 µl Sample (Correction factor 15):	$\frac{\text{24 pg/ml}}{15}$ = 1.6 pg/ml (8.7 pmol/l)

9.2 Specificity (Cross Reactivity)

Structural related components were tested for possible interference:

Substance	**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
Vanilmandelic acid	< 0.002

9.3 Recovery

Increasing amounts of adrenaline 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

EDTA-Plasma			
added	**measured**	**expected**	**% recovery**
0.0	4.5		
8.8	16.6	13.4	124
15.2	18.1	19.7	92
22.4	24.9	26.9	93
34.4	34.8	38.9	89
45.5	43.9	50.0	88
88.2	83.5	92.8	90
151.5	127.9	156.0	82
223.9	219.8	228.4	96
384.6	333.1	389.1	86
		mean:	93

Cell culture medium			
added	**measured**	**expected**	**% recovery**
0.0	5.1		
8.8	14.3	14.0	103
15.2	25.6	20.3	126
22.4	29.2	27.5	106
34.4	42.1	39.5	107
45.5	50.8	50.6	100
88.2	90.1	93.4	97
151.5	148.9	156.6	95
223.9	229.2	229.0	100
384.6	355.7	389.7	91
		mean:	103

9.4 Reproducibility

Intra-Assay

The reproducibility of the ELISA method was investigated by determing 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

Sample	N =	mean value	sd	cv (%)
EDTA-Plasma	16	116.4	7.12	**6.1**
Cell culture medium	24	34.6	3.16	**9.2**

10 Changes to declare

Version 8: “Urine” was removed from title of section 5.2

Version 7: IFU has been re-formatted. Parts of sections 5, 6, 7 and 8 and pipetting schemes have been rephrased to provide greater clarity.

No changes have been made to components or execution of protocols.

Pipetting Scheme Sample Preparation

Use Extraction Plate [EX-PLATE]

		Standards	Controls	Samples
[CAL A – F]	µl	20		
[CON 1 & 2]	µl		20	
Samples	µl			1 - 300
Dist. Water	µl	280	280	Fill up to 300
[EX-BUFF]	µl	100	100	100

Cover the plate with adhesive foil;
Shake for 60 minutes at room temperature
Decant plate and tap out residual liquid

[WASH]	ml	1	1	1

Shake for 5 minutes at room temperature (slow shaking rate)
Decant plate and tap out residual liquid

[ACYL-BUFF]	µl	150	150	150
[ACYL-REAG]	µl	50	50	50

Immediately: Shake for 20 minutes at room temperature
Decant plate and tap out residual liquid

[WASH]	ml	1	1	1

Shake for 5 minutes at room temperature (slow shaking rate)
Decant plate and remove residual liquid
Repeat washing 1x, decant plate and tap out residual liquid

[HCl]	µl	125	125	125

Cover the plate with adhesive foil;
Shake for 20 minutes at room temperature
Caution: Do not decant the supernatant

Use Enzyme Plate [ENZYME-PLATE]

		Standards	Controls	Samples
Transfer from [EX-PLATE] to [ENZYME-PLATE]	µl	100	100	100
Enzyme mix (fresh)	µl	20	20	20

Cover the plate with adhesive foil;
Shake for 1 minute at room temperature
Incubate for 90 minutes at 37ºC
Caution: Do <u>not</u> decant the supernatant

Pipetting Scheme ELISA

Use MT-STRIPS [STRIPS-AD]

		Prepared Standards	Prepared Controls	Prepared Samples
Transfer from [ENZYME-PLATE] to [STRIPS-AD]	µl	100	100	100
[AS-AD]	µl	20	20	20

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

[WASH]	µl	250	250	250

Decant or aspirate plate
Repeat washing 3 x, decant plate and tap out residual liquid

[CONJ]	µl	100	100	100

Shake for 60 minutes at room temperature (medium shaking rate)
Discard or aspirate plate

[WASH]	µl	250	250	250

Decant or aspirate plate
Repeat washing 3 x, decant plate and tap out residual liquid

[SUB]	µl	100	100	100

Shake for 35 – 45 minutes at room temperature (medium shaking rate)

[STOP]	µl	100	100	100

Read absorbance at 450 nm within 15 minutes