RECIPE

Instruction Manual

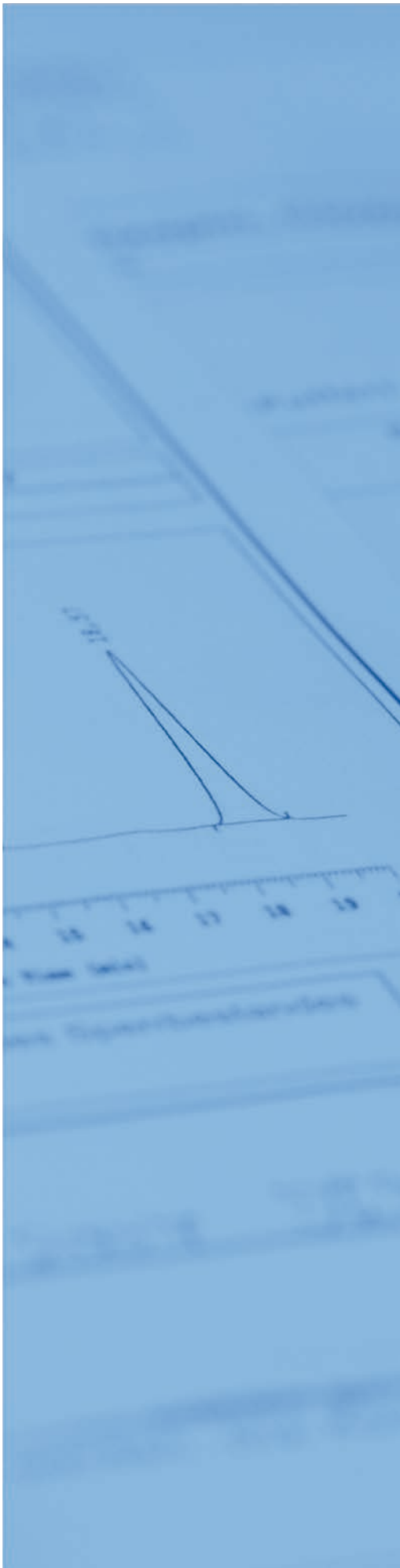
ClinMass® TDM Kit System

Immunosuppressants in Whole Blood

REF MS9000A, MS99200

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC



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1 Introduction

1.1 Information on changes in this instruction manual

This instruction manual (version 1.1) was revised and replaces the previous version 1.0.

Please note the updated intended use of the kit in section 1.2 as well as the updated information on the stability of the prepared samples in section 5.2.3.3.

Please also note the new section 8 (clinical performance).

Changes are marked in the outer margins.

1.2 Intended use

ClinMass® TDM Kit System

The ClinMass® TDM Kit System is based on a universal TDM Platform (order no. MS9000, MS9000A), which can be used with various Add-on Sets for the Therapeutic Drug Monitoring (TDM) by HPLC coupled with tandem mass spectrometry (LC-MS/MS).

The ClinMass® Add-on Set for Immunosuppressants (order no. MS99200) is intended for the quantitative determination in the clinically relevant range and monitoring of the drug level of cyclosporine A, tacrolimus, sirolimus and everolimus from human whole blood.

TDM Platform and Add-on Set are in vitro diagnostic medical devices and are intended for use by professional users in clinical and medical laboratories only.

1.2.1 IVD symbols

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

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

1.3 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 [1–4].

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 [5]. 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 [6].

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.

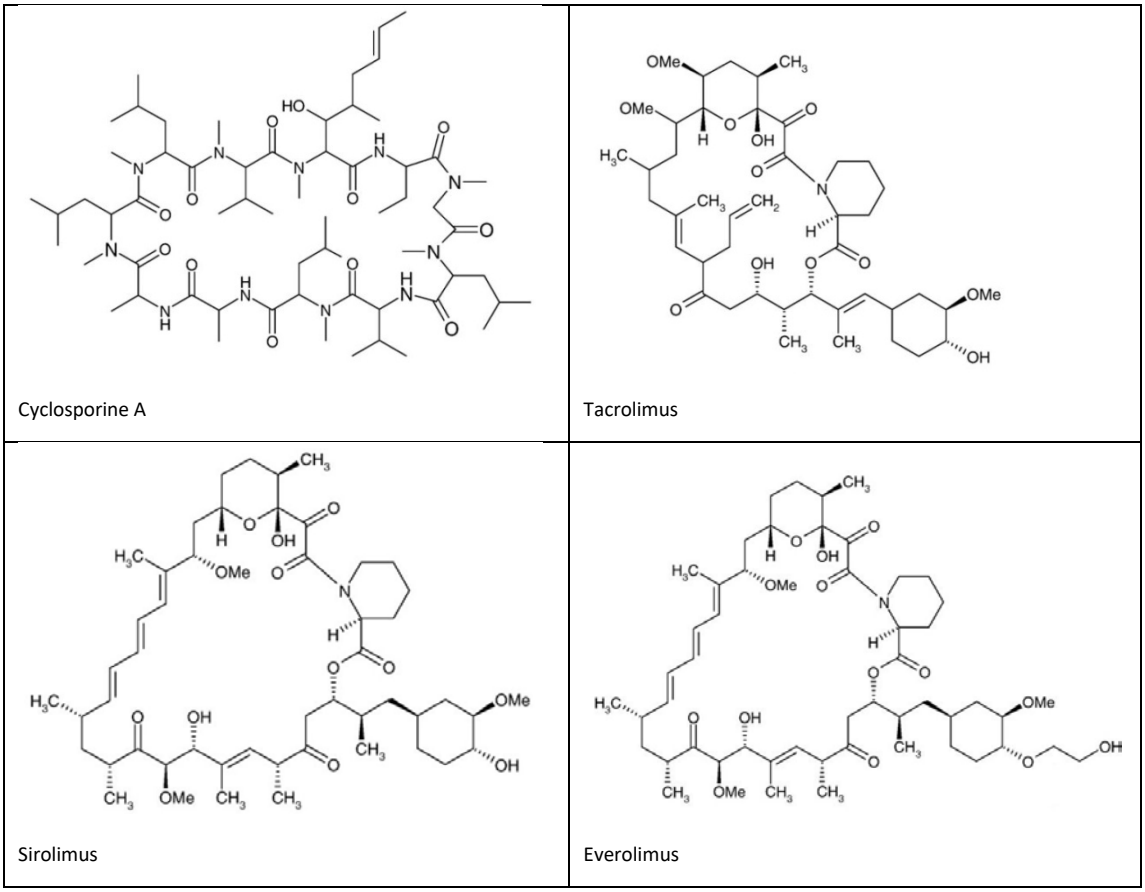


Figure 1. Structural formulas of cyclosporine A, tacrolimus, sirolimus, and everolimus

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, 7].

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 1.4).

1.4 General description of the analytical procedure

The analytical procedure is based on a universal ClinMass® TDM Platform (order no. MS9000, MS9000A), which can be used with various ClinMass® Add-on Sets (ClinMass® TDM Kit System). The TDM Platform MS9000 and MS9000A contains the autosampler washing solution, the mobile phases and the sample preparation reagent (see order information in section 2.1). The order nos. MS9000 and MS9000A differ only in the precipitants for serum (MS9000: precipitant with order no. MS9021) and whole blood (MS9000A: precipitant with order no. MS1021). For the determination of the immunsuppressants in whole blood MS1021 is used.

The ClinMass® Add-on Set with order no. MS99200 contains the analyte-specific components for the determination of cyclosporine A, tacrolimus, sirolimus and everolimus from human whole blood. Analysis is performed by HPLC coupled with tandem mass spectrometry (LC-MS/MS).

Prior to LC-MS/MS analysis a short sample preparation is carried out in order to remove the sample matrix and to spike the samples with an internal standard (see sample preparation section 5.2).

The prepared samples are injected into the LC-MS/MS system for chromatographic separation of the compounds. The analytes are then ionised using electrospray ionisation (ESI).

Electrospray ionisation is a soft ionisation technique where a strong electric field is applied to the liquid passing through the ESI-capillary of the MS-source. The ions are mostly preformed in solution before desorption and then transferred into the ion path of the tandem mass spectrometer which consists of three quadrupoles (two mass selectors connected by a collision cell).

Measurement of the analytes is carried out in MRM mode (MRM: Multiple Reaction Monitoring). In this mode only selected ions (known as “precursor ions”) with a defined mass/charge (*m/z*) ratio are isolated in the first quadrupole and subsequently transferred into the collision cell, where they are fragmented by impact with an inert gas (argon or nitrogen) at defined voltage settings. Among the fragments generated (known as “product ions”) only those with a defined *m/z* ratio can pass the third quadrupole for final detection. In this way the MRM mode ensures a selective identification and quantification of the target analytes.

This analytical method enables a robust and reliable quantitation in complex biological matrices by use of 4 different isotope-labelled internal standards (see section 4.3.2). If required, two mass transitions can be evaluated per analyte (quantifier, qualifier).

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

The calibration of the analytical system is performed by use of ClinCal® Multi-Level Calibrators (see section 5.3.3). For this purpose a 7-level calibrator set is available (level 0–6). For an extended calibration range with an additional, high calibration point, a whole blood calibrator (level 7) is optionally available.

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

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.

Please note:

For a mixed determination of serum and whole blood samples within the ClinMass® TDM-Kit System, the use of the appropriate precipitants is required, i.e. for

- **Serum samples: Precipitant P with order no. MS9021**
- **Whole blood samples: Precipitant P with order no. MS1021**

2 Components of the complete kit and accessories

2.1 Ordering information

Order No.	Description	Quantity
MS9000A	**ClinMass® TDM Platform** for 400 assays	1 pce.
	Content:	
	Autosampler Washing Solution	1 x MS9005
	Mobile Phase A	2 x MS9007
	Mobile Phase B	1 x MS9008
	Sample Preparation Vials	4 x MS9020
	Precipitant P	1 x MS1021
	Separately available components:	
MS9005	Autosampler Washing Solution	1000 ml
MS9007	Mobile Phase A	1000 ml
MS9008	Mobile Phase B	1000 ml
MS9020	Sample Preparation Vials	100 pcs.
MS1021	Precipitant P	80 ml
	Start Accessories:	
MS9030	Analytical Column with test chromatogram	1 pce.
MS9032	Prefilter	1 pce.
MS99200	**ClinMass® Add-on Set** **for Immunosuppressants in Whole Blood** for 200 assays	1 pce.
	Content:	
	Internal Standard IS, lyophil.	2 x MS1412
	Whole Blood Calibrator Set, lyophil. (Level 0 - 6) Manual	1 x 9933
	Separately available components:	
MS1412	Internal Standard IS, lyophil.	3 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 Accessory:	
MS1014	Optimisation Mix 1, lyophil.	2 ml
MS1115	Optimisation Mix 2, lyophil.	2 ml
	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

2.1.1 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).

2.1.2 Storage conditions and lifetime of kit components

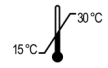
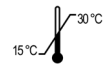
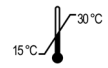
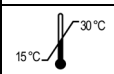
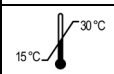
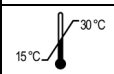
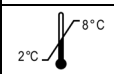
Please unpack the kit components from the transport packaging **immediately upon receipt** and follow the instructions for the storage conditions given on the product labels and Table 1.

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	MS9005	Autosampler Washing Solution		Store at 15–30 °C
REF	MS9007	Mobile Phase A		Store at 15–30 °C
REF	MS9008	Mobile Phase B		Store at 15–30 °C
REF	MS9020	Sample Preparation Vials	Store at ambient temperature	
REF	MS1021	Precipitant P		Store at 15–30 °C
REF	MS9030	Analytical Column		Store at 15–30 °C
REF	MS9032	Prefilter		Store at 15–30 °C
REF	MS1412	Internal Standard IS, lyophil.		Store below -18 °C*
REF	9028	Whole Blood Calibrator (Level 7), lyophil.		Store at 2–8 °C*

Order no.		Product description	Storage conditions	
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	MS1014	Optimisation Mix 1, lyophil.		Store below -18 °C*
REF	MS1115	Optimisation Mix 2, lyophil.		Store below -18 °C*
REF	8830, 8831, 8832; 8833	Whole Blood Controls, lyophil., Level I Whole Blood Controls, lyophil., Level II Whole Blood Controls, lyophil., Level III Whole Blood Controls, lyophil. Level I, II, III		Store at 2–8 °C*
REF	8903	Whole Blood Controls, lyophil. Level IV, V		Store at 2–8 °C*

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

2.1.3 Disposal of laboratory waste

For disposal, laboratory waste should be collected separately 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 sufficient sensitivity and evaluation software. Additionally, the mass spectrometer must be equipped with an internal waste switch. Data regarding the suitability of the various LC-MS/MS systems is available upon request (info@recipe.de).

Required LC modules:

- Autosampler (with cooling function, 4–10 °C)
- Binary HPLC pump (Mobile Phases A and B)
- Column heater (70 °C)
- Degasser (optional)

- For sample preparation the following laboratory instruments are required:
- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

4 Operation of the analytical system

4.1 Flushing of the LC system

Connect the LC modules **with exception** of the column. Put the outlet capillary 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 a 50 : 50 mixture of the Mobile Phases A and B.

Connect the analytical column in the column heater and make sure that the flow direction follows the arrow marking on the column!

Please also make sure to use the proper fittings for the connection to the column. A new fitting should be used and adapted to the column. For information regarding the proper connection please contact RECIPE.

4.2 Equilibration of the LC system

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

- Set the HPLC pump at a flow rate of 0.9 ml/min and set the column heater at the temperature of 70 °C. Equilibrate the column with 10 ml of a 90 : 10 mixture of the Mobile Phases A and B. This corresponds to the start conditions of the gradient programme (see Table 3).
- Then **stop the pump** and connect the outlet capillary of the analytical column with the tandem mass spectrometer.

4.3 Starting the analytical system

The following sections provide the parameters for the LC system (see section 4.3.1) and the tandem mass spectrometer (see section 4.3.2). Please consult section 5.3 regarding optimisation, equilibration, test run and calibration of the LC-MS/MS.

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

4.3.1 LC parameters

Table 2. LC parameters

HPLC pump (Mobile Phases A, B):	Gradient programme of the binary pump: See Table 3 Make sure the bottles are closed well to avoid alteration of the retention times, which could occur due to evaporation of the mobile phase components.
Analytical column:	The analytical column and the prefilter are installed in the column heater (70 °C). At a flow rate of 0.9 ml/min the backpressure of the analytical column should not exceed 300 bar. The prefilter should be renewed after 300 injections at the latest. It should also be replaced if the backpressure of prefilter plus column is increased by 10 % (at a flow rate of 0.9 ml/min).
Autosampler:	Set the autosampler cooling function to 4–10 °C Injection volume: 5–50 µl* Injection interval: 1.9 min Needle washing: The injection needle needs to be flushed after sample injection (minimisation of sample carryover). Please refer to the recommended needle wash settings in the instruction manual of the autosampler manufacturer. For flushing please use the autosampler washing solution with order no. MS9005. *depending on the sensitivity of the mass spectrometer in use

The following gradient programme is used for the binary HPLC pump**:

Table 3. Gradient programme

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]	Flow rate [ml/min]
0.01	90	10	0.9
0.7	90	10	0.9
0.8	5	95	0.9
1.7	5	95	0.9
1.8	90	10	0.9
1.9	90	10	0.9

**Note:
Please note that according to the dead volume of the HPLC system in use an adaptation of the gradient might be necessary.

4.3.2 MS/MS parameters

The mass transitions of the analytes and of the respective isotope-labelled substances in the ClinMass® Internal Standard IS are contained in Table 4.

The assignments of the isotope-labelled substances to the analytes are contained in Table 5.

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 5.3.1).

Table 4. Mass transition of the analytes and the isotope-labelled substances in the IS (ESI positive mode)

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

Table 5. Assignment of the analytes to the isotope-labelled substances in the IS

Analyte	**RT [min]**	**Internal Standard IS (order no. MS1412)**	**RT [min]**
Cyclosporine A	1.35	d_{12}-Cyclosporine A	1.34
Tacrolimus	1.29	$^{13}Cd_2$-Tacrolimus	1.29
Sirolimus	1.30	$^{13}Cd_3$-Sirolimus	1.29
Everolimus	1.30	$^{13}C_2d_4$-Everolimus	1.29

In order to prevent the ion source from unnecessary contamination it is mandatory to divert the LC flow into the waste both at the start and at the beginning of the measurement. For this purpose the internal switching valve of the MS system is set to the positions indicated below (see Table 6).

Table 6. Switching times of the internal valve

Time [min]	**Position**
0	waste
1.0	MS
1.7	waste

4.3.2.1 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).

4.4 Standby mode

When the analytical system is not in use, the pumps have to be switched off. The mobile phases may remain in 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 analytical column should be disconnected and stored tightly closed. The LC system should then be flushed with a water/acetonitrile mixture (1 + 1).

5 Implementation of the analytical procedure

5.1 Collection and storage of whole blood samples

The analysis is performed from whole blood (EDTA or citrate):

At room temperature (15–30 °C) the samples can be stored for one day and at temperatures between 2–8 °C for 7 days. Below -18 °C the samples can be stored for 28 days (multiple freeze-thaw cycles should be avoided).

5.2 Sample preparation

5.2.1 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 sheet.

5.2.2 Preparation of mixture IS/P

Internal Standard IS and Precipitant P (order no. MS1021) are pre-mixed (mixture IS/P) depending on the number of samples (see Table 7). 220 µl* of the mixture IS/P is then used for the matrix precipitation as described in section 5.2.3.1.

*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.

Table 7. Preparation of mixture IS/P depending on the number of samples

Number of samples	Internal Standard IS [ml]	Precipitant P (order no. MS1021) [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 at room temperature (15–30 °C) for three hours and at temperatures between 2–8 °C for 18 hours.

Please note:

The TDM platform MS9000 and MS9000A contain different precipitants. MS9000A contains Precipitant P with order no. MS1021. Please ensure to use MS1021 for the precipitation of whole blood samples.

5.2.3 Work flow

Sample preparation:

Precipitation:

220 µl Mixture IS/P*	100 µl Whole blood (calibrator, control, patient)

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

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

LC-MS/MS analysis:

Inject 5–50 µl** of the supernatant

*Note: For precipitation, a mixture containing Internal Standard IS and Precipitant P (mixture IS/P) is used (see section 5.2.2).

**depending on the sensitivity of the mass spectrometer

5.2.3.1 Precipitation

Pipette 220 µl mixture IS/P (for preparation see section 5.2.2) and 100 µl of the well homogenised whole blood sample (calibrator, control, patient) into a sample preparation vial. Mix for 30 sec on a vortex mixer and afterwards incubate for 5 min at room temperature (15–30 °C). Next mix again for 10 sec (vortex mixer) and centrifuge for 5 min at 10000 x g.

5.2.3.2 LC-MS/MS analysis

Transfer the centrifuge supernatant to a sample vial, which is suitable for the autosampler in use. Inject, depending on the sensitivity of the mass spectrometer, 5–50 µl of the supernatant into the LC-MS/MS system.

5.2.3.3 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 for 7 days and 15 days at temperatures below -18 °C.

5.3 LC-MS/MS Analysis

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

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

5.3.1 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 sheet. If necessary, Optimisation Mix 1 and 2 should be diluted with Mobile Phase B 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).

5.3.2 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 A (order no. MS9007)

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

5.3.3 Calibration run

For calibration, a ClinCal® 7-Level Whole Blood Calibrator Set (level 0–6, order no. 9933) is 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.

Please always consider the information provided in the product data sheet of the calibrator lot in use.

After reconstitution the calibrators must be prepared as described for the patient samples (see section 5.2).

For each analytical series, freshly prepared calibrators are required.

5.3.4 Quality control

For internal quality assurance of the analytical measurements ClinChek® Whole Blood Controls are available in five different concentrations (order no. 8833: level I, II and III; as well as order no. 8903; level IV and V).

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 5.3.3).

These controls are lyophilised and, subsequently to reconstitution, must be prepared as described for the patient samples (see sections 5.2.1 to 5.3).

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

5.3.5 Example chromatogram

Example chromatogram of the ClinCal® Whole Blood Calibrator (order no. 9933), level 2:

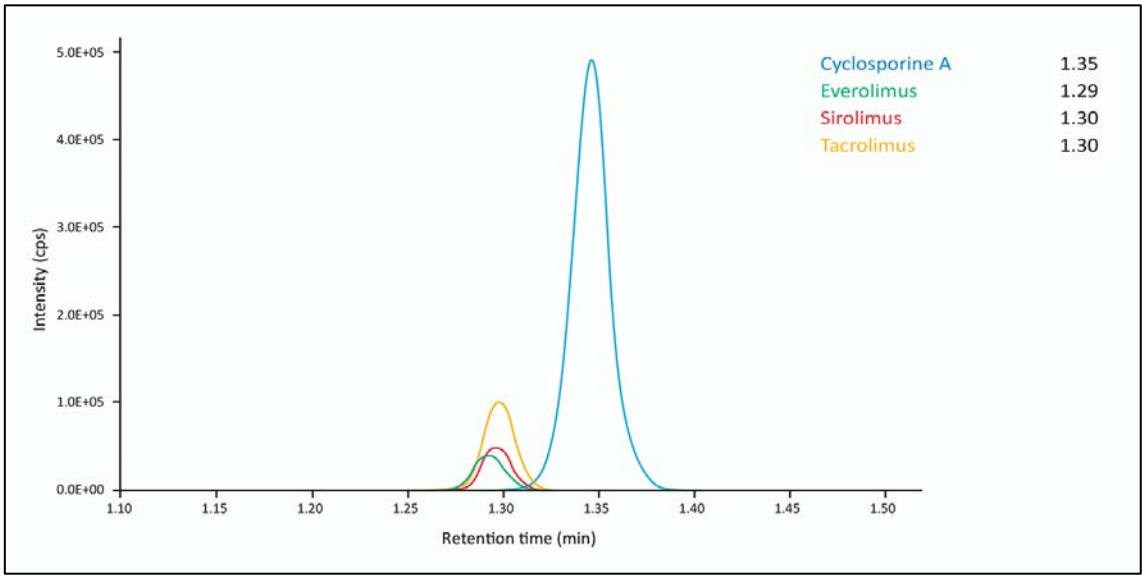


Figure 2. Chromatogram of the ClinCal® Whole Blood Calibrator, level 2

6 Evaluation

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

The evaluation of the analyte concentration is performed 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 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 8.

Table 8. 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 Validation data

The results were obtained with the MS/MS-system AB SCIEX API4500.

7.1.1 Linearity, detection limit and quantitation limit

Linearity, lower detection limit and lower quantitation limit are listed in Table 9.

Table 9. Linearity, lower detection limit and lower quantitation limit

Analyte	LLOD [µg/l]	LLOQ [µg/l]	Linearity [µg/l]
Cyclosporine A	2.92	8.77	8.77–2228
Tacrolimus	0.144	0.433	0.433–80.5
Sirolimus	0.101	0.302	0.302–93.1
Everolimus	0.0967	0.290	0.290–92.7

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

7.1.2 Trueness

For cyclosporine A, tacrolimus, sirolimus, and everolimus, recovery rates between 86-112 % were obtained.

7.1.3 Precision

For the evaluation of the intra- and interassay precision samples with two different concentrations were used. Results are listed in Table 10.

Table 10. Precision results for MS99200

	Concentration [µg/l]		Intraassay Precision [%]		Interassay Precision [%]	
Analyte	Level		Level		Level	
	I	II	I	II	I	II
Cyclosporine A	57.0	237	7.1	4.1	4.8	6.0
Tacrolimus	3.46	14.6	7.0	5.0	4.9	5.3
Sirolimus	3.69	20.3	8.4	2.1	7.4	9.3
Everolimus	3.68	19.3	3.5	5.4	8.6	9.5

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.

Table 11 gives an overview of the therapeutical ranges, determined by different methods [1]*. Similar values are published in review [8].

Table 11. Therapeutical ranges of the immunosuppressants [µg/l]

Cyclosporine (trough)		100–300
	Initial therapy (approx. ≤ 3 months after transplantation)	Maintenance therapy
Kidney	150–225	100–150
Liver	225–300	100–150
Heart	250–350	150–250
Stem cells	200–250 (300)	150–200
Tacrolimus (trough)		4–15
	Initial therapy (approx. ≤ 3 months after transplantation)	Maintenance therapy
Kidney	9.0–13.0	4.0–9.0
Liver	9.0–13.0	4.0–9.0
Heart	9.0–15.0	7.0–13.0
Stem cells	4.0–10.5	4.0–10.5
Sirolimus (trough)		
Kidney transplantation:		
Triple therapy with cyclosporine, corticosteroids, and sirolimus		4.0–12.0
Dual therapy with sirolimus and corticosteroids		12.0–20.0
Liver transplantation:		
Therapy with cyclosporine or tacrolimus, sirolimus, + / - corticosteroids		3.0–6.0
Therapy with sirolimus + / - corticosteroids		5.0–8.0
Everolimus (trough)		
Triple therapy with cyclosporine, corticosteroids, and everolimus		3.0–8.0

*Note:

Edition 8 of textbook „Labor und Diagnose“ with editor Lothar Thomas is the last print version. The current version is published in electronical form and available as an app for the operating systems iOS and Android (iOS: version 2.0, 2016). In the corresponding table 40-2 of the app there are no changes regarding edition 8 of the print version.

7.3 Interferences

The quantifier and qualifier MRMs were tested for potential interferences by relevant pharmacological substances.

No interferences were observed within the retention time windows of the corresponding analytes and internal standards.

Detailed information about the tested substances is available upon request (info@recipe.de).

8 Clinical performance

Clinical performance is determined by parameters such as diagnostic sensitivity and specificity, positive and negative predictive value and likelihood ratio. This requires that there is a relationship between the analyte to be measured and the clinical as well as physiological or pathological condition, e.g. an injury or illness. Such a link exists, for example, in the case of viruses or bacteria that cause a disease, or substances whose presence, reduced or increased concentration in the body act as markers for certain diseases. Most drugs considered in TDM are not related to a clinical or physiological/pathological condition, so data on performance parameters cannot be collected and are not known.

This also applies to the immunosuppressants to be determined with this kit, so that in contrast to the analytical performance, no information on clinical performance can be given.

9 References

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10 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

11 EC-Declaration of Conformity

The EC-Decleration of Conformity is available upon request (info@recipe.de)

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