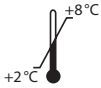
Manual

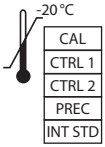
25-OH-Vitamin D_3/D_2 LC-MS/MS Kit

For the determination of 25-OH vitamin D_3 and 25-OH vitamin D_2 in plasma and serum

Valid from 2015-02-04

REF **KMR5000**





RUO

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 849430
e.mail: info@immundiagnostik.com www.immundiagnostik.com

Table of Contents

1. INTENDED USE

The reversed phase HPLC application is intended for the quantitative determination of 25-OH vitamin D_3 and 25-OH vitamin D_2 in serum and plasma. This assay is designed for research use only. Not for use in diagnostic procedures.

2. SUMMARY AND EXPLANATION OF THE TEST

D vitamins and calciferols arise from provitamins by the UV radiation of sunlight catalysed splitting of the B ring of the steran backbone. The two most important D vitamins are vitamin D_3 and vitamin D_2. Contrary to vitamin D_2, which has to be added via food, vitamin D_3 can be produced in the liver.

In the skin formed or together with vitamin D_2 by food ingested vitamin D_3 is bound to a vitamin D binding protein in the plasma, transported into the liver and hydroxylated in position 25 to form 25-OH-D. More than 95 % of 25-OH-D is 25-OH-D_3. 25-OH-D_2 is only detectable in subjects with supplementation of vitamin D_2.

3. PRINCIPLE OF THE TEST

For the determination of 25-OH vitamin D_3 and 25-OH vitamin D_2 samples, we developed three sample extraction methods depending on your LC-MS/MS-system (sample preparation 1, 2, 3).

Sample preparation 1:

Suitable for a LC-MS/MS-system with high sensitivity.

Sample preparation 2 and 3:

Suitable for all LC-MS/MS-systems. The test procedure is a SPE via 1 ml cartridges for test procedure 2 and for test procedure 3 a SPE via 96-well-plate.

4. MATERIAL SUPPLIED

For sample preparation 1:

Determination of 25-OH vitamin D_3/D_2 by *protein precipitation*

Cat. No	Content	Kit Components	Quantity
KMR5000LA	MOPHA A	Mobile Phase A	1000 ml
KMR5000LB	MOPHA B	Mobile Phase B	1000 ml
KMR5000KA	CAL	Calibrator (lyoph. 1,2 ml; concentration is given on the label)	5 vials
KMR5000KO	CTRL1 CTRL2	Control 1 and 2 (lyoph. 0,6 ml; for concentration see product specification)	2 x 5 vials each
KMR5000RE	RECSOL	Reconstitution solution	10 ml
KMR5000AC	ACTSOL	Activation reagent	2,5 ml
KMR5000FR	PREC	Precipitation reagent (-20 °C)	45 ml
KMR5000SO	SOL A	Solution A	2 x 50 ml
KMR5000IS	INT STD	Internal Standard	1,1 ml

For the preparation of the 25-OH vitamin D_3/D_2 application, tuning is necessary to find the ideal LC-MS/MS settings. The tuning solution (KMR5000TU, 25-OH vitamin D_3/D_2 and INT STD as pure substances), the column (KMR5000RP) and all other individual components can be ordered separately. Please ask for our single component price list.

For sample preparation 2:

Determination of 25-OH vitamin D_3/D_2 by *purification via cartridges (Waters Oasis HLB 30 mg, 1cc)*

Cat. No	Content	Kit Components	Quantity
KMR5000LA	MOPHA A	Mobile Phase A	1000 ml
KMR5000LB	MOPHA B	Mobile Phase B	1000 ml
KMR5000KA	CAL	Calibrator (lyoph. 1,2 ml; concentration is given on the label)	5 vials
KMR5000KO	CTRL1 CTRL2	Control 1 und 2 (lyoph. 0,6 ml; for concentration see product specification)	2 x 5 vials each
KMR5000RE	RECSOL	Reconstitution solution	10 ml
KMR5000AC	ACTSOL	Activation reagent	2,5 ml
KMR5000FR2	PREC	Precipitation reagent (-20 °C)	55 ml
KMR5000SO2	SOL A	Solution A	25 ml
KMR5000WL2	WASHSOL	Washing solution	160 ml
KMR5000EL2	ELUSOL	Elution solution (contains acetonitril)	210 ml
KMR5000IS	INT STD	Internal Standard	1,1 ml

For the preparation of the 25-OH vitamin D_3/D_2 application, tuning is necessary to find the ideal LC-MS/MS settings. The tuning solution (KMR5000TU, 25-OH vitamin D_3/D_2 and INT STD as pure substances), the column (KMR5000RP), Oasis® cartridges HLB 30 mg, 1cc (KMR5000CK) and all other individual components can be ordered separately. Please ask for our single component price list.

For sample preparation 3:

Determination of 25-OH vitamin D_3/D_2 by *purification via 96-well plate (Oasis HLB µElution-plate)*

Attention: number of determinations is limited to 96.

Cat. No	Content	Kit Components	Quantity
KMR5000LA	MOPHA A	Mobile Phase A	1000 ml
KMR5000LB	MOPHA B	Mobile Phase B	1000 ml
KMR5000KA	CAL	Calibrator (lyoph. 1,2 ml; concentration is given on the label)	5 vials
KMR5000KO	CTRL1 CTRL2	Control 1 und 2 (lyoph. 0,6 ml; for concentration see product specification)	2 x 5 vials each
KMR5000RE	RECSOL	Reconstitution solution	10 ml
KMR5000AC	ACTSOL	Activation reagent	2,5 ml
KMR5000FR3	PREC	Precipitation reagent (-20 °C)	20 ml
KMR5000SO3	SOL A	Solution A	10 ml
KMR5000WL3	WASHSOL	Washing Solution	20 ml
KMR5000EL3	ELUSOL	Elution solution (contains acetonitril)	10 ml
KMR5000IS	INT STD	Internal Standard	1,1 ml

For the preparation of the 25-OH vitamin D_3/D_2 application, tuning is necessary to find the ideal LC-MS/MS settings. The tuning solution (KMR5000TU, 25-OH vitamin D_3/D_2 and INT STD as pure substances), the column (KMR5000RP), Oasis® 96-well-µElution plate HLB (KMR5000PL) and all other individual components can be ordered separately. Please ask for our single component price list.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Centrifuge (4 °C)
- Vortex mixer
- 1.5 ml reaction tubes (Eppendorf)
- Various pipettes
- LC-MS/MS equipment
- Glass tubes, LC-MS/MS suitable
- 500 ml graduated cylinder, LC-MS/MS suitable
- UPLC column, e. g. Acquity BEH C18, 1.7 µm

For sample preparation 2 you also need:

- Methanol p. a.
- Ultra pure water*
- Oasis HLB cartridges 30 µm, 1cc (KMR5000CK)
- Sample evaporation unit

For sample preparation 3 you also need:

- Methanol p. a.
- Ultra pure water*
- Oasis HLB 96-well µElution plate (KMR5000PL)
- Sample evaporation unit for µElution plate

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

6. PREPARATION AND STORAGE OF REAGENTS

Internal Standard (**INT STD**) and precipitation reagent (**PREC**) are stable at **-20 °C** until the expiry date stated on the label.

Mobile phase and solution A

Before use, a 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

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.

Calibrators and controls

- The **lyophilized calibrator** (CAL), plasma with a specific concentration of 25-OH vitamin D_3 and 25-OH vitamin D_2, must be stored at **-20 °C** until use. The content of 25-OH vitamin D_3/D_2 changes slightly from lot to lot, the exact concentration is stated on the label. Before use, the calibrator has to be **reconstituted in 1.2 ml reconstitution solution** (RECSOL). Reconstituted calibrator **cannot be stored**.
- The **lyophilized controls** (CTRL1, CTRL2) must be stored at **-20 °C** until use. Before use, they have to be **reconstituted** in each **0.6 ml of RECSOL**. Reconstituted controls **cannot be stored**.

All other test reagents are stable until the expiry date (see label of test package) when stored at **2**–**8 °C**.

7. SPECIMEN COLLECTION AND PREPARATION

Serum and plasma could be used. The samples should be stored at -20 °C until testing. Samples are stable for at least four weeks when stored at -20 °C.

8. ASSAY PROCEDURE

Sample preparation 1

Determination of 25-OH vitamin D_3/D_2 by *protein precipitation*.

1.	Pipet **200 µl** of calibrator (**CAL**), controls (**CTRL1**, **CTRL2**) or sample in 1.5 ml reaction tubes
2.	Add **10 µl** of internal standard (**INT STD**) and mix
3.	Add **400 µl** of precipitation reagent (**PREC**) (ice-cold)
4.	Vortex for **1 min**
5.	Incubate **10 min** at **4 °C**
6.	Centrifuge for **10 min** at 10 000 rpm at **4 °C**
7.	Dilute supernatant **1:10** with **activated solution A** (SOL A) (e. g. 100 µl supernatant + 900 µl SOL A)
8.	Inject **50 µl** into the LC-MS/MS system

Sample preparation 2

Determination of 25-OH vitamin D_3/D_2 by *purification via cartridges (Waters Oasis HLB 30 mg, 1cc).*

1.	Equilibrate cartridges with 1 ml methanol and 1 ml ultra pure water, respectively
2.	Pipet **500 µl** of calibrator (**CAL**), controls (**CTRL1**, **CTRL2**) or **sample** in 1.5 ml reaction tubes
3.	Add **10 µl** of internal standard (**INT STD**) and mix
4.	Add **500 µl** of precipitation reagent (**PREC**) (ice-cold)
5.	Vortex for **1 min**
6.	Incubate **10 min** at **4 °C**
7.	Centrifuge for **10 min** at 10 000 rpm at **4 °C**
8.	Pipet **500 µl** of **supernatant** in cartridges
9.	Wash **3 x** with **500 µl** washing solution (**WASHSOL**)
10.	Elute **2 x** with **1 ml** elution solution (**ELUSOL**)
11.	Evaporate eluent
12.	Pipet **200 µl** of **activated solution A** (SOL A) and vortex for 1 min
13.	Inject **50 µl** into the LC-MS/MS system

Sample preparation 3

Determination of 25-OH vitamin D_3/D_2 by *purification via 96-well-plate (Oasis HLB µElution plate)*

1.	Equilibrate µElution plate with 0.2 ml methanol and 0.2 ml ultra pure water, respectively 0.2 ml water, respectively
2.	Pipet **200 µl** of calibrator (**CAL**), controls (**CTRL1**, **CTRL2**) or **sample** in 1.5 ml reaction tubes
3.	Add **10 µl** of internal standard (**INT STD**) and mix
4.	Add **200 µl** of precipitation reagent (**PREC**) (ice-cold)
5.	Vortex for **1 min**
6.	Incubate **10 min** at **4 °C**
7.	Centrifuge for **10 min** at 10 000 rpm at **4 °C**
8.	Pipet **200 µl** of **supernatant** in µElution wells
9.	Wash **3 x** with **200 µl** washing solution (**WASHSOL**)
10.	Elute with 100 µl elution solution (**ELUSOL**)
11.	Add **100 µl** of **activated solution A** (SOL A) to the eluent
12.	Inject **50 µl** into the LC-MS/MS system

9. CHROMATOGRAPHIC CONDITIONS

Column material:	e. g. Acquity BEH C18; 1.7 µm
Column dimension:	2.1 x 50 mm
Flow:	0.3 ml/min
Column temperature:	45 °C
Inject volume:	50 µl
Run time:	5 min

Gradient:

Time	% A	% B
0 min	100	0
2 min	0	100
2,6 min	0	100
2,9 min	100	0
4,0 min	100	0

After analysis, the separation column can be stored in the mobile phase, tightly closed.

10. MS/MS METHOD (LISTED AS AN EXAMPLE FOR A WATERS QUATTRO PREMIER XE TANDEM MASS SPECTROMETER)

Mode:	MRM
Polarity:	ESI+
Capillary (kV):	3
Cone (V):	var.
Extracor (V):	4
RF Lens (V):	0
Source temperature (°C):	130
Desolvation Temperatur (°C):	450
Cone Gas Flow (l/h):	50
Desolvation Gas Flow (l/h):	950
Collision Gas Flow (ml/min):	0,15

MRM transitions (m/z)

25-OH vitamin D_3

401.5 > 383.2	Cone voltage: 22	Collision energy: 10
401.5 > 159.0	Cone voltage: 22	Collision energy: 28

25-OH vitamin D_2

413.4 > 395.3	Cone voltage: 20	Collision energy: 10
413.4 > 83.1	Cone voltage: 20	Collision energy: 25

Internal standard (25-OH-vitamin-D_3-d6)

407.5 > 389.4	Cone voltage: 22	Collision energy: 10
407.5 > 159.1	Cone voltage: 22	Collision energy: 28

11. RESULTS

Calculation

$$\frac{\text{Peak height sample} \times \text{Calibrator concentration}}{\text{Peak height internal standard in the sample}} \times F = \text{Concentration of the sample}$$

$$F = \frac{\text{Peak height internal standard in the calibrator}}{\text{Peak height calibrator}}$$

Tip: Alternatively, the peak area instead of the peak height can be used for quantification.

Conversion factors:

for 25-OH vitamin D_3	*for 25-OH vitamin D_2*
1 ng/ml = 2,5 nmol/l	1 ng/ml = 2,42 nmol/l
1 nmol/l = 0,4 ng/ml	1 nmol/l = 0,412 ng/ml

12. LIMITATIONS

This test cannot be used with whole blood samples.

13. QUALITY CONTROL

Immundiagnostik recommends the use of external controls for internal quality control, if possible.

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 ranges

We recommend each laboratory to establish its own reference range.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

25-OH vitamin D_3

Intra-Assay (n = 6)

Sample	25-OH vitamin D_3 [nmol/l]	CV [%]
1	84.4	2.9

Inter-Assay (n = 18)

Sample	25-OH vitamin D_3 [nmol/l]	CV [%]
1	83.4	6.6

25-OH vitamin D_2

Intra-Assay (n = 6)

Sample	25-OH vitamin D_2 [nmol/l]	CV [%]
1	43.9	7.5

Inter-Assay (n = 18)

Sample	25-OH vitamin D_2 [nmol/l]	CV [%]
1	48.2	12.1

Sensitivity

Detection limit of 25-OH vitamin D_3: 0,64 nmol/l

Detection limit of 25-OH vitamin D_2: 5,4 nmol/l

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

15. DISPOSAL

The mobile phases (MOPHA A, MOPHA B), solution A (SOL A), precipitation reagent (PREC), activation reagent (ACTSOL), washing aolution (WASHSOL) and elution reagent (ELUSOL) must be disposed as non-halogenated solvent. Please refer to the appropriate national guidelines.

16. PRECAUTIONS

- All reagents in the kit package are for research use only.
- Control samples should be analyzed with each run.
- This product contains human source material which was tested and found to be non-reactive to HBsAg, anti-HIV-1/2, and anti-HCV. Since no method can offer complete assurance that hepatitis B virus, HIV-1/2, HVC or other infectious agents are absent, these reagents should be handled as if potentially infectious.
- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.

17. 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 kit label.
- The assay should always be performed according the enclosed manual.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- 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 incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

19. REFERENCES

1. Chapuy, M. C. et al. Healthy elderly French women living at home have secondary hyperparathyroidism and high bone turnover in winter. EPIDOS Study Group. *The Journal of clinical endocrinology and metabolism* **81**, 1129–33 (1996).
2. Scharla, S. H. Prevalence of subclinical vitamin D deficiency in different European countries. *Osteoporosis international* **8** Suppl 2, S7–12 (1998).
3. Grant, W. B. & Holick, M. F. Benefits and requirements of vitamin D for optimal health: a review. *Alternative medicine review : a journal of clinical therapeutic* **10**, 94–111 (2005).
4. Visser, M., Deeg, D. J. H., Puts, M. T. E., Seidell, J. C. & Lips, P. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. *The American journal of clinical nutrition* **84**, 616–22; quiz 671–2 (2006).
5. Wicherts, I. S. et al. Vitamin D status predicts physical performance and its decline in older persons. *The Journal of clinical endocrinology and metabolism* **92**, 2058–65 (2007).
6. Wielders, J. P. M. & Wijnberg, F. A. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. *Clinical chemistry* **55**, 1584–5 (2009).
7. Soldin, O. P., Sharma, H., Husted, L. & Soldin, S. J. Pediatric reference intervals for aldosterone, 17alpha-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry. *Clinical biochemistry* **42**, 823–7 (2009)

Used symbols:

 Temperature limitation

REF Catalogue Number

RUO For research use only

→REF To be used with

 Manufacturer

 Contains sufficient for <n> tests

LOT Lot number

 Use by