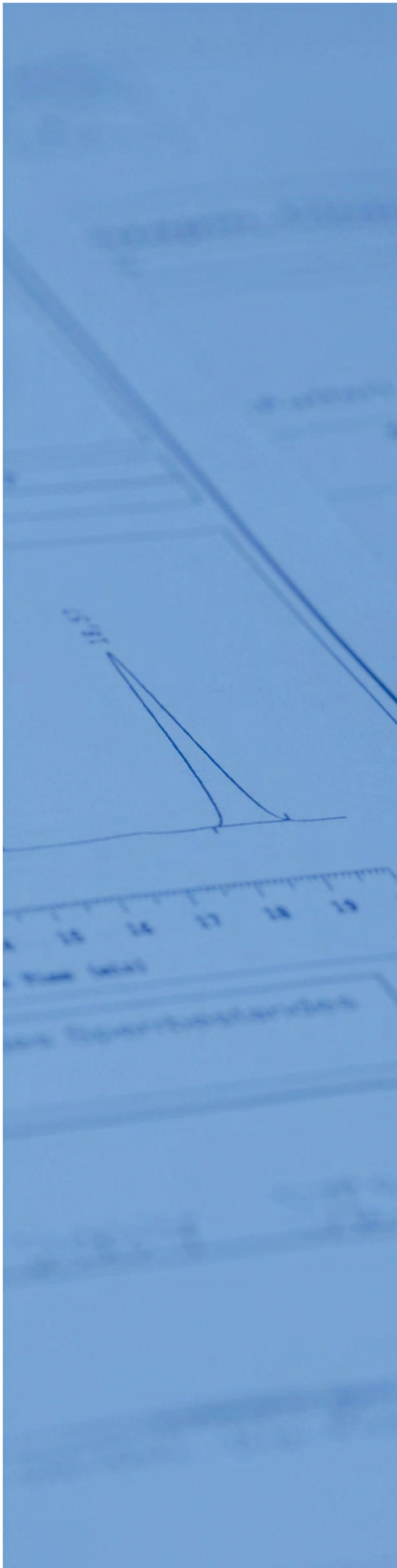
RECIPE®

Instruction Manual

ClinMass® LC-MS/MS Complete Kit,
advanced

Immunosuppressants in Whole Blood – On-line Analysis – automated On-line Analysis



REF MS1100, MS1200

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

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1 Introduction

1.1 Intended use

The present analytical procedure is intended for the determination of cyclosporine A, tacrolimus, sirolimus and everolimus from human whole blood by on-line SPE HPLC with electrospray-tandem mass spectrometry. The sample pretreatment can be performed manually (see section 5.3) or automated (see section 5.4). For this purpose, two separate complete kits with order nos. MS1100 and MS1200 are available (see section 2).

The kit components have to be used in accordance with this user manual. The kit is not designed for combination with components by 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:

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

1.2 Clinical background

Cyclosporine A, tacrolimus, sirolimus, and everolimus (see figure 1) are immunosuppressive drugs used after organ transplantation. The goal of the therapy is to prevent an acute allograft rejection by inhibition of the immunological defence of the recipient with, as far as possible, minimal effect on the immunological resistance towards infections [1-3].

Immunosuppressive drugs function through various mechanisms. Cyclosporine A and tacrolimus are calcineurin inhibitors and block the interleukin-2 production, leading to a decrease in T lymphocyte proliferation. Sirolimus and everolimus act at a later stage than the calicineurin inhibitors by inhibiting the interleukin-2-stimulated cell cycle progression [4]. Due to the complementary mechanisms of action, these two classes of agents are often combined in patient treatment, whereby taking advantage of the synergistic effects [5].

The administration of immunosuppressants requires accurate therapeutic drug monitoring (TDM) within a narrow therapeutic concentration range. Overdose of these drugs increases the risks of severe side effects, whilst underdose can result in immunological rejection and organ loss or damage. Both can significantly reduce the lifespan of the organ recipients.

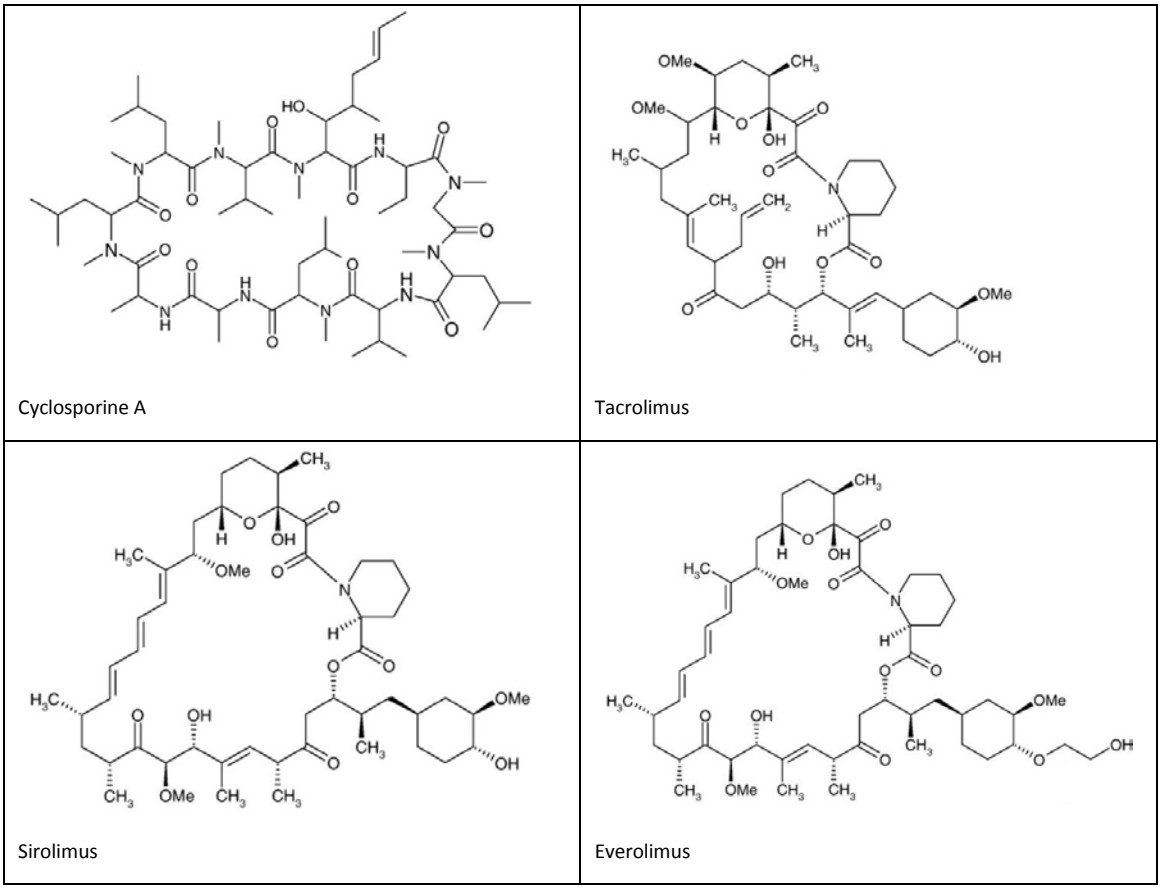


Figure 1: Structural formulas of cyclosporine A, tacrolimus, sirolimus, and everolimus

The pharmacokinetics of immunosuppressants, in general, characteristically shows poor bioavailability and a large intra- and inter-individual variation. Consequently, the correlation

between drug dosage and blood concentration is poor and results in a need to individualise the dose regimen for different recipients.

To avoid over- or under-administration, dosage regimens are usually adjusted according to the whole blood concentration. For the dose adjustment, the trough concentrations (termed as C-0) are often used, i.e. the values, which are measured before the next medication. For cyclosporine A, the C-2 level (blood level measured 2 hours after dose administration) is preferred, due to a better correlation with the pharmacological effect [2, 6].

For the therapeutic drug monitoring, liquid chromatography (LC) based methods are considered the methodology of choice. Immunological methods, although widely used in clinical laboratories, lack in analytical specificity. Cross reactions between drug and drug metabolites can result in an overestimation of the measured drug, with unacceptable biases in some clinical situations (see e.g. [2]). The use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows a highly selective quantification of the main drug independently from its metabolites (see section 1.3).

1.3 General description of the analytical procedure

In this analytical method, cyclosporine A, tacrolimus, sirolimus, and everolimus are determined from human whole blood by on-line SPE HPLC with electrospray-tandem mass spectrometry.

Conventional HPLC methods require manual sample preparation, performed prior to the sample injection. With on-line HPLC methods, the sample preparation is performed within the LC-MS/MS configuration by column switching, using a multiple port valve and a SPE-column (on-line sample preparation).

Prior to the on-line analysis, a precipitation step is required in order to remove the sample matrix and to spike with the internal standards (sample pretreatment, see sections 5.3 and 5.4).

This pretreatment step can be performed either manually or automated by liquid handling systems from 96-well plates. For this purpose different ClinMass® Complete Kits with **order nos. MS1100** (for kit components see section 2.1.1) and **MS1200** (for kit components see section 2.1.2) are available.

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

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

In the present 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') 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 selectively appropriate voltage settings. Among the fragments generated (known as the 'product'), 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 highly characteristic mass transitions for the compound of interest.

ClinMass® Optimisation Mixes are provided for the optimisation of the MS/MS parameters (see section 5.6.1) and for the test run of the analytical system (see section 5.6.2).

The calibration of the analytical system is performed by use of ClinCal® Multi-Level Calibrators (see section 5.6.3). For this purpose a 4-level calibrator set (level 0-3, order no. 9033) as well as a 7-level calibrator set (level 0-6, order no. 9933) are available. For an extended calibration range with an additional, high calibration point (level 7), the whole blood calibrator with order no. 9028 is optionally available.

Quality control is performed by use of ClinChek® Whole Blood Controls. These controls are available in five different concentrations (see section 5.6.4).

2 Components of the complete kit and accessories

2.1 Ordering information

2.1.1 On-line analysis with manual sample pretreatment

Order No.	Description	Quantity
MS1100	**ClinMass® Complete Kit, advanced for Immunosuppressants in Whole Blood** for 400 assays	1 pce.
	Contents:	
	Autosampler Washing Solution	1 x MS1005
	SPE - Buffer	2 x MS1009
	Mobile Phase	1 x MS1010
	IS Internal Standard, lyophil.	3 x MS1412
	Sample Pretreatment Vials	4 x MS1020
	P Precipitant	1 x MS1021
	Manual	
	Separately available components:	
MS1005	Autosampler Washing Solution	1000 ml
MS1006	Reagent MP	8 ml
MS1009	SPE - Buffer	1000 ml
MS1010	Mobile Phase	800 ml
MS1014	Optimisation Mix 1, lyophil.	2 ml
MS1020	Sample Pretreatment Vials	100 pcs.
MS1021	P Precipitant	80 ml
MS1115	Optimisation Mix 2, lyophil.	2 ml
MS1412	IS Internal Standard, lyophil.	3 ml
5013	Whole Blood Calibrator, lyophil. (single point)	5 x 2 ml
9028	Whole Blood Calibrator, lyophil. (additional level for order nos. 9033 and 9933)	2 x 2 ml
9033	Whole Blood Calibrator Set, lyophil. (Level 0 - 3)	4 x 1 x 2 ml
9933	Whole Blood Calibrator Set, lyophil. (Level 0 - 6)	7 x 1 x 2 ml
	Start Accessories:	
MS1030	Analytical Column with test chromatogram	1 pce.
MS1031	SPE - Column	1 pce.
MS1032	Guard Column Holder incl. 1 Guard Column	1 pce.
MS1033	Guard Columns	5 pcs.
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)	1 pce.
	Accessories:	
FK1102	Switching valve Sykam (6-port / 3-channel incl. electr. drive, PEEK)	1 pce.
FK7330	Endfittings	2 pcs.
FK7340	Sealings and sieves	4 pcs. each
FK7350	Endstoppers	2 pcs.
	ClinChek® Controls:	
8830	Whole Blood Control, lyophil. Level I	5 x 2 ml
8831	Whole Blood Control, lyophil. Level II	5 x 2 ml
8832	Whole Blood Control, lyophil. Level III	5 x 2 ml
8833	Whole Blood Control, lyophil. Level I, II, III	3 x 2 x 2 ml
8903	Whole Blood Control, lyophil. Level IV, V	2 x 2 x 2 ml

2.1.2 On-line analysis with automated sample pretreatment

Order No.	Description	Quantity
MS1200	**ClinMass® Complete Kit, advanced for Immunosuppressants in Whole Blood** for 1200 assays	1 pce.
	Contents:	
	Autosampler Washing Solution	3 x MS1005
	SPE - Buffer	6 x MS1009
	Mobile Phase	3 x MS1010
	IS Internal Standard, lyophil.	9 x MS1412
	96-Well-Plates (1200 µl)	3 x FK6511
	P Precipitant	3 x MS1021
	Manual	
	Separately available components:	
MS1005	Autosampler Washing Solution	1000 ml
MS1006	Reagent MP	8 ml
MS1009	SPE - Buffer	1000 ml
MS1010	Mobile Phase	800 ml
MS1014	Optimisation Mix 1, lyophil.	2 ml
FK6511	96-Well-Plates (1200 µl)	5 pcs.
MS1021	P Precipitant	80 ml
MS1115	Optimisation Mix 2, lyophil.	2 ml
MS1412	IS Internal Standard, lyophil.	3 ml
5013	Whole Blood Calibrator, lyophil. (single point)	5 x 2 ml
9028	Whole Blood Calibrator, lyophil. (additional level for order nos. 9033 and 9933)	2 x 2 ml
9033	Whole Blood Calibrator Set, lyophil. (Level 0 - 3)	4 x 1 x 2 ml
9933	Whole Blood Calibrator Set, lyophil. (Level 0 - 6)	7 x 1 x 2 ml
	Start Accessories:	
MS1030	Analytical Column with test chromatogram	1 pce.
MS1031	SPE - Column	1 pce.
MS1032	Guard Column Holder incl. 1 Guard Column	1 pce.
MS1033	Guard Columns	5 pcs.
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)	1 pce.
	Accessories:	
FK1102	Switching valve Sykam (6-port / 3-channel incl. electr. drive, PEEK)	1 pce.
FK6501	Protective sheets for 96-well-plate (PE/PP foil, 80 x 140 mm)	50 pcs.
FK6514	96-Well-Plates (300 µl)	5 pcs.
FK7330	Endfittings	2 pcs.
FK7340	Sealings and sieves	4 pcs. each
FK7350	Endstoppers	2 pcs.
	ClinChek® Controls:	
8830	Whole Blood Control, lyophil. Level I	5 x 2 ml
8831	Whole Blood Control, lyophil. Level II	5 x 2 ml
8832	Whole Blood Control, lyophil. Level III	5 x 2 ml
8833	Whole Blood Control, lyophil. Level I, II, III	3 x 2 x 2 ml
8903	Whole Blood Control, lyophil. Level IV, V	2 x 2 x 2 ml

2.1.3 Safety information

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

The calibrator and control materials are prepared from human whole blood. 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 given in the appropriate Safety Data Sheet (SDS).

2.1.4 Storage conditions and lifetime of kit components

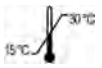
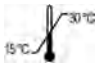
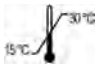
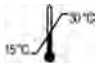
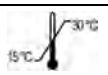
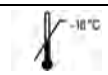
Please unpack the kit components from the transport packaging **immediately upon receipt** and follow the instructions for the storage conditions given on the product labels and table 1.

Unused components, stored under appropriate conditions can be used until the expiry date given 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 life times of ClinMass® Internal Standard and Optimisation Mixes as well as for ClinCal® Calibrators and ClinChek® Controls (lyophilised / after reconstitution) please also refer to the appropriate product data sheets.

Table 1: Storage conditions of kit components

Order no.		Product description	Storage conditions	
REF	MS1005	Autosampler Washing Solution		Store at 15 - 30 °C
REF	MS1006	Reagent MP		Store at 15 - 30 °C
REF	MS1009	SPE-Buffer		Store at 15 - 30 °C
REF	MS1010	Mobile Phase		Store at 15 - 30 °C
REF	MS1014	Optimisation Mix 1, lyophil.		Store below - 18 °C*
REF	MS1020	Sample Pretreatment Vials	Store at ambient temperature	
REF	FK6511	96-Well-Plates (1200 µl)	Store at ambient temperature	
REF	MS1021	P Precipitant		Store at 15 - 30 °C
REF	MS1115	Optimisation Mix 2, lyophil.		Store below - 18 °C*

REF	MS1412	IS Internal Standard, lyophil.		Store below - 18 °C*
REF	5013	Whole Blood Calibrator, lyophil.		Store at 2 - 8 °C*
REF	9028	Whole Blood Calibrator (additional level for order nos. 9033 and 9933), lyophil.		Store at 2 - 8 °C*
REF	9033	Whole Blood Calibrator Set (Level 0-3), lyophil.		Store at 2 - 8 °C*
REF	9933	Whole Blood Calibrator Set (Level 0-6), lyophil.		Store at 2 - 8 °C*
REF	MS1030	Analytical Column		Store at 15 - 30 °C
REF	MS1031	SPE-Column		Store at 15 - 30 °C
REF	MS1032, MS1033	Guard Column		Store at 15 - 30 °C
REF	FK7400	Inline-Filter	Store at ambient temperature	
REF	FK1102	Switching valve Sykam (6-port / 3-channel)	Store at ambient temperature	
REF	FK6501	Protective sheets for 96-well-plate (PE/PP foil, 80 x 140 mm)	Store at ambient temperature	
REF	FK6514	96-Well-Plates (300 µl)	Store at ambient temperature	
REF	FK7330	Endfittings	Store at ambient temperature	
REF	FK7340	Sealings and sieves	Store at ambient temperature	
REF	FK7350	Endstoppers	Store at ambient temperature	
REF	8830 - 8833	Whole Blood Controls, Level I, II, III, lyophil.		Store at 2 - 8 °C*
REF	8903	Whole Blood Controls, Level IV, V, lyophil.		Store at 2 - 8 °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 with regard to its different chemical properties. Recommendations for the disposal of the product and of the packaging are given in section 13 of the appropriate Safety Data Sheet (SDS).

3 Required instruments

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

Required LC modules:

- Autosampler (with cooling function, 4 °C)
- Isocratic HPLC pump 1 (SPE-buffer)
- Isocratic HPLC pump 2 (mobile phase)
- 6-port-3-channel-switching valve (e.g. RECIPE, order no. FK1102)
- Column heater (60 °C)
- Degasser (optional)

The following laboratory instruments are required for the manual sample pretreatment:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

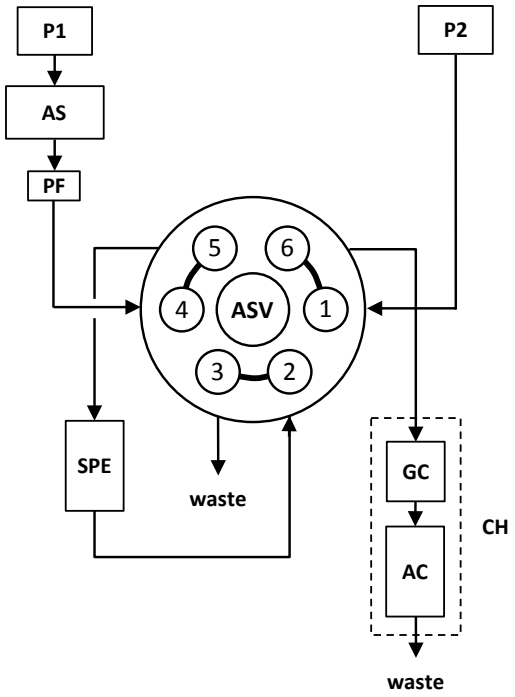
The following laboratory instruments are required for the automated sample pretreatment via liquid handling systems and 96-well plates:

- Liquid handling system with shaker for 96-well plates
- Centrifuge for well plates

4 Operation of the analytical system

4.1 Configuration of the LC system

Connect the LC modules (P1, P2, AS) and the automatic switching valve (ASV) as shown in the figure below, **with exception** of the columns (SPE, GC, AC). Put the outlet capillaries (from ASV to waste) into a safe waste container.



Abbreviations:
P1: HPLC pump 1; P2: HPLC pump 2; AS: autosampler; PF: inline-filter (order no.: FK7400); ASV: automatic switching valve (high pressure switching valve, 6-port/3-channel, order no.: FK1102); SPE: SPE-column; GC: guard column; AC: analytical column; CH: column heater

Figure 2: Configuration of the LC system

4.1.1 Reagent MP

For operation with an Agilent MS/MS system, Reagent MP (order no. MS1006) must be added to the mobile phase.

Add the whole amount of Reagent MP (8 ml) to a freshly opened bottle of mobile phase.

4.2 Flushing of the LC system

Set both HPLC pumps (P1, P2) at a flow rate of 1 ml/min and flush the LC system with 10 ml SPE-buffer and mobile phase, respectively.

Afterwards connect the SPE-column (SPE), the guard column (GC), and the analytical column (AC) as shown in figure 2. The guard column and the analytical column have to be installed within the column heater (CH).

When connecting the SPE-column and the analytical column, please take care that the flow direction follows the arrow marking on the columns!

4.3 Equilibration of the LC system

After flushing the system (see section 4.2) the equilibration is performed as follows:

- Set both HPLC pumps (P1, P2) to a flow rate of 0.5 ml/min, set the column heater to 60 °C, and allow approximately 10 ml SPE-buffer and mobile phase, respectively, to flow through the columns.
- After this, **stop the HPLC pumps** and connect the outlet capillary of the analytical column (AC) with the tandem mass spectrometer.

4.4 Starting the analytical system

The following sections provide the parameters for the LC system (see section 4.4.1) and the tandem mass spectrometer (see section 4.4.2). For optimisation, equilibration and testing, as well as for calibration of the LC-MS/MS system please refer to section 5.6.

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.

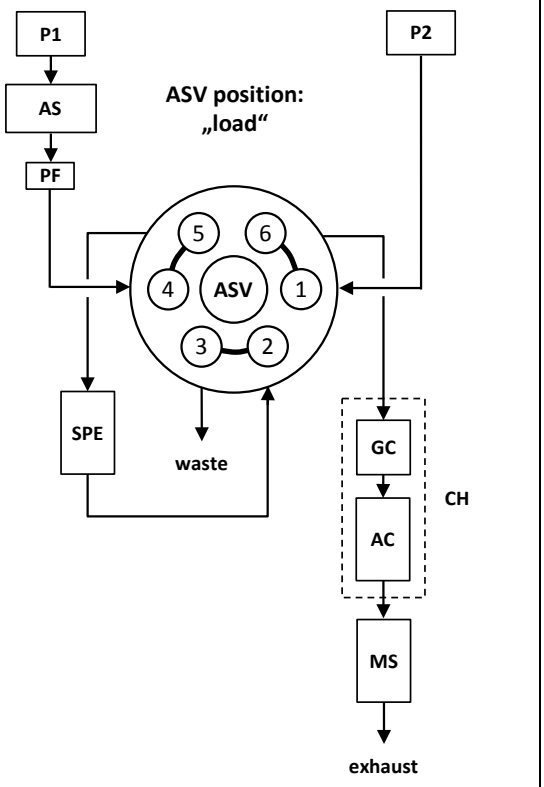
4.4.1 LC parameters

Table 2: LC parameters

Isocratic HPLC pump 1 (SPE-buffer):	Flow rates: 0.1 ml/min, 2.5 ml/min; see section 4.4.1.1, table 3
Isocratic HPLC pump 2 (mobile phase):	Flow rates: 0.5 ml/min, 1.0 ml/min; see section 4.4.1.1, table 3
SPE-buffer / mobile phase:	Make sure that the bottles are closed well to avoid alteration of the retention times through evaporation of components of the SPE-buffer and mobile phase.
Reagent MP:	For operation with an Agilent MS/MS system, Reagent MP must be added to the mobile phase (see section 4.1.1).
Columns:	The analytical column (AC) and the guard column (GC) are installed within the column heater (60° C). At a flow rate of 0.5 ml/min, the backpressure of the analytical column should not exceed 80 bar. At a flow rate of 2.5 ml/min, the backpressure of the SPE column should not exceed 150 bar. For the complete HPLC system, the backpressure should not exceed 250 bar. The sealings and frits of the inline filter (PF) and the guard column should be replaced after every 500 injections. These parts should also be replaced, if the backpressure of the inline filter exceeds 10 bar (at a flow rate of 2.5 ml/min) or if that of the guard column exceeds 10 bar (at a flow rate of 0.5 ml/min).
Column heater:	60 °C
Autosampler:	Set the autosampler cooling function to 4 °C. The autosampler washing solution (order no. MS1005) is available for the flushing of the injection system. Please also refer to the user manual of the autosampler manufacturer. Injection volume: 20 - 50* µl Injection interval: 2 min *depending on the sensitivity of the mass spectrometer
Automatic switching valve:	See section 4.4.1.1

4.4.1.1 Automatic switching valve (on-line analysis)

The SPE sample clean up is performed on-line by use of a 6-port-3-channel automatic switching valve. A description of the working principle is given in figure 3:



Abbreviations:
P1: HPLC pump 1; P2: HPLC pump 2; AS: autosampler; PF1: inline-filter (order no.: FK7400); ASV: automatic switching valve (high pressure switching valve, 6-port/3-channel, order no.: FK1102); SPE: SPE-column; GC: guard column; AC: analytical column; CH: column heater; MS: tandem mass spectrometer

ASV position "load": switching interval: 0 - 0.50 min:

The sample is injected into the LC system and is transferred to the SPE column, which extracts the analytes selectively from the sample matrix. The matrix components are washed through the SPE column and thus are eluted to the waste. Meanwhile, the analytical column is reequilibrated from the previous injection cycle.

ASV position "inject": switching interval: 0.50 - 1.65 min:

The automatic switching valve switches to the "inject" position. The extracted analytes are eluted from the SPE column with mobile phase under backflush mode. They are transferred to the analytical column, on which they are chromatographically separated.

ASV position "load": switching interval: 1.65 - 2 min:

The automatic switching valve switches back to the "load" position. Both columns (SPE- and analytical column) are reequilibrated for the next injection.

Figure 3: Automatic switching valve and configuration of the LC system

The switching times and positions of the automatic switching valve (ASV) and the flow rates of the HPLC pumps P1 and P2 are programmed according to the following table.

Table 3: Switching times and positions (ASV), flow rates of the HPLC pumps (P1, P2)

Time [min]	ASV position	Pump P1 flow rate [ml/min]	Event SPE-column	Pump P2 flow rate [ml/min]	Event analytical column
0.00	load	0.1		0.5	
0.01		2.5	loading		equilibration
0.50	inject	2.5	elution		loading
0.51		0.1			separation
1.30				0.5	
1.35				1.0	
1.50		0.1			
1.51		2.5			
1.55				1.0	
1.65	load		equilibration	0.5	equilibration
1.99		2.5			
2.00		0.1		0.5	

4.4.1.2 Peak assignment and retention times

For analyte / internal standard assignments see table 4.

Table 4: Analyte / internal standard assignments and corresponding LC retention times (RT)

Analyte	RT [min]	Internal Standard IS (order no. MS1412)	RT [min]
Cyclosporine A	1.42	d_{12}-Cyclosporine A	1.42
Tacrolimus	1.24	$^{13}Cd_2$-Tacrolimus	1.24
Sirolimus	1.29	$^{13}Cd_3$-Sirolimus	1.29
Everolimus	1.30	$^{13}C_2d_4$-Everolimus	1.31

4.4.2 MS/MS parameters

4.4.2.1 Mass transitions

Table 5 contains the mass transitions for the analytes and the internal standards. The indicated mass transition parameters should be regarded 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 5.6.1).

Table 5: Mass transitions

Substance	**Precursor [amu]**	**Product [amu]**
Cyclosporine A (Quantifier)	1219.7	1202.8
Cyclosporine A (Qualifier)	1219.7	1184.8
d_{12}-Cyclosporine A (Quantifier)	1232.0	1215.0
d_{12}-Cyclosporine A (Qualifier)	1232.0	1197.1
Tacrolimus (Quantifier)	821.5	768.4
Tacrolimus (Qualifier)	821.5	576.3
$^{13}Cd_2$-Tacrolimus (Quantifier)	824.5	771.5
$^{13}Cd_2$-Tacrolimus (Qualifier)	824.5	579.3
Sirolimus (Quantifier)	931.5	864.5
Sirolimus (Qualifier)	931.5	882.5
$^{13}Cd_3$-Sirolimus (Quantifier)	935.6	864.7
$^{13}Cd_3$-Sirolimus (Qualifier)	935.6	882.5
Everolimus (Quantifier)	975.6	908.4
Everolimus (Qualifier)	975.6	926.7
$^{13}C_2d_4$-Everolimus (Quantifier)	981.6	914.7
$^{13}C_2d_4$-Everolimus (Qualifier)	981.6	932.6

4.4.2.2 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).

4.5 Standby mode

When the analytical system is not in use, the pumps have to be switched off. The SPE-buffer and the mobile phase 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.

For a longer operation pause, the SPE-Column and the analytical column should be disconnected and stored tightly closed. The LC system should then be flushed with a water/methanol mixture (1:1).

5 Implementation of the analytical procedure

5.1 Collection and storage of whole blood samples

The analysis is performed from EDTA whole blood.

If the determination is performed within the same day, the samples may be stored at room temperature (15 - 30 °C). At temperatures between 2 - 8 °C, the samples may be stored for up to 7 days. If the samples shall be stored for a longer period of time, the samples must be stored at temperatures below -18 °C (multiple freeze-thaw cycles should be avoided).

5.2 Reconstitution of the lyophilised whole blood calibrators / controls

ClinCal® Whole Blood Calibrators and ClinChek® Whole Blood Controls (see section 2.1) are lyophilised and thus must be reconstituted before use. Information regarding reconstitution, along with analyte concentrations and information about storage and stability, is given in the appropriate product data sheets.

5.3 Manual sample pretreatment (order no. MS1100)

5.3.1 Work flow

Sample pretreatment:

Precipitation:

20 µl IS Internal Standard	200 µl P Precipitant	100 µl Whole blood (calibrator, control, patient)

mix for 30 sec (vortex mixer), ⇩ incubate (5 min, room temp.)

mix for 10 sec (vortex mixer), ⇩ centrifuge (5 min, 10000 x g)

LC-MS/MS analysis:

Inject 20 - 50* µl of the supernatant

*depending on the sensitivity of the mass spectrometer

5.3.1.1 Precipitation

Pipette 20 µl Internal Standard IS, 200 µl Precipitant P, and 100 µl of the well homogenised whole blood sample (calibrator, control, patient) into a sample pretreatment vial. Mix for 30 sec on a vortex mixer and afterwards incubate for 5 min at room temperature (15 - 30 °C). After this, mix again for 10 sec (vortex mixer) and centrifuge for 5 min with at 10000 x g.

N.b.: Internal Standard IS and Precipitant P may be pre-mixed (mixture IS/P: mixing ratio: 20+200) in accordance to the actual daily amount required. 220 µl* of this mixture is then mixed with 100 µl whole blood sample.

The mixture IS/P can be stored for up to 18 hours at temperatures between 2 - 8 °C.

*Multipettes: with some multipettes the setting of 220 µl may not be possible. In these cases a volume between 200 - 240 µl can be set alternatively.

5.3.1.2 LC-MS/MS analysis

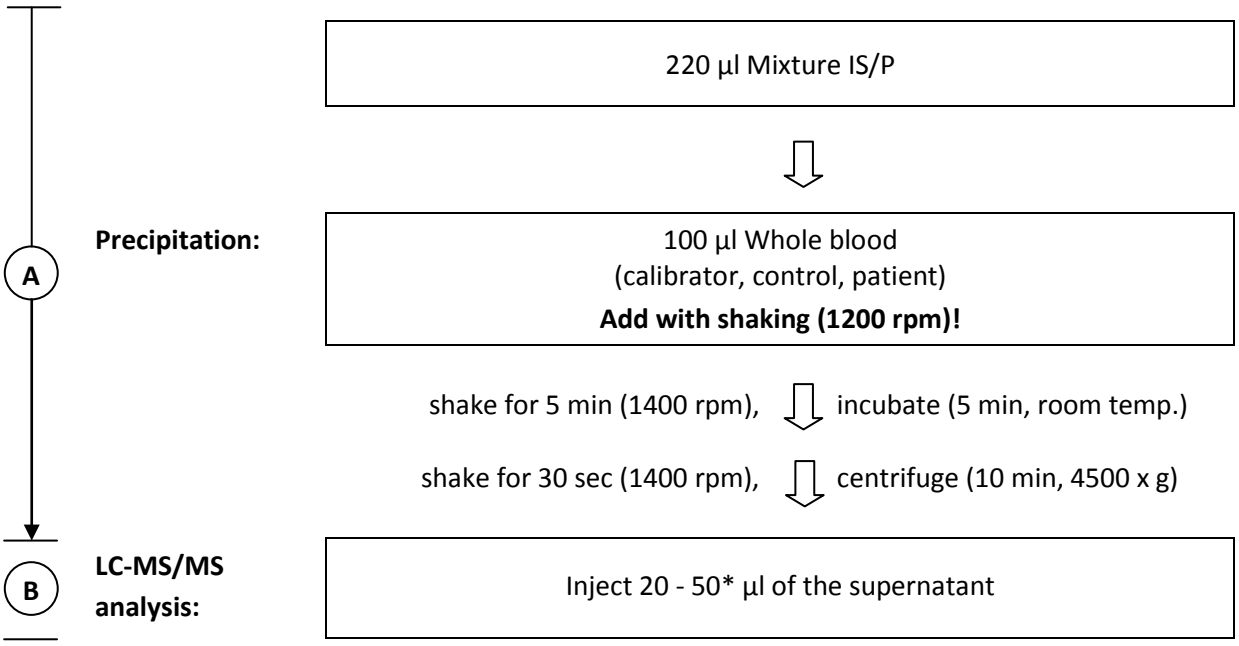
Transfer the centrifuge supernatant to a sample vial, which is suitable for the autosampler in use (brown glass vials are recommended). Inject, depending on the sensitivity of the mass spectrometer, 20 - 50 µl of the supernatant into the LC-MS/MS system.

5.4 Automated sample pretreatment (order no. MS1200)

For the automated sample preparation with liquid handling systems of various manufacturers, device-specific data is available on request (info@recipe.de).

5.4.1 Work flow

Sample pretreatment:



*depending on the sensitivity of the mass spectrometer

A: Use of 96-well plates (96/1200 µl, order no. FK6511)
B: Use of 96-well plates (96/300 µl, order no. FK6514)

5.4.1.1 Preparation of mixture IS/P

Internal Standard IS and Precipitant P are pre-mixed (mixture IS/P) depending on the number of samples (see table 6). Mixture IS/P is then used for the matrix precipitation as described in section 5.4.1.2.

Table 6: Preparation of mixture IS/P depending on the number of samples

Number of samples	Internal Standard IS [ml]	Precipitant P [ml]	Total volume of mixture IS/P [ml]
100	2.0	20.0	22.0
200	4.0	40.0	44.0
400	8.0	80.0	88.0

The mixture IS/P can be stored for up to 18 hours at 2 - 8 °C.

5.4.1.2 Precipitation

Pipette first 220 µl mixture IS/P into each well of the 96-well plate (96/1200 µl, order no. FK6511). Then add 100 µl well homogenised whole blood sample (calibrator, control, patient) **with shaking** (1200 rpm).

Afterwards shake the plate for 5 min at 1400 rpm and incubate for 5 min at room temperature (15 - 30 °C). Then shake again for 30 sec at 1400 rpm and centrifuge for 10 min at 4500 x g.

5.4.1.3 LC-MS/MS analysis

Transfer 150 µl of the supernatant in a 96-well plate* (96/300 µl, order no. FK6514) and cover the plate with protective sheets (order no. FK6501) in order to protect the samples. Inject, depending on the sensitivity of the mass spectrometer, 20 - 50 µl from the covered well plate into the LC-MS/MS system.

*Injection of the supernatant into the LC-MS/MS system can also be performed directly from the covered 96-well plate (96/1200µl) if there are no concerns regarding a blockage of the injection system.

5.5 Stability of the pretreated samples

At temperatures between 2 - 8 °C, pretreated samples are stable for at least 12 hours.

5.6 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.

5.6.1 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 the optimisation of the MS/MS system parameters Optimisation Mix 1 and 2 (order nos. MS1014 and MS1115) are provided.

Optimisation Mix 1 contains the analytes, i.e. cyclosporine A, tacrolimus, sirolimus, and everolimus. Optimisation Mix 2 contains the internal standards, i.e. d_{12}-cyclosporine A, $^{13}Cd_2$-tacrolimus, $^{13}Cd_3$-sirolimus and $^{13}C_2d_4$-everolimus.

Optimisation Mix 1 and 2 are lyophilised and thus have to be reconstituted before use. Information regarding the reconstitution is given in the appropriate product data sheets. If necessary, Optimisation Mix 1 and 2 should be diluted with mobile phase according to 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).

5.6.2 Equilibration of the analytical system and test run

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

In order to confirm the performance of the analytical system, repeatedly inject a mixture of the Optimisation Mix 1 and 2 (see preparation below), until two consecutive chromatograms, comparable in retention times and peak areas, are obtained.

The mixture is prepared from:

- 50 µl Optimisation Mix 1 (order no. MS1014)
- 100 µl Optimisation Mix 2 (order no. MS1115)
- 850 µl Mobile Phase (order no. MS1010)

A further dilution of the mixture with mobile phase may be required, depending on the sensitivity of the MS/MS system in use.

5.6.3 Calibration run

For calibration, a ClinCal® 4-Level Whole Blood Calibrator Set (level 0 - 3, order no. 9033) and a ClinCal® 7-Level Whole Blood Calibrator Set (level 0 - 6, order no. 9933) are available. For an extended calibration range with an additional, high calibration point (level 7), the whole blood calibrator with order no. 9028 is optionally available.

After reconstitution (see section 5.2), the calibrators must be pretreated as described for the patient samples (see sections 5.3 and 5.4).

For each analytical series, freshly prepared calibrators are required.

5.6.4 Accuracy control

For the quality control of the analytical measurements, ClinChek® Whole Blood Controls are available in five different concentrations (level I, order no. 8830; level II, order no. 8831; level III, order no. 8832; level I - III, order no. 8833 as well as level IV - V, order no. 8903).

Please note:
The usage of control levels IV and V (order no. 8903) requires an extension of the calibration range with level 7 of the whole blood calibrator with order no. 9028 (see section 5.6.3).

These controls are lyophilised and, subsequently to reconstitution, must be pretreated as described for the patient samples (see sections 5.3 and 5.4).

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

5.6.5 Example chromatogram

Example chromatogram of the ClinCal® Whole Blood Calibrator (order no. 9933), level 4:

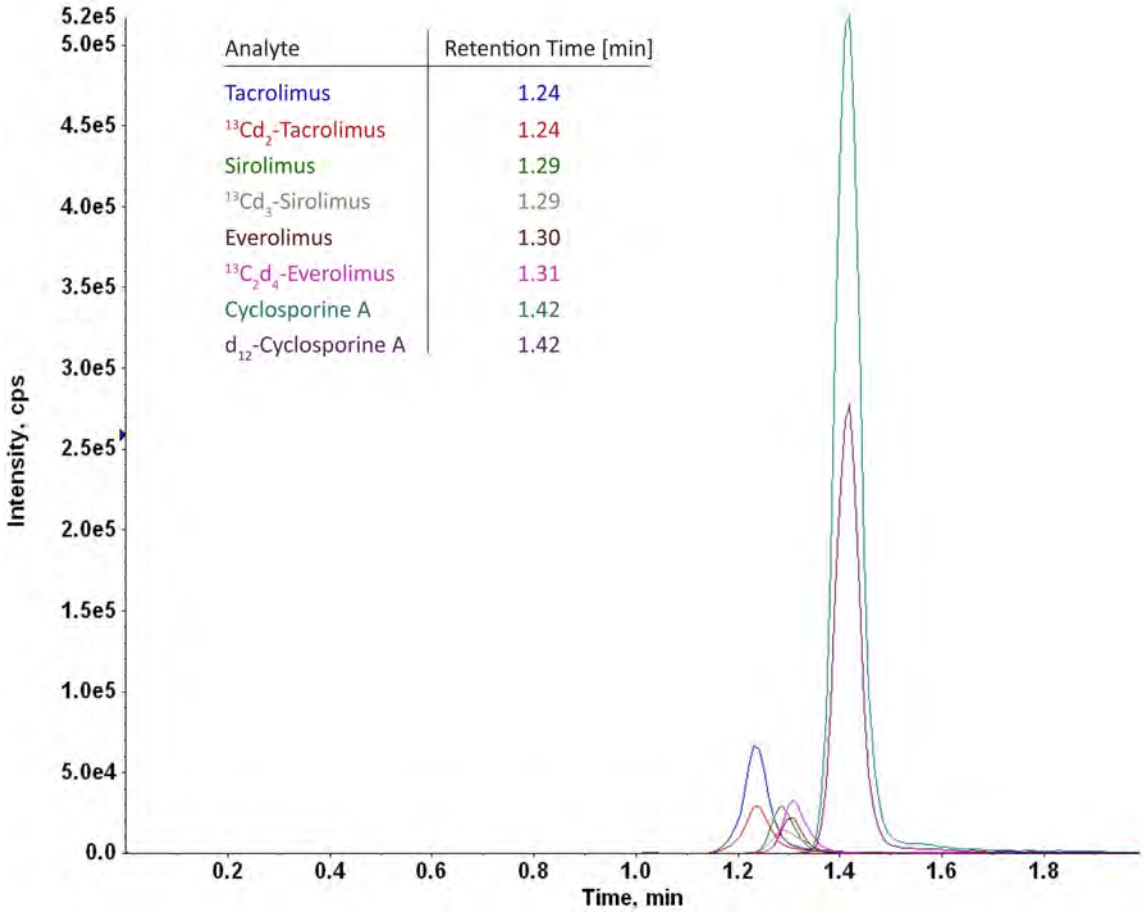


Figure 4: Chromatogram of the ClinCal® Whole Blood Calibrator, level 4

6 Evaluation

The analyte detection is achieved using compound specific mass transitions (see section 4.4.2)

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

The respective calibration curve is obtained from the calibrators by plotting the ratio of *peak area „analyte / internal standard“* against the ratio of c*oncentration „analyte*“.

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 [µmol/l], and vice versa, the analytical results have to be multiplied with the factors shown in table 7.

Table 7: Conversion factors

Analyte	**Molecular weight [g/mol]**	**Conversion factor: µmol/l --> µg/l**	**Conversion factor: µg/l --> µmol/l**
Cyclosporine A	1202.63	1202.63	8.315×10^{-4}
Tacrolimus	804.15	804.15	1.244×10^{-3}
Sirolimus	914.17	914.17	1.094×10^{-3}
Everolimus	958.24	958.24	1.044×10^{-3}

7 Test data

7.1 Test performance

The results were obtained with the MS/MS-systems Agilent 6460, AB SCIEX API4000 (on-line analysis) and Shimadzu LCMS-8050 (automated on-line analysis).

The use of 96-well plates for the automated sample pretreatment has no influence on linearity, LLOD, LLOQ and recovery. For the precision data, please refer to sections 7.1.3.1 and 7.1.3.2 for both manual and automated on-line analysis.

7.1.1 Linearity, quantitation limit, detection limit

	Cyclosporine A	Tacrolimus	Sirolimus	Everolimus
LLOD [µg/l]*	0.011	0.004	0.073	0.126
LLOQ [µg/l]**	0.037	0.014	0.244	0.420
Linearity [µg/l]	0.037 - 1750	0.014 - 65.00	0.244 - 71.70	0.420 - 66.80

*LLOD: Lower limit of detection, S/N=3, **LLOQ: Lower limit of quantitation, S/N=10; S: Signal, N: Noise

7.1.2 Recovery

For cyclosporine A, tacrolimus, sirolimus, and everolimus, mean recovery rates between 95 - 112 % were obtained.

7.1.3 Precision

7.1.3.1 On-line analysis (manual sample pretreatment)

For the evaluation of the intra- and interassay precision, 3 samples with the following concentrations were used:

	Cyclosporine A [µg/l]	Tacrolimus [µg/l]	Sirolimus [µg/l]	Everolimus [µg/l]
Sample 1	58.4	3.67	4.08	3.84
Sample 2	118	7.69	12.7	12.5
Sample 3	232	15.5	21.7	20.5

7.1.3.1.1 Intraassay

For the determination of the intraassay precision the samples were measured in 3 analytical series, each by 6-fold determination (n = 18; n: number of values per sample). The following coefficients of variation (CV) were obtained (mean values):

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	2.4	2.8	4.6	4.9
Sample 2	2.4	3.1	4.1	3.5
Sample 3	1.4	2.4	3.3	3.5

7.1.3.1.2 Interassay

For the determination of the interassay precision the samples were measured in 8 analytical series, each by double determination (n = 16; n: number of values per sample). The following coefficients of variation (CV) were obtained:

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	5.5	4.7	5.1	4.8
Sample 2	4.0	4.5	5.6	3.1
Sample 3	2.1	4.2	4.7	3.7

7.1.3.2 Automated on-line analysis

7.1.3.2.1 Intraassay

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

	Cyclosporine A [µg/l]	Tacrolimus [µg/l]	Sirolimus [µg/l]	Everolimus [µg/l]
Sample 1	55.8	3.55	3.52	3.71
Sample 2	110	7.17	11.3	12.1
Sample 3	217	14.1	19.3	19.4

For the determination of the intraassay precision the samples were measured in one analytical series by 8-fold determination (n = 8; n: number of values per sample). The following coefficients of variation (CV) were obtained (mean values):

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	3.09	7.05	7.71	6.50
Sample 2	3.90	2.59	6.80	5.72
Sample 3	3.23	7.79	2.22	6.05

7.1.3.2.2 Interassay

For the evaluation of the intra- and interassay precision, 3 samples with the following concentrations were used:

	Cyclosporine A [µg/l]	Tacrolimus [µg/l]	Sirolimus [µg/l]	Everolimus [µg/l]
Sample 1	56.4	3.46	4.02	3.73
Sample 2	112	7.64	11.7	11.5
Sample 3	218	14.8	20.0	19.3

For the determination of the interassay precision the samples were measured in 5 analytical series, each by double determination (n = 10; n: number of values per sample). The following coefficients of variation (CV) were obtained:

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	2.0	3.8	9.2	4.9
Sample 2	1.7	6.5	5.3	2.8
Sample 3	3.6	6.8	3.3	5.4

7.2 Reference ranges

The therapeutical ranges depend on several factors, such as the type of transplantation, time after graft and co-medication with other immunosuppressive agents.

For this reason, general therapeutical ranges cannot be given but must be established individually for each patient.

8 References

[1] L. Thomas (Ed.), Labor und Diagnose: Indikation und Bewertung von Laborbefunden, 7. Auflage, TH-Books, Verlagsgesellschaft, Frankfurt/Main 2008, p. 1214-1225.

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[4] A.L. Taylor, C.J. Watson, J.A. Bradley: Immunosuppressive agents in solid organ transplantation: mechanism of action and therapeutic efficacy, Critical Reviews in Oncology/Hematology 2005; 56, 23-46.

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9 Troubleshooting

Problem	Possible cause	Corrective measure
Alteration of retention times	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 methanol or inject 10 x mobile phase • Check flushport solvent level • Clean/replace injection needle and needle seat assembly
	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 configured correctly	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
	Shift of mass calibration	Recalibrate MS/MS system
	Mass resolution too high	Optimise the mass resolution
	Leakage of injection valve	Check the injector

Problem	**Possible cause**	**Corrective measure**
High fluctuations of signals	Spray instable	Check the spray needle capillary and clean or exchange, if necessary
	Gas flow rate instable	Check the gas pipes
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 of 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
80992 Munich / Germany

declares, that the CE labelled products

ClinMass® Complete Kit, *advanced*, for Immunosuppressants
– On-Line Analysis (order no. MS1100)
– Automated On-Line Analysis (order no. MS1200)

meet 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, 09.07.2014

Alfred Bauer
General Manager

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Zertifiziert nach /
Certified acc. to
ISO 9001
ISO 13485

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