# Methylmalonic Acid in serum/plasma/urine

1000 M MMA

Instruction manual for LC-MS/MS assay
for in vitro diagnostic use

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CE 98/79/EC - IVD Medical Devices

# Contents

# 1. Introduction

## 1.1 Intended Use

The LC-MS/MS kit is intended for the determination of Methylmalonic Acid in serum/plasma/ urine.

The components in de kit must be used as stated in the user manual.

## 1.2 Intended User

This kit is designed for (healthcare) laboratory professional use. Diagnotix recommends that users adhere to ISO 15189 Medical Laboratories.

## 1.3 Notice Regarding Serious Incidents

Following (EU) 2017/746 Annex I, Chapter III, 20.4.1 af), any serious incident that has occurred in relation to this device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

## 1.4 IVD symbols

REF Order Number

LOT Lot Number

IVD For in vitro diagnostic use


See instructions for use


Manufacturer


Temperature limits


Contains sufficient for < n > tests


Expiry date

## 1.2 Clinical background

Methylmalonic acid is an organic acid of which the blood levels are usually raised in case of a B12 deficiency. Adenosylcobalamin – one of the two active forms of B12 – is a cofactor of the enzyme L-methylmalonyl-CoA- mutase, which converts L-methylmalonyl-Coa into succinyl-CoA. If adenosylcobalamin is lacking, excess D-methylmalonyl-CoA (precursor of L-methylmalonyl-CoA) is converted into methylmalonic acid(MMA) which causes raised blood levels of MMA. In short: a B12 deficiency (usually) causes high MMA.

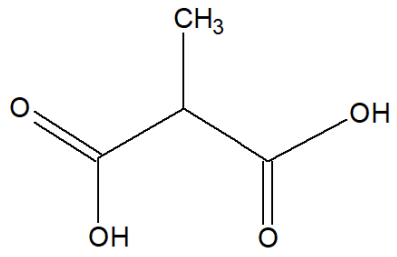
High MMA serum values are also found in people with renal insufficiency, hypovolemia (decreased volume of circulating blood) and intestinal bacterial overgrowth. In these cases MMA levels cannot be used to diagnose B12 deficiency though a B12 deficiency might simultaneously exist. In the case of kidney disease (or hypovolemia) MMA levels in urine can be tested.

MMA values can be false-normal in people with B12 deficiency who take (or recently took) antibiotics, for these destroy the intestinal flora needed to make propionic acid (an organic acid, precursor to MMA). MMA should be tested before starting treatment.

In case of a B12 deficiency treatment will rather quickly lower MMA levels. Testing sometime after starting treatment, for instance after one or two months, may serve as a confirmation of the B12 deficiency diagnosis. This can also be done when people before treatment have a MMA value which is not distinctly above reference values.

The determination of MMA can be performed from serum, plasma and urine.
Serum samples are generally used for MMA determination, as this matrix is used for parallel cobalamin level tests. The advantage of determination from serum therefore is the sample availability. Furthermore nutrition seems to have less influence on the MMA serum level than in case with urine. For determination in urine additional measurement of creatinine is also necessary.

Mass spectrometry based methods have been widely tested for the determination of Methylmalonic Acid. Furthermore chromatographic separation from the structural isomer Succinic Acid (SA) is critical and not elementary. This method can be used for the routine analysis of Methylmalonic Acid in human serum, plasma and urine. Sample preparation is simple and rapid. A six-point lyophilized serum calibrator at clinically relevant levels has been added to the kit. An isotope-labelled internal standard (Methylmalonic Acid D3) is added to compensate for matrix effects and measurement variations.




Methylmalonic Acid

## 1.3 Description of the analytical procedure

Methylmalonic Acid is determined from human serum, plasma or urine by UHPLC with negative ion electrospray LC-MS/MS.

Prior to the LC-MS/MS analysis a sample clean-up is performed to remove the sample matrix and to spike with the internal standard.

After separation by chromatography on an analytical C-18 column, Methylmalonic acid is ionized by electrospray ionization (ESI) and detected by LC-MS/MS.

Measurement of the analytes is carried out in Multi Reaction Monitoring (MRM) mode. In this mode only selected ions (known as precursor ions) with a defined mass/charge (m/z) ratio are isolated in the first quadrupole and subsequently transferred into the collision cell, where they are fragmented by impact with an inert gas (argon or nitrogen) at defined voltage settings. Among the fragments generated (known as product ions) only those with a defined m/z ratio can pass the third quadrupole for final detection.

The analytical method enables a quantitation in biological matrices by use of isotope labelled internal standard.

# 2. Components of the Methylmalonic Acid kit

## 2.1 Ordering information

1000 KIT M MMA Complete Kit for Methylmalonic Acid in serum/plasma/urine

**Contents** (for 300 assays)

| | | |
|---|---|---|
| Methylmalonic Acid Calibrator Set (Calibrator 1 – 6) | 1001 CAL M MMA | 6 x 2 x 1 ml |
| Methylmalonic Acid Internal Standard | 1008 M MMA | 3 x 6 ml |
| Methylmalonic Acid Deproteinization Solution | 1009 M MMA | 3 x 55 ml |
| Methylmalonic Acid Mobile Phase I | 1010 M MMA<br>1010a M MMA | 1 x 500 ml |
| Methylmalonic Acid Mobile Phase II | 1011 M MMA | 1 x 300 ml |
| Methylmalonic Acid Solvent | 1012 M MMA | 3 x 30 ml |
| Methylmalonic Acid Autosampler Washing Solution | 10001 M MMA | 1 x 1000 ml |
| Methylmalonic Acid Manual | | |

**Separately available components:**

| | | |
|---|---|---|
| Methylmalonic Acid Calibrator Set (Calibrator 1 – 6) | 1001 CAL M MMA | 6 x 2 x 1 ml |
| Methylmalonic Acid Internal Standard | 1008 M MMA | 1 x 6 ml |
| Methylmalonic Acid Deproteinization Solution | 1009 M MMA | 1 x 55 ml |
| Methylmalonic Acid Mobile Phase I | 1010 M MMA | 1 x 500 ml |
| Methylmalonic Acid Mobile Phase II | 1011 M MMA | 1 x 300 ml |
| Methylmalonic Acid Solvent | 1012 M MMA | 1 x 30 ml |
| Methylmalonic Acid Diluting Solution for Urine | 1013 M MMA | 1 x 100 ml |
| Methylmalonic Acid Autosampler Washing Solution | 10001 M MMA | 1 x 1000 ml |

| | | |
|---|---|---|
| Analytical Column, Acquity UPLC HSS T3 100 A 1,8 µm 2,1 mm x 100 mm | 186003539 | 1 pcs |
| Methylmalonic Acid Control I | 1014 M MMA | 10 x 1 ml |
| Methylmalonic Acid Control II | 1015 M MMA | 10 x 1 ml |
| Methylmalonic Acid Control III | 1016 M MMA | 10 x 1 ml |
| Methylmalonic Acid Control Set | 1017 CON M MMA | 3 x 3 x 1 ml |

## 2.2 Safety information

Several components are chemical preparations and may contain hazardous substances. For safety information, please consult the Material Safety Data Sheet (MSDS) of each component.

The raw material donor plasma was tested for HBsAg, anti-HIV 1/2 and anti-HCV. However, because no test method can offer complete assurance that products derived from human sources will not transmit infectious agents, it is recommended that this product be handled with the same precautions as patient samples.

## 2.3 Storage conditions and lifetime of kit components

Please unpack the kit components from the transport packaging *immediately upon receipt* and follow the instructions for storage conditions indicated on the product labels.

### 2.3.1 Calibrators and controls

1001 CAL M MMA | Methylmalonic Acid Calibrator Set
1017 CON M MMA | Methylmalonic Acid Control Set
1014 M MMA | Methylmalonic Acid control I
1015 M MMA | Methylmalonic Acid control II
1016 M MMA | Methylmalonic Acid control III

#### 2.3.1.1 Handling

Reconstitute the calibrators and controls as follows:

1. Carefully remove the cap and rubber plug avoiding any loss of contents.
2. Reconstitute Methylmalonic Acid Calibrator Set and Controls with exactly 1 ml distilled or deionised water using a volumetric pipette.
3. Replace the plug and let stand during 15 minutes.
4. Swirl the vial carefully and mix thoroughly making sure that all traces of dry material have dissolved, do not shake. Avoid foaming.
5. Let stand for 15 minutes at room temperature.
6. Swirl the vial carefully, do not shake. Avoid foaming.
7. Use the preparation as a patient sample.

#### 2.3.1.2 Stability and storage

The stability of the calibrators and controls are:

| | | |
|---|---|---|
| Before reconstitution: | 2 - 8 °C | Until expiry date printed on the product label |
| After reconstitution: | 2 - 8 °C | 1 week |
| After reconstitution: | - 20 °C | 2 weeks |

The declared stated stabilities are only valid in case of no bacterial contamination.

### 2.3.2 Internal Standard

1008 M MMA | Methylmalonic Acid Internal Standard

#### 2.3.2.1 Handling

Reconstitute the internal standard as follows:

1. Carefully remove the cap and rubber plug avoiding any loss of contents.
2. Reconstitute Methylmalonic Acid Internal Standard with exactly 6.0 ml distilled or deionised water using a volumetric pipette.
3. Replace the plug and let stand during 15 minutes.
4. Swirl the vial carefully and mix thoroughly making sure that all traces of dry material have dissolved, do not shake. Avoid foaming.
5. Let stand for 15 minutes at room temperature.
6. Swirl the vial carefully, do not shake. Avoid foaming.

#### 2.3.2.2 Stability and storage

| | | |
|---|---|---|
| Before reconstitution: | 2 - 8 °C | Until expiry date printed on the product label |
| After reconstitution: | 2 - 8 °C | 1 week |
| After reconstitution: | - 20 °C | 2 weeks |

The declared stated stabilities are only valid in case of no bacterial contamination.

### 2.3.3 Deproteinization Solution

1009 M MMA | Methylmalonic Acid Deproteinization

#### 2.3.3.1 Handling

The reagent is liquid and ready for use.

#### 2.3.3.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 3 weeks if closed and stored at 2 - 8 °C

### 2.3.4 Mobile Phases

1010 M MMA | Methylmalonic Acid Mobile Phase I
1010a M MMA | Methylmalonic Acid Mobile Phase I (reduced Formic Acid concent)
1011 M MMA | Methylmalonic Acid Mobile Phase II

#### 2.3.4.1 Handling

The reagents are liquid and ready for use.

2.3.4.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 4 weeks on the UHPLC

### 2.3.5 Solvent

1012 M MMA | Methylmalonic Acid Solvent

#### 2.3.5.1 Handling

The reagent is liquid and ready for use.

#### 2.3.5.2 Stability and storage

| | |
|---|---|
| Store at 2 - 8 °C | After first opening the Reagent can be used for 4 weeks if closed and stored at 2 - 8 °C |

### 2.3.6 Dilution Solution for Urine

1013 M MMA | Methylmalonic Acid Dilution Solution for Urine

#### 2.3.6.1 Handling

The reagent is liquid and ready for use.

#### 2.3.6.2 Stability and storage

| | |
|---|---|
| Store at 2 - 8 °C | After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C |

### 2.3.7 Autosampler Washing Solution

10001 M MMA | Methylmalonic Acid Autosampler washing solution

#### 2.3.7.1 Handling

The Reagent is liquid and ready for use.

#### 2.3.7.2 Stability and storage

| | |
|---|---|
| Store at 2 - 8 °C | After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 4 weeks on the UHPLC |

# 3. Required instruments

Using this test kit requires a UHPLC system with tandem mass spectrometer (LC-MS/MS).

## 3.1 Required LC Modules

- Auto sampler
- UHPLC gradient pump
- Column heater
- Degasser

# 4. The analytical system

## 4.1 Preparation of the analytical system

- Flush the LC system excluding the column.
- Set the UHPLC pump at a flow rate of 1 ml/min and flush the system for 10 minutes with Mobile Phase I and II (50 : 50)
- Connect the column with the column heater (see arrow marking on the column)

After flushing the system, the equilibration is performed as follows:

- Set the UHPLC pump to a flow rate of 0.2 ml/min, set the column heater to 30 °C, and equilibrate the column for 15 minutes with Mobile Phase I. Start the program for the gradient and equilibrate for another 10 minutes.

### 4.2.1 Starting the analytical system

- Equilibrate the system
- Check the temperature of the column
- Initialize the injector
- Start the programme on the LC-MS/MS system

## 4.3 LC-MS/MS Parameters and Conditions

### 4.3.1 LC Parameters

| | |
|---|---|
| **UHPLC pump** | Flow rate 0.2 ml/min |
| **Mobile Phases I & II** | Close the bottles to avoid alteration of RT's through evaporation of the mobile phases |

| | |
|---|---|
| **Column** | The column is installed in the column heater 30 °C<br>For the complete UHPLC system the<br>backpressure should not exceed 1000 bar.<br>1 bar = 14.5 PSI |

### 4.3.2 Autosampler Conditions

| | |
|---|---|
| Injection volume: | 10-20 µl |
| Sample temperature: | 10 °C |
| Runtime: | 6.00 min |
| Column temperature: | 30 °C |
| Needle wash: | wash twice for 6 seconds |
| Seal Wash: | 10:90 ACN:H2O |
| Wash Solvent: | Autosampler Washing Solution; 90:10 H2O:MeOH |

### 4.3.3 Gradient

| **Time (min)** | **Flow Rate (mL/min)** | **%A** | **%B** | **Curve** |
|---|---|---|---|---|
| 0.00 | 0.200 | 98.0 | 2.0 | Initial |
| 0.75 | 0.200 | 98.0 | 2.0 | 6 |
| 3.00 | 0.200 | 45.0 | 55.0 | 6 |
| 3.70 | 0.200 | 0.0 | 100.0 | 6 |
| 5.00 | 0.200 | 98.0 | 2.0 | 1 |
| 6.00 | 0.200 | 98.0 | 2.0 | 1 |

Please note that the gradient is dependent on the analyser used. End users will need to define the optimal gradient for the analyser in use.

### 4.3.4 MS Conditions (e.g. Waters Xevo TQS)

| **Substance** | **Precursor** | **Product** |
|---|---|---|
| Methylmalonic Acid | 117 | 73 |
| Methylmalonic Acid D3 | 120 | 76 |

The mass transitions are an indication, the optima can differ slightly between different LC-MS/MS systems.

# 5. Sample

## 5.1 Sample material

### 5.1.1 Serum and plasma

Use serum or plasma (EDTA- and Heparin-plasma)

Samples can be stored: 3 days at room temperature (15 - 25 °C)
7 days at (2 - 8 °C)
1 month (- 20 °C)

Avoid freeze-thaw cycles.

### 5.1.2 Urine

In the cases of patients with impaired renal functions, the analysis is performed from an urine sample.

The stability of urine samples is identical to those of serum and plasma.

Samples can be stored: 3 days at room temperature (15 - 25 °C)
7 days at (2 - 8 °C)
1 month (- 20 °C)

## 5.2 Sample preparation

### 5.2.1 Reconstitution of the lyophilised Calibrators / Controls.

See 2.3.1.1 and also the product data sheets.

### 5.2.2 Sample preparation serum/plasma

Precipitation:

- 100 µl serum / plasma (Calibrator, Control, Patient sample)
- Add 50 µl Methylmalonic Acid Internal Standard
- Mix and add 500 µl Methylmalonic Acid Deproteinization Solution
- Mix for 30 seconds on a vortex mixer
- Centrifuge (5 min, 10000 x g or more)
- Use 400 µl supernatant and evaporate to dryness under
- Nitrogen at 50 °C
- Reconstitute in 200 µl solvent
- Inject 10 µl in the LC-MS/MS

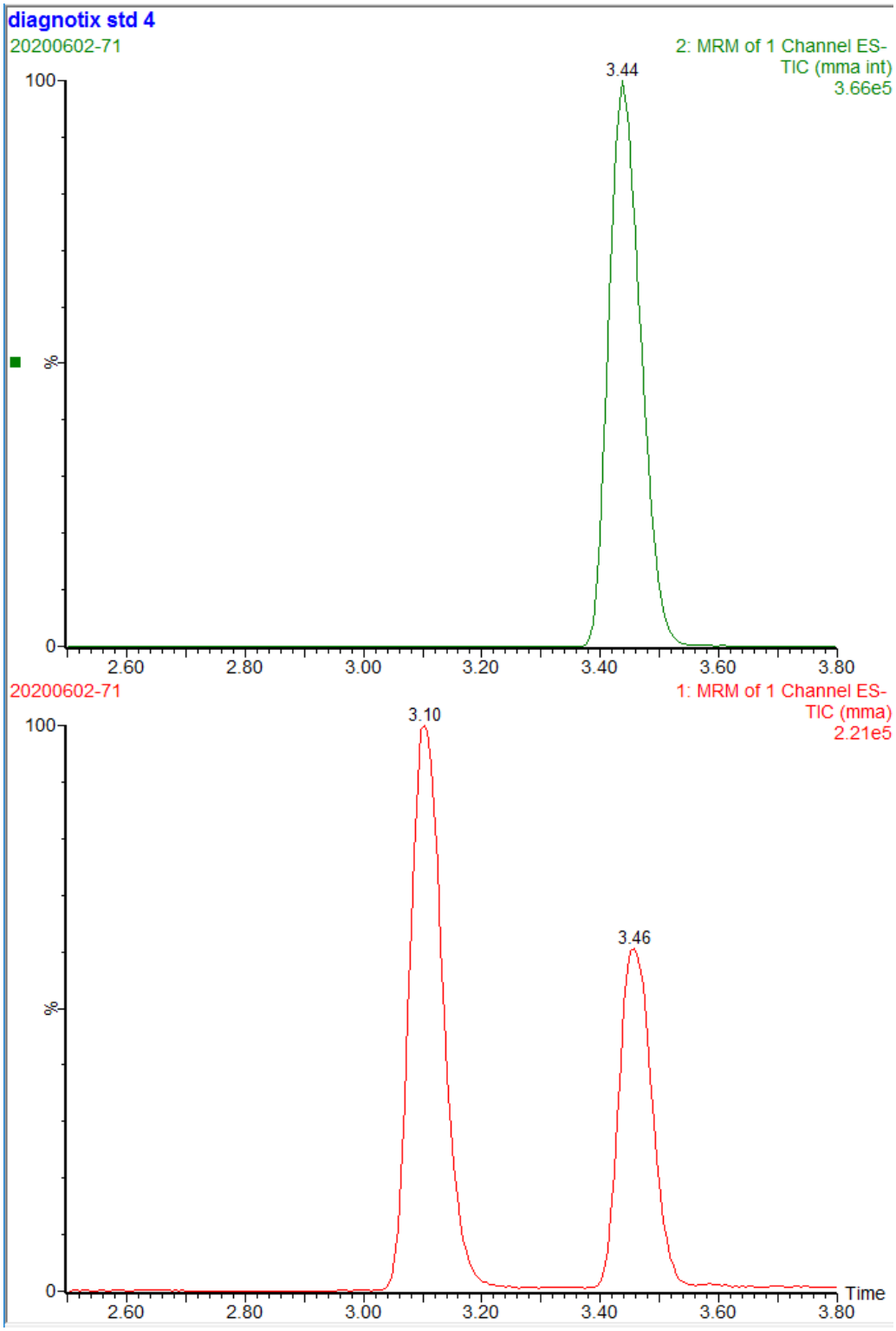
### 5.2.3 Sample preparation urine

For urine samples dilute 50 µl of the urine sample with 1000 µl Diluting Solution.

Precipitation:

- 100 µl Diluted urine
- Add 50 µl Methylmalonic Acid Internal Standard
- Mix and add 500 µl Methylmalonic Acid Deproteinization Solution
- Mix for 30 seconds on a vortex mixer
- Centrifuge (5 min, 10000 x g or more)
- Use 400 µl supernatant and evaporate to dryness under Nitrogen at 50 °C
- Reconstitute in 200 µl solvent
- Inject 10 µl in the LC-MS/MS

## 5.3 Example chromatogram, recorded with the Waters LC-MS/MS TQS system



# 6. Test data (Validation report)

## 6.1 Linearity

| | nmol/l |
|---|---|
| Methylmalonic Acid | 2000 |

## 6.2 Limit of quantification

| | nmol/l |
|---|---|
| Methylmalonic Acid | 37.8 |

## 6.3 Repeatability

| **Item** | **Measured value (nmol/l)** | **Standard Deviation (nmol/l)** | **CV (%)** | **N** |
|---|---|---|---|---|
| Level I | 211.3 | 14.1 | 6.7 | 25 |
| Level II | 350.0 | 12.5 | 3.6 | 25 |
| Level III | 1114.7 | 27.1 | 2.4 | 25 |

## 6.4 Reference Ranges

| | |
|---|---|
| Plasma, serum | 0 – 350 nmol/l |
| Urine | < 3.6 mmol/mol Creatinine |

The indicated reference ranges are taken from scientific literature.
It is recommended that each laboratory establishes its own normal range.

# 7. References


- L. Thomas, labor und diagnose: Indikation und Bewertung von laborbefunden für die medizinische Diagnostik, TH-Books Verlagsgesellschaft, Frankfurt/Main 2012, page 714 *
- Norman, E.J. Urinary Methylmalonic Acid Test May have greater value than the total Homocysteine assay for screening elderly individuals for Cobalamin Deficiency, Clinical Chemistry 2004, 50 (8), 1482-1483. **