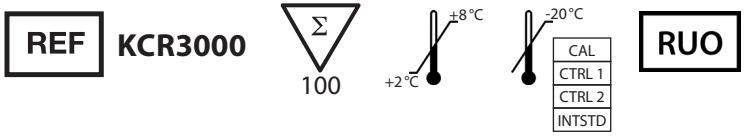
Manual

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 2017-12-11



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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 research use only. Not for use in diagnostic procedures.

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.

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
KCR3000	MOPHA	Mobile phase (contains acetonitrile)	1000 ml
KCR3000	CAL	Calibrator (lyoph. 1.2 ml; see specification for concentration)	3 vials
KCR3000	RECSOL	Reconstitution solution	15 ml
KCR3000	PREC	Precipitation reagent	50 ml
KCR3000	EXTSOL	Extraction solution (contains acetonitril)	40 ml
KCR3000	CTRL1 CTRL2	Control 1 and 2 (0.6 ml lyophilized; see specification for concentration)	2 x 3 vials
KCR3000	INTSTD	Internal standard	1 ml

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

HPLC column (KCR3000RP) 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 KCR3000RP

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. 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.
- Reagents should not be used beyond the expiration date shown on kit label.

8. 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.

9. ASSAY PROCEDURE

Procedural notes

- Quality control guidelines should be observed.
- 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.
- The assay should always be performed according the enclosed manual.

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

10. 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).

11. 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

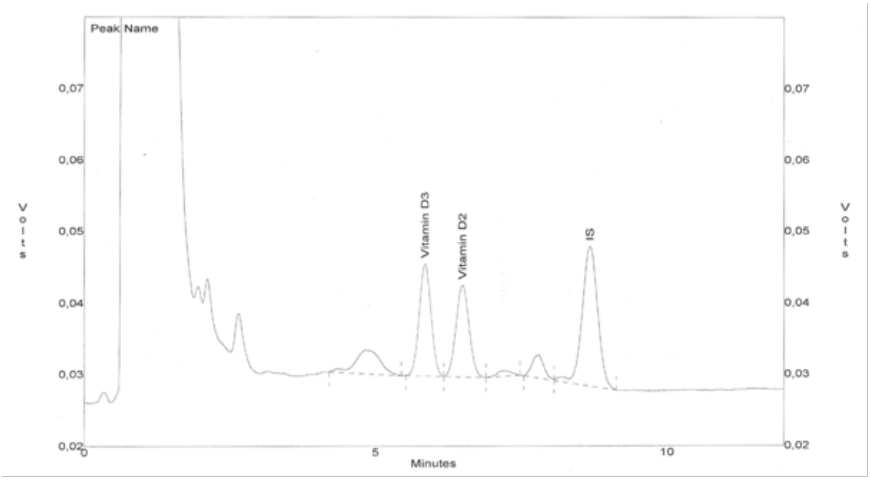
1 nmol/l = 0.4 ng/ml

for 25-OH vitamin D_2

1 ng/ml = 2.42 nmol/l

1 nmol/l = 0.412 ng/ml

Typical chromatogram



12. LIMITATIONS

Do not use whole blood.

13. QUALITY CONTROL

Reference ranges for 25-OH vitamin D

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 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 sample may not be valid.

14. 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

15. DISPOSAL

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

16. 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
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

Problem	Possible reason	Solution
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

17. REFERENCES

1. 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.
2. 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.
3. 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.
4. 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.
5. 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.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the test package are for research use only.

- Reagents should not be used beyond the expiration date shown on the kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- Quality control guidelines should be observed.
- 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.

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