DIAGNOSTIKA GMBH

Instructions for Use

Homoarginine ELISA

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

REF EA205/96

12 x 8

2 – 8 °C

DLD Gesellschaft für Diagnostika und medizinische Geräte mbH
Adlerhorst 15 • 22459 Hamburg • Germany
Tel +49-40-555 87 10 • Fax +49-40-555 87 111
Internet: http://www.dld-diagnostika.de • E-Mail: contact@dld-diagnostika.de

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1. Introduction and Principle of the Test

Homoarginine is a non-essential cationic amino acid, which is formed from lysine. In vitro and in vivo, homoarginine shows characteristics similar to arginine. Epidemiological investigations in two large independent cohorts, namely the German diabetes dialysis (4D) - study and the Ludwigshafen Risk and Cardiovascular Health (LURIC) - study have identified homoarginine as useful predictor of cardiovascular events and mortality.

Beyond that homoarginine concentrations are directly correlated with kidney function and are significantly associated with the progression of chronic kidney disease (CKD). Low homoarginine concentrations might be an early indicator of kidney failure and a potential target for the prevention of disease progression which needs further investigations. Furthermore homoarginine could be a useful marker for the monitoring of hemodialysis patients.

Cited: J Lab Med, 2011; 35 (3): 153–159

We offer a competitive Homoarginine-ELISA using the microtiter plate format. The correlation of the ELISA method to LC-MS is exceptionally good. No interferences with any therapeutic drugs are observed. The ELISA method allows the measurement of large series of patient samples.

Homoarginine as a biomarker for the risk of mortality is applied for a patent. EP2533653A1 and US20130143240.

The competitive Homoraginine-ELISA uses the microtiter plate format. Homoarginine is bound to the solid phase of the microtiter plate. Homoarginine in the samples is acylated and competes with solid phase bound Homoarginine for a fixed number of rabbit anti-Homoarginine 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 Homoarginine is detected by anti-rabbit/peroxidase. The substrate TMB / peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase Homoarginine is inversely proportional to the Homoarginine concentration of the sample.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative (<0.1%). Avoid skin contact.
- 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.

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 labels.
Do not mix various lots of any kit component within an individual assay.

4. Contents of the Kit

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

4.2 **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

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

4.4 **Acylation Buffer** **ACYL-BUFF** 1 vial
3.5 ml, ready for use

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

4.6 **Solvent** **SOLVENT** 2 vials
5.5 ml, contains DMSO

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

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

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

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

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

4.12 **Reaction Plate** **ACYL-PLATE** 1 piece
for acylation

4.13 **Equalizing Reagent** **EQUA-REAG** 1 vial
lyophilized, dissolve content with 21 ml dist. water,
dissolve carefully to minimize foam formation

4.14 **Foil** **FOIL** 2 pieces
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)

5. Sample Collection

Repeated freezing and thawing should be avoided.

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

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

6. Preparation of Reagents

Microtiter strips STRIPS

Before opening the packet of strip wells, 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.

Wash Buffer WASH

Dilute the content 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. For longer use until expiry date of the kit store frozen at -20 °C.

Equalizing Reagent EQUA-REAG

Dissolve the content 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.

Acylation Reagent ACYL-REAG

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

7. Test Procedure

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

7.1 Test Procedure for Plasma and Serum

Preparation of Samples (Acylation)

The wells of the reaction 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, each 20 µl control 1 & 2 and each 20 µl patient sample into the respective wells of the Reaction Plate.

2. Pipette 20 µl Acylation Buffer into all wells.

3. Pipette 200 µl Equalizing Reagent into all wells and mix the reaction plate for 10 seconds.

4. Pipette 50 µl of freshly prepared Acylation Reagent each into all wells, continue with point 5. immediately. Colour change 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 sample for the Homoarginine-ELISA.

ELISA for Plasma and Serum

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

1. Pipette each 20 µl prepared Standards, controls and samples into the respective wells of the coated microtiter strips.

2. Pipette each 50 µl Antiserum into all wells.

3. Cover the plate with adhesive 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. 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 into all wells.

6. Incubate for 25 minutes at room temperature on an orbital shaker with medium frequency.

7. Repeat step 4.

8. Pipette each 100 µl Substrate into all wells.

9. Incubate for 25 ± 5 minutes at room temperature on an orbital shaker with medium frequency.

10. Pipette each 100 µl Stop Solution into all wells and mix briefly.

11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

7.2 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.

Preparation of Samples (Acylation)

The wells of the reaction 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, each 20 µl control 1 & 2 and each 20 µl cell culture sample into the respective wells of the Reaction Plate.

2. Pipette 20 µl standard 1 in all wells containing cell culture samples (compensation for matrix).

3. Pipette 20 µl cell culture medium into all wells containing standards and controls (compensation for matrix). Do not pipette into wells with cell culture samples.

4. Pipette 20 µl Acylation Buffer into all wells.

5. Pipette 200 µl Equalizing Reagent into all wells and mix the reaction plate for 10 seconds.

6. Pipette 50 µl of freshly prepared Acylation Reagent each into all wells, 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.

ELISA for Cell Culture Samples

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

1. Pipette each 20 µl prepared Standards, controls and samples into the respective wells of the coated microtiter strips.

2. Pipette each 50 µl Antiserum into all wells.

3. Cover the plate with adhesive 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. 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 into all wells.

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 into all wells.

9. Incubate for 30 ± 5 minutes at room temperature on an orbital shaker with medium frequency.

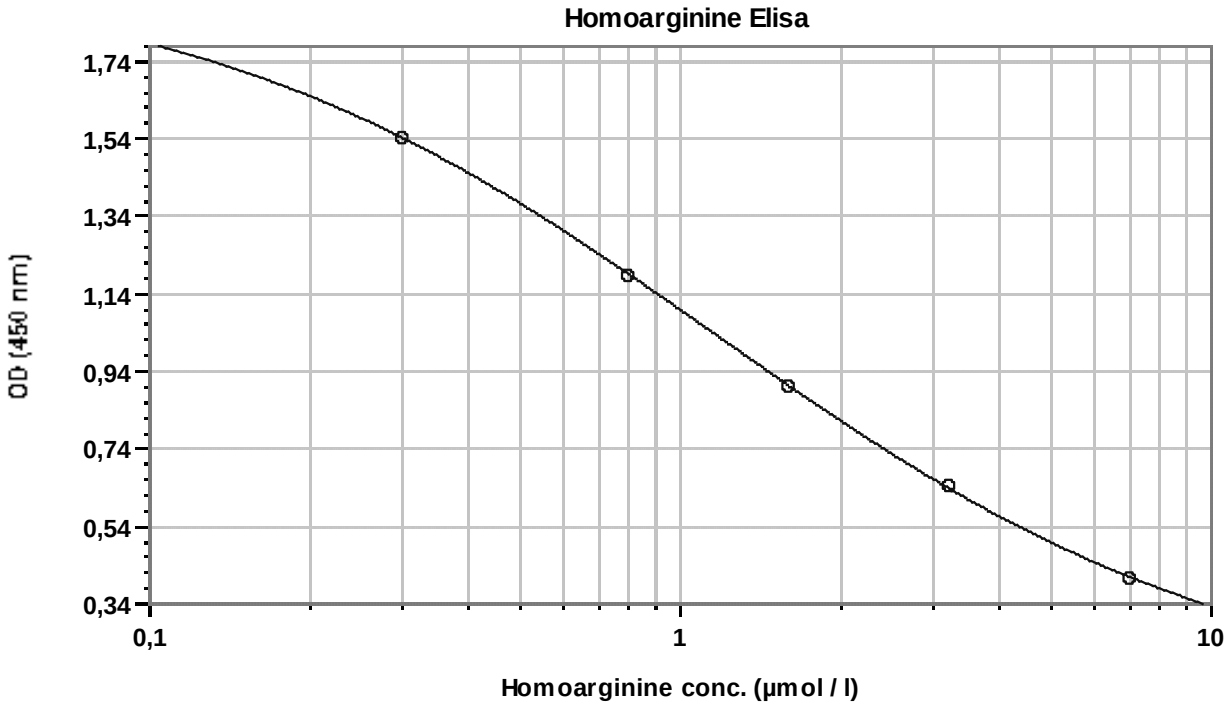
10. Pipette each 100 µl Stop Solution into all wells and mix briefly.

11. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

8. Calculation of the Results

On a semilogarithmic graph paper 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:



	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.

9. Assay Characteristics

Expected Values

The reference range given serves as a guideline. Each laboratory has to establish its own reference values.

matrix	reference range
EDTA-plasma, serum	2.0 ± 0.7 µmol / l

Sensitivity

lower limit of detection	calculation
0.05 µmol / l	OD_{Cal1} – 3 x SD

Specificity (Cross Reactivity)

substance	cross reactivity (%)
homoarginine	100
arginine	0.025
ADMA	< 0.025
SDMA	< 0.025
monomethylarginine (NMMA)	< 0.025

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

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

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 %

Method Comparison

matrix	method	correlation
EDTA-plasma	LC/MS	Y = 0.98 x LC/MS + 0.12; R = 0.998; N = 25

matrix	comparison	correlation
Serum	plasma	Y = 1.00 x plasma + 0.11; R = 0.965; N = 12

10. Literature

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Pipetting Scheme Sample Preparation Plasma and Serum

		Standard	Control	Plasma	Serum
Standard 1 - 6	µl	20			
Control 1 & 2	µl		20		
Plasma	µl			20	
Serum	µl				20
Acylation Buffer	µl	20	20	20	20
Equalizing Reagent	µl	200	200	200	200

shake for 10 seconds

Acylation Reagent	µl	50	50	50	50

immediately shake for 15 minutes at room temperature

take 20 µl for the ELISA

Pipetting Scheme ELISA for Plasma und Serum

		Standard	Control	Sample
Acyl. Standard	µl	20		
Acyl. Control	µl		20	
Acyl. Sample	µl			20
Antiserum	µl	50	50	50

cover plate with foil.
shake for 90 minutes at room temperature

wash 4 x

Enzyme Conj.	µl	100	100	100

shake for 25 minutes at room temperature

wash 4 x

Substrate	µl	100	100	100

shake for 25 ± 5 minutes at room temperature

Stop Solution	µl	100	100	100

Reading of absorbance at 450 nm

Pipetting Scheme Sample Preparation for Cell Culture Samples

		Standard	Control	Cell Culture Sample
Standard 1 - 6	µl	20		
Control 1 & 2	µl		20	
Cell Culture Sample	µl			20
Standard 1	µl			20
Cell Culture Medium	µl	20	20	
Acylation Buffer	µl	20	20	20
Equalizing Reagent	µl	200	200	200

shake plate for 10 seconds

Acylation Reagent	µl	50	50	50

immediately shake for 15 minutes at room temperature

take 20 µl for the ELISA

Pipetting Scheme ELISA for Cell Culture Samples

		Standard	Control	Sample
Acyl. Standard	µl	20		
Acyl. Control	µl		20	
Acyl. Sample	µl			20
Antiserum	µl	50	50	50

cover plate with foil.
shake for 90 minutes at room temperature

wash 4 x

Enzyme Conj.	µl	100	100	100

shake for 30 minutes at room temperature

wash 4 x

Substrate	µl	100	100	100

shake for 30 ± 5 minutes at room temperature

Stop Solution	µl	100	100	100

Reading of absorbance at 450 nm