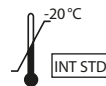
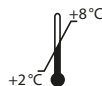


Vitamin D combi ImmuTube[®] LC-MS/MS Kit

*For the determination of
25-hydroxyvitamin D₃/D₂
1,25-dihydroxyvitamin D₃/D₂
24,25-dihydroxyvitamin D₃
in plasma and serum*

Valid from 2019-05-28

REF KMR1200



RUO



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1. INTENDED USE

The described ImmuTube® LC-MS/MS application is intended for the quantitative determination of 25-hydroxyvitamin D₃/D₂, 1,25-dihydroxyvitamin D₃/D₂ and 24,25-dihydroxyvitamin D₃ in serum and plasma. For research use only. Not for use in diagnostic procedures.

2. INTRODUCTION

D vitamins and calciferols arise from provitamins in the skin (UV radiation of sunlight catalyses the splitting of the B ring of the steran backbone) or taken up from nourishment. The two most important D vitamins are vitamin D₃ and vitamin D₂. Contrary to vitamin D₂, which has to be added via food, provitamin D₃ can be produced in the liver.

Vitamin D₃ formed in the skin or ingested together with vitamin D₂ is bound to the vitamin D binding protein in the plasma, transported into the liver and hydroxylated in position 25 to form the storage form of vitamin D, 25-hydroxyvitamin-D₃ or 25-hydroxyvitamin D₂, respectively. More than 95 % of 25-hydroxyvitamin-D is 25-hydroxyvitamin-D₃. 25-hydroxyvitamin-D₂ is only measured in subjects with vitamin D₂ supplementation.

The hormone 1,25-dihydroxyvitamin D (D hormone) is formed in a second hydroxylation step in the kidney. The responsible enzyme, 1 α -hydroxylase, is subjected to a rigid control through hormones (especially parathyroid hormone) and its activity is influenced by the serum concentrations of calcium and phosphate.

If 25-hydroxyvitamin D₃ is not metabolised by 1 α -hydroxylase, but by 25-hydroxyvitamin-D₃-24-hydroxylase, 24,25-dihydroxyvitamin D is formed. Its function is not secured yet, but there is evidence for its importance in bone metabolism and chronic kidney disease. In addition, Wagner et al. showed that the ratio of 24,25-dihydroxyvitamin D to 25-hydroxyvitamin D is predictive of 25-hydroxyvitamin D₃ response to vitamin D₃ supplementation.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KMR1200	MOPHA A	Mobile phase A	1000 ml
KMR1200	MOPHA B	Mobile phase B	1000 ml
KMR1200	CAL 1 CAL 2 CAL 3	Calibrator 1, 2 and 3 (lyophilized, reconstitute in 600 µl RECSOL; see product specification for concentration)	3 x 5 vials
KMR1200	CTRL1 CTRL2	Control 1 and 2 (lyophilized, reconstitute in 600 µl RECSOL; see product specification for concentration)	2 x 5 vials
KMR1000	RECSOL	Reconstitution solution	2 x 15 ml
KMR1200	SOL A	Solution A	25 ml
KMR1000	ACTSOL	Activation reagent	2.5 ml
KMR1200	INT STD	Internal standard	600 µl
KMR1200	DER	Derivatisation solution, ready-to-use	20 ml

For reorders of single components, use the catalogue number followed by the label as product number.

As preparation for the application of the Immundiagnostik vitamin D combi LC-MS/MS kit, tuning is necessary to estimate the optimal LC-MS/MS settings as well as to assess the sufficiency of the sensitivity. The tuning solutions (cat. no. KMR1201TU [5 ml], KMR1201IS [2 ml]), the UPLC separation column (cat. no. KMR1200, label: column) as well as all other individual components can be ordered separately from Immundiagnostik AG. Please ask for our single component price list.

4. CONTENT OF THE EXTRACTION KIT

Cat. No.	Label	Kit components	Quantity
KMR1100	COLUMNS	ImmuTube®-Columns for isolation of vitamin D metabolites from the sample	50 columns
KMR1100	ELUREAG	Elution reagent for ImmuTube®, ready to use	20 ml
KMR1100	WASHSOL	Wash solution for ImmuTube®	80 ml

For reorders of single components, use the catalogue number followed by the label as product

number.

The extraction kit can also be ordered separately from Immundiagnostik AG as catalog number KMR1100.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Glass tubes; LC-MS/MS-suitable
- Precision pipettors and disposable tips to deliver 10–1000 µl
- 500 ml graduated cylinder, LC-MS/MS suitable
- A repeating dispenser
- Centrifuge (10000 g) for 1.5 ml Eppendorf reaction tubes and 550 g for glass tubes, respectively
- Vortex mixer
- Vacuum centrifuge or nitrogen distributor
- Standard laboratory disposable plastic reagent vials
- Upside-down shaker
- LC-MS/MS equipment
- RP-C18 column, e.g. Luna Omega® 1.6 µm (2.1 x 100 mm)

6. PREPARATION AND STORAGE OF REAGENTS

- The **internal standard** (INT STD) has to be stored at **-20°C** and used until the expiration date stated on the label.
- Before use, **0.1% activation reagent** (ACTSOL) must be added to the **mobile phases** (MOPHA A, MOPHA B) and **solution A** (SOL A), e. g.

500 ml MOPHA + 500 µl ACTSOL

12,5 ml SOL A + 12,5 µl ACTSOL

The prepared solutions can be used within 2 weeks. For this reason, it is recommended to prepare only the amount necessary for each assay.

WARNING: The activation reagent (ACTSOL) must be added under the **fume hood**. All vials to be used must be absolutely clean, detergent-free and preferably made of a LC-MS/MS suitable glass.

- Before use, dissolve **calibrators** (CAL 1, CAL 2 and CAL 3) and **controls** (CTRL 1 and CTRL 2) in each **600 µl** of **reconstitution solution** (RECSOL).
- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2–8°C**.

7. SAMPLE PREPARATION

Serum and plasma samples are suited for the assay. The samples must be centrifuged before use (minimum 5 min at 10 000 *g*).

Control samples should be analyzed with each run.

1.	Prior to use in the assay, allow all samples and reagents to come to room temperature (18–26 °C). Mix samples and reagents well before use.
2.	Vortex ImmuTubes® carefully, place them in a suitable rack and make sure that no suspension remained on the ImmuTubes® cover. For this purpose, we recommend to shortly centrifuge the tubes (30s at 500–1000 rpm).
3.	Label the covers of ImmuTubes®, open ImmuTubes®, add quickly 500 µl of CAL/SAMPLE/CTRL (calibrator/sample/control), add 10 µl of INT STD (internal standard) in each ImmuTube®, close ImmuTubes® and mix gently.
4.	“Mix-rotate” (end-over-end rotation) intensively for 1 h at RT. Allow the remaining separation material on the inner side of the cover to flow down.
5.	Insert closed ImmuTubes® in plastic reagent vials, centrifuge for 1 min at 550 <i>g</i> .
6.	Open first the cover and then the outlet the ImmuTubes® and centrifuge for 2 min at 550 <i>g</i> to dryness. Discard flow-through.
7.	Add 500 µl of WASHSOL And centrifuge for 2 min at 550 <i>g</i> to dryness. Discard flow-through. Repeat this step twice.
8.	Label fresh glass tubes, place ImmuTubes® in the labeled glass tubes.
9.	Add 250 µl of ELUREAG (elution reagent for ImmuTubes®), centrifuge for 2 min at 550 <i>g</i> and collect the vitamin D metabolites in the glass tubes.
10.	Evaporate the eluate under a nitrogen stream at 37 °C or in a vacuum centrifuge.
11.	Vortex the remainder for 1 min in 50 µl derivatisation solution (DER).
12.	Add 150 µl activated solution A, mix; inject 50 µl in the UPLC system.

8. CHROMATOGRAPHIC CONDITIONS

Column material:	e. g. Luna Omega®, 1.6 µm C18 (2.1 x 100 mm)
Flow rate:	0.28 ml/min
Column temperature:	40 °C
Injection volume:	50 µl
Run-time:	12 min
Gradient:	

Time [min]	A [%]	B [%]
0	60	40
1	60	40
6.5	25	75
6.7	25	75
6.9	5	95
9.0	5	95
9.1	60	40
12.0	60	40

This gradient serves as orientation. It is dependent of the system and the column. 1,25-dihydroxyvitamin D and 24,25-dihydroxyvitamin D have the same molecular masses and transitions and compulsory have to be separated chromatographically.

9. MS/MS METHOD

Waters Quattro Premier XE

Mode:	MRM
Polarity:	ESI ⁺
Capillary (kV):	3
Cone (V):	30
Extractor (V):	4
RF Lens (V):	0
Source temperature (°C):	130
Desolvation temperature (°C):	450
Cone gas flow (l/h):	50
Desolvation gas flow (l/h):	950
Collision gas flow (ml/min):	0.2

Sciex Qtrap 5500

Mode:	MRM
Polarity:	ESI ⁺
Curtain gas (psig):	40
Collision gas (psig):	9
Ion spray voltage (V):	5 500
Temperature (°C):	550
Ion source gas 1 (psig):	50
Ion source gas 2 (psig):	70
Declustering potential (V):	100
Entrance potential (V):	10

*MRM transitions (m/z)***1,25-(OH)₂-vitamin D₂ (molar mass: 428.65 Da)**

586.2 > 313.9	Collision energy Waters Quattro:	20
	Collision energy Sciex Qtrap:	21

1,25-(OH)₂-vitamin D₃ (molar mass: 416.64 Da)

574.2 > 313.9	Collision energy Waters Quattro:	15
	Collision energy Sciex Qtrap:	21

24,25-(OH)₂-vitamin D₃ (molar mass: 416.64 Da)

574.2 > 298.0	Collision energy Waters Quattro:	20
	Collision energy Sciex Qtrap:	27

25-OH-vitamin D₂ (molar mass: 412.65 Da)

570.2 > 298.0	Collision energy Waters Quattro:	20
	Collision energy Sciex Qtrap:	27

25-OH-vitamin D₃ (molar mass: 400.64 Da)

558.2 > 298.0	Collision energy Waters Quattro:	20
	Collision energy Sciex Qtrap:	27

Internal standard:***isotopically labelled 1,25-dihydroxyvitamin D₃-d₆ (molar mass: 422.65 Da)***

580.2 > 313.9	Collision energy Waters Quattro:	15
	Collision energy Sciex Qtrap:	21

isotopically labelled 25-hydroxyvitamin D₃-d₆ (molar mass: 406.67 Da)

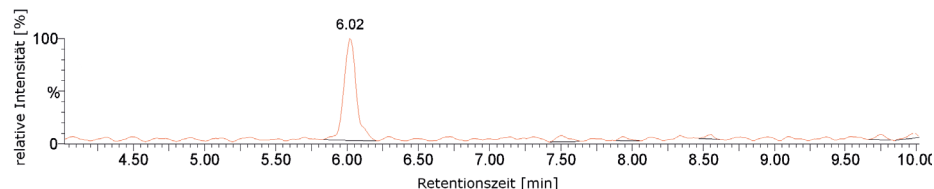
564.4 > 298.0	Collision energy Waters Quattro:	15
	Collision energy Sciex Qtrap:	27

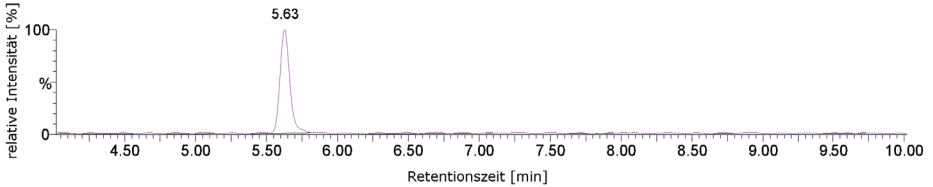
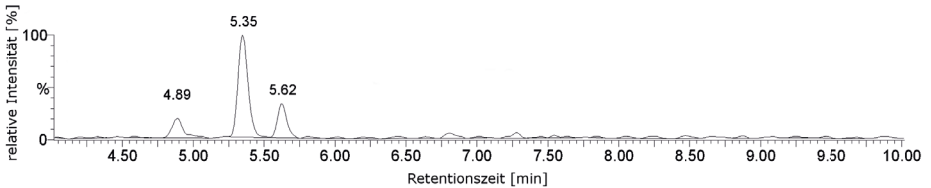
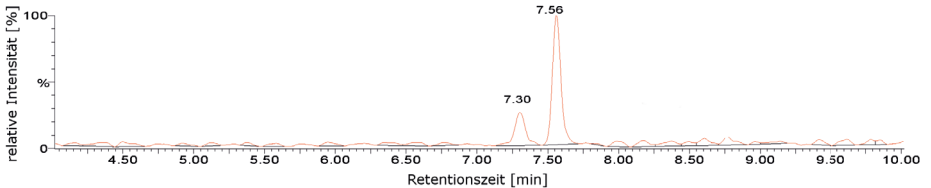
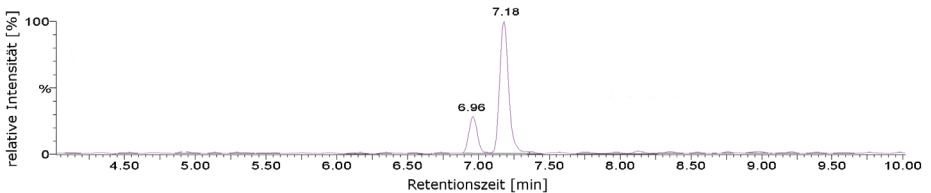
Derivatisation adds a mass of 175.15 Da. The listed masses of the derivatised metabolites of the MRM method correlate to the loss of water.

These parameters only serve for orientation. Each laboratory should optimise the MS/MS method for the measurement. When appropriately optimised, this method is also suitable for other mass spectrometers with sufficient sensitivity.

10. CALCULATION

Linear regression can be used as model for result evaluation. The two calibrator concentration points are connected by a straight line. The samples can be calculated using the obtained line.

11. EXAMPLE CHROMATOGRAMS***Example chromatogram for 1,25-dihydroxyvitamin D₂ (standard)***

Example chromatogram for 1,25-dihydroxyvitamin D₃ (standard)*Example chromatogram for 24,25-dihydroxyvitamin D₃ (standard)**Example chromatogram for 25-hydroxyvitamin D₂ (standard)**Example chromatogram for 25-hydroxyvitamin D₃ (standard)*

12. QUALITY CONTROL

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid, if within the same

assay one or more values of the quality control sample are outside the acceptable limits.

Reference ranges

We recommend each laboratory to establish its own reference range.

13. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

1,25-dihydroxyvitamin D₂

Inter-Assay (n = 15)

Sample	1,25-dihydroxyvitamin D ₂ [pg/ml]	CV [%]
1	164.0	14.0

Intra-Assay (n = 5)

Sample	1,25-dihydroxyvitamin D ₂ [pg/ml]	CV [%]
1	80.8	6.9

1,25-dihydroxyvitamin D₃

Inter-Assay (n = 15)

Sample	1,25-dihydroxyvitamin D ₃ [pg/ml]	CV [%]
1	55.0	10.2

Intra-Assay (n = 5)

Sample	1,25-dihydroxyvitamin D ₃ [pg/ml]	CV [%]
1	62.3	8.7

24,25-dihydroxyvitamin D₃***Inter-Assay (n = 15)***

Sample	24,25-dihydroxyvitamin D ₃ [ng/ml]	CV [%]
1	13.1	8.3

Intra-Assay (n = 5)

Sample	24,25-dihydroxyvitamin D ₃ [ng/ml]	CV [%]
1	63.3	8.0

25-hydroxyvitamin D₂***Inter-Assay (n = 15)***

Sample	25-hydroxyvitamin D ₂ [ng/ml]	CV [%]
1	64.7	6.5

Intra-Assay (n = 5)

Sample	25-hydroxyvitamin D ₂ [ng/ml]	CV [%]
1	62.8	4.1

25-hydroxyvitamin D₃***Inter-Assay (n = 15)***

Sample	25-hydroxyvitamin D ₃ [ng/ml]	CV [%]
1	65.6	4.8

Intra-Assay (n = 5)

Sample	25-hydroxyvitamin D ₃ [ng/ml]	CV [%]
1	60.3	3.4

Sensitivity

The detection limit was calculated with the formulas for linear calibration from DIN 32645. 6 concentrations in the range of 6.25–200 pg/ml were measured 5 times.

Detection limit 1,25-dihydroxyvitamin D ₂ :	12,5 pg/ml
Detection limit 1,25-dihydroxyvitamin D ₃ :	12,5 pg/ml
Detection limit 24,25-dihydroxyvitamin D:	6,25 pg/ml
Detection limit 25-hydroxyvitamin D ₂ :	6,25 pg/ml
Detection limit 25-hydroxyvitamin D ₃ :	6,25 pg/ml

14. PRECAUTIONS

- For research use only.
- The quality control guidelines should be followed.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.

15. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay.
- Reagents should not be used beyond the expiration date stated on the kit label.
- The assay should always be performed according to the enclosed manual.

16. DISPOSAL

Mobile phases (MOPHA A, MOPHA B), activation reagent (ACTSOL) and elution reagent for ImmuTube® (ELUREAG) must be disposed as non-halogenated solvents.

17. GENERAL NOTES ON THE TEST AND TEST PROCEDURE












- The guidelines for laboratories should be followed.
- ImmuTube® is a trademark of Immundiagnostik AG.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be sent to Immundiagnostik AG along with a written complaint.

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Used symbols:

	Temperature limitation		Catalogue Number
	For research use only		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by
	Attention		Consult instructions for use
	Consult specification data sheet		