Manual

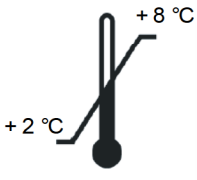
ADMA ELISA Kit

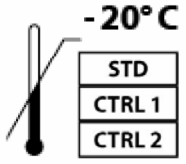
For the determination of ADMA in EDTA-plasma and serum of rodents during experimental studies and in cell culture media

For research use only

Valid from 09.12.2009

K 3001





1. INTENDED USE

The ADMA ELISA Kit is intended for the quantitative determination of asymmetric dimethylarginine (ADMA) in rodent EDTA-plasma or serum and in cell culture media. It is for *research* use only.

2. INTRODUCTION

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO-synthase. It is formed during proteolysis of methylated proteins and removed by renal excretion or metabolic degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Several cell types, including human endothelial and tubular cells are capable of synthesizing and metabolizing ADMA. Elevated ADMA concentrations in the blood are found in numerous diseases associated with endothelial dysfunction. For example, elevated ADMA levels in blood of dialysis patients correlate significantly with the degree of arteriosclerosis and cardiovascular risk. Furthermore, elevated ADMA levels are found in patients with hypercholesterolemia, hypertension, arteriosclerosis, chronic renal failure and chronic heart failure, and are associated with restrictions in endothelial vasodilatation.

During the last years, the important clinical relevance of the regulation of vascular tone and structure by nitric oxide (NO) has been shown. Moreover, there were reports that human endothelial cells produce ADMA as well as nitric oxide, which points to an endogenous endothelial NO-regulation by ADMA. Therefore it was assumed that hypertension, arteriosclerosis and immunological dysfunction in patients with chronic renal failure are connected to a dysfunction of the L-arginin/NO-metabolism and to ADMA accumulation. The reasons for the deregulation of the L-arginin/NO-metabolism could only partially be elucidated. Certainly, there are multiple factors involved in the L-arginin/NO-metabolism regulation as for example elevation of free superoxide radicals (O_2^-), ADMA accumulation and reduced NO-synthase activity.

Prospective clinical studies of the last years demonstrate the increased importance of ADMA as a novel cardiovascular risk factor.

Indication

- Arteriosclerosis
- Hypertension
- Chronic heart failure
- Coronary artery disease
- Hypercholesterolemia
- Chronic renal failure
- Diabetes mellitus
- Peripheral arterial occlusive disease

3. PRINCIPLE OF THE TEST

This assay is based on the method of competitive enzyme linked immunoassays. The sample preparation includes the addition of a derivatization-reagent for ADMA coupling. Afterwards, the treated samples and the polyclonal ADMA-antiserum are incubated in wells of microplate coated with ADMA-derivative (tracer). During the incubation period, the target ADMA in the sample competes with the tracer immobilized on the wall of the microtiter wells for the binding of the polyclonal antibodies. The ADMA in the sample displaces the antibodies out of the binding to the tracer. Therefore the concentration of the tracer-bound antibody is inverse proportional to the ADMA concentration in the sample. During the second incubation step, a peroxidase-conjugated antibody is added to each microtiter well to detect the anti-ADMA antibodies. After washing away the unbound components, tetramethylbenzidine (TMB) is added as a substrate for peroxidase. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color is inverse proportional to the ADMA concentration in the sample; this means high ADMA concentration in the sample reduces the concentration of tracer-bound antibodies and lowers the photometric signal.

A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standard. ADMA present in the patient samples is determined directly from this curve.

4. MATERIAL SUPPLIED

Catalog No	Content	Kit Components	Quantity
K3001MTP	PLATE	One holder with precoated strips	12 x 8 wells
K3001ST	STD	Standards (diluted in reaction buffer)	6 x 1 vial
K3001KO	CTRL 1+ CTRL 2	Controls (diluted in reaction buffer)	2 x 1 vial
K3001WP	WASHBUF	Wash buffer concentrate (10 fold)	2 x 100 ml
K3001AK	AB	ADMA antibody (lyophilized)	2 x 1 vial
K3001K	2.AB	POD antibody (concentrate)	120 µl
K3001CSP	2.ABDIL	Conjugate stabilizing buffer	24 ml
K3001RP	DERBUF	Reaction buffer	25 ml
K3001DR	DER	Derivatization reagent	2 x 50 mg
K3001LM	DMSO	Dimethylsulfoxid (DMSO)	7 ml
K3001SL	CODIL	Dilution buffer for coupling	28 ml
K3001TMB	SUB	TMB substrate	25 ml
K3001AC	STOP	Stop solution	15 ml
K3001MC	CFD	Centrifugal filter device	40 devices

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Bidistilled water (aqua bidist.)
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 14000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm
  (reference wave length 620 or 690 nm)

6. PREPARATION AND STORAGE OF REAGENTS

- To run assay more than once, ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each assay**. The kit can be used up to 2 times within the expiry date stated on the label.

- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.

- The **Wash buffer concentrate (WASHBUF)** should be diluted with aqua bidist. **1:10** before use (100 ml concentrate + 900 ml aqua bidist.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or at 37°C using a water bath before dilution of the buffer solutions. The **buffer concentrate** is stable at **2-8°C** until the expiry date stated on the label. Diluted **buffer solution** can be stored in a closed flask at **2-8°C for one month**.

- **Standards (STD) and Controls (CTRL1, CTRL2)** are already diluted in the **reaction buffer (DERBUF).** Store Standards and Controls frozen at -20°C, thaw before use in the test, and re-freeze immediately after use. Standards and Controls can be re-frozen up to 3 times. Standards and controls must not be ultrafiltrated.

- **DMSO** could crystallize at 4°C. Dissolve the crystals at 20-25°C in a water bath.

- The content of one vial of **derivatization reagent (DER) (50 mg)** must be dissolved in **3 ml DMSO.** Put the vial on a horizontal shaker for 5 min. After use, the rest of the reagent should be discarded. DER must be **prepared immediately before use**. The ELISA kit can be separated into two performances by the two DER vials. Please note: **DMSO attacks all plastics but not polypropylene products and laboratory glass.**

- The content of one vial with **ADMA antibody (AB)** must be dissolved in **5.6 ml of diluted wash buffer**. Therefore, at first, the content of one AB vial is reconstituted with 0.6 ml of diluted wash buffer for 5 minutes. Then the obtained AB solution is quantitatively transferred into a separate vial and 5 ml of diluted wash buffer are added. The ELISA kit can be separated into two performances by the two AB vials. **Diluted ADMA antibody (AB) is stable over a longer period of time. It can be stored at 2-8°C for 4 weeks.**

- The **POD antibody (2.AB)** must be diluted **1:200** in **conjugate stabilizing buffer (2.ABDIL)** (110 µl 2.AB + 22 ml 2.ABDIL). The undiluted POD antibody (2.AB) is stable at **2-8°C** until the expiry date stated on the label. **Diluted POD antibody (2.AB) is not stable over a longer period. It can be stored at 2-8°C for only 5 days.**

- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2-8°C.

7. PRECAUTIONS

- For *research* use only.
- Human materials used in kit components were tested and found to be negative for HIV and Hepatitis B. However, for safety reasons, all kit components should be treated as if potentially infectious.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

EDTA-plasma and serum from animal studies and cell culture media

- Venous fasting blood is suited for this test system. Samples are stable for one week at 2-8°C. For longer storage samples should be frozen at –20°C up to the measurement.
- Lipemic or hemolytic samples may give erroneous results and should not be used for analysis.
- Plasma, serum and cell culture medium must be ultrafiltrated before analyzing.

  For sample preparation, a DER for coupling of ADMA is added (details are given in the sample preparation procedure).

9. ASSAY PROCEDURE

Procedural notes

- Quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results. Immundiagnostik AG can therefore not be held responsible for any damage resulting from this.
- The assay should always be performed according to the enclosed manual.

Sample preparation procedure

Coupling of standards (STD), controls (CTRL) and samples (SAMPLE) are carried out in single analysis.

1.	Bring all reagents and samples to room temperature (18-26°C)
2.	Pipette 100 µl of sample (SAMPLE) into a centrifugal filter device (CFD) and add 300 µl of reaction buffer (DERBUF)
3.	Centrifuge for 20-30 min at 14000 x g and 4°C until 250-300 µl filtrate is collected; the residual solution remains in the centrifugal filter device

4.	Dilute 200 µl filtrate with 200 µl DERBUF
5.	Add 200 µl of ready to use standards (STD), 200 µl of ready to use controls (CTRL) and 200 µl of the filtrated and diluted samples (SAMPLE) in the corresponding vial
6.	Add **50 µl** of freshly prepared **derivatization reagent (DER)** into each vial (standards, controls and samples), mix well and incubate **for 45 min on a shaker (180-240 rpm) at room temperature (18-26°C)**
7.	Afterwards add **250 µl of dilution buffer (CODIL)** into each vial, mix well and incubate for **45 min on a shaker (180-240 rpm) at room temperature (18-26°C)**

2 x 100 µl of each treated sample (STD, CTRL, SAMPLE) are used in the ELISA in duplicate.

Test procedure

8.	Mark the positions of **standards (STD)/controls (CTRL)/ samples (SAMPLE)** in duplicate on a protocol sheet
9.	Take as many microtiter strips (PLATE) as needed from kit. Store unused strips covered at 2-8°C. Strips are stable until the expiry date stated on the label
10.	Wash each well **5 times** by dispensing **250 µl** of diluted **wash buffer** into each well. After the final washing step, the inverted microtiter plate (PLATE) should be firmly tapped on absorbent paper to remove excess solution
11.	For the analysis in duplicate, take **2 x 100 µl of standard (STD)/control (CTRL)/samples (SAMPLE)** out of the vial and add into the respective well of the microtiter plate (PLATE)

12.	Add **100 µl** diluted **ADMA antibody (AB)** into each well. Cover the plate tightly
13.	Incubate overnight **(15-20 hours) at 2-8°C**
14.	Aspirate the contents of each well. Wash each well **5 times** by dispensing **250 µl** of diluted **wash buffer** into each well. After the final washing step, the inverted microtiter plate (PLATE) should be firmly tapped on absorbent paper to remove excess solution
15.	Add **200 µl** diluted **POD antibody (2. AB)** into each well
16.	Cover plate tightly and incubate for **1 hour at room temperature (18-26°C)** on a horizontal shaker (180-240 rpm)
17.	Aspirate the contents of each well. Wash each well **5 times** by dispensing **250 µl** of diluted **wash buffer** into each well. After the final washing step, the inverted microtiter plate (PLATE) should be firmly tapped on absorbent paper to remove excess solution
18.	Add **200 µl** of **TMB substrate (SUB)** into each well
19.	Incubate for **6-10 min at room temperature (18-26°C)** in the dark*
20.	Add **100 µl of stop solution (STOP)** into each well, mix thoroughly
21.	Determine absorption **immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference

*The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation

10. EVALUATION OF RESULTS

If the test is performed in strict compliance with the manufacturer's instructions, e.g. with the exact volumes for standards, controls and samples/sample treatment a **dilution factor of 2 is required for calculation of the results.**

The following algorithms can be used alternatively to calculate the results. We recommend to use the "4-parameter-algorithm".

1. 4-parameter-algorithm

   It is recommended to use a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.01).

2. Point-to-point-calculation

   We recommend a linear ordinate for optical density and a linear abscissa for concentration.

3. Spline-algorithm

   We recommend a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.01).

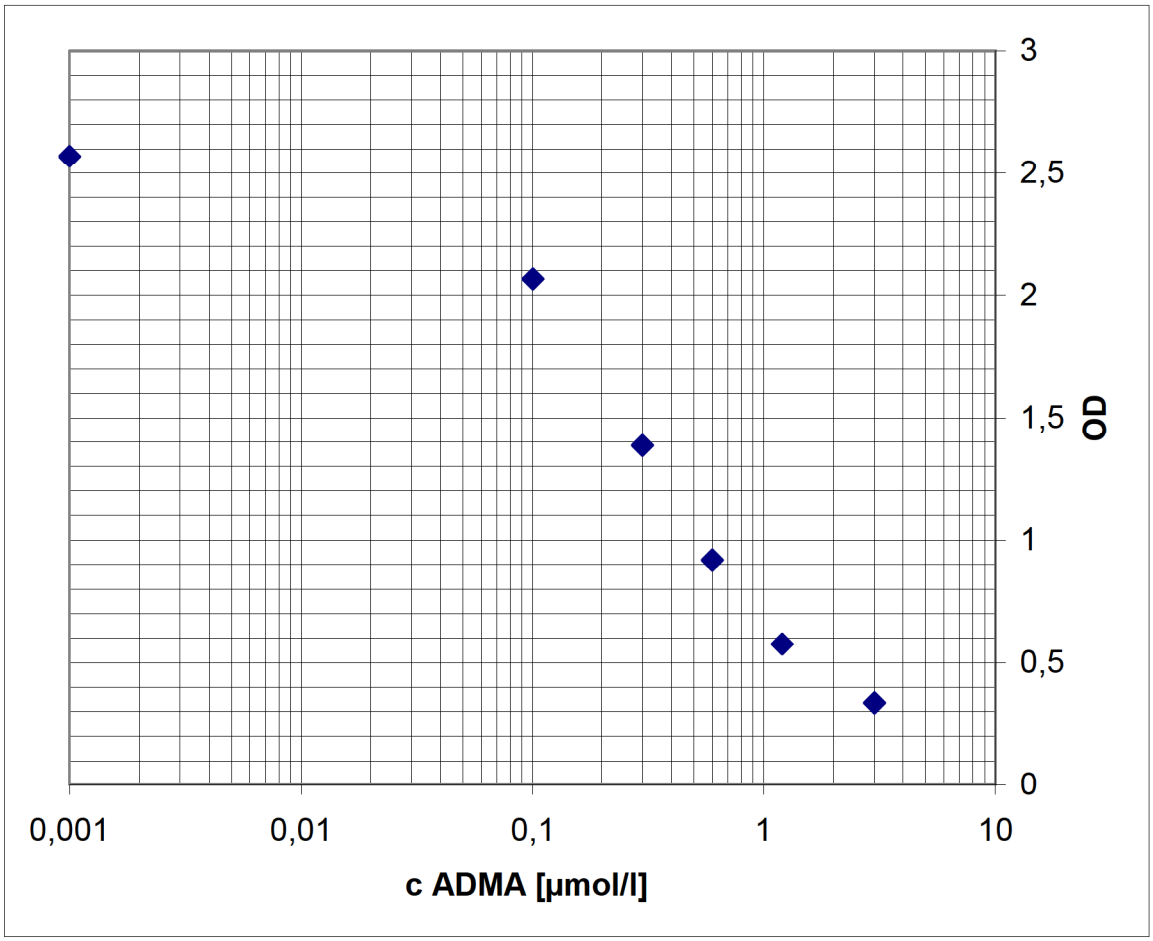
   The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Controls

Control samples or serum pools should be analyzed with each run. Results, generated from the analysis of the control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

The concentration of controls and patient samples can be determined directly from calibration curve. In the following an example of a calibration curve is given.

Example of a calibration curve



11. PERFORMANCE CHARACTERISTICS

Cross reactivity

SDMA < 0,5 % NMMA < 0,5 % L-Arginin < 0,02 %

Precision and reproducibility

Serum

Intra-Assay (n=4)		
sample	ADMA [µmol/l]	Standard variation (SD) [%]
1	0,33	7
2	0,67	6,5

Inter-Assay (n=4)		
sample	ADMA [µmol/l]	Standard variation (SD) [%]
1	0,34	7
2	0,67	6,5

Cell culture media

Intra-Assay (n=4)		
sample	ADMA [µmol/l]	Standard variation (SD) [%]
1	0,54	6,6
2	1,02	4,8

Inter-Assay (n=4)		
Probe	ADMA [µmol/l]	Standard variation (SD) [%]
1	0,54	7,7
2	0,99	5,5

Sensitivity

The sensitivity was set as B_0 + 1SD. The zero-standard was measured 6 times.

Sample	ADMA mean value [OD]	Standard variation (SD) [%]	Detection limit [µmol/l]
0	2,45	0,08	0,05

Recovery

EDTA-Plasma and serum

One serum sample was spiked with different ADMA concentrations and measured using this assay. The analytical recovery rate was determined by the expected and measured ADMA levels. The expected levels were calculated as the sum of the measured ADMA concentration in the original sample and the spiked ADMA amount. The mean recovery rate for all concentrations was 96 % (n=4).

Spike [µmol/l]	ADMA expected [µmol/l]	ADMA measured [µmol/l]	Recovery [%]
0	x	x=0,55	100
0,5	0,5+x=1,05	0,978	93
1,0	1,0+x=1,55	1,41	91

Cell culture media

One sample of cell culture medium was spiked with different ADMA concentrations and measured using this assay. The analytical recovery rate was determined by the expected and measured ADMA levels. The expected levels were calculated as the sum of the measured ADMA concentration in the original sample and the spiked ADMA amount. The mean recovery rate for all concentrations was 103 % (n=4).

Spike [µmol/l]	ADMA expected [µmol/l]	ADMA measured [µmol/l]	Recovery [%]
0	x	x=0,0	100
0,5	0,5+x=0,5	0,55	110
1,0	1,0+x=1,0	0,98	98

Linearity

Serum

The linearity of the ELISA was determined by the dilution of a spiked patient sample. The mean linearity was 96%.

Dilution	Measured [µmol/l]	Expected [µmol/l]	Recovery [%]
original	1,513	1,513	100
1+1	0,713	0,756	94
1+3	0,360	0,378	95

Cell culture media

The linearity of the ELISA was determined by the dilution of a spiked patient sample. The mean linearity was 92 %.

Dilution	Measured [µmol/l]	Expected [µmol/l]	Recovery [%]
original	0,98	0,98	100
1+1	0,44	0,49	90
1+3	0,21	0,245	86

12. LIMITATIONS

Strong hemolytic and lipemic samples often show wrong concentrations. Do not to measure hemolytic and lipemic samples.

13. REFERENCES

1. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of NO synthesis in chronic renal failure. Lancet 1992; 339: 572 – 575
2. Kielstein JT, Böger RH, Bode-Böger SM, et al. Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: Relationship to treatment method and atherosclerotic disease. J. Am. Soc. Nephrol. 1999; 10: 594 – 600
3. Böger RH, Bode-Böger SM, Szuba A, Tangphao O, Tsao PS, Chan JR, Blaschke TF, Cooke JP. Asymmetric dimethylarginine: a novel risk factor for endothelial dysfunction. Its role in hypercholesterolemia. Circulation 1998; 98: 1842 – 1847
4. Stühlinger M, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP, Reaven GM, Tsao PS. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. J. Am. Med. Assoc. 2002; 287: 1420-1426
5. Zoccali C, Bode-Böger SM, Mallamaci F, Benedetto FA, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frölich JC, Böger RH. Asymmetric dimethylarginine (ADMA): An endogenous inhibitor of nitric oxide synthase predicts mortality in end-stage renal disease (ESRD). Lancet 2001; 358: 2113-2117
6. Nijveldt RJ, Teerlink T, Van der Hoven B, Siroen MP, Kuik DJ, Rauwerda JA, van Leeuwen PA. Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality. Clin. Nutr. 2003; 22: 23-30
7. Savvidou MD, Hingorani AD, Tsikas D, Frolich JC, Vallance P, Nicolaides KH. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. Lancet 2003; 361: 1511-1517
8. Böger RH. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc. Res. 2003; 59: 824-833
9. Lu TM, Ding YA, Lin SJ, Lee WS, Tai HC. Plasma levels of asymmetrical dimethylarginine and adverse cardiovascular events after percutaneous coronary intervention. Eur Heart J. 2003; 24: 1912-1919.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- Test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the test package are for research use only.
- Reagents should not be used after the date of expiry stated on the label.
- Single components with different lot numbers should not be mixed or exchanged.
- Guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.

18.12.2009

Used symbols:

Temperature limitation

REF Catalogue Number

RUO For research use only

Contains sufficient for <n> tests

Manufacturer

Use by

LOT Lot number