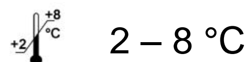
Instruction for use

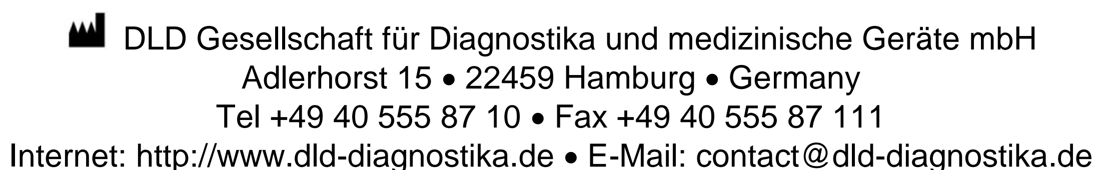
ADMA / Arginine ELISA

Enzyme Immunoassay
for the Quantitative Determination of
Endogenous Asymmetric Dimethylarginine (ADMA)
and L-Arginine in Serum and EDTA-Plasma

REF EA207/192

2 x 96


2 – 8 °C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1. Introduction and Principle of the Test

Nitric oxide (NO which is formed in the vascular endothelium plays a crucial role in the regulation of vascular structure and function. NO has been named an “endogenous anti-atherogenic molecule” due to its diverse regulatory functions in vascular homeostasis.

NO is formed by the enzyme NO synthase (NOS) from the amino acid precursor L-arginine. NOS activity is inhibited by asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS.

The effects of ADMA on NO synthesis and NO-mediated pathophysiological processes have been described in numerous experimental and clinical studies, including patients with hyper-cholesterolemia, hypertension, chronic heart failure, chronic renal failure and other internal disorders.

Elevated ADMA levels are a risk factor for future cardiovascular events and total mortality, as evidenced by prospective clinical studies comprising more than 10,000 participants. Thus, ADMA has diagnostic relevance as a novel cardiovascular risk marker.

Importantly, high ADMA levels and low L-arginine/ADMA ratio were both independent predictors of death in the community-based Framingham Offspring Study. As ADMA competes with L-arginine for binding to NO synthase, many scientists suggest that the L-arginine/ADMA ratio is a better index of NOS substrate availability and, thus, functional integrity of the NOS pathway, than L-arginine levels alone. Furthermore the measurement of both L-arginine and ADMA plasma concentrations is suitable for treatment surveillance of subjects during nutritional L-arginine supplementation.

The competitive ADMA-Arginine ELISA uses the microtiter plate format. Antigen is bound to the solid phase of the microtiter plate. Antigen in the samples is acylated and competes with solid phase bound antigen 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 ADMA and Arginine, respectively are detected by anti-rabbit/peroxidase. The substrate TMB / peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase antigen is inversely proportional to the antigen concentration of the sample.

2. Precautions

- For in vitro use only.
- Disposable gloves should be used.
- 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.

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-ADMA** 12 strips
8 wells each, break apart
precoated with ADMA; blue coloured

4.2 **MT-Strips** **STRIPS-ARG** 12 strips
8 wells each, break apart
precoated with L-Arginine; yellow coloured

4.3 **Standards 1 - 6** **CAL 1 – 6** 6 vials
Each 4 ml, ready for use
Concentrations:

Standard	1	2	3	4	5	6
ADMA µmol/l	0	0.2	0.45	0.7	1.0	3.0
Arginine µmol/l	5	15	35	70	120	300

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

4.5 **Acylation Buffer** ACYL-BUFF 1 bottle
3.5 ml, ready for use, blue coloured

4.6 **Acylation Reagent** ACYL-REAG 3 vials
lyophilised, dissolve content
in 3 ml Solvent before use; if required
combine the contents of the vials

4.7 **Antiserum ADMA** AS-ADMA 1 vial
7 ml, ready for use
Rabbit-anti-N-acyl-ADMA; blue coloured

4.8 **Antiserum Arginine** AS-ARG 1 vial
7 ml, ready for use
Rabbit-anti-N-acyl-Arginine; yellow coloured

4.9 **Enzyme Conjugate** CONJ 2 vials
13 ml, ready for use
Goat anti-rabbit-IgG-peroxidase

4.10 **Wash Buffer** WASH 2 bottles
20 ml, concentrated
Dilute content with dist. water to 1,000 ml total volume.

4.11 **Substrate** SUB 2 vials
13 ml TMB solution, ready for use

4.12 **Stop Solution** STOP 2 vials
13 ml, ready for use
Contains 0.3 M sulphuric acid, not corrosive

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

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

4.15 **Solvent** SOLVENT 2 vials
5 ml, contains DMSO
(please note that Solvent reacts with many plastic materials including plastic trays; Solvent does not react with normal pipette tips and with glass devices)

4.16 **Self-adhesive Foil** FOIL 4 pieces
ready for use

Additional materials and equipment required but not provided:

- Pipettes (10, 20, 25, 50, 100 and 200 µl)
- Multichannel pipette
- Orbital shaker
- Multipette
- Microplate washing device
- Microplate photometer (450 nm)
- Vortex mixer
- Roll mixer

5. Sample Collection

EDTA-Plasma and Serum

The test can be performed with EDTA plasma and serum.

Haemolytic 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

Repeated freezing and thawing should be avoided.

6. Preparation of Reagents and Samples

Microtiter strips **STRIPS-ADMA** **STRIPS-ARG**

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 of one bottle with dist. water to a total volume of 1,000 ml. The diluted wash buffer has to be stored at 2 - 8 °C for a maximum of 4 weeks. For storage 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 stable until expiry date of the kit.

6.4. Acylation Reagent **ACYL-REAG**

Dissolve the content of one bottle in 3 ml Solvent and shake for 10 minutes on a orbital shaker. The Acylation Reagent has always to be prepared immediately before use and is stable for 3 hours. The second and third bottles allow a second and third run, respectively, of the test. 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. After use the reagent has to be discarded.

Please note that Solvent reacts with many plastic materials including plastic trays. Solvent does not react with normal pipette tips and with glass devices.
It is recommended to use an Eppendorf multipette, or similar device. Fill the syringe directly from the vial (using a yellow tip) with dissolved Acylation Reagent and add well by well.

All other reagents are ready for use.

6.5. 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 to avoid repeated use.

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.
   Mix the reaction plate for 10 seconds.

4. Prepare Acylation Reagent just before use and pipette 50 µl prepared Acylation Reagent each into all wells, mix **immediately.**
   Colour changes to violet.
   It is recommended to use an Eppendorf multipette with a yellow tip (or similar device). Fill the syringe directly from the vial with dissolved Acylation Reagent and add well by well.

5. Incubate for 20 minutes at room temperature (approx. 20 °C) on an orbital shaker with medium speed.

Take each 25 µl for the ADMA-ELISA.

Take each 10 µl for the Arginine-ELISA.

7. Test Procedure ELISA

7.1. Preparation of Reagents

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

7.2 ADMA-ELISA

1. **Sample Incubation**
   Pipette each 25 µl prepared Standards 1 to 6, 25 µl prepared controls and 25 µl prepared samples into the respective wells of the coated microtiter strips (blue coloured; duplicates are recommended).

   Pipette each 50 µl ADMA-Antiserum **AS-ADMA** into all wells.

   Cover the plate with adhesive foil and incubate Microtiter Strips for 90 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium speed.

2. **Washing**
   Discard or aspirate the contents of the wells and wash thoroughly with each 300 µl prepared Wash Buffer (Shake shortly on an orbital shaker). Repeat the washing procedure 4 times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.

3. **Conjugate Incubation**
   Pipette each 100 µl enzyme conjugate into all wells.
   Incubate for 30 minutes at room temperature on an orbital shaker with medium speed.

4. **Washing**
   Repeat step 7.2.

5. **Substrate Incubation**
   Pipette each 100 µl Substrate into all wells and incubate for 25 ± 5 minutes at room temperature on an orbital shaker.

6. **Stopping**
   Pipette each 100 µl Stop Solution into all wells.

7. **Reading**
   Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

7.3 Arginine-ELISA

1. **Sample Incubation**
   Pipette each 10 µl prepared Standards 1 to 6, 10 µl prepared controls and 10 µl prepared samples into the respective wells of the coated microtiter strips (yellow coloured; duplicates are recommended).

   Pipette each 50 µl Arginine-Antiserum **AS-ARG** into all.

   Cover the plate with adhesive foil and incubate Microtiter Strips for 90 minutes at room temperature (20 – 25 °C) on an orbital shaker with medium speed.

2. **Washing**
   Discard or aspirate the contents of the wells and wash thoroughly with each 300 µl prepared Wash Buffer (Shake shortly on an orbital shaker). Repeat the washing procedure 4 times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.

3. **Conjugate Incubation**
   Pipette each 100 µl enzyme conjugate into all wells.
   Incubate for 30 minutes at room temperature on an orbital shaker with medium speed.

4. **Washing**
   Repeat step 7.2.

5. **Substrate Incubation**
   Pipette each 100 µl Substrate into all wells and incubate for 25 ± 5 minutes at room temperature on an orbital shaker.

6. **Stopping**
   Pipette each 100 µl Stop Solution into all wells.

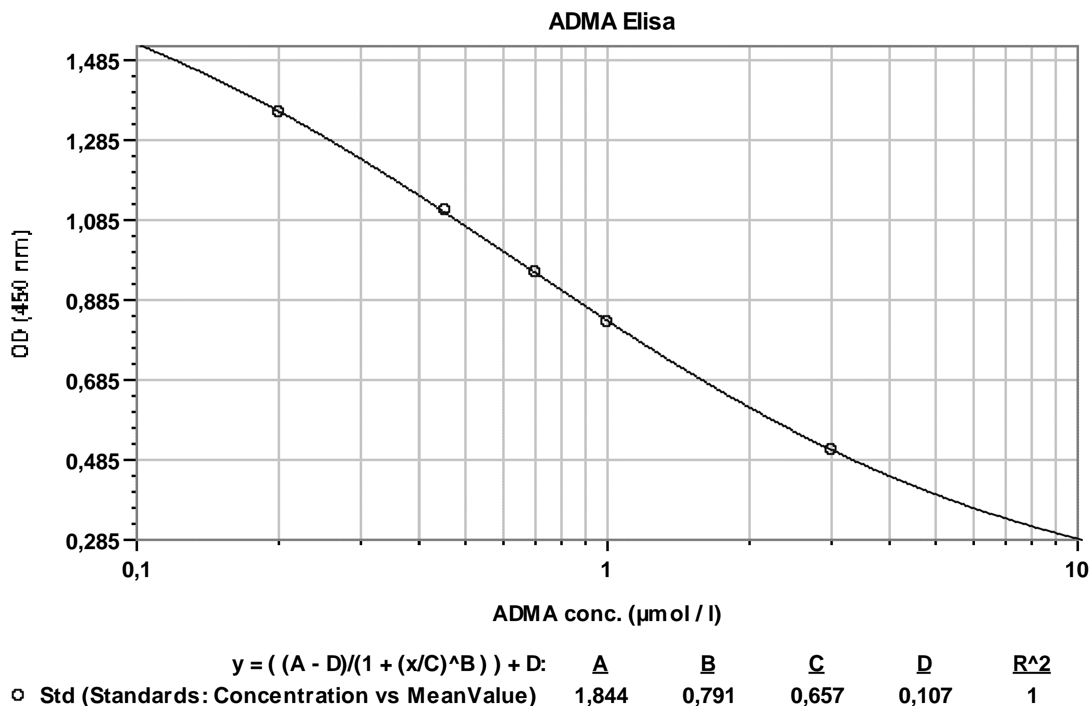
7. **Reading**
   Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

8. Calculation of the Results

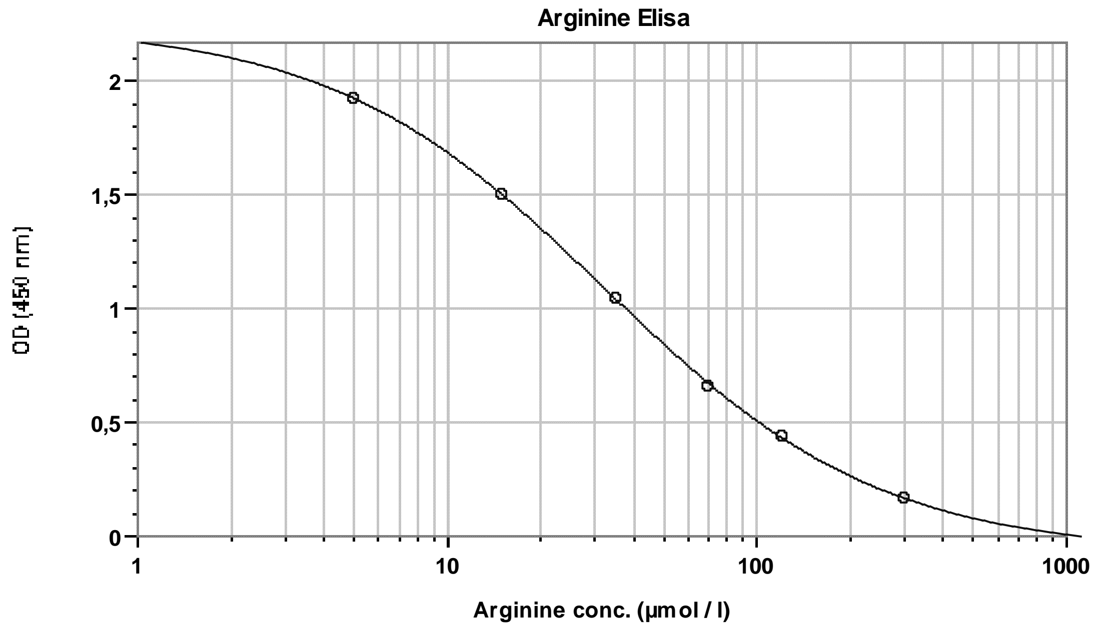
On a semilogarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Cubic spline, 4 parameter or similar iteration procedures are recommended for evaluation of the standard curve.
The concentration of the controls and samples can be read directly from this standard curve by using their average optical density.

Conversion: ADMA: 1 µmol / l = 202 ng / ml
Arginin: 1 µmol / l = 174 ng / ml

Typical Example for ADMA ELISA



Typical Example for Arginine ELISA



y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
o Std (Standards: Concentration vs MeanValue)	2,248	0,975	32,2	-0,071	1

9. Assay Characteristics

9.1 Test Characteristics ADMA

Reference Range

The reference ranges given above should only be taken as a guideline. It is recommended that each laboratory should establish its own reference values.

	Reference Range
EDTA-Plasma, Serum	0.40 – 0.75 µmol / l

Sensitivity

Lower Limit of Detection	**Calculation**
0.03 µmol / l	OD_{Cal1} – 3 x SD

Specificity (Cross Reactions)

Substance	**Cross Reactivity (%)**
ADMA	100
SDMA	0.05
Monomethylarginine (NMMA)	1.93
Homoarginine	< 0.01
Arginine	0.03

Recovery

	Range (µmol / l)	**Mean (%)**	**Range (%)**
EDTA-Plasma	0.43 – 1.55	99	90 - 107
Serum	0.54 – 1.72	92	87 - 102

Linearity

	Range (µmol / l)	**Highest Dil.**	**Mean (%)**	**Range (%)**
EDTA-Plasma	0,23 – 1,53	1 : 6 with water	99	92 - 105

Reproducibility

	Range (µmol / l)	**Intra-Assay-CV**
EDTA-Plasma	0.58 – 1.04	4.9 – 5.4 %

	Range (µmol / l)	**Inter-Assay-CV**
EDTA-Plasma	0.57 – 1.34	4.3 – 9.6 %

Method Comparison

	Method	**Correlation**
Serum + Plasma	LC/MS	Y = 0.99 x LC/MS + 0.02; R = 0.983; N = 32

9.2 Test Characteristics Arginine

Reference Range

The reference ranges given above should only be taken as a guideline. It is recommended that each laboratory should establish its own reference values.

	Reference Range
EDTA-Plasma, Serum	20 – 80 µmol / l

Sensitivity

Lower Limit of Detection	Calculation
6 µmol / l	OD_{Cal1} – 3 x SD

Specificity (Cross Reactions)

Substance	Cross Reactivity (%)
Arginine	100
ADMA	< 0.37
Homoarginine	2.92
SDMA	0.88

Recovery

	Range (µmol / l)	Mean (%)	Range (%)
EDTA-Plasma	48 – 163	97	93 - 100
Serum	82 – 211	100	96 - 103

Linearity

	Range (µmol / l)	Highest Dil.	Mean (%)	Range (%)
EDTA-Plasma	28 – 193	1 : 6 with water	102	94 - 106

Reproducibility

	Range (µmol / l)	Intra-Assay-Vk
EDTA-Plasma	56 – 125	3.6 – 2.3 %

	Range (µmol / l)	Inter-Assay-Vk
EDTA-Plasma	53 – 170	3.2 – 6.3 %

Method Comparison

	Method	Correlation
Serum + Plasma	LC/MS	Y = 0.95 x LC/MS - 0.68; R = 0.991; N = 32

10. Literature

Literature using the ADMA-ELISA from DLD Diagnostika

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The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor
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Plasma levels of asymmetrical dimethylarginine and adverse cardiovascular events after percutaneous coronary intervention.
Eur Heart J. 2003; 24: 1912-1919

Pipetting Scheme
Sample Preparation
(ADMA and Arginine)

		Standards	Control	Sample
Standard 1 - 6	µl	20		
Control 1 & 2	µl		20	
Patient Sample	µl			20
Acylation Buffer	µl	20	20	20
Equalizing Reagent	µl	200	200	200

shake for 10 seconds

freshly prepared Acylation Reagent	µl	50	50	50

mix immediately and incubate for 20 minutes
at room temperature on an orbital shaker

Take each 25 µl for the ADMA-ELISA.

Take each 10 µl for the Arginine-ELISA.

Pipetting Scheme ADMA-ELISA

		Standard	Control	Patient-Sample
Standard 1 - 6	µl	25		
Control 1 & 2	µl		25	
Patient Sample	µl			25
Antiserum	µl	50	50	50

cover plate with foil and incubate 90 minutes at room temperature

wash 4 x with each 300 µl Wash Buffer

Enzyme Conjugat	µl	100	100	100

shake for 30 minutes at room temperature

wash 4 x with each 300 µl Wash Buffer

Substrate	µl	100	100	100

shake for 25 ± 5 minutes at room temperature

Stop Solution	µl	100	100	100

read absorbance at 450 nm

Pipetting Scheme Arginine-ELISA

		Standard	Control	Patient-Sample
Standard 1 - 6	µl	10		
Control 1 & 2	µl		10	
Patient Sample	µl			10
Antiserum	µl	50	50	50

cover plate with foil and incubate 90 minutes at room temperature

wash 4 x with each 300 µl Wash Buffer

Enzyme Conjugat	µl	100	100	100

shake for 30 minutes at room temperature

wash 4 x with each 300 µl Wash Buffer

Substrate	µl	100	100	100

shake for 25 ± 5 minutes at room temperature

Stop Solution	µl	100	100	100

read absorbance at 450 nm