DIAGNOSTIKA GMBH

Instructions for Use

Homoarginine ELISA

Enzyme Immunoassay for the
Quantitative Determination of
Homoarginine in EDTA-Plasma, Serum and Cell Culture Samples

RUO

For Research Use Only
Not for Use in Diagnostic Procedures

REF	EA205/96
Σ	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 chapter 4 Contents of the Kit.

1 Warning

ATTENTION: IMPORTANT PATENT NOTICE (German part of the European Patent EP 2 533 653 B1)

The Homoarginine ELISA kit offered/supplied by us is suitable for use in a method for determining the mortality risk of a patient, in which the amount of homoarginine or its metabolic precursors in a sample from the patient is determined and the determined amount is compared with a reference amount.

We expressly point out that

COMPARING THE AMOUNT OF HOMOARGININE OR A METABOLIC PRECURSOR THEREOF, WHICH WAS DETERMINED WITH THE HOMOARGININE ELISA-KIT, WITH A REFERENCE AMOUNT, WHEREBY THE MORTALITY RISK IN THE PATIENT IS DETERMINED

lies within the scope of the German part of the European patent EP 2 533 653 B1 and is protected for the patent proprietor (Immundiagnostik AG) and **therefore requires the separate consent of the patent proprietor.**

Use of this Homoarginine ELISA kit for the described mortality risk determination method without the consent of the patent holder constitutes a patent infringement.

2 Introduction and Principle of the Test

The Homoarginine ELISA kit contains reagents for the quantitative determination of derivatized homoarginine in EDTA-plasma, serum and cell culture samples. Derivatization takes place during sample preparation. Homoarginine is quantitatively converted into N-acylhomoarginine by the acylation reagent.

The Homoarginine ELISA is a competitive enzyme immunoassay. Antigen bound to the solid phase and free antigen in solution compete for a defined number of antibody binding sites. When the system is in equilibrium, the unbound antigen-antibody complex is removed in a wash step and the corresponding bound complex is detected by means of a anti-rabbit-IgG-peroxidase conjugate and determined via the turnover of tetramethylbenzidine (TMB). The TMB/POD reaction is stopped and measured at 450 nm. The concentration of the antigen-antibody complex bound to the solid phase is inversely proportional to the concentration of the antigen in the sample.

3 Precautions

- For research use only. Not for use in diagnostic procedures. For professional use only.
- Observe the Important Patent Notice.
- Before carrying out the test, the valid instructions for use, as included in this kit, should be read completely and the content understood.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.
- Individual components of different lots and 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 safety goggles while performing the test.
- Some of the components of this kit contain hazardous substances. These components bear the appropriate hazard symbol on their label. Further information can be found in 4. Contents of the Kit and on the relevant safety data sheets.
- Avoid any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.
- Avoid contact with individual reagents, as these can cause irritation and chemical burns.
- Dispose of waste according to state and local environmental protection regulations.
- Broken glass can cause injury. Be cautious with glass vials.

4 Changes to declare

Version _5: Additions/changes are highlighted in grey

Version _4: Inclusion of Important Patent Notice.

Version _3: Additions/changes are highlighted in grey

Version _2: IFU has been re-formatted. Section Precautions was up-dated. Component names as printed on labels were included in sections 7 and 8 and pipetting schemes to provide greater clarity. No changes have been made to components or execution of protocols.

5 Storage and Stability

The kit is shipped at ambient temperature. Upon arrival, store the kit at 2 - 8 °C. Once opened the kit is stable until its expiry date. The shelf life of the ready-to-use reagents is indicated on the respective bottle label. For stability of prepared reagents refer to Preparation of Reagents.

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

6 Contents of the Kit

MT-Strips **STRIPS** 12 strips
8 wells each, break apart, precoated with Homoarginine

Standards (1 - 6) **CAL 1 - 6** 6 vials
each 4 ml, ready for use

Control 1 & 2 **CON 1 & 2** 2 vials
each 4 ml, ready for use, Range: see QC certificate

Acylation Reagent **ACYL-REAG** 3 vials
lyophilised, dissolve content in Solvent before use

Acylation Buffer **ACYL-BUFF** 1 vial
3.5 ml, ready for use, color-coded blue  Warning

Solvent **SOLVENT** 2 vials
 Danger
10 ml, ready for use, contains DMSO
 Warning

Antiserum **AS** 1 vial
7 ml, ready for use, Rabbit-anti-N-acyl-Homoarginine  Warning

Enzyme Conjugate **CONJ** 1 vial
13 ml, ready for use, goat anti-rabbit-IgG-peroxidase  Warning

Wash Buffer **WASH** 1 vial
20 ml, concentrated, (50 x)

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

Stop Solution **STOP** 1 vial
13 ml, ready for use, contains 0.3 M sulphuric acid, not corrosive

Reaction Plate **ACYL-PLATE** 1 piece
For acylation

Equalizing Reagent **EQUA-REAG** 1 vial
lyophilized, dissolve content with dist. water

Foil **FOIL** 2 Stück
Ready for use

Additional materials and equipment required, but not provided:

- Pipettes (20, 50, 100 and 200 µl)
- Multichannel pipette or microplate washing device
- Multipette
- Distilled water
- Microplate photometer (450 nm)
- Orbital shaker
- Vortex mixer and roller mixer
- Paper towels, pipette tips, timer

7 Sample Collection

Repeated freezing and thawing should be avoided.

7.1 EDTA-Plasma and Serum

The test can be performed with serum as well as with EDTA plasma.

Hemolytic, ikteric and lipemic samples should not be used.

The samples can be stored up to 6 hours at 2 – 8 °C. For a longer storage (up to 18 months) the samples must be kept frozen at –20 °C

7.2 Cell Culture Media

Cell culture media like DMEM and RPMI have been tested successfully. Other media have to be tested by the user.

8 Preparation of Reagents

8.1 Microtiter strips

Before opening the packet of strip wells STRIPS, allow it to stand at room temperature for at least 10 minutes. After opening, keep any unused wells in the original foil packet with the desiccant provided. Reseal **carefully** and store at 2 – 8 °C.

8.2 Wash Buffer

Dilute the content of WASH with dist. water to a total volume of 1,000 ml, mix briefly. The diluted wash buffer has to be stored at 2 - 8°C and can be used for 4 weeks.

Should the kit be used in several runs, then prepare only the required amount of wash buffer for each run.

8.3 Equalizing Reagent

Dissolve the content of EQUA-REAG with 21 ml dist. water, mix shortly and leave on a roll mixer or orbital shaker for 20 minutes. Handle carefully in order to minimize foam formation. The reconstituted Equalizing Reagent should be stored at -20 °C and is stable until the expiry date.

8.4 Acylation Reagent

Remove the required amount of vials of Acylation Reagent ACYL-REAG from the foil pouch, leave the remaining vials inside together with the desiccant and close the pouch carefully. Dissolve the content of one bottle with 3 ml Solvent SOLVENT and shake for at least 10 minutes on a rollmixer or orbital shaker. The Acylation Reagent should be freshly prepared immediately before use and is stable for at least 3 hours. The second and third bottle allow a second and third run of the test, respectively. If the whole kit is to be used in one run it is recommended to pool the dissolved contents of two vials of Acylation Reagent. Discard the remaining reconstituted reagent after use.

All other reagents are ready for use.

9 Test Procedure

Duplicates are recommended for standards, controls and samples.

Bring all reagents to room temperature and mix them carefully, avoid development of foam.

The wells of the reaction plate ACYL-PLATE can be used only once. Therefore, mark the respective wells before use (marker).

9.1 Test Procedure for EDTA-Plasma and Serum

9.1.1 Preparation of EDTA-Plasma and Serum Samples (Acylation)

1. Pipette each 20 µl standard 1 - 6 CAL 1 - 6, each 20 µl control 1 & 2 CON 1 & 2 and each 20 µl sample into the respective wells of the reaction plate ACYL-PLATE.
2. Pipette 20 µl Acylation Buffer ACYL-BUFF into each well.
3. Pipette 200 µl Equalizing Reagent EQUA-REAG (see 8.3) into each well and shake the reaction plate for 10 seconds on an orbital shaker with medium frequency.
4. Pipette 50 µl of freshly prepared Acylation Reagent ACYL-REAG (see 8.4) each into each well, continue with the next step, immediately. Color changes to violet.

   Attention: Please note that Acylation Reagent reacts with many plastic materials including plastic trays. It does not react with normal pipette tips and with glass devices. Use an Eppendorf multipette or similar, fill the syringe directly from the vial and add well by well.
5. Incubate for 15 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.

   Take each 20 µl of the acylated samples for the Homoarginine ELISA.

9.1.2 ELISA with EDTA-Plasma and Serum Samples

1. Pipette each 20 µl of prepared Standards, controls and samples into the respective wells of the coated microtiter strips [STRIPS].
2. Pipette 50 µl Antiserum [AS] into each well.
3. Seal the plate with adhesive foil [FOIL] and incubate for 90 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.
4. Discard or aspirate the contents of the wells and wash with each 300 µl prepared Wash Buffer [WASH] (see 8.2). Discard or aspirate the contents of the wells and remove residual liquid by 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 25 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.
7. Repeat step 4.
8. Pipette 100 µl Substrate [SUB] into each well.
9. Incubate for 25 ± 5 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.
10. Pipette 100 µl Stop Solution [STOP] into each well. Shake on an orbital shaker for minimum 10 seconds.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) with a microplate photometer.

9.2 Test Procedure for Cell Culture Samples

The sample preparation of cell culture samples and subsequent ELISA has to be done separately and cannot be performed in parallel to the EDTA-plasma and serum samples.

9.2.1 Preparation of Cell Culture Samples (Acylation)

1. Pipette each 20 µl standard 1 - 6 [CAL 1 - 6], each 20 µl control 1 & 2 [CON 1 & 2] and each 20 µl cell culture sample into the respective wells of the Reaction Plate [ACYL-PLATE].
2. Pipette 20 µl Standard 1 [CAL 1] in each well containing cell culture samples (to harmonize matrices).
3. Pipette 20 µl cell culture medium into each well containing standards and controls (to harmonize matrices). Do not pipette into wells with cell culture samples.
4. Pipette 20 µl Acylation Buffer [ACYL-BUFF] into each well.
5. Pipette 200 µl Equalizing Reagent [EQUA-REAG] (see 8.3) into each well and shake the reaction plate for 10 seconds on an orbital shaker with medium frequency.
6. Pipette 50 µl of freshly prepared Acylation Reagent [ACYL-REAG] (see 8.4) each into each well, continue with the next step, immediately. Colour changes to violet.

   Attention: Please note that Acylation Reagent reacts with many plastic materials including plastic trays. It does not react with normal pipette tips and with glass devices. Use an Eppendorf multipette or similar, fill the syringe directly from the vial and add well by well.
7. Incubate for 15 minutes at room temperature (20-25 °C) on an orbital shaker with medium frequency.

   Take each 20 µl of the acylated samples for the Homoarginine ELISA.

9.2.2 ELISA with Cell Culture Samples

1. Pipette each 20 µl of prepared standards, controls and samples into the respective wells of the coated microtiter strips [STRIPS].
2. Pipette 50 µl Antiserum [AS] into each well.
3. Cover the plate with adhesive foil [FOIL] and incubate for 90 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.
4. Discard or aspirate the contents of the wells and wash with each 300 µl prepared Wash Buffer [WASH] (see 8.2). Discard or aspirate the contents of the wells and remove residual liquid by 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 (20 – 25 °C) on an orbital shaker with medium frequency.
7. Repeat step 4.
8. Pipette 100 µl Substrate [SUB] into each well.
9. Incubate for 30 ± 5 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.
10. Pipette 100 µl Stop Solution [STOP] into each well. Shake on an orbital shaker for minimum 10 seconds.
11. Read the optical density at 450 nm (reference wavelength between 570 nm and 650 nm) in a microplate photometer.

10 Calculation of the Results

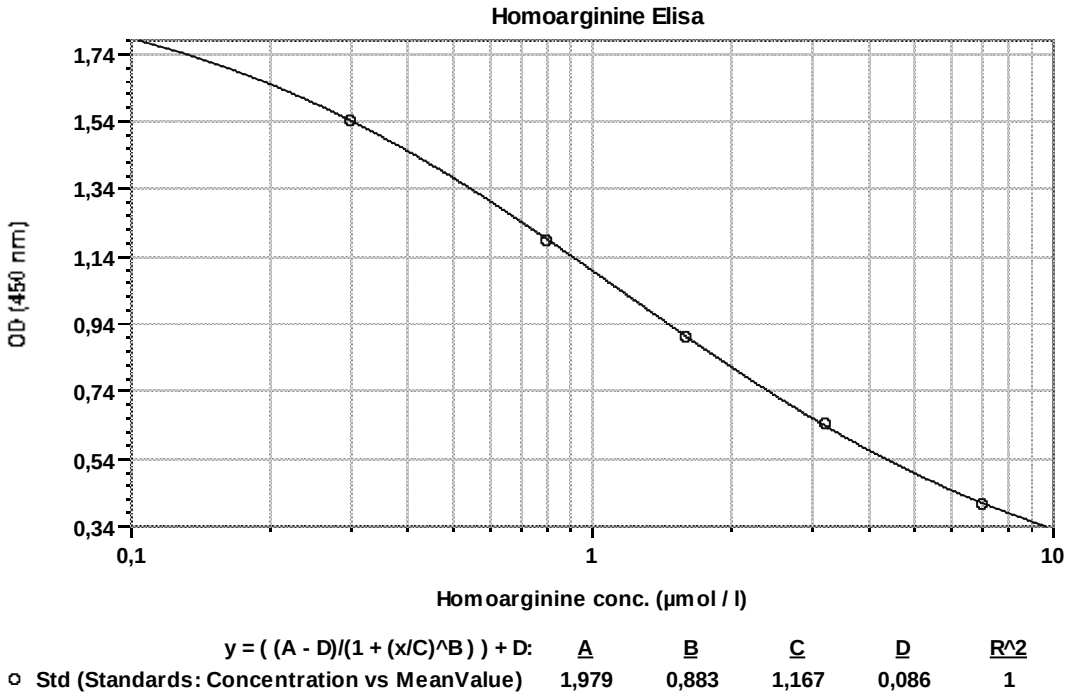
Standard	1	2	3	4	5	6
µmol / l	0	0.3	0.8	1.6	3.2	7
ng / ml	0	56	151	301	602	1,318

Conversion factor: Homoarginine: 1 µmol/l = 188.23 ng/ml

The concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

The concentration of the controls and samples in µmol/l can be read directly from the standard curve by using their average optical density.

Typical standard curve (do not use for calculation of results)**:**



Quality Control: The controls included in the kit have to give results within the target range (see QC certificate). Otherwise the assay results are invalid and the test has to be repeated.

11 Assay Characteristics

11.1 Sensitivity

Lower Limit of Detection	Calculation
0.05 µmol / l	ODCal1 – 3 x SD

11.2 Specificity (Cross Reactivity)

Substance	Cross Reactivity (%)
homoarginine	100
arginine	0.025
ADMA	< 0.025
SDMA	< 0.025
monomethylarginine (NMMA)	< 0.025

11.3 Recovery after Spiking

Matrix	Range (µmol / l)	Mean (%)	Range (%)
EDTA-plasma	0.66 – 6.70	95	87 - 104
serum	1.51 – 5.10	103	97 - 107
cell culture medium	0.52 – 4.12	96	87 - 100

11.4 Linearity

Matrix	Range (µmol / l)	Highest dil.	Mean (%)	Range (%)
EDTA-plasma	0.48 – 3.76	1 : 7 with water	99	89 - 105
serum	0.39 – 2.68	1 : 7 with water	103	96 - 109
cell culture medium	0.30 – 3.30	1 : 10 with water	101	91 - 108

11.5 Reproducibility

Matrix	Range (µmol / l)	Intra Assay CV
EDTA-plasma	0.83 – 2.23	6.1 – 3.3 %
serum	1.30 – 2.73	4.6 – 5.6 %
cell culture medium	1.59 – 3.33	6.2 – 4.7 %

11.6 Method Comparison

Matrix	Method or Matrix	Correlation
EDTA-plasma	LC/MS	Y = 0.98 x LC/MS + 0.12; R = 0.998; N = 25
Serum	EDTA-plasma	Y = 1.00 x plasma + 0.11; R = 0.965; N = 12

11.7 Calibration

The calibration is carried out by weighing the pure substance.

11.8 Limitations of Method

The results are for research use only. Samples measured above the highest standard must be diluted with distilled water (see 11.4) and reassayed. The dilution factor must then be used for calculation of the final results.

11.9 Interferences

Hemolytic, lipemic and icteric samples should not be used.

12 Literature

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Pipetting Scheme – EDTA-Plasma and Serum

Sample Preparation

		Standards	Controls	Plasma	Serum
ACYL-PLATE:					
CAL 1 – 6	µl	20			
CON 1 & 2	µl		20		
EDTA-Plasma	µl			20	
Serum	µl				20
ACYL-BUFF	µl	20	20	20	20
EQUA-REAG	µl	200	200	200	200

Shake for 10 seconds

ACYL-REAG	µl	50	50	50	50

Immediately, shake for 15 minutes at room temperature

ELISA

		Acyl. Standards	Acyl. Controls	Acyl. Samples
STRIPS:				
Transfer from ACYL-PLATE into STRIPS	µl	20	20	20
AS	µl	50	50	50

Cover plate with FOIL, shake for 90 minutes at room temperature
Wash 4 x

CONJ	µl	100	100	100

Shake for 25 minutes at room temperature
Wash 4 x

SUB	µl	100	100	100

Shake for 25 ± 5 minutes at room temperature

STOP	µl	100	100	100

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

Pipetting Scheme - Cell Culture Samples

Sample Preparation

		Standards	Controls	Cell Culture Sample
ACYL-PLATE:				
CAL 1 – 6	µl	20		
CON 1 & 2	µl		20	
Cell Culture Sample	µl			20
CAL 1	µl			20
Cell Culture Medium	µl	20	20	
ACYL-BUFF	µl	20	20	20
EQUA-REAG	µl	200	200	200

Shake plate for 10 seconds

ACYL-REAG	µl	50	50	50

Immediately, shake for 15 minutes at room temperature

ELISA

		Acyl. Standards	Acyl. Controls	Acyl. Samples
STRIPS:				
Transfer from ACYL-PLATE into STRIPS	µl	20	20	20
AS	µl	50	50	50

Cover plate with FOIL, shake for 90 minutes at room temperature
Wash 4 x

CONJ	µl	100	100	100

Shake for 30 minutes at room temperature
Wash 4 x

SUB	µl	100	100	100

Shake for 30 ± 5 minutes at room temperature

STOP	µl	100	100	100

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