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Manual

25-OH VITAMIN D_3/D_2 RP-HPLC KIT

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

Valid from 2022-09-07

REF **KC3000**

Σ 100

+8 °C
+2 °C

-20 °C
CAL
CTRL 1
CTRL 2
INTSTD

IVD CE

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1. INTENDED USE

This 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 *in vitro* diagnostic use only.

2. INTRODUCTION

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

Vitamin D_3, formed in the skin or ingested together with vitamin D_2 in food, 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 patients with vitamin D_2 medication.

3. PRINCIPLE OF THE TEST

For the determination of 25-OH vitamin D_3 and 25-OH vitamin D_2 samples, a simple sample preparation is used, a combination of precipitation and extraction.

The HPLC separation works with an isocratic method at 30 °C with a „reversed phase" column. Chromatograms are detected by an UV detector. The separation takes 12 minutes for each run. Results are quantified by the provided calibrator and calculated by the „external standard-method" by determination of the peak height.

Summary

This complete kit includes all reagents for analytical HPLC separation and preparation of the samples.

As with many other parameters, the advantage of HPLC analytic is the simultaneous handling of many analytes in one test. The HPLC complete system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical-chemical routines quickly and without difficulties. Mostly a one-point calibration is sufficient for calibrating the test system – unlike immunoassays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results, so that even higher numbers of samples can be handled nearly without control. With short test series, the one-point calibration is much more economic than 6-point calibration for immunoassays.

4. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 0005.15	RECSOL	Reconstitution solution	15 ml
KC3000	MOPHA	Mobile phase (contains acetonitrile; important: do not recirculate)	1000 ml
	CAL	Calibrator; lyophilised (see specification data sheet for concentration)	4 x
	PREC	Precipitation reagent	50 ml
	EXTSOL	Extraction solution (contains acetonitrile)	40 ml
	CTRL1	Control1; lyophilised	4 x
	CTRL2	Control2; lyophilised	4 x
	INTSTD	Internal standard	1 ml

HPLC column (KC3000RP) as well as individual components can be ordered separately from Immundiagnostik. Please ask for the price list of the individual components.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Centrifuge
- Vortex mixer
- 2 ml reaction tubes (Eppendorf)
- Various pipettes
- HPLC with UV detector
- HPLC column vitamin D KC3000RP

6. STORAGE AND PREPARATION OF REAGENTS

Preparation

All test reagents provided except calibrator and controls are in solution and ready to use:

- The **calibrator** (CAL), plasma containing a defined amount of 25-OH vitamin D_3/D_2, have to be reconstituted directly before use in **1.2 ml RECSOL** (reconstitution solution): allow the vial content to dissolve for 10 min, then mix well.

The exact amount of 25-OH vitamin D_3/D_2 changes slightly from lot to lot, the exact amount is stated in the specification data sheet.

- The **controls** (CTRL1, CTRL2) have to be reconstituted directly before use in **0.6 ml RECSOL**: allow the vial content to dissolve for 10 min, then mix well. The exact amount of 25-OH vitamin D_3/D_2 changes slightly from lot to lot, the exact amount is stated in the specification data sheet.

Storage

- The internal standard (**INTSTD**), the lyophilised controls (**CTRL1**, **CTRL2**) and the lyophilised calibrator (**CAL**) have to be stored at **-20 °C** until expiry date stated on the label.

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. SPECIMEN COLLECTION AND PREPARATION

Serum and plasma can 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

Test procedure

1.	Pipet **500 µl precipitation reagent** (PREC) in 2 ml reaction tubes.
2.	Add **500 µl calibrator** (CAL), **control** (CTRL1, CTRL2) or **sample.**
3.	Add **10 µl internal standard**.
4.	Add **400 µl** of **cold extraction solution** (EXTSOL) under the fume hood.
5.	Vortex for **1 min.**
6.	Centrifuge for **10 min** at 10 000 *g*.
7.	Inject **100 µl** of the **supernatant** into the HPLC system.

Chromatographic conditions

Column dimension:	125 × 4 mm
Flow rate:	1 ml/min (the exact flow rate is given in the corresponding data sheet)
Temperature:	30 °C
UV detector:	264 nm
Injection volume:	100 µl
Running time / chromatogram:	15 minutes

9. TREATMENT OF THE COLUMN

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

Before use, the system including the column should be equilibrated with about 30 ml MOPHA (mobile phase).

10. RESULTS

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

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

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

Conversion factors:

for 25-OH vitamin D_3

1 ng/ml = 2.5 nmol/l

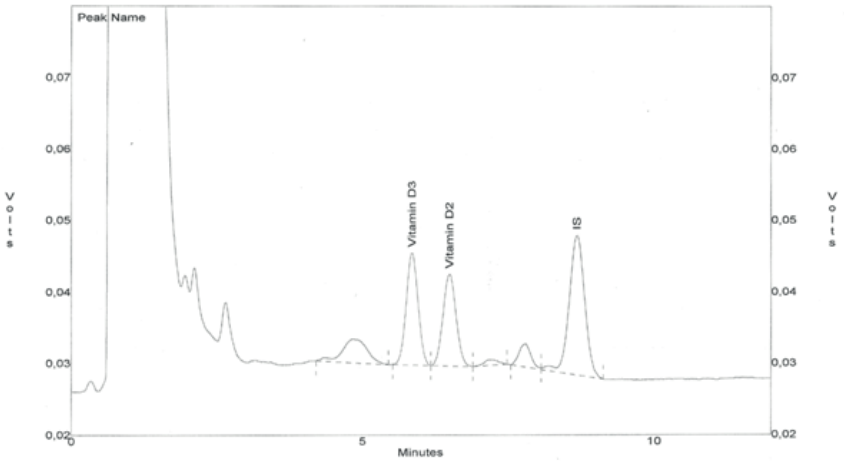
1 nmol/l = 0.4 ng/ml

for 25-OH vitamin D_2

1 ng/ml = 2.42 nmol/l

1 nmol/l = 0.412 ng/ml

Typical chromatogram



11. LIMITATIONS

Do not use whole blood.

12. QUALITY CONTROL

Reference ranges for 25-OH vitamin D

Information from ABMR 2011

Deficiency (seriously deficient)	< 20 ng/ml	or	< 50 nmol/l
Insufficiency (deficient)	20–29 ng/ml	or	50–74 nmol/l
Sufficiency (adequately supplied)	> 30 ng/ml	or	> 75 nmol/l

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http://osteologie-sachsen.de/aktuelles_vitamin_d.htm

Note

The vitamin D production in the skin is high variable and depends on the season and time of day, degree of latitude, age, sun protection etc.

The reference ranges depend on the method used (e. g. vitamin D release from the vitamin D binding protein, VDBP) and only serve as orientation.

We recommend each laboratory to establish its own reference range.

Controls

Control samples or serum pools should be analyzed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the patient sample may not be valid.

Literature references

1. Visser, M. et al., 2006. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. *The American journal of clinical nutrition*, **84**(3), pp.616–22; quiz 671–2.
2. Grant, W.B. & Holick, M.F., 2005. Benefits and requirements of vitamin D for optimal health: a review. *Alternative medicine review : a journal of clinical therapeutic*, **10**(2), pp.94–111.
3. Wicherts, I.S. et al., 2007. Vitamin D status predicts physical performance and its decline in older persons. *The Journal of clinical endocrinology and metabolism*, **92**(6), pp.2058–65.

13. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Inter-Assay (n = 5)

Analyte	Sample	[nmol/l]	CV [%]
25-OH vitamin D_3	1	121.4	4.5
	2	345.1	3.3
25-OH vitamin D_2	1	146.0	4.2
	2	420.2	3.3

Intra-Assay (n = 11)

Analyte	Sample	[nmol/l]	CV [%]
25-OH vitamin D_3	1	138.7	6.4
	2	428.9	6.7
25-OH vitamin D_2	1	201.4	2.9
	2	463.2	3.2

Linearity

The linearity for 25-OH vitamin D_3/D_2 is given up to 1250 nmol/l.

Detection limit

25-OH vitamin D_3	7.0 nmol/l
25-OH vitamin D_2	9.4 nmol/l

Recovery

25-OH vitamin D_3	109.8 %	n = 14
25-OH vitamin D_2	102.8 %	n = 14

14. DISPOSAL

The extraction solution (EXTSOL) must be disposed as non-halogenated solvent. Please refer to the appropriate national guidelines.

15. TROUBLESHOOTING

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Doublepeaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol

Problem	Possible reason	Solution
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min
Baseline is drifting	Detector lamp did not yet reach working temperature	Wait
	Detector lamp is too old	Renew lamp
	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flow cell is dirty	Clean flow cell

16. PRECAUTIONS

- 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 extraction solution (EXTSOL) and the mobile phase (MOPHA) contain acetonitril and must be handled carefully. Acetonitril is highly flammable and toxic by inhalation or contact the skin. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.
- As a precaution, it is recommended that the human material used is always considered potentially infectious.

- The precipitation reagent (PREC) contains acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.

17. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- All reagents in the test package are for *in vitro* diagnostic use only.
- Do not interchange different lot numbers of any kit component within the same assay.
- The guidelines for medical laboratories should be followed.
- Control samples should be analysed with each run.
- Serious incidents are to be reported to Immundiagnostik AG and the national regulatory authorities.
- 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.
- Plugs and caps of different reagents should not be swapped.
- 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.
- Reagents should not be used beyond the expiration date stated on kit label.
- The assay should always be performed according the enclosed manual.

18. REFERENCES

4. Merke, J., Ritz, E. & Schettler, G., 1986. New viewpoints on the role of vitamin D. Current knowledge and outlook. *Deutsche medizinische Wochenschrift* (1946), **111**(9), pp.345–349.
5. Reichel, H., Koeffler, H.P. & Norman, A.W., 1989. The role of the vitamin D endocrine system in health and disease. *The New England journal of medicine*, **320**(15), pp.980–991.
6. Visser, M. et al., 2006. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. *The American journal of clinical nutrition*, **84**(3), pp.616–22; quiz 671–2.
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Used symbols: