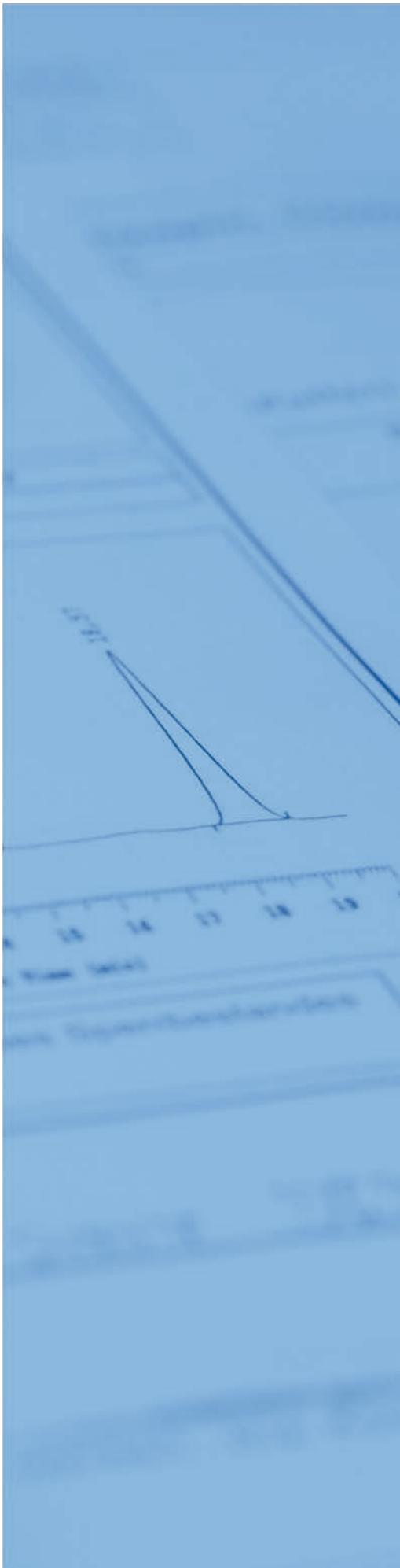
RECIPE



Instruction Manual

ClinSpot® LC-MS/MS Complete Kits

Amino Acids and Acylcarnitines in Dried Blood Spots (DBS)

REF MS10000, MS10100

IVD For in vitro diagnostic use

CE 2797 IVDD, 98/79/EC

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3, 80992 München / Germany
Phone: +49 / 89 / 54 70 81 - 0
Fax: +49 / 54 70 81 - 11
info@recipe.de
www.recipe.de

REF MS10000, MS10100

IVD For in vitro diagnostic use

Document version:	3.1
Replaces:	3.0
Date of revision:	07.08.2019
File name:	MS10000-MS10100_m_e_V3-1.docx

Contents

Contents

1 Introduction

1.1 Information on changes in this instruction manual

This instruction manual (version 3.1) replaces the previous version 3.0.

Please note the updated information regarding the stability of prepared samples in section 5.2.4

Version 3.0 (previous version)	**This version 3.1**
Stability of the prepared samples at room temperature (15–30 °C): 7 days	Stability of the prepared samples at room temperature (15–30 °C): 3 days, at temperatures between 2–8 °C: 7 days

The changes are marked on the page margin.

1.2 Intended use

The ClinSpot® Complete Kits are intended for the semi-quantitative determination of amino acids and acylcarnitines for neonatal screening. The determination is performed with LC-MS/MS from dried blood spot specimen.

The components of the complete kit are intended to be used according to the instructions in this manual. The kit is not designed for combination with components of other manufacturers.

1.2.1 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:

For in vitro diagnostic use


Manufacturer

Order number

Lot number


Upper temperature limit: … °C


Temperature limits: … °C to … °C


Expiry date: …


See instructions for use

1.3 Clinical background

Neonatal screening is an important population-wide preventive measure to detect congenital metabolism disorders at a very early stage. The range and demands on the screening programme vary in the different European countries, usually regulated within the respective national legislation.

In Germany newborns have a legal right on the screening of currently 13 target diseases (chapter A §1 and C.I. of the „Kinder-Richtlinien“ [1]). Of these target diseases those listed in Table 1 are screened by tandem mass spectrometry. The determination of amino acids and acylcarnitines is used for the screening of aminoacidopathies, fatty acid oxidation defects, carnitine cycle defects and organoacidemias.

Table 1. Target diseases, incidence, prevalence and diagnostic parameters, prepared according to [2, 3]

Target diseases	**Prevalence[1]**	**Incidence[2]**	**Diagnostic Parameters**
Aminoacidopathies			
Phenylketonuria (PKU) and hyperphenylalaninemia (HPA)	1 : 10000 1 : 7000	1 : 5584	Phenylalanine, Phe/Tyr quotient
Maple syrup urine disease (MSUD)	1 : 160000	1 : 162.273	Leucine/isoleucine, valine
Tyrosinemia type I	1 : 135000	–	Succinylaceton[3]
Fatty acid oxidation defects			
MCAD deficiency (medium-chain-acyl-CoA-dehydrogenase deficiency)	1 : 10000	1 : 10.610	Octanoylcarnitine (C8) and other medium-chain acylcarnitines
LCHAD deficiency (long-chain 3-OH-acyl-CoA-dehydrogenase deficiency)	1 : 170000	1 : 212.202	Long-chain hydroxylated acylcarnitines (C14OH, C16OH, C18OH, C18:1OH)
VLCAD deficiency (very-long-chain-acyl-CoA-dehydrogenase deficiency)	1 : 76000	1 : 88.988	Long-chain acylcarnitines, in particular C14:1-Carnitine
Carnitine cycle defects			
CPT-I deficiency (CPT: Carnitine palmitoyl transferase)	– (very rare disease)	1 : 551.727	Free carnitine (C0), Long-chain acylcarnitines
CPT-II deficiency	– (very rare disease)	1 : 919.544	Free carnitine (C0), Long-chain acylcarnitines
CACT deficiency (CACT: Carnitine-acylcarnitine-translocase deficiency)	– (very rare disease)	–	Long-chain acylcarnitines
Organoacidemias			
Glutaraciduria type I (GA I)	1 : 130000	1 : 125.392	Glutarylcarnitine (C5DC-Carnitine)
Isovaleric acidemia (IVA)	1 : 95000	1 : 114.943	Isovalerylcarnitine (C5-Carnitine)

[1]Calculated on the basis of 6 million neonates born in Germany between 2006 and 2014 [2]

[2]Data of neonates tested between 2005 and 2008 (n = 2.758.633) according to [3]

[3]The analyte succinylacetone is not an element of the method of the kits with order nos. MS10000 and MS10100

All diseases are caused by genetic enzyme defects. The enzyme defect leads to extreme metabolism disorders and an accumulation of toxic metabolites, which may cause irreversible organ damage already in the first days of life. The blood withdrawal for the screening must therefore be carried out in the first 36–72 hours after birth. The diagnosis needs to be established within 72 hours after the blood extraction [1].

For the neonatal screening dried blood samples are tested by LC-MS/MS (see general description of the method in section 1.4). For the sample collection the heel is punctured and the blood is applied onto a filter test card and dried (see section 5.1).

Due to the high analytical sensitivity and selectivity of the tandem mass spectrometry the samples can be tested in a time-saving way without HPLC separation (injection interval: 1.0–1.6 min). The samples however are determined semi-quantitatively only as the blood volume varies depending on the used filter paper and the haematocrit level of the dried blood sample.

The screening on its own therefore does not allow to establish the diagnosis, it just gives reason to suspect the presence of a disease. Thus, any positive screening result needs to be verified by additional tests (confirmation diagnosis) [2, 3] such as molecular-genetic tests, the analysis of amino acids and acylcarnitines in plasma as well as organic acids in urine [4].

1.4 General description of the analytical method

This analytical method directly determines the following amino acids and acylcarnitines, without chromatographic separation on an HPLC column, via tandem mass spectrometry (MS/MS) at a constant flow rate:

Table 2. Determination of amino acids and acylcarnitines with the complete kits MS10000/MS10100

Amino acids		**Acylcarnitines (C0 – C18-Carnitine)**	
Ala	Alanine	C0	Free Carnitine
Arg	Arginine	C2	Acetylcarnitine
Asp	Aspartic acid	C3	Propionylcarnitine
Cit	Citrulline	C4	Butyrylcarnitine
Glu	Glutamic acid	C5	Isovalerylcarnitine
Gly	Glycine	C5DC	Glutarylcarnitine
Leu	Leucine	C6	Hexanoylcarnitine
Met	Methionine	C8	Octanoylcarnitine
Orn	Ornithine	C10	Decanoylcarnitin
Phe	Phenylalanine	C12	Dodecanoylcarnitine
Pro	Proline	C14	Tetradecanoylcarnitine (Myristoylcarnitine)
Tyr	Tyrosine	C16	Hexadecanoylcarnitine (Palmitoylcarnitine)
Val	Valine	C18	Octadecanoylcarnitine (Stearoylcarnitine)

The analysis is performed semi-quantitatively from dried blood samples (heel blood, see section 5.1).

A sample preparation is carried out prior to the LC-MS/MS analysis. The sample is spiked with the Internal Standard IS, the analytes are extracted from the dried blood matrix and derivatised (see section 5.2).

The ClinMass® Internal Standard IS contains the isotope labelled analytes (internal standards) of the amino acids and acylcarnitines to be determined. The correct assignments (e.g. Carnitine / $^{2}H_{9}$-Carnitine) are listed in the tables in section 4.2.2.1.

In the derivatisation step the analytes are transferred into the corresponding n-Butylester (see Figure 1 and Figure 2).

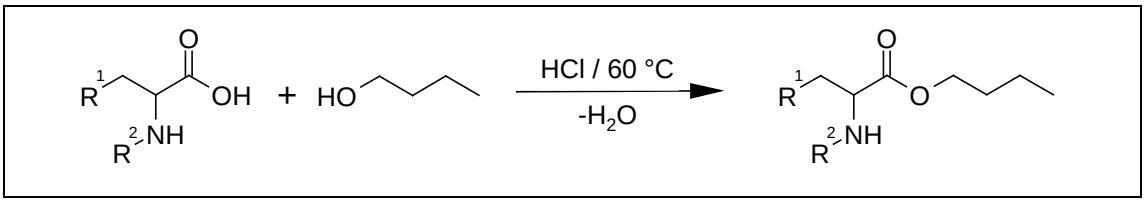


Figure 1. Derivatisation of the amino acids (R = side chain of the amino acids)

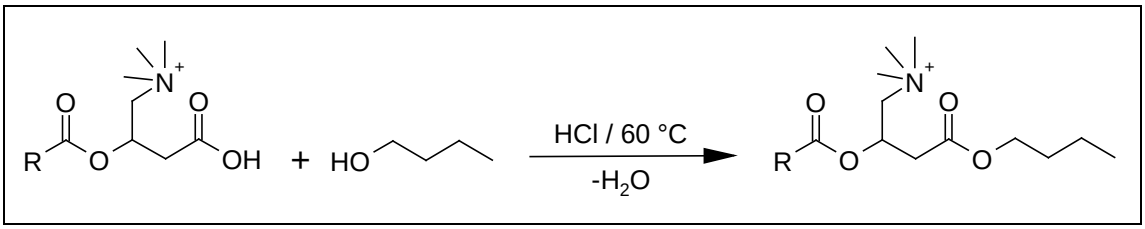


Figure 2. Derivatisation of the acylcarnitines (R = C0 – C18; carnitine (C0), acetylcarnitine (C2), …)

After the sample preparation the samples are injected in the LC-MS/MS system, where the analytes are detected via electrospray ionisation (ESI).

During the electrospray ionisation the sample components are ionised and then transferred into the gas phase. Subsequently they are transferred into the MS/MS system consisting of two quadrupoles, which are connected through a collision cell.

The measurement of the analytes in this method is carried out in MRM mode (MRM: Multiple Reaction Monitoring). In this mode only selected ions (known as the “precursor ions”) with a defined ratio mass/charge (*m/z*) are isolated in the first quadrupole and subsequently transferred into the collision cell. There the ions are fragmented by impact with an inert gas (argon or nitrogen) at selected appropriate 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 analyte concentration is calculated with the ClinMass® Internal Standard IS (see evaluation in section 6).

Quality control is performed with the ClinChek® Dried Blood Spot Controls (DBS) in two different concentrations (see section 5.3.4).

2 Components of the complete kit and accessories

2.1 Ordering information

2.1.1 Sample preparation with microtiter plates (without filter-plates), order no. MS10000

Order No.	Description	Quantity
MS10000	**ClinSpot® Complete Kit for Amino Acids and Acylcarnitines in Dried Blood Spots (DBS)** for 960 assays	1 pce.
	Content:	
	Autosampler Washing Solution	1 x MS10005
	Mobile Phase	1 x MS10010
	Internal Standard IS, lyophil.	1 x MS10012A
	Reagent A	1 x MS10021
	Reagent B	1 x MS10022
	Reagent C	1 x MS10023
	96-Well-Plates (370 µl)	6 x MS10040
	Covers for 96-Well-Plates	2 x MS10041
	Dried Blood Spot Control, Level I, II	1 x MS10182
	Manual	
	Separately available components:	
MS10005	Autosampler Washing Solution	1000 ml
MS10010	Mobile Phase	1000 ml
MS10012	Internal Standard IS, lyophil.	25 ml
MS10012A	Internal Standard IS, lyophil.	100 ml
MS10014	Optimisation Mix, lyophil.	2 x 1 x 5 ml
MS10021	Reagent A	100 ml
MS10022	Reagent B	50 ml
MS10023	Reagent C	100 ml
MS10040	96-Well-Plates (370 µl)	5 pcs.
MS10041	Covers for 96-Well-Plates	15 pcs.
	Accessories:	
MS10042	Protective Sheets for 96-Well-Plates (PE/PP foil, 80 x 140 mm)	50 pcs.
MS10045	Backpressure regulator, PEEK version, 34 bar	1 pce.
MS10046	Replacement cartridge gold coat, 34 bar	1 pce.
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)	1 pce.
FK7340	Sealings and sieves for order no. FK7400	4 pcs. each
	ClinChek® Controls:	
MS10182	Dried Blood Spot Control, Level I, II	2 x 1 x 3 spots

2.1.2 Sample preparation with filter-plates, order no. MS10100

Order No.	Description	Quantity
MS10100	**ClinSpot® Complete Kit for Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) with Filter-Plates** for 960 assays	1 pce.
	Content:	
	Autosampler Washing Solution	1 x MS10005
	Mobile Phase	1 x MS10010
	Internal Standard IS, lyophil.	1 x MS10012A
	Reagent A	1 x MS10021
	Reagent B	1 x MS10022
	Reagent C	1 x MS10023
	96-Well-Plates (370 µl)	3 x MS10040
	Covers for 96-Well-Plates	1 x MS10041
	96-Well-Filter-Plates (500 µl) with covers	3 x MS10140
	Dried Blood Spot Control, Level I, II	1 x MS10182
	Manual	
	Separately available components:	
MS10005	Autosampler Washing Solution	1000 ml
MS10010	Mobile Phase	1000 ml
MS10012	Internal Standard IS, lyophil.	25 ml
MS10012A	Internal Standard IS, lyophil.	100 ml
MS10014	Optimisation Mix, lyophil.	2 x 1 x 5 ml
MS10021	Reagent A	100 ml
MS10022	Reagent B	50 ml
MS10023	Reagent C	100 ml
MS10040	96-Well-Plates (370 µl)	5 pcs.
MS10041	Covers for 96-Well-Plates	15 pcs.
MS10140	96-Well-Filter-Plates (500 µl) with covers	5 pcs.
	Accessories:	
MS10042	Protective Sheets for 96-Well-Plates (PE/PP foil, 80 x 140 mm)	50 pcs.
MS10045	Backpressure regulator, PEEK version, 34 bar	1 pce.
MS10046	Replacement cartridge gold coat, 34 bar	1 pce.
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)	1 pce.
FK7340	Sealings and sieves for order no. FK7400	4 pcs. each
	ClinChek® Controls:	
MS10182	Dried Blood Spot Control, Level I, II	2 x 1 x 3 spots

2.1.3 Safety information

Several of the kit components such as mobile phase and reagents are chemical preparations and may contain hazardous substances. For safety information please consult the respective safety data sheet (SDS) of each component.

The control materials were prepared from human plasma. Although the products are tested for the absence of common infection markers, they should still 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 SDS.

2.1.4 Storage conditions and lifetime of the kit components

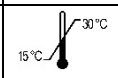
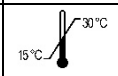
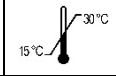
Please unpack the kit components from the transport packaging **immediately upon receipt** and follow the instructions for storage conditions indicated on the product labels and in Table 3.

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

After the use of ClinSpot® Reagents and ClinSpot® Mobile Phase the bottles must be closed tightly and stored immediately under the required conditions. Provided that 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 ClinChek® Controls, of the ClinMass® Internal Standard IS as well as the ClinMass® Optimisation Mix, please also refer to the respective product data sheets.

Table 3. Storage conditions of kit components

Order no.		Product description	Storage conditions	
REF	MS10005	Autosampler Washing Solution		Store at 15–30 °C
REF	MS10010	Mobile Phase		Store at 15–30 °C
REF	MS10012A	Internal Standard IS, lyophil.		Store below -18 °C*
REF	MS10012	Internal Standard IS, lyophil.		Store below -18 °C*
REF	MS10014	Optimisation Mix, lyophil.		Store below -18 °C*
REF	MS10021	Reagent A		Store at 15–30 °C
REF	MS10022	Reagent B		Store at 2–8 °C
REF	MS10023	Reagent C		Store at 15–30 °C
REF	MS10040	96-Well-Plates (370 µl)	Store at ambient temperature	
REF	MS10041	Covers for 96-Well-Plates	Store at ambient temperature	

REF	MS10042	Protective Sheets PE/PP for 96-Well-Plate (80 x 140 mm)	Store at ambient temperature	
REF	MS10045	Backpressure Regulator, PEEK-version, 34 bar	Store at ambient temperature	
REF	MS10046	Replacement Cartridge gold coat, 34 bar	Store at ambient temperature	
REF	MS10140	96-Well-Filter-Plates (500 µl)	Store at ambient temperature	
REF	FK7400	Inline-Filter	Store at ambient temperature	
REF	FK7340	Sealings and sieves	Store at ambient temperature	
REF	MS10182	Dried Blood Spot Control, Level I+II		Store below -18 °C

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

2.1.5 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 safety data sheet (SDS).

3 Required instruments

The use of this test kit requires an LC system with tandem mass spectrometer (LC-MS/MS) of appropriate sensitivity and evaluation software. Data regarding the suitability of the various LC-MS/MS systems is available upon request (info@recipe.de).

Required LC modules:

- Autosampler
- Isocratic HPLC pump
- Degasser

The following laboratory instruments and equipment are required for sample preparation:

- Pipettes (50 µl, 100 µl), pipette tips
- 25 ml volumetric flask for MS10012, 100 ml volumetric flask for MS10012A
- Punching device (device for punching-out from filter plates, 3.2 mm or ⅛ inch)
- Heated evaporator, e.g. Glas-Col® ZipVap 96
- Laboratory fume cupboard

Additional requirements:

- Shaker, heatable (60 °C), for well plates (recommended)

or

- Shaker, not heatable (60 °C), for well plates and
- Drying oven

4 Operation of the analytical system

4.1 Preparation of the LC system

Connect the LC modules 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 of the mobile phase.

After flushing, a restrictor (order no. MS10045) may optionally be used between the HPLC pump and the autosampler. Please consult the manufacturer's instruction manual whether a restrictor is required for your HPLC pump at a flow rate of 0.1 ml/min.

After installing the restrictor, set the HPLC pump at a flow rate of 0.1 ml/min and equilibrate the system with 1 ml of the mobile phase. Then **stop the pump** and connect the autosampler outlet capillary with the tandem mass spectrometer.

4.2 Operation of the analytical system

The following sections provide the parameters for the LC system (section 4.2.1) and the tandem mass spectrometer (section 4.2.2). Please see section 5.3 for optimisation, equilibration and test run of the system as well as the measurement of the samples.

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

4.2.1 LC parameters

Table 4: LC parameters

HPLC pump:	Flow rate: 0.1 ml/min
Mobile phase:	Make sure the bottles are closed well in order to avoid evaporation of the mobile phase components.
Autosampler:	The injection needle has to be flushed after sampling (minimising sample carryover). For this purpose, please use the settings recommended by the manufacturer of the autosampler in use. Injection volume: 2–20 µl* Injection interval: 1.0–1.6 min** *depending on the sensitivity of the mass spectrometer in use *depending on the autosampler in use

4.2.2 MS/MS parameters

4.2.2.1 Mass transitions

The following tables contain the mass transitions of the amino acids and acylcarnitines as well as the corresponding isotope labelled substances in the ClinMass® Internal Standard IS.

The indicated mass transitions should be considered as starting points for optimisation. The optima may vary between different MS/MS systems and should therefore be optimised for the system to be used (see section 5.3.1).

Table 5. Mass transitions of the amino acids and the isotope labelled substances in the IS

Analyte	**Precursor [amu]**	**Product [amu]**	**Internal Standard IS**	**Precursor [amu]**	**Product [amu]**
Alanine	146	44	$^{13}C_3{}^{15}N$-Alanine	150	47
Arginine	231	70	$^{13}C_6$-Arginine	237	74
Aspartic acid	246	144	$^{13}C_4$-Aspartic acid	250	147
Citrulline	232	113	$^{2}H_7$-Citrulline	239	120
Glutamic acid	260	158	$^{13}C_5$-Glutamic acid	265	162
Glycine	132	76	2-$^{13}C^{15}N$-Glycine	134	78
Leucine	188	86	$^{2}H_3$-Leucine	191	89
Methionine	206	104	$^{2}H_3$-Methionine	209	107
Ornithine	189	70	$^{2}H_6$-Ornithine	195	76
Phenylalanine	222	120	$^{13}C_6$-Phenylalanine	228	126
Proline	172	116	$^{13}C_5$-Proline	177	121
Tyrosine	238	136	$^{13}C_6$-Tyrosine	244	142
Valine	174	72	$^{2}H_8$-Valine	182	80

Table 6. Mass transitions of the acylcarnitines and the isotope labelled substances in the IS

Analyte	**Precursor [amu]**	**Product [amu]**	**Internal Standard IS**	**Precursor [amu]**	**Product [amu]**
Carnitine	218	85	$^{2}H_{9}$-Carnitine	227	85
C2-Carnitine	260	85	$^{2}H_{3}$-C2-Carnitine	263	85
C3-Carnitine	274	85	$^{2}H_{3}$-C3-Carnitine	277	85
C4-Carnitine	288	85	$^{2}H_{3}$-C4-Carnitine	291	85
C5-Carnitine	302	85	$^{2}H_{9}$-C5-Carnitine	311	85
C5DC-Carnitine	388	85	$^{2}H_{9}$-C5DC-Carnitine	397	85
C6-Carnitine	316	85	$^{2}H_{3}$-C6-Carnitine	319	85
C8-Carnitine	344	85	$^{2}H_{3}$-C8-Carnitine	347	85
C10-Carnitine	372	85	$^{2}H_{3}$-C10-Carnitine	375	85
C12-Carnitine	400	85	$^{2}H_{3}$-C12-Carnitine	403	85
C14-Carnitine	428	85	$^{2}H_{3}$-C14-Carnitine	431	85
C16-Carnitine	456	85	$^{2}H_{3}$-C16-Carnitine	459	85
C18-Carnitine	484	85	$^{2}H_{3}$-C18-Carnitine	487	85

The mass transitions of additional acylcarnitines, for which no corresponding isotope labelled analytes are available, are indicated in Table 7. These are determined by the use of alternative isotope labelled substances. For these analytes however the data are not validated and must therefore be considered as recommendations only, based on the experience of many screening centres.

Table 7. Mass transitions of additional acylcarnitines with no corresponding isotope labelled analytes available

Analyte	**Precursor [amu]**	**Product [amu]**	**Recommended Internal Standard**	**Precursor [amu]**	**Product [amu]**
C3DC-Carnitine	360	85	$^{2}H_{3}$-C3-Carnitine	277	85
C4OH-Carnitine	304	85	$^{2}H_{3}$-C4-Carnitine	291	85
C4DC-Carnitine	374	85	$^{2}H_{3}$-C4-Carnitine	291	85
C5:1-Carnitine	300	85	$^{2}H_{9}$-C5-Carnitine	311	85
C5OH-Carnitine	318	85	$^{2}H_{9}$-C5-Carnitine	311	85
C6DC-Carnitine	402	85	$^{2}H_{3}$-C6-Carnitine	319	85
C8:1-Carnitine	342	85	$^{2}H_{3}$-C8-Carnitine	347	85
C10:2-Carnitine	368	85	$^{2}H_{3}$-C10-Carnitine	375	85

C10:1-Carnitine	370	85	$^2H_3$-C10-Carnitine	375	85
C12:1-Carnitine	398	85	$^2H_3$-C12-Carnitine	403	85
C14:2-Carnitine	424	85	$^2H_3$-C14-Carnitine	431	85
C14:1-Carnitine	426	85	$^2H_3$-C14-Carnitine	431	85
C14OH-Carnitine	444	85	$^2H_3$-C14-Carnitine	431	85
C16:1-Carnitine	454	85	$^2H_3$-C16-Carnitine	459	85
C16:1OH-Carnitine	470	85	$^2H_3$-C16-Carnitine	459	85
C16OH-Carnitine	472	85	$^2H_3$-C16-Carnitine	459	85
C18:2-Carnitine	480	85	$^2H_3$-C18-Carnitine	487	85
C18:1-Carnitine	482	85	$^2H_3$-C18-Carnitine	487	85
C18:2OH-Carnitine	496	85	$^2H_3$-C18-Carnitine	487	85
C18:1OH-Carnitine	498	85	$^2H_3$-C18-Carnitine	487	85
C18OH-Carnitine	500	85	$^2H_3$-C18-Carnitine	487	85

4.2.2.2 Device-specific settings of various MS/MS systems

Device-specific data for the various MS/MS systems by different suppliers is available upon request (info@recipe.de).

4.3 Standby mode

When the analytical system is not in use, the HPLC pump should be switched off. The mobile phase can be left inside 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 the multiplier, the MS/MS system should be switched into standby mode.

5 Implementation of the analytical procedure

5.1 Collection and storage of samples

Acylcarnitines and amino acids are determined from dried blood spots (DBS).

For the analysis heel blood is applied onto suitable filter paper (e.g. Whatman 903) and put to dry in a horizontal position at room temperature. **Detailed instructions regarding sample collection, storage and transportation are available in the respective national regulations for neonatal screening!** (Germany: Kinder-Richtlinie dd 19 October 2017 [1]).

The international CLSI standard for the collection of dried blood specimen (CLSI Standard NBS01-A6 [5]) contains the following core aspects:

1.) General precautions for sample collection, in particular the regulations for the documentation on filter paper test cards, need to be followed.

2.) Preparation and disinfection of the heel puncture site. The skin needs to be dry at the moment of blood collection.

3.) Puncture with a sterile lancet device. Wipe away the first drop of blood with a dry and sterile gauze pad (the first drop of blood is unsuitable for the laboratory analysis).

4.) Allow a new drop of blood to form and completely fill a preprinted circle on the filter paper. Blood should only be applied to the front side of the filter paper segment of the filter card, however both sides of the filter paper should be examined to ensure that the blood evenly penetrated and saturated the paper. Representative samples regarding the sample quality are available in the appendix of the above mentioned CLSI standard.

5.) Put the blood specimen to air dry in a horizontal position for at least **three hours** at an ambient temperature of 18–25 °C. High humidity (> 50 % relative humidity) may cause longer drying times. The dried blood samples need to be stacked without touching other surfaces. Keep the specimen away from cross-contamination, direct sunlight and draughts.

6.) As soon as the samples are dry, they need to be properly packaged for transportation and analysis the same day. In case regulatory instructions require airtight packaging (e.g. plastic bag or foil), suitable desiccants with indicator have to be included in the transportation box in order to avoid humidity.

5.1.1.1 Storage of the dried blood samples during and after analysis

After receipt of the samples they can be stored at room temperature (15–30 °C) before analysis, which however should take place within 24–48 hours. For longer storage (> 48 hours) the samples need to be stored at temperatures below -18 °C. The samples have to be protected from humidity (e.g. condensed water) and stored at low humidity (< 30 %) [5].

Residual samples also need to be stored at temperatures below -18 °C and at low humidity for longer periods of storage. Breathable plastic bags with desiccants and humidity indicator are recommended for storage [5].

Dried blood samples need to be protected from humidity. In order to protect cooled samples from condensed water, allow the samples to warm up to room temperature before taking them out of their protection cover.

5.2 Sample preparation

The sample preparation can be performed either with **two well plates** (see section 5.2.2) or with **a well plate and a filter plate** (see section 5.2.3).

In the sample preparation with filter plate no sample transfer is necessary (cf. work flow descriptions in sections 5.2.2.1 and 5.2.3.1).

5.2.1 Reconstitution of the lyophilised internal standard IS (MS10012A, MS10012)

The ClinMass® Internal Standard IS is lyophilised and needs to be reconstituted with Reagent A (order no. MS10021) prior to use. Information regarding reconstitution, analyte concentrations, storage and stability is available in the respective product data sheets.

Annotation:
The ClinMass® Complete Kits with order nos. MS10000 and MS10100 contain the Internal Standard IS with a package size 1 x 100 ml (order no. MS10012A). A package size 1 x 25 ml (order no. MS10012) is available separately.

5.2.2 Sample preparation with well-plates (without filter-plates, MS10000)

5.2.2.1 Work flow

Sample preparation:

Extraction/ Spiking with IS:	*Well plate 1*: 3.2 mm disc (control, patient)	100 µl Internal Standard IS (reconstituted with Reagent A)
	Close *Well plate 1* (cover), ⇩ extract while shaking (30 min, at 700 rpm)	
Transfer:	Remove cover, transfer supernatant into *Well plate 2*	
	Evaporate to **complete** dryness ⇩ (ca. 30 min, at 40 °C)	
Derivatisation:	Add 50 µl Reagent B into *Well plate 2*	
	1.) Close *Well plate 2* (new cover), ⇩ incubate sample (20 min, at 60 °C)	
	2.) Evaporate to **complete** dryness ⇩ (ca. 30 min, at 40 °C)	
Reconstitution:	Add 100 µl Reagent C	
	Close *Well plate 2* (protective ⇩ sheet), dissolve while shaking (5 min, at 700 rpm)	
LC-MS/MS Analysis:	Inject 2–20 µl	

5.2.2.1.1 Extraction / Spiking with Internal Standard IS

Punch out a 3.2 mm (⅛ inch) dried blood disc of the filter test card (control, patient) and transfer it in a defined well of the 96/370 µl Well-Plate ("Well plate 1", order no. MS10040). Then pipette 100 µl Internal Standard IS (reconstituted with Reagent A, see section 5.2.1) on the sample.

For the sample extraction close the Well plate 1 with the cover (order no. MS10041) and extract while shaking for 30 min at 700 rpm (shaker for well-plates).

5.2.2.1.2 Transfer

Remove the cover and transfer the sample supernatant into another well-plate („Well-plate 2").

Evaporate to **complete** dryness by blowing off air (ca. 30 min, at 40 °C).

Please note:
During the evaporation of the sample harmful vapours are escaping (see also respective information in the safety data sheet of Reagent A). Therefore the evaporation needs to be performed under a laboratory fume cupboard.

5.2.2.1.3 Derivatisation

Pipette 50 µl Reagent B on the sample in Well plate 2. Close the well plate with a new cover (order no. MS10041) and incubate the well plate for 20 min at 60 °C. Use a drying oven or alternatively a heatable shaker for well plates.

After incubation please remove the cover and evaporate to **complete** dryness by blowing off with air (ca. 30 min, at 40 °C)**.**

Please note:
During the evaporation of the sample harmful and irritating vapours are escaping (see also respective information in the safety data sheet of Reagent B). Therefore the evaporation needs to be performed under a laboratory fume cupboard.

5.2.2.1.4 Reconstitution

Pipette 100 µl Reagent C on the sample. Close Well plate 2 with a suitable protective sheet (PE/PP protective sheet, order no. MS10042, separately available) and dissolve while shaking for 5 min at 700 rpm (shaker for well-plates).

5.2.2.1.5 LC-MS/MS analysis

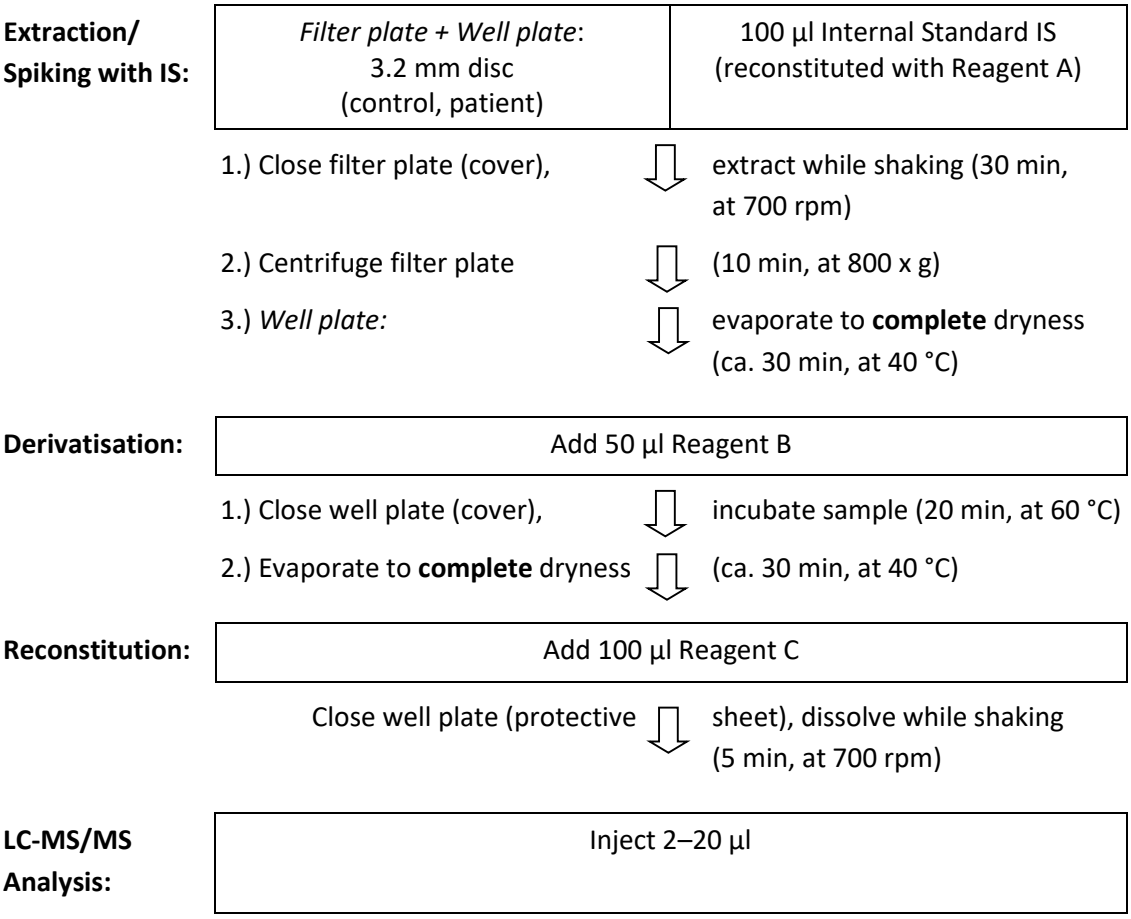
Inject 2–20 µl of the supernatant into the LC-MS/MS system.

The injection volume needs to be selected with respect to the sensitivity of the tandem mass spectrometer in use.

5.2.3 Sample preparation with filter-plates (MS10100)

5.2.3.1 Work flow

Sample preparation:



5.2.3.1.1 Extraction / Spiking with the Internal Standard IS

Put the 96/500 µl filter-plate (order no. MS10140) on the 96/370 µl well-plate (order no. MS10040).

Punch out a 3.2 mm (⅛ inch) disc of dried blood from the filter test card (control, patient) and transfer it in a defined well of the filter-plate. Then pipette 100 µl Internal Standard IS (reconstituted with Reagent A, see section 5.2.1) on the sample.

For the sample extraction close the filter-plate with the cover (use the cover contained together with the filter plate within order no. MS10140) and extract while shaking for 30 min at 700 rpm (shaker for well-plates). Then centrifuge for 10 min at 800 x g.

Remove the filter-plate and evaporate the samples to **complete** dryness by blowing off air (ca. 30 min, at 40 °C).

Please note:
During the evaporation of the sample harmful vapours are escaping (see also respective information in the safety data sheet of Reagent A). Therefore the evaporation needs to be performed under a laboratory fume cupboard.

5.2.3.1.2 Derivatisation

Pipette 50 µl Reagent B on the sample. Close the well-plate with the cover (order no. MS10041, included within the kit) and incubate the samples for 20 min at 60 °C.

After the incubation remove the cover and evaporate the sample to **complete** dryness (ca. 30 min, at 40 °C) by blowing off air.

Please note:
During the evaporation of the sample harmful and irritating vapours are escaping (see also information in the safety data sheet of Reagent B). Therefore the evaporation needs to be performed under a laboratory fume cupboard.

5.2.3.1.3 Reconstitution

Pipette 100 µl Reagent C on the sample. Close the well plate with a suitable protective sheet (PE/PP protective sheet, order no. MS10042, separately available) and dissolve while shaking for 5 min at 700 rpm.

5.2.3.1.4 LC-MS/MS analysis

Inject 2–20 µl of the supernatant into the LC-MS/MS system.

The injection volume needs to be selected with respect to the sensitivity of the tandem mass spectrometer in use.

5.2.4 Stability of the prepared samples

The prepared samples can be stored at room temperature (15–30 °C) for three days, at temperatures between 2–8 °C the samples can be stored for 7 days.

5.3 LC-MS/MS analysis

Regardless of the method of analysis we recommend to regularly check the mass accuracy of the tandem mass spectrometer. If necessary please recalibrate the instrument.

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

5.3.1 Optimisation of the tandem mass spectrometer

The optimisation of the MS/MS system includes the optimisation of the ion source parameters and the compound-specific mass transitions.

For this purpose the ClinMass® Optimisation Mix (order no. MS10014, containing one bottle for each amino acids and acylcarnitines) is used. Spike each bottle with 500 µl Reagent B (order no. 10022) and after a brief shaking carry out the derivatisation (20 min, 60 °C). Then evaporate to complete dryness and reconstitute each with 5 ml Reagent C (order no. 10023). Dilute the solutions further, if necessary. The concentrations of both solutions needed for the optimisation is depending on the sensitivity of the MS/MS system in use. Device-specific information for various LC-MS/MS systems is available upon request (info@recipe.de).

Alternatively the optimisation can be performed also with the ClinChek® Dried Blood Spot Controls. In this case the controls need to be prepared as described for patient samples (see section 5.2).

5.3.2 Equilibration of the analytical system and test run

Equilibrate the entire analytical system for at least 30 min prior to sample injection.

Three „Blank-Injections“ need to be carried out at the beginning of each analytical series (injection volume: 0 µl or injection of mobile phase). This procedure facilitates reproducible analytical results already from the first sample injection.

For the test run please use the prepared ClinChek® Controls, i.e. Level I and Level II of the Dried Blood Spot Control (included in order no. MS10182). Inject the prepared controls several times until two consecutive chromatograms comparable in retention times and peak areas are obtained.

5.3.3 Measurement of the samples

The Internal Standard IS is used for the calculation of the analyte concentration of the sample. For this purpose the sample is spiked with the Internal Standard IS (see sample preparation, section 5.2).

The Internal Standard IS contains the corresponding isotope labelled analytes (internal standards). The assignment to the respective analytes and mass transitions is contained in the tables shown in section 4.2.2.1. The concentrations of the isotope labelled analytes are indicated in the data sheet of the Internal Standard IS (order no. MS10012A or MS10012).

5.3.4 Quality control

For assay performance please run ClinChek® Dried Blood Spot Controls (DBS) in two different concentrations with each analytical series (order no. MS10182). The controls need to be prepared as described for patient samples (see section 5.2).

6 Evaluation

The analyte concentration is calculated with the following formula:

$$C\ (Analyte)[\mu mol/l] = \frac{A\ (Analyte) \times C\ (IS)\ [\mu mol/l]}{A\ (IS)}\ x\ \frac{V\ (IS)\ [\mu l]}{V\ (A)\ [\mu l]}$$

Abbreviations:

C (Analyte):	Analyte concentration in the sample
A (Analyte):	Analyte peak area in the sample
C (IS):	Concentration of the isotope labelled analyte in the Internal Standard IS (indication in the product data sheet)
A (IS):	Peak area of the isotope labelled analyte in the Internal Standard IS
V (IS):	Volume of the Internal Standard IS
V (A):	Volume of the sample

If the sample preparation is performed as described (see sections 5.2.2 and 5.2.3), the formula can be simplified as follows:

$$C\ (Analyte)[\mu mol/l] = \frac{A\ (Analyte) \times C\ (IS, sample)\ [\mu mol/l]}{A\ (IS)}$$

Abbreviation:

C (IS, sample):	Concentration of the isotope labelled analyte in the sample (indication in the product data sheet)

The basis for this calculation is a volume of 100 µl Internal Standard IS and a sample disc diameter of 3.2 mm (⅛ inch). The concentration of the isotope labelled analytes in the Internal Standard IS is converted into the concentration of the isotope labelled analytes in the sample. A medium sample volume of 3.1 µl is presumed for whole blood controls on certified filter paper with 50 % haematocrit and a diameter of 3.2 mm [6].

The de facto volumes of the sample however may vary from case to case. This method therefore does not serve the purpose of exact quantification of acylcarnities and amino acids but the **semi-quantitative** determination within the screening.

In order to ensure a correct evaluation please consult the software manual of the MS/MS manufacturer.

For the conversion of mass concentrations [mg/l] into molar concentrations [µmol/l] and vice versa, the analytical results should be multiplied with the factors indicated in the following tables.

Table 8. Conversion factors for amino acids

Analyte	Molecular weight [g/mol]	Conversion: µmol/l → mg/l	Conversion: mg/l → µmol/l
Alanine	89.1	0.0891	11.224
Arginine	174.2	0.1742	5.7405
Aspartic acid	133.1	0.1331	7.5130
Citrulline	175.2	0.1752	5.7082
Glutamic acid	147.1	0.1471	6.7967
Glycine	75.1	0.0751	13.322
Leucine	131.2	0.1312	7.6235
Methionine	149.2	0.1492	6.7019
Ornithine	132.2	0.1322	7.5665
Phenylalanine	165.2	0.1652	6.0537
Proline	115.1	0.1151	8.6858
Tyrosine	181.2	0.1812	5.5191
Valine	117.2	0.1172	8.5363

Table 9. Conversion factors for acylarnitines

Analyte	Molecular weight [g/mol]	Conversion: µmol/l → mg/l	Conversion: mg/l → µmol/l
Carnitine	161.2	0.1612	6.2035
C2-Carnitine	203.2	0.2032	4.9204
C3-Carnitine	217.3	0.2173	4.6027
C4-Carnitine	231.3	0.2313	4.3236
C5-Carnitine	245.3	0.2453	4.0764
C5DC-Carnitine	275.3	0.2753	3.6324
C6-Carnitine	259.3	0.2593	3.8560
C8-Carnitine	287.4	0.2874	3.4795
C10-Carnitine	315.4	0.3154	3.1701
C12-Carnitine	343.5	0.3435	2.9112
C14-Carnitine	371.6	0.3716	2.6914
C16-Carnitine	399.6	0.3996	2.5025
C18-Carnitine	427.7	0.4277	2.3383

Table 10. Conversion factors of additional acylcarnitines

Analyte	Molecular weight [g/mol]	Conversion: µmol/l → mg/l	Conversion: mg/l → µmol/l
C3DC-Carnitine	247.2	0.2472	4.0446
C4OH-Carnitine	247.3	0.2473	4.0439
C4DC-Carnitine	261.3	0.2613	3.8274
C5:1-Carnitine	243.3	0.2433	4.1102
C5OH-Carnitine	261.3	0.2613	3.8268
C6DC-Carnitine	289.3	0.2893	3.4563
C8:1-Carnitine	285.4	0.2854	3.5041
C10:2-Carnitine	311.4	0.3114	3.2111
C10:1-Carnitine	313.4	0.3134	3.1905
C12:1-Carnitine	341.5	0.3415	2.9284
C14:2-Carnitine	367.5	0.3675	2.7209
C14:1-Carnitine	369.5	0.3695	2.7061
C14OH-Carnitine	387.6	0.3876	2.5803
C16OH-Carnitine	397.6	0.3976	2.5151
C16:1-Carnitine	413.6	0.4136	2.4178
C16:1OH-Carnitin	415.6	0.4156	2.4061
C18:2-Carnitine	423.6	0.4236	2.3606
C18:1-Carnitine	425.6	0.4256	2.3494
C18:2OH-Carnitine	439.6	0.4393	2.2746
C18:1OH-Carnitine	441.6	0.4416	2.2643
C18OH-Carnitine	443.7	0.4437	2.2540

7 Test data

7.1 Analytical Performance

7.1.1 Linearity, detection and quantitation limit

The data for linearity and detection and quantitation limits were determined with the MS/MS system AB SCIEX API3000™.

For the linearity determination dried blood samples with analyte concentrations in the clinically relevant range were spiked and measured according to the CLSI document NBS04-A [6]. The lower detection limit (LOD) and the lower quantitation limit (LOQ) were determined by dilution of prepared dried blood samples with Reagent A / Internal Standard IS.

Table 11. Linearity, detection and quantitation limits of the amino acids

Analyte	**LOD [µmol/l]**	**LOQ [µmol/l]**	**Measured linearity range [µmol/l]**
Alanine	1.8	5.4	329–2829
Arginine	0.7	2.1	11.4–2511
Aspartic acid	3.8	11.4	53.0–2553
Citrulline	2.0	6.0	50.8–2551
Glutamic acid	8.2	24.6	474–2974
Glycine	3.0	9.0	267–2767
Leucine	1.5	4.5	152–2652
Methionine	1.4	4.2	11.6–2512
Ornithine	1.7	5.1	67.7–2568
Phenylalanine	2.6	7.8	68.1–2568
Proline	0.75	2.25	101–2601
Tyrosine	1.1	3.3	66.9–2567
Valine	3.3	9.9	213–2813

Table 12. Linearity, detection and quantitation limits of the acylcarnitines

Analyte	**LOD [µmol/l]**	**LOQ [µmol/l]**	**Measured linearity range [µmol/l]**
Carnitine	0.20	0.60	23–273
C2-Carnitine	0.06	0.18	1.94–252
C3-Carnitine	0.04	0.12	0.49–75
C4-Carnitine	0.04	0.12	0.13–75
C5-Carnitine	0.04	0.12	0.17–75
C5DC-Carnitine	0.10	0.30	0.13–75
C6-Carnitine	0.02	0.06	0.05–75
C8-Carnitine	0.09	0.27	0.05–75
C10-Carnitine	0.04	0.12	0.07–50
C12-Carnitine	0.02	0.06	0.59–51
C14-Carnitine	0.02	0.06	0.51–51
C16-Carnitine	0.03	0.09	1.58–52
C18-Carnitine	0.04	0.12	1.04–51

7.1.2 Recovery

The recovery rates were obtained with commercial available reference materials and the MS/MS systems AB SCIEX API3000™, Shimadzu 8050 and Waters TQD.

Table 13. Recovery rates (REC) of the amino acids

Analyte	**REC [%]**					
	API3000		**Shimadzu 8050**		**Waters TQD**	
	Level I	**Level II**	**Level I**	**Level II**	**Level I**	**Level II**
Alanine	104	108	100	97	110	105
Arginine	137	116	99	109	91	81
Aspartic acid	109	112	97	100	93	97
Citrulline	127	134	93	78	93	99
Glutamic acid	98	102	102	100	100	97
Glycine	93	92	93	89	123	129
Leucine	90	109	96	100	108	118

Methionine	111	118	97	93	97	102
Ornithine	102	97	101	102	96	97
Phenylalanine	105	101	95	93	100	99
Proline	93	97	91	87	111	117
Tyrosine	92	97	82	79	112	117
Valine	103	111	71	72	112	112

Table 14. Recovery rates (REC) of the acylcarnitines

Analyte	**REC [%]**					
	API3000		**Shimadzu 8050**		**Waters TQD**	
	Level I	**Level II**	**Level I**	**Level II**	**Level I**	**Level II**
Carnitine	98	97	86	82	111	114
C2-Carnitine	121	103	74	96	109	103
C3-Carnitine	87	96	96	97	92	96
C4-Carnitine	90	96	95	94	98	100
C5-Carnitine	98	105	102	102	99	96
C5DC-Carnitine	96	92	97	96	48	49
C6-Carnitine	90	98	97	92	115	112
C8-Carnitine	88	96	88	85	98	97
C10-Carnitine	89	83	90	78	90	87
C12-Carnitine	95	96	99	95	110	100
C14-Carnitine	96	100	90	85	107	99
C16-Carnitine	107	110	117	114	79	78
C18-Carnitine	98	106	104	102	88	91

7.1.3 Precision

The precision was determined with commercial available reference material and the MS/MS systems ABSCIEX API3000™ and Shimadzu 8050 and performed according to CLSI document NBS04-A [6].

7.1.3.1 Intraassay

The obtained precision data are indicated in Table 15 for amino acids and Table 16 for acylcarnitines.

Table 15. Intraassay precisions of the amino acids

Analyte	**Intraassay CV [%]**			
	API3000		**Shimadzu 8050**	
	Lev. I	**Lev. II**	**Lev. I**	**Lev. II**
Alanine	4.6	5.1	5.6	6.6
Arginine	8.4	6.4	7.9	5.3
Aspartic acid	4.8	8.3	6.5	6.6
Citrulline	4.5	4.4	5.7	2.8
Glutamic acid	4.5	2.5	3.6	3.5
Glycine	4.6	3.5	5.6	5.3
Leucine	4.9	5.4	5.6	4.5
Methionine	4.4	3.4	4.7	5.3
Ornithine	5.7	4.0	3.5	5.2
Phenylalanine	4.3	3.5	4.6	5.0
Proline	3.0	2.9	9.0	5.9
Tyrosine	4.3	5.7	4.2	4.1
Valine	5.6	5.0	4.7	5.0

Table 16. Intraassay precisions of the acylcarnitines

Analyte	**Intraassay CV [%]**			
	API3000		**Shimadzu 8050**	
	Lev. I	**Lev. II**	**Lev. I**	**Lev. II**
Carnitine	4.2	5.9	5.6	6.3
C2-Carnitine	5.8	3.9	1.5	9.5
C3-Carnitine	5.4	5.6	5.3	5.7

C4-Carnitine	4.0	5.0	4.9	5.2
C5-Carnitine	7.2	7.0	5.3	5.3
C5DC-Carnitine	7.3	7.7	3.1	4.4
C6-Carnitine	7.5	4.1	5.1	4.8
C8-Carnitine	6.7	5.9	4.5	5.0
C10-Carnitin	5.8	5.1	5.1	5.2
C12-Carnitine	7.1	4.8	5.7	5.5
C14-Carnitine	7.5	6.8	5.0	5.2
C16-Carnitine	4.8	4.9	7.3	8.9
C18-Carnitine	8.9	7.0	5.9	7.1

7.1.3.2 Interassay

The obtained precision data are indicated in Table 17 for amino acids and Table 18 for acylcarnitines.

Table 17. Interassay precisions of the amino acids

Analyte	**Intraassay CV [%]**				
		API3000		**Shimadzu 8050**	
		Level I	**Level II**	**Level I**	**Level II**
Alanine	Total	7.1	8.2	8.1	7.3
	Repeatability	2.4	5.1	2.3	3.9
	Between-day	6.7	6.4	7.8	6.2
Arginine	Total	25.7	25.7	11.9	14.1
	Repeatability	11.1	5.3	7.5	3.4
	Between-day	23.2	25.2	9.3	13.7
Aspartic acid	Total	16.4	19.2	9.5	10.6
	Repeatability	6.9	10.3	2.7	5.6
	Between-day	14.9	16.2	9.1	9.0
Citrulline	Total	15.0	15.8	7.9	3.5
	Repeatability	7.9	3.5	4.1	1.3
	Between-day	12.8	15.4	6.7	3.3

Glutamic acid	Total	12.5	10.5	7.0	7.8
	Repeatability	4.1	3.1	1.9	3.6
	Between-day	11.9	10.0	6.7	6.9
Glycine	Total	7.9	9.3	6.3	6.8
	Repeatability	2.5	3.4	2.8	4.0
	Between-day	7.5	8.7	5.6	5.5
Leucine	Total	7.4	8.2	7.2	6.3
	Repeatability	4.7	8.2	2.9	2.9
	Between-day	5.7	0.0	6.6	5.6
Methionine	Total	6.5	5.6	7.8	5.7
	Repeatability	3.8	5.6	2.4	2.5
	Between-day	5.3	0.4	7.5	5.1
Ornithine	Total	23.0	19.5	8.4	11.9
	Repeatability	4.5	4.5	2.6	4.7
	Between-day	22.6	18.9	8.0	11.0
Phenylalanine	Total	6.3	6.3	7.8	5.8
	Repeatability	2.8	6.0	3.3	3.1
	Between-day	5.6	2.0	7.1	4.9
Proline	Total	8.3	8.1	8.1	8.9
	Repeatability	2.2	3.5	4.5	5.0
	Between-day	8.0	7.3	6.8	7.4
Tyrosine	Total	5.6	5.7	5.5	5.9
	Repeatability	4.2	4.7	2.5	3.9
	Between-day	3.7	3.4	4.9	4.4
Valine	Total	8.6	5.9	5.2	5.0
	Repeatability	4.6	5.4	1.8	2.7
	Between-day	7.4	2.4	4.9	4.2

Table 18. Interassay precisions of the acylcarnitines

Analyte	**Intraassay CV [%]**				
		API3000		**Shimadzu 8050**	
		Level I	**Level II**	**Level I**	**Level II**
Carnitine	Total	7.0	7.4	7.4	9.1
	Repeatability	3.4	2.7	2.0	3.0
	Between-day	6.1	6.9	7.2	8.6
C2-Carnitine	Total	17.6	4.8	2.2	8.0
	Repeatability	6.7	3.8	1.7	1.9
	Between-day	16.3	2.8	1.3	7.7
C3-Carnitine	Total	6.3	7.4	8.1	7.1
	Repeatability	6.3	5.9	3.9	2.9
	Between-day	0.0	4.5	7.1	6.5
C4-Carnitine	Total	5.2	6.1	7.2	7.3
	Repeatability	2.8	5.3	3.2	3.4
	Between-day	4.3	2.9	6.4	6.4
C5-Carnitine	Total	6.2	6.3	7.8	6.8
	Repeatability	4.8	3.3	3.6	3.3
	Between-day	3.9	5.4	6.9	6.0
C5DC-Carnitine	Total	9.8	5.9	9.0	9.0
	Repeatability	5.4	2.9	2.2	3.8
	Between-day	8.1	5.2	8.7	8.2
C6-Carnitine	Total	7.5	6.3	6.8	6.8
	Repeatability	3.7	4.5	2.4	3.5
	Between-day	6.6	4.5	6.3	5.9
C8-Carnitine	Total	6.7	5.4	7.1	6.1
	Repeatability	5.6	4.7	2.3	1.9
	Between-day	3.7	2.5	6.7	5.7
C10-Carnitine	Total	8.2	5.0	7.6	7.4
	Repeatability	4.1	4.4	3.2	3.7
	Between-day	7.1	2.4	6.8	6.4

C12-Carnitine	Total	5.3	7.7	11.5	7.1
	Repeatability	3.4	4.4	5.9	3.1
	Between-day	4.1	6.4	9.9	6.4
C14-Carnitine	Total	5.8	6.8	9.3	6.6
	Repeatability	4.3	5.1	3.6	2.7
	Between-day	3.9	4.5	8.6	6.1
C16-Carnitine	Total	6.6	7.4	14.0	9.9
	Repeatability	3.7	3.6	7.0	4.6
	Between-day	5.5	6.4	12.1	8.8
C18-Carnitine	Total	6.6	8.2	10.5	8.8
	Repeatability	3.6	8.2	4.2	4.7
	Between-day	5.5	0.0	9.6	7.4

7.2 Reference ranges (cut-off values)

The screening lab in the paediatric department of the General Hospital in Vienna (AKH Wien) carried out a pilot study with this analytical method. The cut-off values were determined as 99.5 % percentiles on two different analytical systems (Waters TQD, Thermo TSQ Quantum Ultra) and from two patient groups with 1254 and 1520 samples. The obtained cut-off values are listed in the following tables.

Please note that the values may vary among different patient groups and different MS/MS systems in use. Each screening lab therefore needs to determine its own cut-off values within a pilot study. The indicated values do not reflect any recommendation by the kit manufacturer but may provide guidance for the determination of own values.

Table 19. Cut-off values of the amino acids as 99.5 % percentiles

Analyte	**Waters TQD, n = 1254**	**Thermo TSQ Quantum Ultra n = 1520**
	Cut-off [µmol/l]	**Cut-off [µmol/l]**
Alanine	679	436
Arginine	20.3	21.3
Aspartic acid	345	225*
Citrulline	26.2	29.7
Glutamic acid	498	465
Glycine	1142	859
Leucine	291	257
Methionine	41.2	100
Ornithine	263	273
Phenylalanine	102	101
Proline	441	513
Tyrosine	259	281
Valine	319	297

*n=266

Table 20. Cut-off values of the acylcarnitines as 99.5 % percentiles

Analyte	**Waters TQD, n = 1254**	**Thermo TSQ Quantum Ultra n = 1520**
	Cut-off [µmol/l]	**Cut-off [µmol/l]**
Carnitine	76.1 (10.8)*	87.7 (12.6)*
C2-Carnitine	49.0	8.04
C3-Carnitine	5.20	5.21
C4-Carnitine	0.751	0.761
C5-Carnitine	0.452	0.381
C5DC-Carnitine	0.140	0.289
C6-Carnitine	0.120	0.105
C8-Carnitine	0.150	0.205
C10-Carnitine	0.220	0.269
C12-Carnitine	0.601	0.477
C14-Carnitine	0.481	0.466
C16-Carnitine	5.98	8.14
C18-Carnitine	1.84	2.24

*Lower cut-off value as 0.5 %-Percentile

Please note:

Each screening laboratory needs to determine its own cut-off values in a pilot study. Therefore the indicated values do not reflect any recommendation by the kit manufacturer but may provide guidance for the determination of own values.

7.3 Influence of external factors on the analysis

7.3.1 Nutrition and medication

External factors such as nutrition (parenteral nutrition, special formulations), blood transfusions or the administration of medication may lead to a false diagnosis.

A general description of various factors and recommended actions are provided in the CLSI standard NBS-04A for the performance of neonatal screening [6].

These factors need to be considered in the diagnostic assessment.

7.3.2 Improper handling of dried blood samples

The improper handling of dried blood samples (collection, contamination, heat, humidity) may falsify the analytical results.

A description of various factors and recommendations is provided in the CLSI standard NBS01-A6 regarding the collection of dried blood samples [5].

7.3.3 Unsuitable sample vials

Additives in plastic containers (well-plates and covers, filter-plates, protective foil) may interfere with the analysis. We therefore recommend the use of the products tested by RECIPE (see ordering information in sections 2.1.1 and 2.1.2).

7.4 Interferences

Isoleucine / Leucine

Isoleucine and leucine are isobar substances with identical fragmentation and are detected together. The concentration measured in the sample therefore is the sum of both compounds.

Hydroxyproline / Leucine

Hydroxyproline and leucine are isobar substances with identical fragmentation and are detected together. However even in the rare cases of hyperhydroxyprolinemia no false-positive MSUD results are to be expected [7].

Asparagine / Ornithine

This method was tested for a possible interference of asparagine with ornithine. 20 % of asparagine is detected as ornithine.

Glutamic acid / C2-Carnitine

The method was tested for a possible interference of glutamic acid and C2-carnitine (acetylcarnitine). Around 2 % of glutamic acid are detected as C-2 carnitine. In the reverse case no interference could be determined.

7.5 Limitations of the method

The ClinSpot® LC-MS/MS Complete Kits with the order nos. MS10000 und MS10100 are in vitro screening tests. They are not intended for follow-up or confirmatory diagnostic procedures.

There are no isotope labelled substances commercially available for the acylcarnitines listed in Table 7. This method is therefore not validated for these analytes. It is common practice in many screening laboratories to use alternative internal standards for the measurement of these acylcarnitines (see recommendations in Table 7.) and to perform a validation in a pilot study in order to determine the cut-off values (see section 7.2).

8 References

[1] Richtlinie des Gemeinsamen Bundesausschusses über die Früherkennung von Krankheiten bei Kindern (Kinder-Richtlinien), dd 19. Oct 2017, published in Bundesanzeiger AT 15.03.2018 B2, entered into force on 16. March 2018.

[2] AWMF-Leitlinie 024/012 - Neugeborenen-Screening auf angeborene Stoffwechselstörungen, Endokrinopathien und Mukoviszidose, dd 07.02.2019. Available on: https://www.awmf.org/leitlinien/detail/ll/024-012.html, [viewed on: 30.07.2019].

[3] Harms E, Olgemöller B. National screening for metabolic and endocrine disorders. Dtsch Arztebl Int 2001;108(1-2):11–22; DOI: https://doi.org/10.3238/arztebl.2011.0011.

[4] Dietzen DJ, Rinaldo P, Whiteley RJ, Rhead WJ, Hannon WH, Garg UC et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: Follow-Up Testing for Metabolic Disease Identified by Expanded Newborn Screening Using Tandem Mass Spectrometry; Executive Summary. Clin Chem 2009;55(9):1615–1625; DOI: https://doi.org/10.1373/clinchem.2009.131300.

[5] CLSI Document NBS01-A6: Blood Collection on Filter Paper for Neonatal Screening Programs, Approved Standard - Sixth Edition, Vol. 33. No. 9, July 2013.

[6] CLSI Document NBS04-A: New Born Screening by Tandem Mass Spectrometry, Approved Standard, Vol. 30 No. 16, July 2010.

[7] Fingerhut R. Recall rate and positive predictive value of MSUD screening is not influenced by hydroxyproline. Eur J Pediatr 2009;168:599–604; DOI: https://doi.org/10.1007/s00431-008-0804-0.

9 Troubleshooting

Problem	**Possible cause**	**Corrective measure**
Alteration of elution times	Defective HPLC pump	Check pumps
	Air in the system	Degas the mobile phases and flush the HPLC system
	Fluctuations of the flow rate	Check pumps
Interference signals	Injection system contaminated	• Rinse with methanol or inject 10 x mobile phase • Flushport: check solvent level • Clean/replace injection needle and needle seat assembly
	Interference due to unsuitable sample vials	Use RECIPE sample vials (see section 7.3.3)
	Mobile phase contaminated	Replace mobile phase and flush system
	Mass resolution too low	Optimise mass resolution
	System not installed correctly	Check all connections
No signals	Defective injector	Check injector
	Defective HPLC pump	Check pump
	MS/MS system not ready for operation	Check MS/MS system
Decrease of sensitivity	Ion source contaminated	Clean ion source
	Mass spectrometer contaminated	Clean mass spectrometer
	Shift of mass calibration	Recalibrate MS/MS
	Mass resolution too high/low	Optimise mass resolution
	Injection valve leaking	Check injector

Problem	Possible cause	Corrective measure
Fluctuations in signal intensity	Spray instable	Check the spray needle capillary and clean, if necessary
	Gas flow rate instable	Check the gas lines
No vacuum	Defective vacuum pumps	Check pre- and high-vacuum pumps
	Vacuum system leaking	Check vacuum tubes and fittings
No gas supply	Defective nitrogen generator	Check nitrogen generator
	Defective compressor	Check compressor
	Gas bottle is empty	Replace gas bottle
	Inlet gas pressures not within specified range	Regulate the inlet gas pressures

10 EC-Declaration of Conformity

The EC-Declaration of Conformity according to Annex IV of the IVD directive 98/79/EC is available upon request (info@recipe.de).

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
80992 München

Tel. +49 89 54 70 81 - 0
Fax. +49 89 54 70 81 - 11

info@recipe.de
www.recipe.de

Zertifiziert nach /
Certified acc. to
ISO 13485