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Research
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ADMA ELISA

Cat. No.: REA201/96

IVD CE

Enzyme Immunoassay for the Quantitative Determination of Endogenous Asymmetric Dimethylarginine (ADMA) in Serum or Plasma

1. Introduction and principle of the Test

The vascular endothelium plays a central role in the regulation of vascular structure and function, mainly due to the formation of endothelium-derived nitric oxide (NO). 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 synthetase (NOS) from the amino acid precursor L-arginine. NOS activity can be downregulated 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 studies. Moreover, elevated ADMA levels in plasma have been found in clinical studies including patients with hypercholesterolemia, hypertension, chronic heart failure, chronic renal failure and other internal disorders.

Recent prospective and cross-sectional studies indicated that elevated ADMA levels are a risk factor for future cardiovascular events and total mortality. ADMA may have diagnostic relevance as a novel cardiovascular risk marker.

The competitive ADMA-ELISA uses the microtiter plate format. ADMA is bound to the solid phase of the microtiter plate. ADMA in the samples is acylated and competes with solid phase bound ADMA for a fixed number of rabbit anti-ADMA 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 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 ADMA is inversely proportional to the ADMA 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 th e 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 ADMA

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	0.1	0.3	0.6	1.0	5.0

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 bottle

3,5 ml, ready for use

4.5 **Acylation Reagent** **ACYL-REAG** 2 vials
lyophilised, dissolve content
in 2.8 ml Solvent before use; if required
combine the contents of both vials (see also 6.4.)

4.6 **Antiserum** **AS** 1 vials
5.5 ml, ready for use
Rabbit-anti-N-acyl-ADMA

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

4.8 **Wash Buffer** **WASH** 1 bottle
20 ml, concentrated
Dilute content with dist. water to 500 ml total volume.

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

4.10 **Stop Solution** **STOP** 1 vial
lyophilzed, dissolve content with 20.5 ml dist. water,
dissolve carefully to minimize foam formation

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

4.12 **Equalizing Reagent** **EQUA-REAG** 1 vial
lyophilzed, dissolve content with 5 ml dist. water,
dissolve carefully to minimize foam formation

4.13 **Solvent** **SOLVENT** 1 vial
6 ml, contains acetone/ DMSO
(please note that Solvent reacts with many plastic materials including plastic trays; Solvent does not react with normal pipete tips and with glass devices)

Additional materials and equipment required but not provided:

- Pipettes (20, 25, 50, 100 and 250 µl,)
- Orbital shaker
- Microplate washing device
- Microplate photometer (450 nm)
- Vortex mixer
- Roll mixer

5. Sample Collection

5.1 Serum and Plasma

The test can be performed with serum as well as with EDTA plasma.
Hemolytic and lipemic samples should not be used.
The samples can be stored up to 24 hours at 2 - 8 °C. For a longer storage (up to 24 months) the samples must be frozen at -20 °C.
Repeated freezing and thawing should be avoided.

6. Preparation of Reagents and Samples

6.1 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.

6.2 Wash Buffer **WASH**

Dilute the content with dist. water to a total volume of 500 ml.
The diluted wash buffer has to be stored at 2 - 8 °C.

6.3 Equalizing Reagent **EQUA-REAG**

Dissolve the content with 20.5 ml dist. water, mix shortly and leave on a roll mixer or orbital shaker for 30 minutes. Handle carefully in order to minimize foam formation. The reconstituted Equalizing Reagent should be stored frozen at -20 °C and is stable for a minimum of 1 year.

6.4 Acylation Reagent ACYL-REAG

Dissolve the content of one bottle in 2.8 ml Solvent and shake for 5 minutes on a orbital shaker. After use the reagent has to be discarded.

The Acylation Reagent has always to be prepared immediately before use. The second bottle allows a second run of the test. If the whole kit is to be used in one run it is recommended to pool the dissolved contents of the two vials of Acylation Reagent.

Please note that Solvent reacts with many plastic materials including plastic trays. Solvent does not react with normal pipette tips and with glass devices.

Attention

Solvent is volatile and the dissolved Acylation Reagent evaporates quickly. Therefore, please do not use a tray with big surface together with a multichannel pipette for pipetting Acylation Reagent. Rather, 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 25 µl Acylation Buffer into all wells.
3. Pipette 200 µl Equalizing Reagent into all wells.
4. Mix the reaction plate for 10 seconds.
5. Prepare Acylation Reagent just before use and pipette 50 µl prepared Acylation Reagent each into all wells, mix immediately.

   Attention

   Solvent is volatile and the dissolved Acylation Reagent evaporates quickly. Therefore, please do not use a tray with big surface together with a multichannel pipette for pipetting Acylation Reagent.

   Rather, 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.
6. Incubate for 90 minutes at room temperature (approx. 20 °C) on an orbital shaker. Do not cover the wells or the plate; leave the plate open on the shaker.

Take each 50 µl for the ADMA-ELISA.

7. Test procedure ELISA

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

7.1 Sample Incubation

Pipette each 50 µl prepared Standards 1 to 6, 50 µl prepared controls and 50 µl prepared samples into the respective wells of the coated microtiter strips (duplicates are recommended).
Pipette each 50 µl Antiserum into all wells and shake shortly on an orbital shaker.
Cover the plate with adhesive foil and incubate Microtiter Strips for 15 –20 hours (overnight) at 2 – 8 °C.

7.2 Washing

Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl 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.

7.3 Conjugate Incubation

Pipette each 100 µl enzyme conjugate into all wells.
Incubate for 60 minutes at room temperature on an orbital shaker.

7.4 Washing

Repeat step 7.2.

7.5 Substrate Incubation

Pipette each 100 µl Substrate into all wells and incubate for 20 to 30 minutes at room temperature on an orbital shaker.

7.6 Stopping

Pipette each 100 µl Stop Solution into all wells.

7.7 Reading

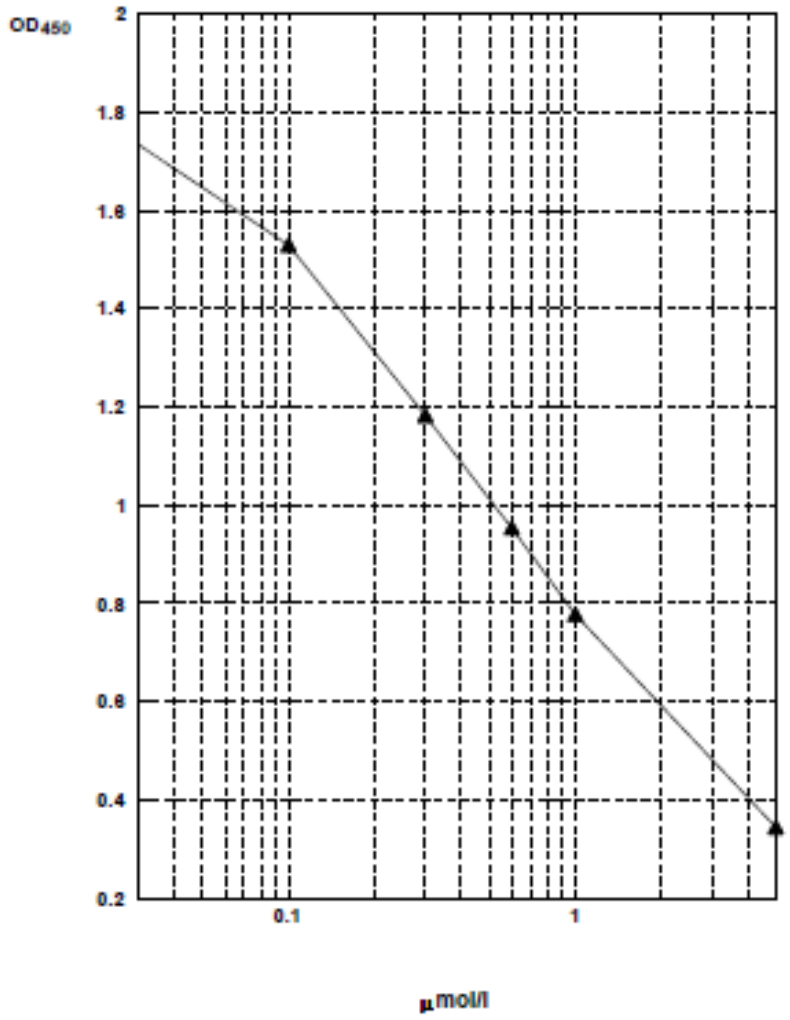
Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

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:



9. Assay Characteristics

Expected Values

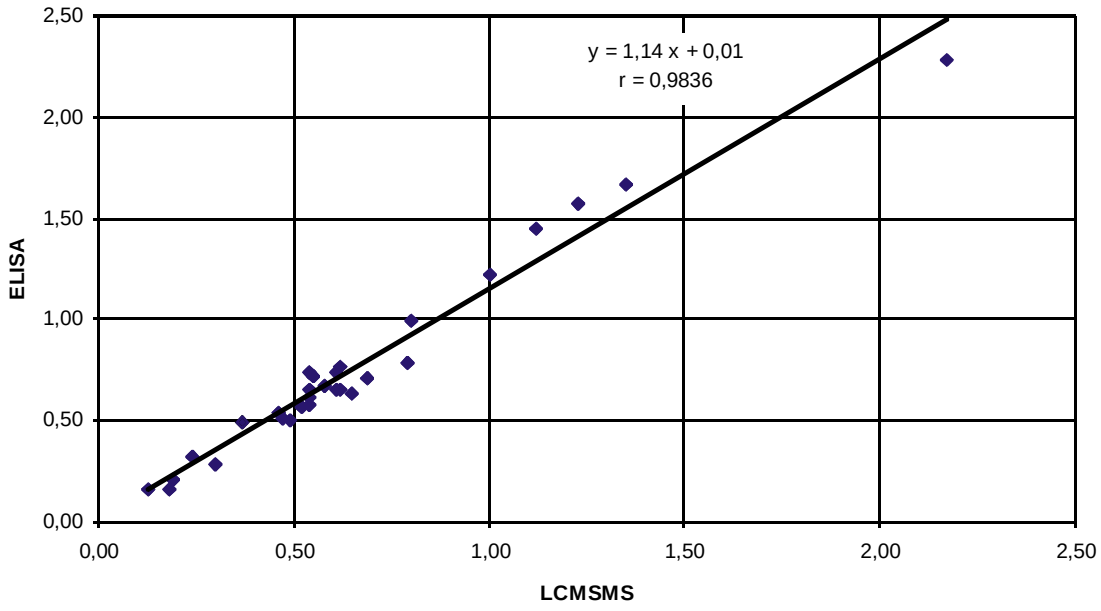
0,4 – 0,75 µmol/l (80 – 150 ng/ml)

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

Sensitivity

0.05 µmol/l

Correlation to LC-MS-MS



The figure shows the correlation to the LC-MS-MS method. More data with higher number of patient samples are evaluated at the moment. Comparative investigations to HPLC without subsequent MS lead to inconsistent results.

Specificity (Cross Reactivity)

Structural related components were tested for possible interference with the antisera against ADMA used in the ELISA method. The tested compounds were Arginine, Monomethylarginine (NMMA) und SDMA.

Substance	ED-50-Value (ng/ml)	Cross Reactivity (%)
ADMA	126	100
Arginin	660,000	< 0.02
NMMA	12,200	1.0
SDMA	10,500	1.2

Recovery

Increasing amounts of ADMA were added to a serum sample. Each spiked sample was assayed. The analytical recovery of ADMA was estimated at twelve different concentrations by using the theoretically expected and the actually measured values. The mean recovery from all concentrations was 97% (90 - 104%).

added	measured	expected	recovery %
0	0.43		
0.1	0.55	0.53	104
0.19	0.61	0.62	98
0,28	0.67	0.71	94
0.37	0.79	0.80	99
0.45	0.83	0.88	97
0.73	1.08	1.16	93
0.97	1.33	1.40	95
1.20	1.60	1.63	98
1.42	1.67	1.85	90
1.92	2.24	2.35	95
2.33	2.76	2.76	100
2.80	3.32	3.23	103
	mean value		**97**

concentrations in µmol/l

Linearity

The linearity of the ELISA method was investigated using nine different dilutions of a serum sample. The mean linearity from all dilutions was 97% (88 - 112%)

dilution	measured	recalculated value	recovery %
orig.	2.819		
3 + 1	2.090	2.787	99
2 + 1	1.745	2.618	93
1 + 1	1.236	2.472	88
1 + 2	0.860	2.580	91
1 + 4	0.535	2.675	95
1 + 7	0.361	2.888	102
1 + 10	0.286	3.146	112
1 + 15	0.178	2.848	101
1 + 20	0.104	2.704	96
		mean recovery	97

concentrations in µmol/l

Reproducibility

The reproducibility of the ELISA method was investigated by determing the intra- und inter-assay-coefficients of variation (cv) by repeated measurements of different serum samples with different ADMA concentrations.

Intra-Assay Variation

sample	n =	mean value	sd	cv (%)
1	42	0.66	0.037	5.7
2	42	1.01	0.066	6.4

Inter-Assay Variation

sample	n =	mean value	sd	cv (%)
1	28	0.63	0.07	10.3
2	28	1.01	0.10	9.8
3	28	1.38	0.13	9.4
4	28	2.26	0.19	8.3

10. Reference

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Pipetting Scheme
Sample Preparation

		Standards	Control	Sample
Standard 1 -6	µl	20		
Control 1 & 2	µl		20	
Patient Sample	µl			20
Acylation Buffer	µl	25	25	25
Equalizing Reagent	µl	200	200	200

10 seconds shaking

freshly prepared Acylation Reagent	µl	50	50	50

incubate for 90 minutes at room temperature on an orbital shaker

do not cover wells or plate, leave the plate open on the shaker

Pipetting Scheme ELISA

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

shake shortly on an orbital shaker

incubate 15 – 20 hours (overnight) at 2 - 8 °C cove red with foil

wash 4 x with each 250 µl Wash Buffer

Enzyme Conjugat	µl	100	100	100

shake for 60 minutes at room temperature

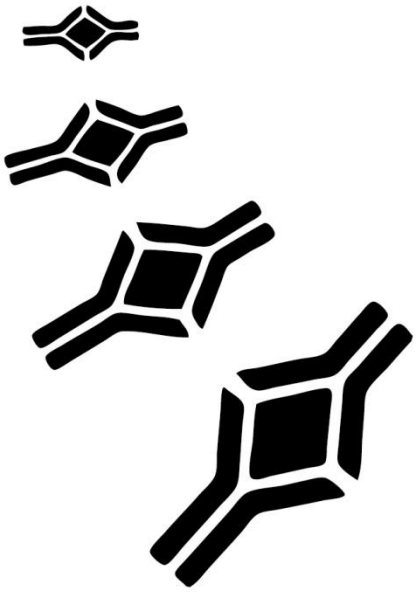
wash 4 x with each 250 µl Wash Buffer

Substrate	µl	100	100	100

shake for 20 - 30 minutes at room temperature

Stop Solution	µl	100	100	100

read absorbance at 450 nm



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