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

MASSDETECT™ MMA

INSTRUCTIONS FOR USE FOR
THE IN VITRO DETERMINATION OF METHYLMALONIC ACID IN BLOOD

CE

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

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Content

1. METHYLMALONIC ACID LC-MS/MS KIT 2
2. INTENDED USE 2
3. INTRODUCTION 3
4. PRINCIPLES OF THE PROCEDURE 3
5. WARNING AND PRECAUTIONS 3
6. HEALTH AND SAFETY PRECAUTIONS 3
7. KIT CONTENTS 3
8. STORAGE CONDITIONS 4
9. MATERIAL REQUIRED BUT NOT SUPPLIED 4
10. PREPARATION OF MOBILE PHASES 4
11. CHROMATOGRAPHIC CONDITIONS LC-MS/MS METHOD 4
12. TRANSITION 5
13. LC-GRADIENT 5
14. WASH SOLUTION 5
15. STARTUP - OPTIMIZATION OF THE PARAMETERS FOR THE ANALYTES 5
16. SAMPLE STORAGE AND TRANSPORTATION 5
17. PREPARATION OF INTERNAL STANDARD IN EXTRACTION SOLUTION 5
18. SAMPLE PREPARATION 6
19. PREPARATION OF CALIBRATOR CURVE 6
20. DETERMINATION OF MMA CONCENTRATION 6
21. QUALITY CONTROL 7
22. PERFORMANCE CHARACTERISTICS 8
23. REFERENCE INTERVAL 8
24. REFERENCE 8
25. APPENDIX 1 9

1. METHYLMALONIC ACID LC-MS/MS KIT

Art. No. 40-2002, 100 analyzes including column.

Art. No. 40-2001, 100 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 blood/plasma.

The method is used to determine elevated levels of methylmalonic acid, which is a measure of B_{12} vitamin deficiency

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 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 blood 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, the 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 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 response as unlabeled MMA. Thus can D_3-MMAbe used for the calculations of MMA concentrations.

5. WARNING AND PRECAUTIONS

Materials included in this kit should not be used past the expiration date on the kit label.

Reagents or substrates included in this kit should not be mixed or substituted with reagents or substrates from other kits.

Reagents in this kit could be harmful while ingested, is breath in of is absorbed through the skin. Carefully read the information for each reagent in the safety data sheet before performing the analysis

6. HEALTH AND SAFETY PRECAUTIONS

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 100 Determinations, including column

Art. No. 40-2002, 100 Determinations, replacement kit

Component	Quantity
Calibrator, D_3-MMA 50 µM	0.5 mL
Internal standard $^{13}C_4D_3$-MMA, 100 µM	0.2 mL
Extraction solution including	40 mL
Reconstitution solution	20 mL
Tuning solution, MMA, $^{13}C_4D_3$-MMA, D_3-MMA and succinic acid, (0.5 µM of each)	0.5 ml
Column	1 pcs.

8. STORAGE CONDITIONS

The reagents should be stored at +2-8^{O}C

The Calibrator when diluted in whole blood should be stored frozen at -20^{O}C.

9. MATERIAL REQUIRED BUT NOT SUPPLIED

- LC-MS/MS Equipment
- Mobil phase A
- Mobil phase B
- Vortex-Mixer
- Centrifuge
- Pre-column or filter
- Injection vials
- 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. CHROMATOGRAPHIC CONDITIONS LC-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. TRANSITION

Compound	$M+H^+$ > 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

Follow the manufacturer's recommendations for the autoinjector. Since the mobile phases contain methanol, water and acid, a mixture of them is preferred.

15. STARTUP - OPTIMIZATION OF THE PARAMETERS FOR THE ANALYTES

Use the supplied MMA tuning solution (TUN) to find the exact transitions of the MRM tracks when installing 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.

16. SAMPLE STORAGE AND TRANSPORTATION

The analysis is made on blood/plasma. Use EDTA tubes. Invert the tube immediately after sampling 6–10 times. Keep the tube upright for 30 min after sampling (to avoid hemolysis). Avoid placing the test tubes in direct sunlight.

Durability during storage and transport from sampling to arrival at the analyzing laboratory.
Uncentrifuged sample:

Room temperature 15–23 °C, maximum 4 hours

Centrifugation:

Centrifuge the sample for 10 min at 2000 G, unless otherwise specified in the sampling instructions.

Room temperature 15–23 °C, maximum 1 day

Refrigerated 4 - 8 °C, maximum 3 days

Freeze the sample, -20 °C, if stored for longer than 3 days

17. PREPARATION OF INTERNAL STANDARD IN EXTRACTION SOLUTION

Centrifuge the ampule containing internal standard $^{13}C_4D_3$-MMA (2000 RCF, 2 min), open the ampule and add 0.5 ml Ext. sol., transfer the solution in its entirety to the Ext. sol. bottle, repeat 2 times to quantitatively transfer the internal standard to the Ext. sol. bottle. The final concentration of internal

standard $^{13}C_4D_3$-MMA in Ext. sol. is 0.5 µM.

18. SAMPLE PREPARATION

Handle the patient and calibration samples the way

1. To 50 µL blood add 250 µL extraction solution containing the internal standard. Mix well, vortex
2. Centrifuge 10 min,
3. Supernatant, evaporate to dryness.
4. Add 100 µL of reconstitution solution.
5. Transfer to injection vials

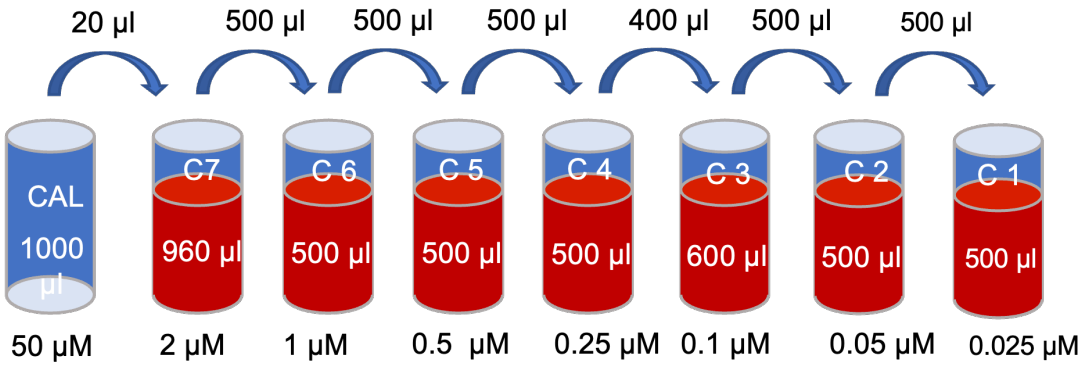
If the MS peaks become too high, dilute the samples with an appropriate volume of REC solution.

19. PREPARATION OF CALIBRATOR CURVE

The calibration curve is constructed using labelled D_3-MMA.

Dilutions in series: 40 µL 50 µM calibration solution, D_3-MMA, is added to 960 µL blood to get a concentration of 2.0 µM (C7 in table). Continue with the other calibration points as is indicated below.

Standard	Calibrator konc. (µM)	Stock	Volume (µL)	Volume (serum/ blood) (µL)	Total volume (µL)	Final volume (µL)
C7	2.0	CAL	40	960	1000	500
C6	1.0	C7	500	500	1000	500
C5	0.5	C6	500	500	1000	500
C4	0.25	C5	500	500	1000	600
C3	0.1	C4	400	600	1000	500
C2	0.05	C3	500	500	1000	500
C1	0.025	C2	500	500	1000	1000
C0	0			500	500	500



20. DETERMINATION OF MMA CONCENTRATION

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

To make it possible to use a calibration curve based on D_3-MMA for the quantification of MMA in blood, the MS response of both molecules must be identical. 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.

Lower limit of quantification (LOQ) is 0.025 µmol/L.

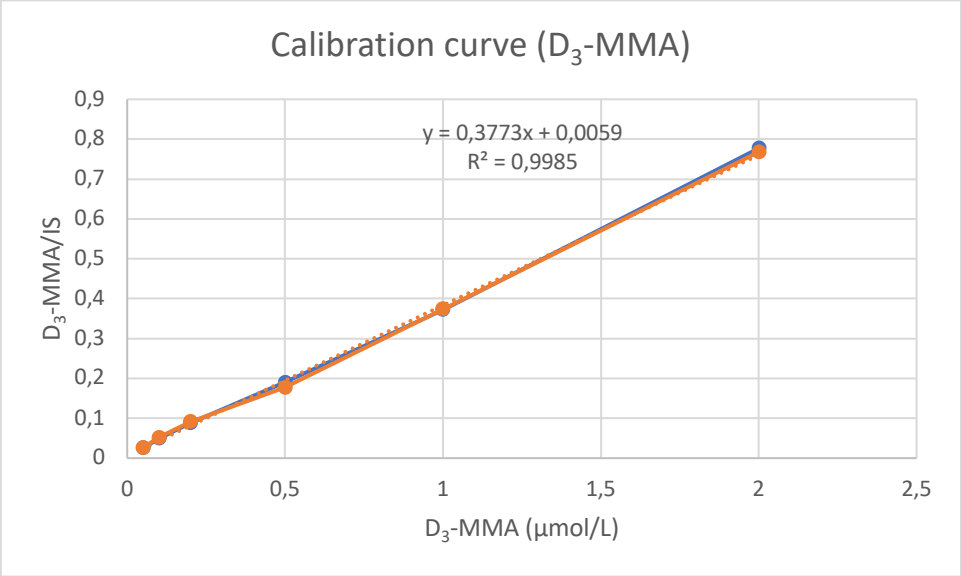


Figure 1 Highest calibration level (2 µmol/L)

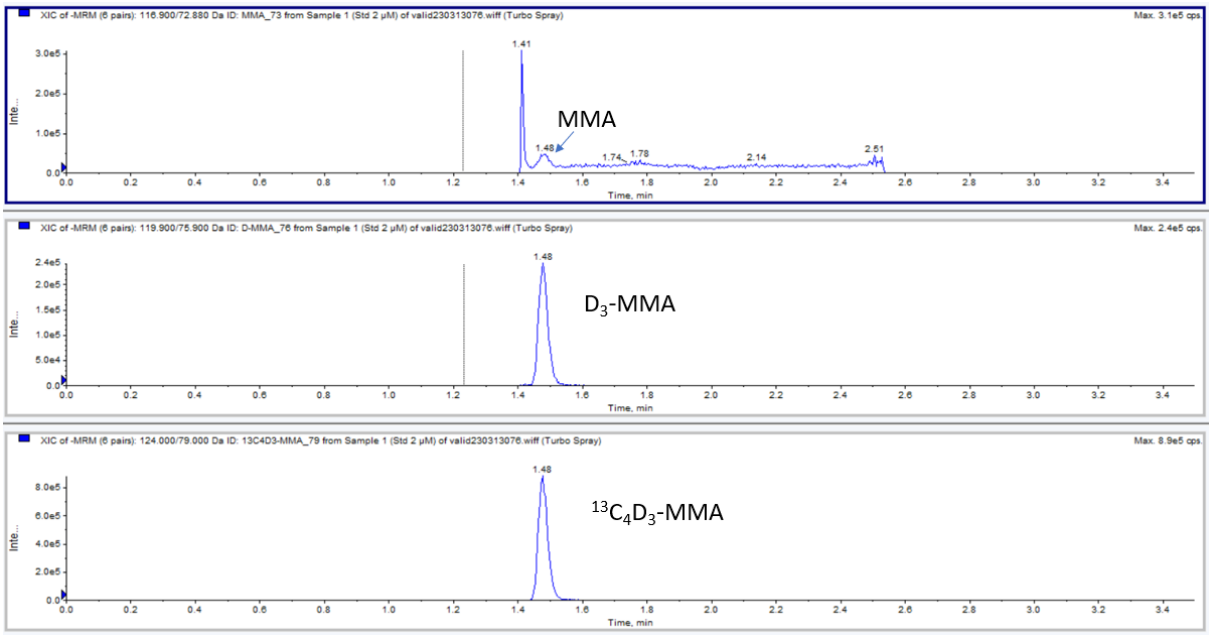


Figure 2. As the peak area of succinic acid in blood samples is much higher than MMA it is difficult to integrate MMA properly. Let the flow go to waste until shortly before the MMA peak elutes, most of the succinic acid peak will not interfere with the integration of MMA as seen in the figure.

21. QUALITY CONTROL

Control samples should 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

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

Control samples can be ordered from redhot diagnostics, Appendix 1

22. PERFORMANCE CHARACTERISTICS

Measuring range

0.025 – 2.0 μM

Samples over 2.0 µM should be diluted and analyzed again

Detection Level (lowest calibrator level)

0.025 µmol/L

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

According to the Karolinska University Hospital

<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 Algoritms 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