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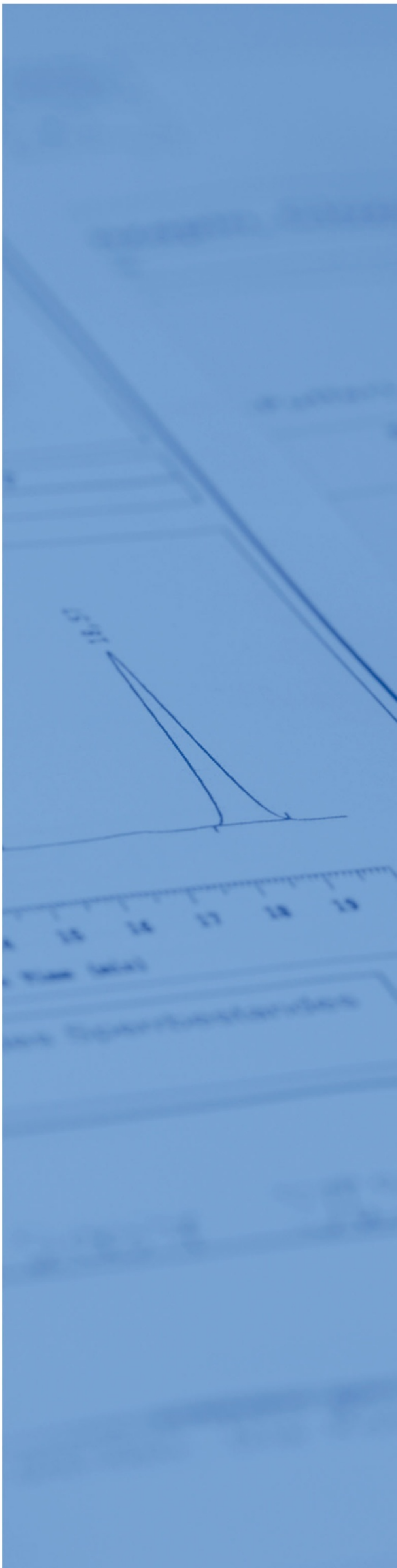

Instruction Manual

ClinMass® LC-MS/MS Complete Kit,
advanced

Methylmalonic Acid in Serum / Plasma / Urine

REF MS5100

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

COSMO BIO CO., LTD.
Inspiration for Life Science

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1 Introduction

1.1 Intended use

This ClinMass® Complete Kit is intended for the determination of methylmalonic acid from human serum, plasma and urine with LC-MS/MS.

The kit components have to be used in accordance with this user manual. The kit is not designed for combination with components from other manufacturers.

1.1.1 IVD symbols

Symbols according to EU directive 98/79/EC for in vitro diagnostic medical devices (IVDD), which are used on the product labels and in this user manual:

IVD	For in vitro diagnostic use	REF	Order number
🏭	Manufacturer	LOT	Lot number
		🌡	Upper temperature limit: … °C
		🌡	Temperature limits: … °C to …°C
		⌛	Expiry date: …
		📖	See instructions for use

1.2 Clinical background

Vitamin B12 (cobalamin) is an essential nutrient and plays an important role for the normal functioning of the human organism.

The coenzyme form of vitamin B12 (coenzyme B12) participates in two metabolic key positions. One of these reactions is the vitamin B12-dependent conversion of methylmalonyl-coenzyme A (CoA) to succinyl-CoA [1]. In cases of Vitamin B12 deficiency methylmalonyl-CoA accumulates and methylmalonic acid (MMA) is subsequently released (see figure 1) [1, 2].

Accordingly vitamin B12 deficiency results in quantitative accumulation of MMA in blood and urine. This occurs already in the early stages of insufficiency, i.e. when vitamin B12 levels still appear “normal” (see below), making MMA a sensitive, early biomarker for intracellular, functional vitamin B12 deficiency [2].

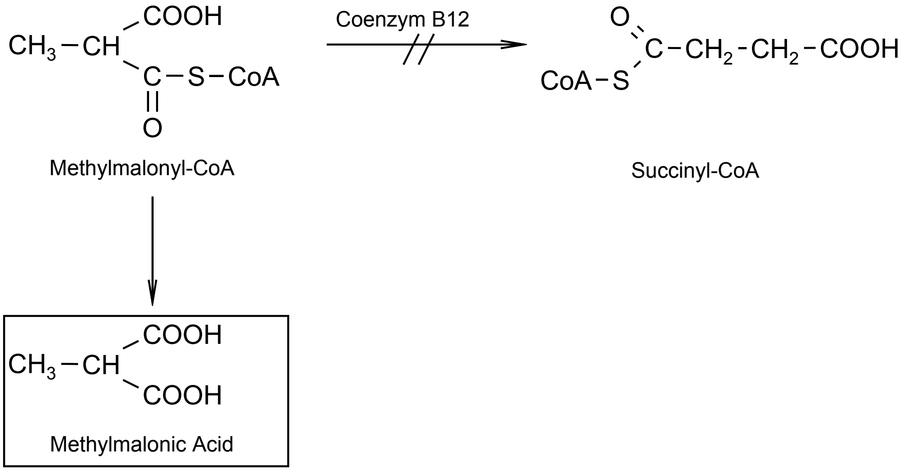

Figure 1: Vitamin B12 deficiency and release of MMA

In contrast, the determination of vitamin B12 in serum (as total vitamin B12), which is frequently used due to its cost efficiency, does not show adequate selectivity and sensitivity at the lower reference level range (below 400 pmol/l) [1]. As such this can lead to potential false negative diagnosis in cases of intracellular functional vitamin B12 deficiency, where vitamin B12 levels appear normal (> 156 pmol/l). In such cases however the serum MMA is already significantly increased (> 300 nmol/l) and clearly indicates the deficiency [1].

In these particular cases holotranscobalamin (Holo TC) and homocysteine* will be determined in addition to total vitamin B12 and MMA. Holo TC is the intracellularly utilised form of vitamin B12 and, as a precursor of coenzyme B12, is required for the conversion of MMA and homocysteine. A metabolically manifested vitamin B12 deficiency will thus be indicated by lowered levels of Holo TC and by increased levels of MMA and homocysteine [1 - 3].

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*For the determination of homocysteine, the following products available:
ClinMass® LC-MS/MS Complete Kit for Homocysteine in Plasma / Serum (order no. MS2000)
ClinRep® HPLC Complete Kit for Homocysteine in Plasma (order no. 23000)

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 is the case with urine [4, 5]. Additional measurement of creatinine is also necessary for the determination from urine, as the MMA/creatinine ratio is required for data interpretation [5].

The advantage of determination from urine however lies in the significantly higher MMA levels, which facilitate the analyses. In cases of patients with impaired renal function serum MMA measurements may provide false positive results due to reduced urinary MMA excretion [6]. However calculation of the urine MMA/creatinine ratio can compensate for this [7].

Mass spectrometry based methods have been widely tested for the determination of MMA.

GC/MS has been routinely applied to the quantitation of MMA, however, due to the requirement of derivatisation prior to analysis an alternative method with less time-consuming sample preparation and hence faster turn-around time is of continued interest.

The application of LC-MS/MS methods to MMA determination has received increased attention in the last few years, which however still bears some challenges due to the low endogenous concentration of MMA, the highly polar nature, low molecular weight, low pKa and dicarboxylic acid structure. Furthermore chromatographic separation from the naturally occurring structural isomer succinic acid (SA), present in physiological concentrations approximately 50 times higher than MMA, is critical and not elementary. Many methods hence require lengthy sample preparation steps such as solid-phase extraction, derivatisation, evaporation and/or ultra-filtration, and can also show sub-optimal resolution from succinic acid [8, 9].

This method was developed for the routine analysis of methylmalonic acid (MMA) in human serum, plasma and urine samples. Sample preparation is simple and rapid, and analogous for the different biological matrices. Calibration is performed using lyophilised serum calibrators at clinically relevant levels. Lyophilised serum controls are also available for quality assurance. An isotope-labelled internal standard (d3-methylmalonic acid) is used in order to compensate for matrix effects and measurement variations. Samples are analysed using negative ion electrospray in MRM mode for maximum sensitivity and selectivity.

1.3 General description of the analytical procedure

In this analytical method MMA is determined from human serum, plasma or urine by HPLC with electrospray-tandem mass spectrometry (LC-MS/MS).

The routine analysis of MMA is primarily performed from serum. However, the methodology presented here can also be applied to plasma (citrate-, EDTA- and heparin-) and urine matrices (see collection and storage of samples, section 5.1). In the case of urine samples (required for patients with renal insufficiency, see section 1.2) the creatinine level must also be quantified and results interpreted from the MMA/creatinine ratio (see evaluation, section 6).

Prior to the LC-MS/MS analysis a short sample clean-up is performed in order to remove the sample matrix and to spike with the internal standard (sample preparation, see section 5.2).

After the chromatographic separation on the analytical column within the HPLC system, MMA is ionised by electrospray ionisation (ESI) and detected by the tandem mass spectrometer (MS/MS).

In electrospray ionisation the sample components are ionised and then transferred to the gas phase, where they subsequently pass into the MS/MS, which is composed of two quadrupoles and connected through a collision cell.

In this analytical method the MS/MS measurement of the analytes is performed in the MRM (Multiple Reaction Monitoring) mode. In this mode only selected ions (known as the ‘precursor ions’) with a defined mass/charge ratio (*m/z*) are isolated in the first quadrupole and subsequently are transferred into the collision cell. These ions are then fragmented by impact with an inert gas (argon or nitrogen) at defined voltage settings. Among the fragments generated (known as the ‘product ions’), only those with a defined *m/z* ratio are isolated in the final quadrupole for subsequent detection. Thus, measurement in MRM mode ensures identification and quantification with high selectivity and sensitivity, with the analyte identification based on characteristic mass transitions for the compound of interest.

The ClinMass® Optimisation Mix is provided for the optimisation of the MS/MS parameters (see section 5.3.1) and for the test run of the analytical system (see section 5.3.2).

The calibration of the analytical system is performed by use of ClinCal® Serum Calibrators. For this purpose a 4-Level Serum Calibrator Set is provided (see section 5.3.3).

Quality control is performed by use of ClinChek® Serum Controls. These controls are available in two different concentrations (see section 5.3.4).

2 Components of the complete kit and accessories

2.1 Ordering information

Order No.	Description	Quantity
MS5100	**ClinMass® Complete Kit, *advanced,* for Methylmalonic Acid in Serum / Plasma / Urine** for 300 assays	1 pce.
	Contents:	
	Autosampler Washing Solution	1 x MS5005
	Mobile Phase A	1 x MS5108
	Mobile Phase B	1 x MS5109
	P Precipitant with Internal Standard	3 x MS5112
	Serum Calibrator Set, lyophil. (Level 0 - 3)	1 x MS5013
	Sample Preparation Vials	3 x MS5020
	Manual	
	Separately available components:	
MS5005	Autosampler Washing Solution	1000 ml
MS5108	Mobile Phase A	1000 ml
MS5109	Mobile Phase B	200 ml
MS5112	P Precipitant with Internal Standard	40 ml
MS5013	Serum Calibrator Set, lyophil. (Level 0 - 3)	4 x 1 x 2 ml
MS5114	Optimisation Mix	2 ml
MS5020	Sample Preparation Vials	100 pcs.
MS5021	D Diluting Solution for Urine	50 ml
	Start Accessories:	
MS5130	Analytical Column with test chromatogram	1 pce.
	ClinChek® Controls:	
MS5080	Serum Control, lyophil., Level I	10 x 2 ml
MS5081	Serum Control, lyophil., Level II	10 x 2 ml
MS5082	Serum Control, lyophil., Level I, II	2 x 5 x 2 ml

Please note:

Apart from the use in sample preparation, Diluting Solution D for Urine (order no. MS5021) is also intended for the optimisation and test run of the analytical system (see sections 5.3.1 and 5.3.2). Diluting Solution D for Urine is therefore also required for analysis of serum and plasma samples.

2.1.1 Safety information

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

The calibrator and control materials are prepared from human serum. Although the products are tested for the absence of common infection markers, they still should be considered as potentially infectious. For this reason we recommend the product to be handled with the same precautions as patient samples. Detailed safety information is indicated in the respective Material Safety Data Sheet (MSDS).

2.1.2 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 and table 1.

Unused components, stored under appropriate conditions can be used until the expiry date indicated on the product label.

After use of ClinMass® Reagents and ClinMass® Mobile Phases, the bottles must be closed tightly and stored immediately under the required conditions. Provided proper use and storage procedures are followed, the lifetime of the reagents is the same as for the unused products.

For storage conditions and lifetime of the ClinMass® Optimisation Mix as well as the ClinCal® Calibrators and ClinChek® Controls (lyophilised / after reconstitution) please also refer to the respective product data sheets.

Table 1: Storage conditions of kit components

Order no.		**Product description**	**Storage conditions**	
REF	MS5005	Autosampler Washing Solution	15°C 30°C	Store at 15 - 30 °C
REF	MS5108	Mobile Phase A	15°C 30°C	Store at 15 - 30 °C
REF	MS5109	Mobile Phase B	15°C 30°C	Store at 15 - 30 °C
REF	MS5112	P Precipitant with Internal Standard	-18°C	Store below - 18 °C
REF	MS5013	Serum Calibrator Set, lyophil., Level 0 - 3	2°C 8°C	Store at 2 - 8 °C*
REF	MS5114	Optimisation Mix	-18°C	Store below - 18 °C
REF	MS5020	Sample Preparation Vials	Store at ambient temperature	
REF	MS5021	D Diluting Solution for Urine	2°C 8°C	Store at 2 - 8 °C

REF	MS5130	Analytical Column	15 °C 30 °C	Store at 15 - 30 °C
REF	MS5080 - MS5082	Serum Controls, lyophil., Level I, II, I+II	2 °C 8 °C	Store at 2 - 8 °C*

*Refers to the lyophilised product. For storage conditions after reconstitution, please refer to the product data sheet.

2.1.3 Disposal of laboratory waste

For disposal, laboratory waste should be collected separately according to the different chemical properties. Recommendations for the disposal of product and packaging are indicated in section 13 of the respective Material Safety Data Sheet (MSDS).

3 Required instruments

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

Requirements for the tandem mass spectrometer:

The tandem mass spectrometer should be of comparable (or higher) sensitivity as the instrument described in section 4.3.2.

Required LC modules:

- Autosampler
- Binary HPLC gradient pump
- Column heater
- Degasser

For sample preparation the following laboratory instruments are required:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

4 Operation of the analytical system

4.1 Flushing of the LC system

Connect the LC modules, **excluding** the column, with the outlet capillary directed into a safe waste container.

Set the HPLC pump at a flow rate of 1 ml/min and flush the LC system with 10 ml Mobile Phase A/B (Mobile Phase A/B = 50:50).

Thereafter connect the analytical column within the column heater.

When connecting the analytical column please make sure the flow direction follows the arrow marking on the column!

Also take care that the fittings used are appropriate to the column. These should be custom-prepared with a new fitting and the column. In case of questions, please contact RECIPE for detailed installation instructions.

4.2 Equilibration of the LC system

After flushing the system (see section 4.1) the equilibration is performed as follows:

- Set the HPLC pump to a flow rate of 0.7 ml/min, set the column heater to 25 °C, and equilibrate the column with approximately 10 ml Mobile Phase A (gradient starting condition).
- Subsequently **stop the HPLC pump** and connect the outlet capillary of the analytical column with the tandem mass spectrometer.

4.3 Starting the analytical system

The following sections provide the parameters for the LC system (see section 4.3.1) and the tandem mass spectrometer (see section 4.3.2). For optimisation, equilibration, testing, and calibration of the LC-MS/MS system, please refer to section 5.3.

Please consult the user manual of the tandem mass spectrometer to ensure appropriate usage. User trainings, provided by the instrument manufacturer, may also be advisable.

4.3.1 LC parameters

Table 2: LC parameters

Binary HPLC gradient pump:	Flow rate: 0.7 ml/min see table 3, table 4 and table 5.
Mobile Phases A and B:	Make sure that the bottles are closed well to avoid alteration of the retention times through evaporation of components of the mobile phases.
Column:	The analytical column* is installed in the column heater (25° C). At a flow rate of 0.7 ml/min the backpressure of the analytical column should not exceed 300 bar. For the complete HPLC system the backpressure should not exceed 400 bar. *Please see section 4.4 for appropriate deinstallation and storage of the analytical column.
Column heater:	25 °C
Autosampler:	Use the recommended needle wash settings (for minimum sample carry over) from the autosampler supplier. E.g.: For the Agilent autosampler set a 6 second needle wash, using the flushport. Injection volume: 2 - 5 µl Injection interval: 3.0 min / 2.5 min / 2.0 min (see table 3, table 4 and table 5)

The Mobile Phase A/B and flow rate gradients of the HPLC pump are programmed according to the tables* below.

* Please note: The gradients indicated are based on the standard configurations of mixer and pulse damper. Use of other configurations will affect retention times and performance of the analytical procedure. The retention time of MMA should be between 1.9 - 2.3 min (see table 3), 1.4 - 1.8 min (see table 4) and 1.3 - 1.7 min (see table 5), respectively.

The parameters shown in table 3 refer to the Agilent 1100/1200 LC-system with mixer.

Table 3: Mobile Phase A/B gradient and flow rate for the LC system Agilent 1100/1200 with mixer

Time [min]	**Mobile Phase A [%]**	**Mobile Phase B [%]**	**Flow rate [ml/min]**
0.00	100	0	0.7
0.01	70	30	0.7
0.05	70	30	0.7
0.10	40	60	0.7
1.10	40	60	0.7
1.11	0	100	0.7
1.20	100	0	0.7
3.00	100	0	0.7

The parameters shown in table 4 refer to the Agilent 1200 LC-system without mixer and the Agilent 1200 SL LC system.

Table 4: Mobile Phase A/B gradient and flow rate for the LC system Agilent 1200 without mixer and Agilent 1200 SL

Time [min]	**Mobile Phase A [%]**	**Mobile Phase B [%]**	**Flow rate [ml/min]**
0.00	100	0	0.7
0.20	100	0	0.7
0.21	70	30	0.7
0.25	70	30	0.7
0.30	40	60	0.7
0.90	40	60	0.7
1.00	0	100	0.7
1.01	100	0	0.7
2.50	100	0	0.7

The parameters shown in table 5 refer to the Agilent 1290 UHPLC system (with the 35 µl JetWeaver mixer).

Table 5: Mobile Phase A/B gradient and flow rate for the UHPLC system Agilent 1290

Time [min]	**Mobile Phase A [%]**	**Mobile Phase B [%]**	**Flow rate [ml/min]**
0.00	100	0	0.7
0.50	100	0	0.7
0.51	70	30	0.7
1.00	70	30	0.7
1.10	40	60	0.7
1.30	40	60	0.7
1.31	0	100	0.7
1.40	100	0	0.7
2.00	100	0	0.7

4.3.2 MS/MS parameters

The MS/MS parameters indicated in the following tables are recommended values only. This particularly applies to the mass transition specific parameters. The values should be regarded as starting points for optimisation. The optima vary between different MS/MS systems and therefore should be optimised for the system to be used. ("Compound optimisation", see section 5.3.1).

The parameters shown in table 6 refer to the MS/MS system API 4000™.

Table 6: MS/MS parameters, API 4000™

	API 4000™
Ion Source	Turbo-ionspray (TIS) ESI
Polarity	negative
Resolution Q1 and Q3	unit (0.7 amu)
Nebuliser Gas/GS 1	60
GS 2	50
Curtain Gas (CUR)	35
Collision Gas (CAD)	5
Ion Spray Voltage (IS)	-1500 V
Source Temperature (TEM)	550 °C
Interface Heater (ihe)	ON
Mass transitions	see table 7

Table 7: Mass transitions, API 4000™

Substance	**Precursor [amu]**	**Product [amu]**	**Dwell time [ms]**	**DP [V]**	**CE [V]**	**CXP [V]**	**EP [V]**
MMA (Quantifier)	116.9	73	100	-35	-12	-3	-10
MMA (Qualifier)	116.9	55	100	-35	-32	-3	-10
MMA-d3 (Quantifier)	119.9	76	100	-40	-12	-3	-10
MMA-d3 (Qualifier)	119.9	58	100	-40	-32	-3	-10

4.4 Standby mode

When the analytical system is not in use, the HPLC pump should be switched off. The mobile phases can be left within the LC system.

The vacuum pumps of the tandem mass spectrometer (MS/MS system) should be in permanent operation. In order to protect the ion source and multiplier, the MS/MS system should be switched into the standby mode.

If the system is not used for more than 2 days, the analytical column should be disconnected and closed tightly.

5 Implementation of the analytical procedure

5.1 Collection and storage of samples

5.1.1 Serum and plasma

The routine analysis of MMA is primarily performed from serum. If serum is not available, plasma (citrate-, EDTA- and heparin-plasma) can also be used.

The samples can be stored at least 3 days at room temperature (15 - 30 °C), at least 7 days at temperatures between 2 - 8 °C and at least 3 months at temperatures below -18 °C (multiple freeze-thaw cycles should be avoided).

5.1.2 Urine

In the cases of patients with impaired renal function, the analysis is performed from the second early morning urine.

The stability of urine samples is identical to those of serum and plasma samples (for storage conditions see section 5.1.1).

5.2 Sample preparation

5.2.1 Reconstitution of the lyophilised serum calibrators / controls

ClinCal® Serum Calibrators and ClinChek® Serum Controls (see section 2.1) are lyophilised and must be reconstituted before use. Information regarding reconstitution, analyte concentrations, storage and stability is indicated in the respective product data sheets.

5.2.2 Serum and plasma

5.2.2.1 Work flow

Sample preparation:

Precipitation:	400 µl P Precipitant (contains Internal Standard)	100 µl serum, plasma (calibrator, control, patient)

⇩ mix for 30 sec (vortex mixer)

⇩ centrifuge (5 min, 10000 x g)

LC-MS/MS analysis:	Inject 2 - 5 µl supernatant

5.2.2.1.1 Precipitation

Pipette 400 µl Precipitant P (contains Internal Standard IS) into a sample preparation vial (order no. MS5020) and then add 100 µl of the serum or plasma sample (calibrator, control, patient). Mix for 30 sec on a vortex mixer and subsequently centrifuge for 5 min at 10000 x g.

5.2.2.1.2 LC-MS/MS analysis

Transfer the centrifuged supernatant to a sample vial, which is suitable for the autosampler in use. Depending on the sensitivity of the LC-MS/MS system, inject 2 - 5 µl of the supernatant.

5.2.2.1.3 Stability of the prepared samples

The prepared samples can be stored at least 7 days at temperatures between 2 - 8 °C and at least 3 months at temperatures below -18 °C (multiple freeze-thaw cycles should be avoided).

5.2.3 Urine

5.2.3.1 Work flow

Sample preparation:

Dilution:	1000 µl D Diluting Solution for Urine	50 µl urine (patient)
	⇩ mix shortly (vortex mixer)	
Addition of IS:	400 µl P Precipitant (contains Internal Standard)	100 µl diluted urine
	⇩ mix shortly (vortex mixer)	
LC-MS/MS analysis:	Inject 2 - 5 µl sample	

5.2.3.1.1 Dilution

For dilution, pipette 1000 µl Diluting Solution D into a sample preparation vial (order no. MS5020) and add 50 µl of the urine sample (patient). Subsequently mix shortly on a vortex mixer.

5.2.3.1.2 Addition of IS

Pipette 400 µl Precipitant P (contains Internal Standard IS) into a sample vial suitable for the autosampler in use. Subsequently add 100 µl of the diluted urine (see section 5.2.3.1.1) and mix shortly on a vortex mixer. Afterwards put the sample into the autosampler.

5.2.3.1.3 LC-MS/MS analysis

Depending on the sensitivity of the LC-MS/MS system in use, inject 2 - 5 µl of the sample into the LC-MS/MS System.

5.2.3.1.4 Stability of the prepared samples

The prepared samples can be stored at least 7 days at temperatures between 2 - 8 °C and at least 3 months at temperatures below -18 °C (multiple freeze-thaw cycles should be avoided).

5.3 LC-MS/MS analysis

Independent from the analytical method, the mass accuracy of the tandem mass spectrometer (MS/MS) should be checked at regular intervals. A mass calibration may be required.

For information regarding the check-up of the MS/MS system, please refer to the documentation provided by the instrument manufacturer.

5.3.1 Compound optimisation (MS/MS)

For the optimisation of the MS/MS system parameters the Optimisation Mix is provided (“compound optimisation”). The Optimisation Mix contains the analyte (methylmalonic acid) and the Internal Standard IS (d_3-Methylmalonic Acid).

The Optimisation Mix should be diluted with Diluting Solution D (order no. MS5021) according to the sensitivity of the MS/MS system in use. For the API 4000™ MS/MS system we recommend a 1+1 dilution of the Optimisation Mix with Diluting Solution D.

The compound optimisation procedure for the MS/MS system in use should then be followed in order to optimise the ionisation source parameters and the compound specific mass transition parameters.

5.3.2 Equilibration of the analytical system and test run

Equilibrate the entire analytical system for at least 30 min before injecting samples.

Before each series of analyses perform a blank-injection (injection volume 0 µl or injection of Mobile Phase A). This procedure provides reproducible results right from the first sample injection.

In order to confirm the performance of the analytical system, repeatedly inject the Optimisation Mix until two consecutive chromatograms, comparable in retention times and peak areas, are obtained.

A dilution of the Optimisation Mix with Diluting Solution D will be required, depending on the sensitivity of the MS/MS system in use. For the API 4000™ MS/MS system we recommend a 1+19 dilution of the Optimisation Mix with Diluting Solution D.

5.3.3 Calibration run

For calibration, a ClinCal® 4-Level Serum Calibrator Set (level 0 - 3, order no. MS5013) is available.

The serum calibrators can also be reliably used for the accurate determination of MMA from plasma and urine samples.

Please note that a scale factor must be considered for the quantitation of urine samples (see section 6).

The calibrators are lyophilised and, subsequent to reconstitution (see section 5.2.1), must be prepared as described for the patient samples (see section 5.2).

For each analytical series freshly prepared calibrators should be used.

5.3.4 Accuracy control

For the quality control of the analytical measurements, ClinChek® Serum Controls in two concentrations are available (level I, order no. MS5080; level II, order no. MS5081; level I + II, order no. MS5082).

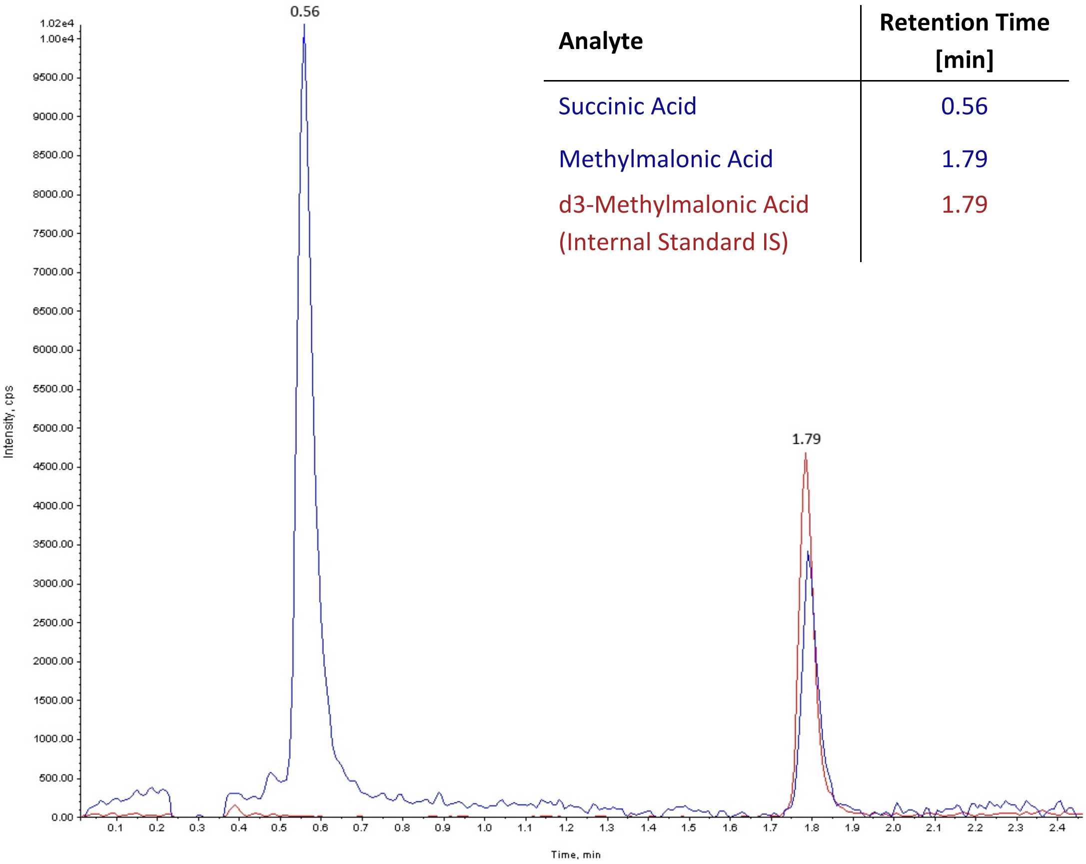
The serum controls can also be reliably used for the accurate determination of MMA from plasma and urine samples.

These controls are lyophilised and, subsequent to reconstitution (see section 5.2.1), must be prepared as described for the patient samples (see section 5.2).

For each analytical series freshly prepared controls must be used. In case of large analytical series we recommend to inject these controls additionally at the end of the series.

5.3.5 Example chromatogram

Example chromatogram of the ClinChek® Serum Control, level I (order no. MS5080), recorded with the Agilent 1200 LC system without mixer and the MS/MS system API 4000™.



Analyte	**Retention Time [min]**
Succinic Acid	0.56
Methylmalonic Acid	1.79
d3-Methylmalonic Acid (Internal Standard IS)	1.79

Figure 2: Chromatogram of the ClinChek® Serum Control, level I (order no. MS5080)

6 Evaluation

The analyte detection is achieved using compound specific mass transitions (see section 4.3.2)

The evaluation of the analyte concentration is performed by the internal standard method using the peak areas.

Calibration curves are achieved for the calibrators by plotting the ratio *analyte peak area/internal standard peak area* against the ratio *analyte concentration/internal standard concentration*.

The analyte concentrations for samples and controls are calculated from the calibration curve.

Please consult the software user manual of the MS/MS manufacturer in order to ensure correct evaluation of the results.

For the calculation of mass concentrations [µg/l] into molar concentrations [nmol/l], and vice versa, the analytical results should be multiplied with the factors shown in table 8.

Table 8: Conversion factors

Analyte	**Molecular weight [g/mol]**	**Conversion factor : nmol/l --> µg/l**	**Conversion factor: µg/l --> nmol/l**
MMA	118.09	0.118	8.468

Urine samples:
In case of urine samples, the creatinine level must be quantified and the results interpreted from the **mol MMA/mol creatinine** ratio.

Due to the calibration with the ClinCal® Serum Calibrator (no dilution within sample preparation), the urine MMA analytical results must be multiplied with the **scale factor = 21**.

7 Test data

7.1 Test performance

The results were obtained with the API 4000™ MS/MS system.

7.1.1 Linearity, detection limit, quantitation limit

	Serum/Plasma		Urine	
	[µg/l]	**[nmol/l]**	**[µg/l]**	**[nmol/l]**
Linearity	3 - 8000	25 - 67745	77 - 168420	651 - 1426200
LLOD	1.8	15	46	391
LLOQ	3.0	25	77	651

LLOD: Lower limit of detection, LLOQ: Lower limit of quantitation

7.1.2 Recovery

For MMA mean recovery rates between 91 - 116 % were obtained.

7.1.3 Precision

7.1.3.1 Intraassay

For the evaluation of the intraassay precision 3 samples with the following concentrations were used:

	MMA [nmol/l]
Sample 1	148
Sample 2	275
Sample 3	614

The samples were measured in 3 analytical series, each by 8-fold determination (n = 24; n: number of values per sample). The following coefficients of variation (CV) were obtained (mean values):

	CV [%]
Sample 1	5.65
Sample 2	3.60
Sample 3	3.77

7.1.3.2 Interassay

For the evaluation of the interassay precision 3 samples with the following concentrations were used:

	MMA [nmol/l]
Sample 1	186
Sample 2	479
Sample 3	686

The samples were measured in 8 analytical series, each by 2-fold determination (n = 16; n: number of values per sample). The following coefficients of variation (CV) were obtained:

	CV [%]
Sample 1	5.76
Sample 2	6.08
Sample 3	3.47

7.2 Reference Ranges

	Plasma, Serum [10]	**Urine** [11]
Normal range	73 - 271 nmol/l	< 3.6 mmol/mol creatinine

The indicated reference ranges are taken from thoroughly selected and current scientific literature. Their actuality corresponds to the printing date of this document. Please note that these ranges do not reflect any recommendations by the manufacturer of this product, but may be used as a guideline for the assessment of the reference range by the clinical laboratory.

8 References

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9 Troubleshooting

Problem	**Possible Cause**	**Corrective Measure**
Gradient profile cannot be generated	Defective HPLC pump	Check the pumps
	Air within the system	Degas the mobile phases and flush and purge the HPLC system thoroughly
	Fluctuation of the flow rate	Check the pumps
Interference signals	Injection system contaminated	• Rinse needle with acidified methanol or inject 10 x Mobile Phase B • Check flushport solvent level • Clean/exchange needle seat assembly and/or injection valve
	Sample vials contaminated	Use new vials
	Vial septum contaminated	Use another septum
	Mobile phase contaminated	Change the mobile phases and flush the system
	Column(s) (guard / analytical column) contaminated	Change the guard / analytical column
	Mass resolution too low	Optimise mass resolution
	System not correctly configured	Check all connections
No signals	Injector defect	Check injector
	Defective HPLC pump	Check the pumps
	MS/MS system not ready for operation	Check the MS/MS system
Decrease of sensitivity	Ion source contaminated	Clean the ion source
	Mass spectrometer contaminated	Clean the mass spectrometer
	Leakage of injection valve	Check the injector
	Shift of mass calibration	Recalibrate MS/MS system
	Mass resolution too high/low	Optimise the mass resolution

Problem	**Possible Cause**	**Corrective Measure**
High fluctuations of signals	Spray instable	Check the spray needle capillary and clean or exchange, if necessary
	Fluctuation of the flow rate	Check the HPLC pumps
	Gas flow rate instable	Check the gas lines
No vacuum	Defective vacuum pumps	Check the pre- and high-vacuum pumps
	Leakage within the vacuum system	Check the vacuum tubes and fittings
No gas supply	Defective nitrogen generator	Check the nitrogen generator
	Defective compressor	Check the compressor
	Gas bottle is empty	Replace the gas bottle
	Inlet gas pressures are not within the specified range	Regulate the inlet gas pressures

10 Appendix: EC-Declaration of Conformity

Declaration of Conformity

for in-vitro diagnostic medical devices, acc. to article 9 (1) of the directive 98/79/EC

The company

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
D-80992 Munich / Germany

declares, that the CE labelled product

ClinMass® Complete Kit, *advanced,* for Methylmalonic Acid (order no. MS5100)

meets all applicable provisions of the directive on in vitro diagnostic medical devices 98/79/EC. The conformity assessment was performed according to annex III. The technical documentation is held according to annex III no. 3.

Munich, 12.09.2013

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Zertifiziert nach /
Certified acc. to
ISO 9001
ISO 13485

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