

1,25-(OH)₂-Vitamin D₃/D₂ ImmuTube[®] LC-MS/MS Kit

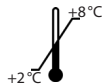
*For the determination of 1,25-(OH)₂ Vitamin D₃/D₂
in plasma and serum*

For Informational/Reference Purposes Only

Valid from 2020-04-30



KMR1000



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1. INTENDED USE

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

2. INTRODUCTION

Vitamin D is either produced in the skin (under the influence of UV light) or taken up from nourishment. The storage type of vitamin D, namely 25-hydroxy vitamin D, is formed in the liver. The hormone 1,25-dihydroxy vitamin D (D hormone) is formed in a second hydroxylation step in the kidney. The responsible enzyme, the kidney 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.

The serum concentration of 1,25-dihydroxy vitamin D normally re-adjusts itself to the demands of metabolism.

Supplemental vitamin D is available in two distinct forms: ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Pharmacopœias have officially regarded these two forms as equivalent and interchangeable, based on studies of rickets prevention in infants. The determination of 1,25 dihydroxy vitamin D₃/D₂ as a measure of 1,25 dihydroxy vitamin D status provides an objective, quantitative measure of the biological response to vitamin D administration.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KMR1000	MOPHA A	Mobile phase A	500 ml
KMR1000	MOPHA B	Mobile phase B	500 ml
KMR1000	CAL 1 CAL 2	Calibrator 1 and 2 (lyophilised, reconstitute in 600 μ l RECSOL; see label for concentration)	2 x 5 vials
KMR1000	CTRL1 CTRL2	Control 1 and 2 (lyophilised, reconstitute in 600 μ l RECSOL; for concentration, see product specification)	2 x 5 vials
KMR1000	RECSOL	Reconstitution solution	15 ml
KMR1000	SOL A	Solution A	25 ml
KMR1000	ACTSOL	Activation reagent	2.5 ml

Cat. No.	Label	Kit components	Quantity
KMR1000	INT STD	Internal Standard	600 µl
KMR1100		Extraction kit	see point 4.

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

As a first step for the application of the Immundiagnostik 1,25-Dihydroxyvitamin D₃/D₂ LC-MS/MS-Kit, a tuning is necessary to estimate the optimal LC-MS/MS-settings as well as to assess the sufficiency of the sensitivity. The UPLC separation column (cat. no. KMR1000, label: column) can be ordered separately from Immundiagnostik AG.

4. CONTENT OF THE EXTRACTION KIT

Cat. No.	Label	Kit components	Quantity
KMR1100	COLUMNS	ImmuTube®-Columns for isolation of 1,25-(OH) ₂ -vitamin D ₃ 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 be ordered separately from Immundiagnostik AG under catalogue 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 capable of 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. Acquity BEH C18, 1.7 µm (2.1 x 50 mm), Zorbax Eclipse Plus C18, 1.8 µm (2.1 x 100 mm)

6. PREPARATION AND STORAGE OF REAGENTS

- The **internal standard** (INT STD) must be stored at **-20°C** and used until the expiration date given 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 desired 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 and CAL 2) 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 WITH IMMUTUBE EXTRACTIONS FOR 50 SAMPLES

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 analysed 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 (30 s at 500–1000 rpm).

3.	Label the covers of Imm Tubes [®] , open Imm Tubes [®] , add quickly 500 µl of CAL/SAMPLE/CTRL (calibrator/sample/control), add 10 µl of INT STD (internal standard) in each Imm Tube [®] , close Imm Tubes [®] and mix gently.
4.	“Mix-rotate” (end-over-end rotation) intensively for 1 h at RT. Let the remaining separation material on the inner side of the cover flow down.
5.	Insert closed Imm Tubes [®] in plastic reagent vials, centrifuge for 1 min at 550 g.
6.	Open the cover and then the outlet the Imm Tubes [®] and centrifuge for 2 min at 550 g to dryness. Discard flow-through.
7.	Add 500 µl of WASH SOL and centrifuge for 2 min at 550 g to dryness. Discard flow-through. Repeat this step twice.
8.	Label fresh glass tubes, place Imm Tubes [®] in the labelled glass tubes.
9.	Add 250 µl of ELUREAG (elution reagent for Imm Tubes [®]), centrifuge for 2 min at 550 g and collect the 1,25-(OH) ₂ vitamin D ₃ /D ₂ eluates 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 165 µl of activated aolution A; inject 50 µl in the UPLC system, respectively 100 µl in the HPLC system.

8. CHROMATOGRAPHIC CONDITIONS

UPLC-Method

Column material:	Zorbax Eclipse Plus C18, 1.8 µm (2.1 x 100 mm) Guard column/filter is recommended (e. g. Waters, 0.2 µm, 2.1 mm, P/N 289002078)
Flow rate:	0.3 ml/min
Column temperature:	45 °C
Injection volume:	50 µl
Gradient:	

Zorbax Eclipse		
0 min	100 % A	0 % B
7.0 min	0 % A	100 % B
7.5 min	0 % A	100 % B
7.6 min	100 % A	0 % B
8.0 min	100 % A	0 % B

Example for a comparable HPLC method (derived from the UPLC method)

Column material:	e.g. xBridge™ C18; 5 µm
Column dimension:	3 x 150 mm
Flow rate:	0.5 ml/min
Column temperature:	45 °C
Injection volume:	100 µl
Running time:	13 min
Gradient:	

0 min	100 % A	0 % B
7.4 min	0 % A	100 % B
7.8 min	0 % A	100 % B
8.2 min	100 % A	0 % B
13 min	100 % A	0 % B

It is recommended that a guard column/filter is used to extend column's life.

After the analysis, the separation column should be washed with ca. 20 ml of 50 % methanol. The column can be stored in 50 % methanol.

9. MS/MS METHOD

The MS/MS method listed here is an example for a Waters Quattro Premier xE tandem mass spectrometer.

Mode:	MRM
Polarity:	ESI ⁺
Capillary (kV):	3
Cone (V):	var.
Extractor (V):	4
RF Lens (V):	0
Source Temperature (°C):	130
Desolvation Temperature (°C):	450
Cone Gas Flow (L/Hr):	50
Desolvation Gas Flow (L/Hr):	950
Collision Gas Flow (mL/Min):	0.15

MRM transitions (m/z)

1,25-(OH)₂ vitamin D₃

399.11 > 134.58	Cone Voltage: 30	Collision Energy: 22
399.11 > 150.64	Cone Voltage: 30	Collision Energy: 22

1.25-(OH)₂ vitamin D₂

411 > 134.8	Cone Voltage: 30	Collision Energy: 23
411 > 150.7	Cone Voltage: 30	Collision Energy: 26

Internal standard (isotopically labelled 1.25-(OH)₂ vitamin-D₃-d6):

405.21 > 134.68	Cone Voltage: 35	Collision Energy: 20
405.21 > 150.62	Cone Voltage: 35	Collision Energy: 23

1,25-(OH)₂ vitamin D₃ has a molecular mass of 416.64 Da, 1,25-(OH)₂ vitamin D₂ 428.65 Da and isotopically labelled 1,25-(OH)₂ vitamin D₃-d6 422.65 Da.

The masses of 399.11 Da, 411 Da and 405.21 Da correspond to a loss of a molecule of water, respectively.

10. CALCULATION

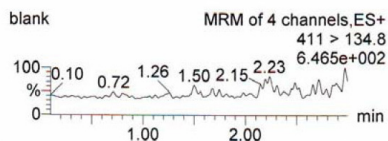
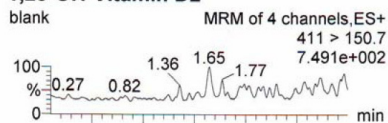
The linear regression can be used as model for evaluation of the results. The two calibrator concentration points are connected by a straight line. The samples can be calculated using the obtained line.

11. EXAMPLES OF CHROMATOGRAMS

Examples of chromatograms for 1,25-(OH)₂ vitamin D₂

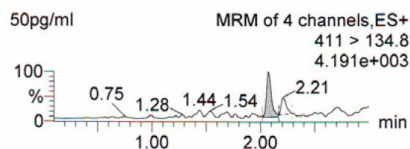
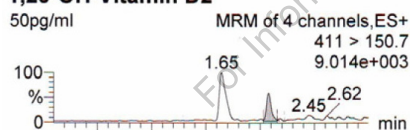
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1,25-OH-Vitamin D2

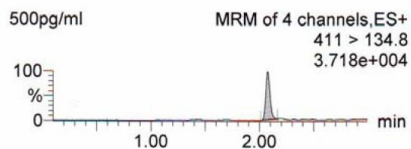
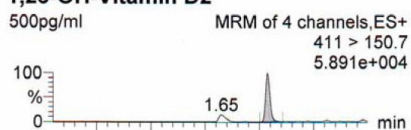


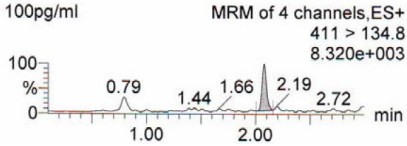
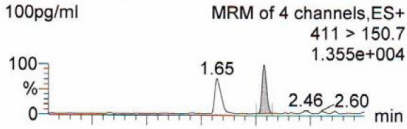
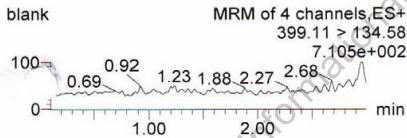
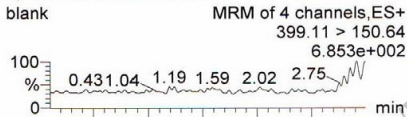
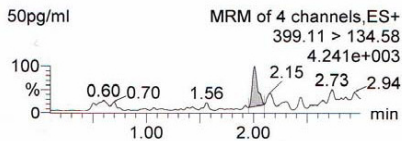
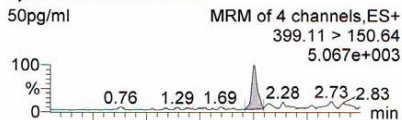
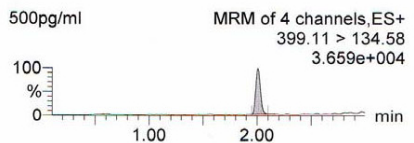
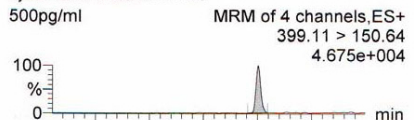
Standards:

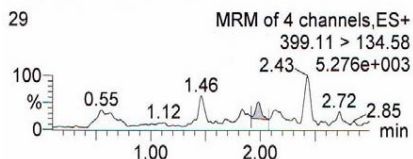
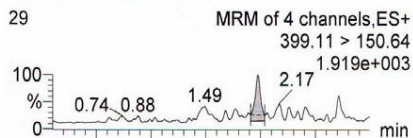
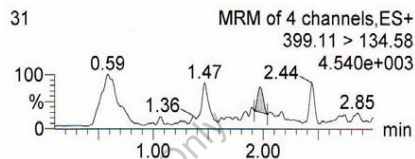
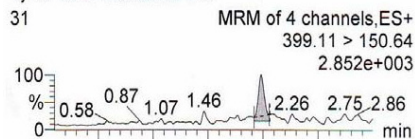
1,25-OH-Vitamin D2



1,25-OH-Vitamin D2



Sample:**1,25-OH-Vitamin D₂***Examples of chromatograms for 1,25-(OH)₂ vitamin D₃***Blank:****1,25-OH-Vitamin D₃****Standards:****1,25-OH-Vitamin D₃****1,25-OH-Vitamin D₃**

Samples:**1,25-OH-Vitamin D3****1,25-OH-Vitamin D3****12. QUALITY CONTROL**

Control samples should be analysed 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 range (plasma or serum)

We recommend each laboratory to establish its own norm concentration range.

13. PERFORMANCE CHARACTERISTICS*Accuracy – Precision***Repeatability (Intra-Assay); n=22**

The repeatability was assessed with 2 plasma samples under constant parameters (same operator, measurement system, day and kit lot).

Sample	1,25-(OH) ₂ -vitamin D ₃ [pg/ml]	CV [%]
1	113.0	7.2
2	338.9	4.0

Sample	1,25-(OH) ₂ -vitamin D ₂ [pg/ml]	CV [%]
1	115.0	5.3
2	367.1	2.7

Reproducibility (Inter-Assay); n=14

The reproducibility was assessed with 2 serum samples under varying parameters (different operators, measurement systems, days and kit lots).

Sample	1,25-(OH) ₂ -vitamin D ₃ [pg/ml]	CV [%]
1	118.8	12.2
2	345.0	9.7

Sample	1,25-(OH) ₂ -vitamin D ₂ [pg/ml]	CV [%]
1	119.1	6.7
2	340.4	8.4

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, 1,25-(OH)₂-vitamin D₃/D₂ spikes with known concentrations were added to a plasma sample.

Spike [pg/ml]	Obtained [pg/ml]	Recovery [%]
30	26.2	87.3
40	49.2	123.0
50	60.2	120.4
75	64.9	86.5
300	238.5	79.5
400	369.0	92.3
500	455.7	91.1
800	634.6	79.3
1000	807.0	80.7

Analytical sensitivity

The detection limit was calculated with the formulas for linear calibration from DIN 32645. 10 concentrations in the range of 10–100 pg/ml were measured 5 times.

Detection limit of 1,25-(OH)₂ vitamin D₃: 5.68 pg/ml

Detection limit of 1,25-(OH)₂ vitamin D₂: 12.01 pg/ml

It should be noted that the determination of the detection limit depends not only on the application method but also on the instrument.

14. PRECAUTIONS

- For research use only.
- The quality control guidelines should be followed.
- Human material used in the kit components was 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 mix different lot numbers of any kit component.
- Reagents should not be used beyond the expiration date shown on the kit label.
- The assay should always be performed according the enclosed manual.

16. DISPOSAL

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

17. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- All reagents in the kit package are for research use only.
- The guidelines for laboratories should be followed.
- 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 wrong use.

- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days after receipt of the product. The product shall be sent to Immundiagnostik AG together with a written complaint.

18. REFERENCES

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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		

For Informational Reference Purposes Only