PROVIDER OF CE-CERTIFIED LC-MS/MS DIAGNOSTIC KIT

MASSDETECT™ MMA

INSTRUCTIONS FOR USE FOR THE IN VITRO DETERMINATION OF METHYLMALONIC ACID IN PLASMA/SERUM

CE

CE-IVD label according to European Directive 98/79/EC

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1. METHYLMALONIC ACID LC-MS/MS KIT

Art. No. 40-2002, 150 analyzes including column.

Art. No. 40-2001, 150 analyzes, replacement kit.

Risk class 1 according to IVDD and risk class A according to IVDR

Global article number (GTIN) 07350143680007

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2. INTENDED USE

The described LC-MS/MS application is intended for the quantitative determination of Methylmalonic acid (MMA) in plasma/serum.

The method is used to determine elevated levels of methylmalonic acid, which is a measure of B_{12} vitamin deficiency. MMA is also a specific marker for a group of diseases collectively called methylmalonic acidemia, which includes at least seven different complementation groups.

The method is intended to be performed by a laboratory professional in clinical laboratories.

For in vitro diagnostics use.

3. INTRODUCTION

Methylmalonic acid (MMA) is a marker of intracellular cobalamin (vitamin B_{12}) deficiency. Cobalamin metabolism is complex and thus difficult to chemically diagnose (1). The analysis is done when investigating suspected cobalamin (vitamin B_{12}) deficiency, e.g. in unclear neuropsychiatric symptoms and megaloblastic anemia. Cobalamin deficiency in tissue leads to increased levels of MMA and homocysteine in blood (2). At low levels of vitamin B_{12}, MMA accumulates in the blood, while normal plasma/serum levels make cobalamin deficiency unlikely. Other causes of abnormal levels of MMA can be a metabolic disorder such as methylmalonic acidemia. This is caused by inherited mutations in one or more genes that code for enzymes and proteins involved in the conversion of methylmalonyl-CoA to succinyl-CoA. Deficiency of vitamin B_{12} is a very important public health problem because a deficiency of B_{12} vitamin causes severe complications if it is not detected and treated. Discovering risk factors and risk groups and educating about vitamin B deficiency would prevent them from getting irreversible complications. In many countries, examinations are therefore carried out on newborns as part of the public health program (3).

4. PRINCIPLES OF THE PROCEDURE

Methylmalonic acid is extracted from plasma/serum by mixing the sample with a precipitation solution including the internal standard, the solution is vortexed and centrifugated. The supernatant is transferred to a new tube, evaporated to dryness, reconstitution solution is added and the then transferred to an injection vial for analysis.

The sample is separated on a LC-column using a binary gradient. The effluent from the column is monitored with electrospray ionization mass spectrometry using multiple reaction monitoring (MRM) in negative mode to follow the respective characteristic transitions for MMA, D_3-MMA and the internal standard, $^{13}C_4D_3$-MMA.

Since no blood is free of MMA, labeled MMA (D_3-MMA) is used as a surrogate molecule. The labeled molecule can, after optimization of MS parameters, give the same MS-response as unlabeled MMA. If it is not possible to achieve the same MS-response a response factor ratio must be used for the calculation. Thus, a calibration curve of D_3-MMA in plasma/serum is used for the calculations of MMA concentrations in samples.

5. WARNING AND PRECAUTIONS

Materials included in this kit should not be used past the expiration date on the kit label.
Solutions included in this kit should not be mixed or substituted with solutions from other kits.

6. HEALTH AND SAFETY PRECAUTIONS KIT CONTENTS

Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to discard residues in accordance with laboratory regulations.

7. KIT CONTENTS

Art. No. 40-2001 150 Determinations, including LC-column.
Art. No. 40-2002, 150 Determinations, replacement kit

Label	Component	Volume
CAL	Calibrator curve in serum 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 och 2.0 µM, D_3-MMA	8 x 0.2 mL
IS	Internal standard $^{13}C_4D_3$-MMA, 50 µM	0.2 mL
EXT	Extraction solution	40 mL
REC	Reconstitution solution	20 mL
TUN	Tuning solution, MMA, $^{13}C_4D_3$-MMA, D_3-MMA, and succinic acid, (1 µM of each)	0.5 ml
COL	LC-column	1 pcs.

8. STORAGE CONDITIONS

The reagents should be stored at +2-8^{o}C.
The Calibrator should be stored frozen at -20^{o}C.

9. MATERIAL REQUIRED BUT NOT SUPPLIED

- Mobil phase A
- Mobil phase B
- Appendix 1 lists products that are available for the MMA method.

10. PREPARATION OF MOBILE PHASES

NOTE! Acetic acid and formic acid should be of high-grade quality, stored in glass bottles.

Mobile Phase A

0.05% acetic acid in 90% methanol	Preparation of 1 000 mL
Acetic acid	0.5 mL
Methanol	900 mL
Milli-Q water	100 mL

Mobile Phase B

0.5% formic acid in 20% methanol	Preparation of 1 000 mL
Formic acid	5 mL
Methanol	195 mL
Milli-Q water	800 mL

11. MS/MS METHOD

Instrument	Sciex API 5500
Ionization	Electrospray
Scan Type	MRM
Polarity	ESI-
Curtain Gas	10
Collision Gas	8
Ion Spray Voltage (kV)	-4500
Temperature	500
Ion Source Gas 1	40
Ion Source Gas 2	40
DP (declustering potential)	30 - 45
CE (collision energy)	10 - 14

12. TRANSITIONS

Analyte	$M+H^+$> fragment
MMA and succinic acid	116.9 > 72.9, 55.2
D_3-MMA	119.9 >75.9, 58.1
$^{13}C_4D_3$-MMA	124.0 > 79.0, 61.0

13. LC-GRADIENT

Flow rate: 0.4 mL/min

Analysis time: 3.5 min

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	100	0
0.5	100	0
1.0	0	100
2.0	0	100
2.1	100	0

14. WASH SOLUTION OF AUTOINJECTOR

Washing solutions must be run between each injection.
First wash phase: Acetonitrile/Methanol/Water to 1/1/1 (Valve and post clean 5 seconds each)
Second wash phase: Mobile phase A (See point 10) (Valve and post clean 5 seconds each)..

15. STARTUP - OPTIMIZATION OF THE MS PARAMETERS FOR THE ANALYTES

Use the supplied MMA tuning solution (TUN) to find the exact transitions of the MRM tracks when using the kit for the first time. Check the accuracy of the mass scales after maintenance of the mass spectrometer, or if the instrument has been affected in other ways that affect the accuracy of the mass spectrometer. It is important that the separation between succinic acid and MMA is sufficient.

NOTE! Special care needs to be taken to adjust the response for the respective MMA variants. It is especially important that the response for D_3-MMA and unlabeled MMA is identical, otherwise use the response factor ratio between D_3-MMA and MMA when calculating the MMA concentrations.

16. SAMPLES – STORAGE AND TRANSPORTATION CONDITIONS

Use plasma/serum tubes with gel and coagulation activator. Invert the tube immediately after sampling 6-10 times. Keep the tube upright for 30 min after sampling.
Durability during storage and transport from sampling to arrival at the analyzing laboratory.
In room temperature maximum 4 hrs., after centrifugation room temperature for maximum 24 hrs. refrigerated, maximum 3 days.
Allow samples and reagents to reach room temperature before use.

17. PREPARATION OF EXTRACTION SOLUTION

Centrifuge the ampoule containing $^{13}C_4D_3$-MMA (IS) (2000 RCF, 2 min), open the tube and transfer the solution in its entirety to EXT bottle. The final concentration of internal standard $^{13}C_4D_3$-MMA in extraction solution is 0.25 µM.

18. SAMPLE PREPARATION

Handle patient and calibration samples in the same way.

1. To 50 µL plasma/serum add 250 µL extraction solution containing the internal standard.

2. Mix well, vortex.
3. Centrifuge at 16 400*g*, for 10 min at +10 °C (preferable).
4. Transfer supernatant to new tube, injection vials/microtiterplate.
5. Evaporate to dryness.
6. Add 100 µL of reconstitution solution.
7. Inject 5 µL

If the MS peaks become too high, dilute the samples with an appropriate volume of REC solution.

19. Preparation of Calibrator curve

The calibrator curve in blood is ready-to-use.

20. Determination of MMA concentration in samples

D_3-MMA will be used as a surrogate analyte for MMA, as plasma/serum already contains MMA.

To make it possible to use a calibration curve based on D_3-MMA for the quantification of MMA in plasma/serum, the MS response of both molecules must be identical, or a response factor ratio must be used. In each measurement point, the peak area for the respective analyte is related to the corresponding peak area for the internal standard ($^{13}C_4D_3$-MMA). These ratios are plotted against the concentration of D_3-MMA to calculate the calibration curve equation, which is used in determining the MMA concentration of the samples. First order linear regression weighted by 1/x is preferred.

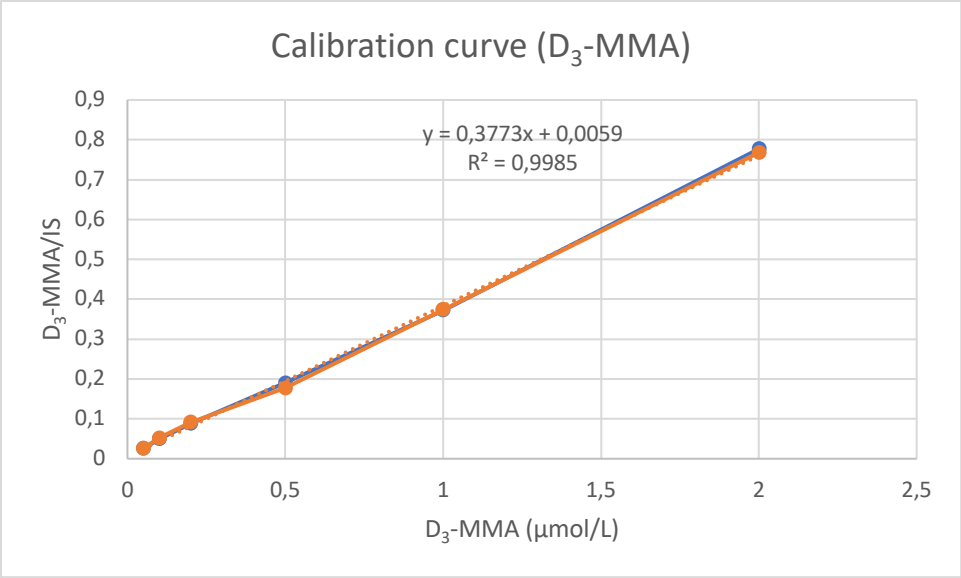

Figure 1 Highest calibration level (2 µmol/L)

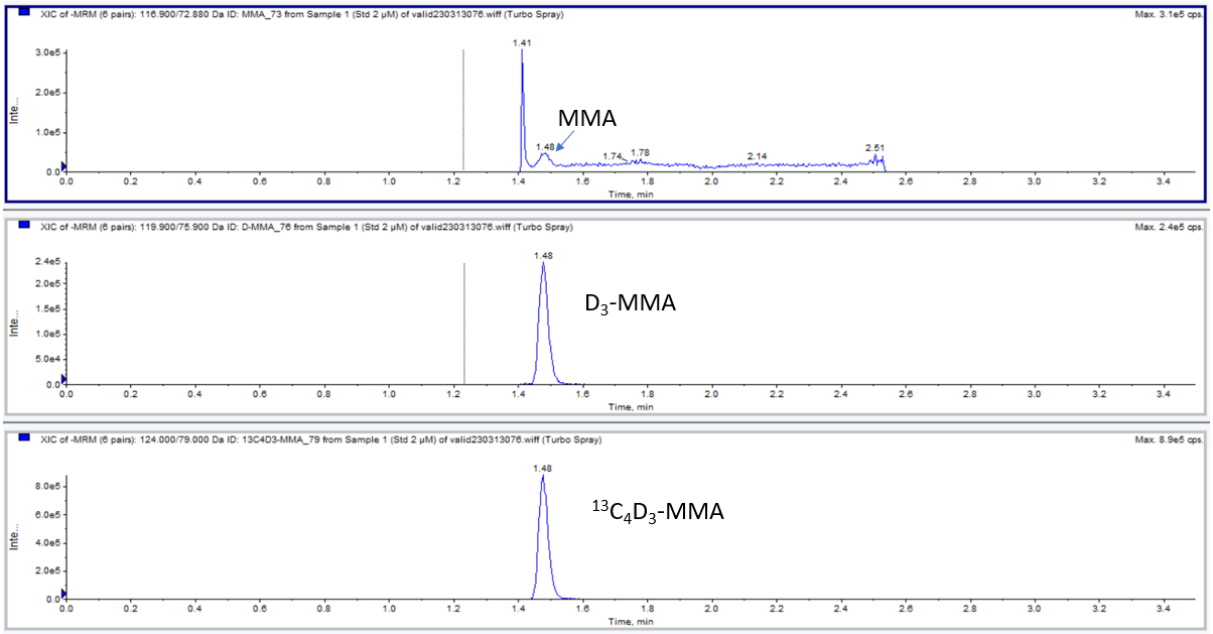

Figure 2. As the peak area (concentration) of succinic acid in blood samples is much higher than MMA it is difficult to integrate MMA properly. Let the flow go to waste until shortly before the MMA peak elutes, most of the succinic acid peak will not interfere with the integration of MMA (as seen in the figure).

21. QUALITY CONTROL

Control samples must be analyzed with each batch of samples. Results generated from the analysis of control samples should be evaluated by statistical methods to ensure that the method shows accurate results.

The MS response (peak areas) of the internal standard should be the same for each sample within an analysis run. Larger deviations are a sign of interference or that an incorrect volume has been added. The labeled internal standard compensates for ion suppression, volume differences after evaporation and injection volume. A systematic decrease in the peak area of the internal standard over one or several different runs of analysis may indicate hardware-related problems, such as a contaminated LC-column or ion source. Individual outliers may indicate problems with the sample or sample preparation.

Carry-over

"Carry over" for MMA after two blank tests is below 1% for both MMA and D3-MMA.
"Carry over" is mainly due to small amounts of the substances sticking to the column and accumulating over time.
It is therefore important to inject the so-called blanks containing e.g. mobile phase A between samples and especially important after high concentrations of calibrator samples.
It may also sometimes be necessary to rinse through the column for a slightly longer time with mobile phase B.

22. PERFORMANCE CHARACTERISTICS

LOQ Level (limit of quantification)

0.025 µmol/L

Measuring range

0.025 – 2.0 μM

Samples above 2.0 µM should be diluted and analyzed again.

Reproducibility of QC samples in blood

D_3-MMA (µmol/L)	% std dev (%) intra assay (n=4)
0.075	8.9
0.75	5.8
1.5	4.0

23. REFERENCE INTERVAL

<0.28 µmol/L for < 50 years of age

< 0.36 µmol/L for ≥ 50 years of age

24. REFERENCE

1. Green, R. et al. Vitamin B12 deficiency. Nature Review. 2017, 3, 1-19
2. Guney, T. et al. Epidemiology of vitamin B12 Deficiency. Epidemiology of Vitamin B12 Deficiency. http://dx.doi.org/10.5772/63760, 2016, Cpt 16, 103-112.
3. Wilsdon, T. et al. A landscape assessment of newborn screening (NBS) in Europe. CRA Insights: Life Science. 2021, 1-12.
4. Hannibal, L. et al. Biomarkers and Algorithms for the Diagnosis of Vitamin B12 Deficiency. Frontiers in Molecular Sciences. 2016, 3, 1- 16.
5. Aparicio-Ugarriza, R. et al. A review of the cut-off points for the diagnosis of vitamin B12 deficiency in the general population. Clin. Chem. Lab Med 2015; 53(8): 1149–1159

25. APPENDIX 1

40–1006 MMA QC low

40–1007 MMA QC mid

40–1008 MMA QC high