DIAGNOSTIKA GMBH

Instructions for Use

Homoarginine ELISA

Enzyme Immunoassay for the
Quantitative Determination of
Homoarginine in 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

Hazard Pictograms

 Warning

 Danger

1 Introduction and Principle of the Test

Homoarginine is an amino acid derivative with a molecular weight of 188.23 and is derived from the amino acid lysine. It is said to increase the availability of nitric oxide (NO) and thus improves endothelial function. Lower concentrations of homoarginine are associated with endothelial dysfunction and increase the risk of coronary heart disease.

NO is an important physiological mediator in the cardiovascular and other organ systems, involved in the regulation of blood pressure and vascular resistance, adhesion and aggregation of platelets, adhesion of leukocytes and monocytes and proliferation of vascular smooth muscle cells. NO also plays an important physiological role in erection. In cardiovascular diseases such as arteriosclerosis, hypercholesterolemia, hypertension, chronic heart failure, metabolic diseases such as diabetes mellitus, pre-eclampsia, erectile dysfunction and other diseases, the biological effects of NO are attenuated, accelerating the progression of these diseases and the accompanying vascular lesions.

This assay is for research use only.

The Homoarginine ELISA kit contains reagents for the quantitative determination of derivatized homoarginine in 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 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.

2 Precautions

- For research use only. Not for use in diagnostic procedures. For professional use only.
- 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 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 safety goggles while performing the test.
- Some of the components of this test 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.

3 Changes to declare

Version _3: Aditions/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.

4 Storage and Stability

On 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.

5 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,
concentrations:

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

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 3 ml Solvent before use

Acylation Buffer **ACYL-BUFF** 1 vial

3.5 ml, ready for use


Warning

Solvent **SOLVENT** 2 vials


Danger

5.5 ml, contains DMSO


Warning

Antiserum **AS** 1 vial

7 ml, ready for use, Rabbit-anti-N-acyl-Homoarginine

Enzyme Conjugate **CONJ** 1 vial

13 ml, ready for use, goat anti-rabbit-IgG-peroxidase

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 21 ml dist. water,
dissolve carefully to minimize foam formation

Foil **FOIL** 2 Stück

Ready for use

Additional materials and equipment required but not provided:

- Pipettes (20, 50, 100 and 200 µl)
- Orbital shaker
- Multichannel pipette
- Microplate washing device
- Microplate photometer (450 nm)

6 Sample Collection

Repeated freezing and thawing should be avoided.

6.1 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

6.2 Cell Culture Media

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

7 Preparation of Reagents

7.1 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.

7.2 Wash Buffer

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

If the kit is to be divided into several runs, prepare for each run only the required amount of wash buffer.

7.3 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 frozen at -20 °C and is until expiry date of the kit.

7.4 Acylation Reagent

Dissolve the content of one bottle of ACYL-REAG with 3 ml Solvent SOLVENT and shake for at least 10 minutes on a rollmixer or orbital shaker. The Acylation Reagent has always to be prepared immediately before use and is stable for at least 3 hours. After use the reagent has to be discarded. The second and third bottle allows 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.

All other reagents are ready for use.

8 Test Procedure

8.1 Test Procedure for Plasma and Serum

Bring all reagents to room temperature and mix them carefully, avoid development of foam. Duplicates are recommended for standards, controls and samples.

8.1.1 Preparation of Plasma and Serum Samples (Acylation)

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

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 into each well and shake the reaction plate for 10 seconds.
4. Pipette 50 µl of freshly prepared Acylation Reagent ACYL-REAG each into each well, continue with point 5., 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.
5. Incubate for 15 minutes at room temperature on an orbital shaker with medium frequency.

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

8.1.2 ELISA for 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. 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]. Discard or aspirate the contents of the wells and remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 4 times.
5. Pipette 100 µl enzyme conjugate [CONJ] into each well.
6. Incubate for 25 minutes at room temperature 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 on an orbital shaker with medium frequency.
10. Pipette each 100 µl Stop Solution [STOP] into each well and mix briefly.
11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

8.2 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 plasma and serum samples. Bring all reagents to room temperature and mix them carefully, avoid development of foam. Duplicates are recommended for standards, controls and samples.

8.2.1 Preparation of Cell Culture Samples (Acylation)

The wells of the reaction plate ACYL-PLATE for the acylation can be used only once. Please mark the respective wells before use (Edding)

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.
2. Pipette 20 µl standard 1 CAL 1 in each well containing cell culture samples (compensation for matrix).
3. Pipette 20 µl cell culture medium into each well containing standards and controls (compensation for matrix). 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 into each well and shake the reaction plate for 10 seconds.
6. Pipette 50 µl of freshly prepared Acylation Reagent ACYL-REAG each into each well, continue with point 7, 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 on an orbital shaker with medium frequency.

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

8.2.2 ELISA for Cell Culture Samples

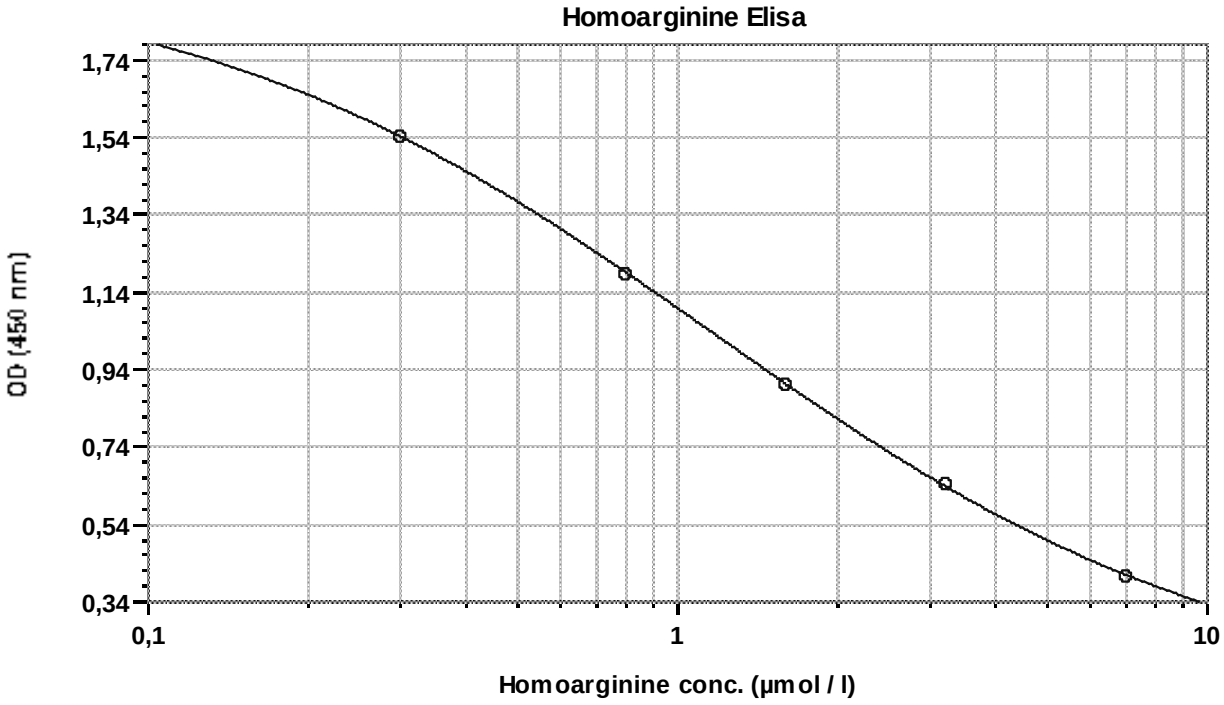
1. Pipette each 20 µl of prepared standards, controls and samples into the respective wells of the coated microtiter strips [STRIPS].
2. Pipette 50 µl Antiserum [AS] into each well.
3. Cover the plate with adhesive foil [FOIL] and incubate for 90 minutes at room temperature (20 – 25 °C) on an orbital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

9 Calculation of the Results

The concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Cubic spline, 4 parameter or similar iteration procedures are recommended for evaluation of the standard curve.

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

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



y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Std (Standards: Concentration vs MeanValue)	1,979	0,883	1,167	0,086	1

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.

10 Assay Characteristics

10.1 Expected Values

This kit is for research use only, the values below are not for use in diagnostic procedures and should only be taken as a guideline. It is recommended that each laboratory establishes its own normal values.

Matrix	Reference Range
EDTA-plasma, serum	2.0 ± 0.7 µmol / l

10.2 Sensitivity

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

10.3 Specificity (Cross Reactivity)

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

10.4 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

10.5 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

10.6 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 %

10.7 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	plasma	Y = 1.00 x plasma + 0.11; R = 0.965; N = 12

10.8 Calibration

The calibration is carried out by weighing the pure substance. The correctness of the method was determined by comparison with the expected values (10.1) and comparison of method (10.7).

10.9 Limitations of Method

Samples measured above the highest standard must be diluted with distilled water (10.5) and reassayed.

10.10 Interferences

Hemolytic, lipemic and icteric samples should not be used.

11 Literature

- Meinitzer, Ch Drechsler. A. Tomaschitz, S. Pilz, V. Krane, Ch. Wanner, W. März
  Homoarginin, ein neuer kardiovaskulärer Risikomarker bei Dialysepatienten
  J. Lab. Med. 2011, 35 (3): 153 -159, copy 2011 by Walter de Gruyter, Berlin, Boston

- W. März A. Meinitzer, Ch. Drechsler, S. Pilz, V. Krane, M.E. Kleber, J. Fischer, B.R. Winkelmann, B.O. Böhm, E. Ritz, Ch. Wanner.
  Homoarginine, Cardiovascular Risk and Mortality
  Circulation 2010, 122: 967-975

- Pietro Ravani, Renke Maas, Fabio Malberti, Paola Pecchini, Maren Mieth, Robert Quinn, Giovanni Tripepi, Francesca Mallamaci, Carmine Zoccali
  Homoarginine and Mortality in Pre-Dialysis Chronic Kidney Disease (CKD) Patients
  Plos One; September 2013, Volume 8, Issue 9: 1-6

- Ch. Drechsel, B. Kolleritz. A, Meinitzer, W. März, E. Ritz, P. König, U. Neyer, S. Pilz, Ch. Wanner, F. Kronenberg
  Homoarginine and Progression of Chronic Kidney Disease: Results from the Mild to Moderate Kidney Disease Study
  May 2013; Plos One, 10, 1371

- A.A. Khalil, D. Tsikas, R. Akolekar, J. Jordan, K.H. Nicolaides
  Asymmetric dimethylarginine, arginine and homoarginine at 11-13 weeks´ gestation and preeclampsia: a case control study.
  J. of Human Hypertension January 2013 27; 38-43

- A. Jazwinska-Kozuba, J. Martens-Lobenhoffer, O. Kruszelnicka, J. Rycaj, B. Chyrchel, A. Surdacki, S. M. Bode-Böger
  Opposite Associations of Plasma Homoarginine and Ornithine with Arginine in Healthy Children and Adolescents
  Int. J. Mol. Sci. 2013, 14, 21819-21832

- Choe CU, Atzler D, Wild PS, Carter AM, Böger RH, Ojeda F, Simova O, Stockebrand M, Lackner K, Nabuurs C, Marescau B, Streichert T, Müller C, Lüneburg N, De Deyn PP, Benndorf RA, Baldus S, Gerloff C, Blankenberg S, Heerschap A, Grant PJ, Magnus T, Zeller T, Isbrandt D, Schwedhelm E
  Homoarginine levels are regulated by L-arginine: glycine amidinotransferase and affect stroke outcome; results from human and murine studies
  Circulation, 2013 Sep 24, 128 (13) 1451-1461

- van der Zwan, L., Davids, M., Scheffer, P.; et al.
  L-Homoarginine and L-arginine are antagonistically related to blood pressure in an elderly population: the Hoorn study
  Journal of Hypertension 2013: 31:1114–1123

- Franczyk-Skóra, B., Gluba, A., Banach, M.; et al. (2012):
  Prevention of sudden cardiac death in patients with chronic kidney disease
  BMC Nephrology 13:162

- Davids, M., Ndika, J., Salomons, G.; et al. (2012):
  Promiscuous activity of arginine:glycine amidinotransferase is responsible for the synthesis of the novel cardiovascular risk factor homoarginine
  FEBS Letters 586 3653–3657

- Valtonen, P., Laitinen, T., Lyyra-Laitinen, T.; et al. (2008):
  Serum L-Homoarginine Concentration is Elevated During Normal Pregnancy and is Related to Flow-Mediated Vasodilatation
  Circulation Journal Vol.72, 1879– 1884

Pipetting Scheme – Plasma and Serum

Sample Preparation

		Standards	Controls	Plasma	Serum
ACYL-PLATE:					
CAL 1 – 6	µl	20			
CON 1 & 2	µl		20		
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
Take 20 µl for the ELISA

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

Reading of 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
Take 20 µl for the ELISA

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

Reading of absorbance at 450 nm (ref 570 – 650 nm)