DIAGNOSTIKA GMBH

## 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

**R U O**
For Research Use Only
Not for Use in Diagnostic Procedures

REF EA207/192

2 x 96

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

## Contents



### Symbols

| | | | |
|---|---|---|---|
| R U O | For Research Use Only | | |
| CONT | Contents | | Expiry Date |
| LOT | Lot Number | | Store |
| | Manufactured by | Σ | Sufficient for |
| REF | Catalogue Number | | Consult Instructions |

### Hazard Pictograms

Danger

Warning

# 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 hypercholesterolemia, 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 research use only. Not for use in diagnostic procedures.
- 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**<br>3.5 ml, ready for use, blue coloured | **ACYL-BUFF** | 1 bottle |
| 4.6 | **Acylation Reagent**<br>lyophilised, dissolve content<br>in 3 ml Solvent before use; if required<br>combine the contents of the vials | **ACYL-REAG** | 3 vials |
| 4.7 | **Antiserum ADMA**<br>7 ml, ready for use<br>Rabbit-anti-N-acyl-ADMA; blue coloured | **AS-ADMA** | 1 vial |
| 4.8 | **Antiserum Arginine**<br>7 ml, ready for use<br>Rabbit-anti-N-acyl-Arginine; yellow coloured | **AS-ARG** | 1 vial |
| 4.9 | **Enzyme Conjugate**<br>13 ml, ready for use<br>Goat anti-rabbit-IgG-peroxidase | **CONJ** | 2 vials |
| 4.10 | **Wash Buffer**<br>20 ml, concentrated<br>Dilute content with dist. Water<br>to 1,000 ml total volume. | **WASH** | 2 bottles |
| 4.11 | **Substrate**<br>13 ml TMB solution, ready for use | **SUB** | 2 vials |
| 4.12 | **Stop Solution**<br>13 ml, ready for use<br>Contains 0.3 M sulphuric acid, not corrosive | **STOP** | 2 vials |
| 4.13 | **Reaction Plate**<br>for acylation | **ACYL-PLATE** | 1 piece |
| 4.14 | **Equalizing Reagent**<br>lyophilzed,<br>dissolve content with 21 ml dist. water,<br>dissolve carefully to minimize foam formation | **EQUA-REAG** | 1 vial |

Warning

4.15 **Solvent** **SOLVENT** 1 vial
10 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)

Danger

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**

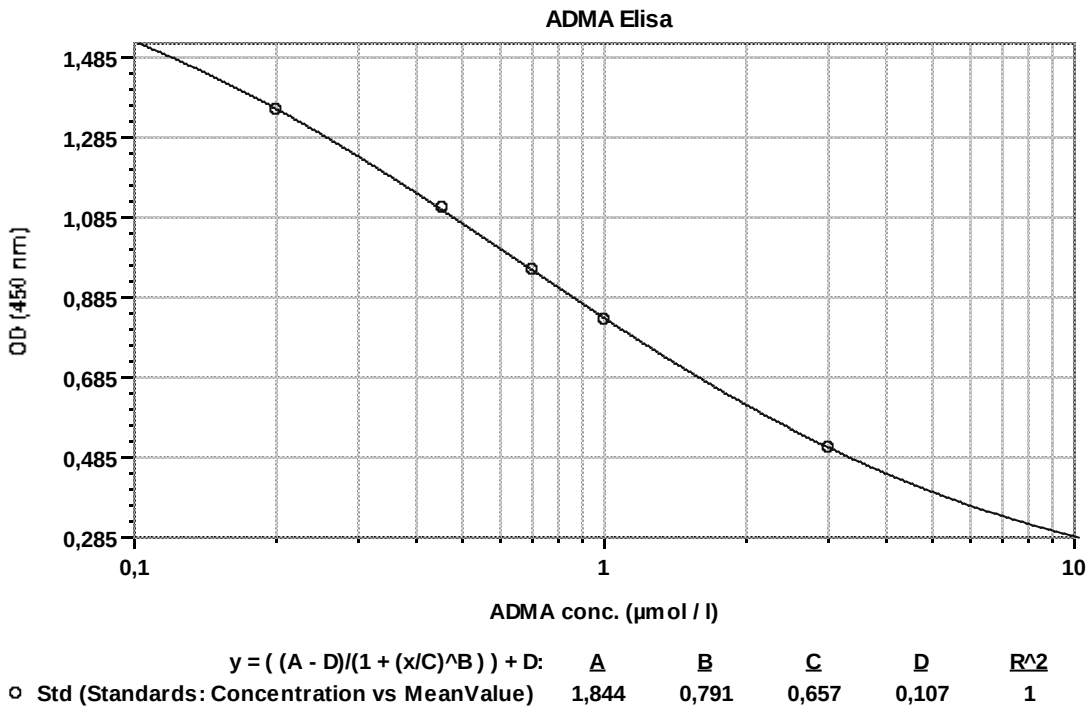
Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

## 8. Calculation of the Results

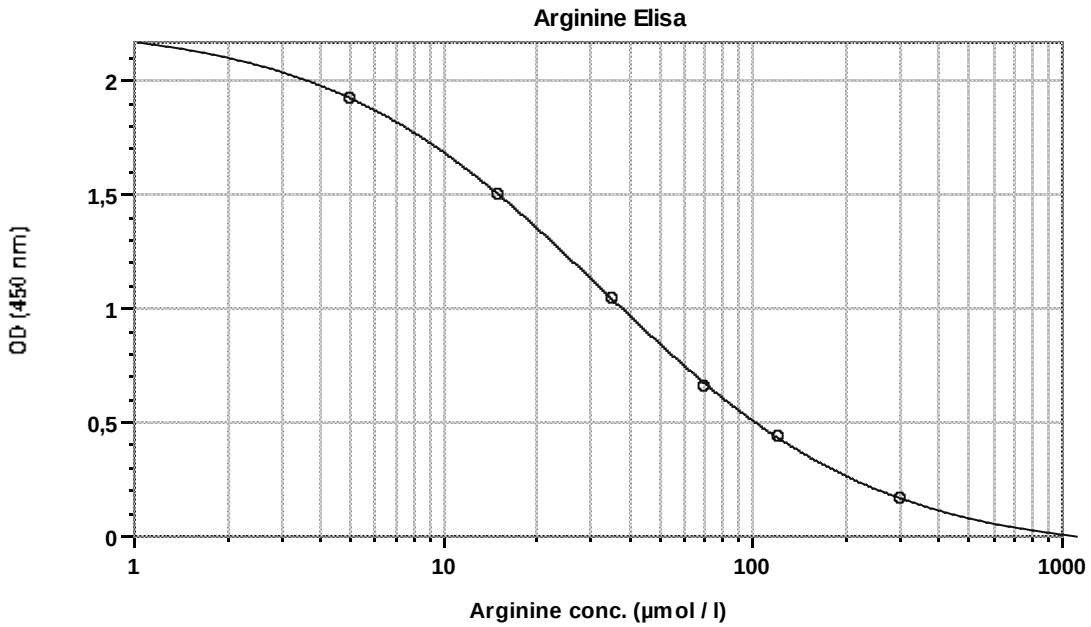
On a semilogarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Cubic spline, 4 parameter or similar iteration procedures are recommended for evaluation of the standard curve.
The concentration of the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


Schulze F, Wesemann R, Schwedhelm E, Sydow K, Albsmeier J, Cooke JP, Böger RH.
**Determination of ADMA using a novel ELISA assay.**
Clin. Chem. Lab. Med. 2004; 42: 1377-1383

Krempl TK, Kähler J, Maas R, Silberhorn L, Meinertz T, Böger RH.
**Elevation of asymmetric dimethylarginine (ADMA) in patients with unstable angina and recurrent cardiovascular events.**
Eur. Heart J. 2005; 26: 1846-1851

Schulze F, Maas R, Freese R, Schwedhelm E, Silberhorn L, Böger RH.
**Determination of a reference value for N,N-dimethyl-L-arginine in 500 subjects.**
Eur. J. Clin. Invest. 2005; 35 : 622-626

Schnabel R, Blankenberg S, Lubos E, Lackner KJ, Rupprecht HJ, Espinola-Klein C, Jachmann N, Post F, Peetz D, Bickel C, Cambien F, Tiret L, Münzel T.
**Asymmetric dimethylarginine and the risk of cardiovascular events and death in patients with coronary artery disease: results from the AtheroGene Study.**
Circ. Res. 2005; 97: e53-59

O`Dwyer MJ, Dempsey F, Crowley V, Kelleher D, McManus R, Ryan T.
**Septic shock correlates with ADMA levels which may be influenced by a polymorphism in DDAH II: a prospective observational study.**
Crit. Care 2006; 10: (5): R139

Antoniades C, Tousoulis D, Marinou K, Vasiliadou C, Tentolouris C, Bouras G, Pitsavos C, Stefanidis C.
**Asymmetrical dimethylarginine regulates endothelial function in methionine-induced but not in chronic homocystinemia in humans: effect of oxidative stress and proinflammatory cytokines**
Am. J. Clin. Nutr. 2006; 84: 781-788

Wang TZ., Chen WJ., Cheng WC., Lin JW., Chen MF., Lee YT.
**Relation of improvement in endothelium-dependent flowmediated vasodilation after Rosiglitazone to changes in asymmetric dimethylarginine, endothelin-1, and C-reactive protein in nondiabetic patients with the metabolic syndrome**
Am. J. Cardiol. 2006; 9: 1057-1062

Wanby P., Nilsson I., Brudin L., Nyhammar I., Gustafsson I., Carlsson M.
**Increased plasma levels of asymmetric dimethylarginine in patients with carotid stenosis: no evidence for the role of the common FABBP2 A54T gene polymorphism**
Acta Neurol. Scand. 2007; 115: 90-96

Konishi H, Sydow K, Cooke JP.
**Dimethylarginine dimethylaminohydrolase promotes endothelial repair after vascular injury**
J. Am. Coll. Cardiol. 2007; 49: 1099-1105

Iribarren C, Husson G, Sydow K, Wang BY, Sidney S, Cooke JP.
**Asymmetric dimethyl-arginine and coronary artery calcification in young adults entering middle age: the CARDIA Study**
Eur. J. Cardiovasc. Prev. Rehabil. 2007; 14:222-229

Melikian N, Wheatcroft SB, Ogah OS, Murphy C, Chowienczyk PJ, Wierzbicki AS, Sanders TA, Jiang B, Duncan ER, Shah AM, Kearney MT.
**Asymmetric dimethylarginine and reduced nitric oxide bioavailability in young Black African men**
Hypertension 2007; 49: 873-877

Horowitz JD, Heresztyn T.
**An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: Methodological considerations.**
J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2007; epub ahead of print

Korish AA, Arafah MM.
**Catechin combined with vitamins C and E ameliorates insulin resistance (IR) and atherosclerotic changes in aged rats with chronic renal failure (CRF)**
Arch. Gerontol. Geriatr. 2007; in press

Charitidou C, Farmakiotis D, Zournatzi V, Pidonia I, Pegiou T, Karamanis N, Hatzistilianou M, Katsikis I, Panidis D.
**The administration of estrogens, combined with anti-androgens, has beneficial effects on the hormonal features and asymmetric dimethyl-arginine levels, in women with the polycystic ovary syndrome**
Atherosclerosis 2007; in press

## General Literature

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

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

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

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

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

Böger RH.
**The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor**
Cardiovasc. Res. 2003; 59: 824-833

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

## 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