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Manual

25-OH Vitamin D₃ / D₂ HPLC Kit

*For the determination of 25-OH Vitamin D₃ and
25-OH Vitamin D₂ in plasma and serum*

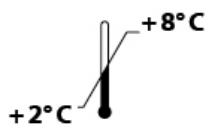
Valid from 20.07.2010

REF

KC 3420



+2°C



CAL
CTRL 1
CTRL 2
STD 2
STD 3

CE

IVD

1. INTENDED USE

The assay is intended for the quantitative determination of 25-(OH) Vitamin D₃ and 25-(OH) Vitamin D₂ in serum and plasma. This assay is designed for *in vitro* diagnostic use only.

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 D₃ and vitamin D₂. In the contrary to vitamin D₂ which has to be added via food, vitamin D₃ can be produced in the liver.

In the skin formed or together with vitamin D₂ by food ingested vitamin D₃ 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-D₃. 25-OH-D₂ is only detectable in patients with medication of vitamin D₂.

3. PRINCIPLE OF THE TEST

For the determination of 25-OH vitamin D₃ and 25-(OH) Vitamin D₂ samples have to be prepared as follows. To get rid of high molecular weight substances the samples are precipitated and solid phase extracted with C₁₈-cartridges. The eluat is evaporated with nitrogen, suspended in mobile phase and injected into the HPLC system.

The HPLC separation works with an isocratic method at 30 °C with a "normal phase" column. Chromatograms are detected by an UV-detector. The separation takes 20 minutes for each run. Results are quantified by the delivered calibrator and calculated by the "external standard-method" by integration of the peak area. The ethanol solutions of standards are used for the recognition of the peaks.

Summary

This complete kit includes all reagents for analytical HPLC separation and for the extraction of the samples but no columns for the 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 immuno assays 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 immuno assays.)

4. MATERIAL SUPPLIED

Cat. No	Content	Kit Components	Quantity
KC3420LM	MOPHA	Mobile Phase	1000 ml
KC3420KA	CAL	Calibrator (lyoph. 6 ml; Concentration is given on the label)	2 vials
KC3420ST	STD2 STD3	Ethanollic Standard D ₂ Ethanollic Standard D ₃	each 1 ml
KC3420RE	RECSOL	Reconstitution solution	20 ml
KC3420FR	PREC	Precipitation reagent (contains acetonitril)	50 ml
KC3420WL	WASHSOL	Washing solution	300 ml
KC3420EL	ELUSOL	Elution solution (contains acetonitril)	400 ml
KC3420KO	CTRL1 CTRL2	Control 1 and 2; 0.6 ml lyophilized	2 x 3 vials

HPLC column (KC 3420RP) 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
- Pointed-bottomed glass tubes
- 1.5 ml reaction tubes (Eppendorf)
- Various pipettes
- HPLC with UV-detector
- Sample evaporation unit
- Solid phase extraction unit
- Methanol p.A.
- Silica column, 4 µm, 125 x 4 mm
- Sep-Pack C₁₈ cartridges, available by Immundiagnostik (KC3420CK).

6. PREPARATION AND STORAGE OF REAGENTS

- Reconstitute **calibrator** (CAL) in 6 ml, and **controls** (CTRL1, CTRL2) in 600 µl reconstitution solution (RECSOL), divide calibrator (CAL) in several aliquots and store at -20 °C. Avoid thawing and freezing several times. The content of 25-OH vitamin D₃ and 25-OH vitamin D₂ might have minor changes from lot to lot.
- STD2 and STD3 should be stored at -20 °C. All other test reagents are stable at 2-8 °C up to the date of expiry stated on the label.

7. PRECAUTIONS

- For *in vitro* diagnostic use only.
- 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, HCV or other infectious agents are absent, these reagents should be handled as if potentially infectious.
- The precipitating reagent (PREC) and the elution solution (ELUSOL) 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.

- The mobile phase (MOPHA) contains n-hexan. Hexan is highly flammable and toxic if inhaled or contact the skin. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. 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 could be used. The samples should be stored at -20°C until testing.

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 wrong use.
- The assay should always be performed according the enclosed manual.

Preparation of the SPE-cartridges

Rinse the cartridge with 5 ml methanol and 5 ml aqua dest.

Repeated use of the SPE-cartridges

Dry the cartridges after the sample preparation very well and store in a closed plastic sack. Before the next sample preparation, prepare the cartridges as mentioned above. One cartridge can be used five times.

Sample and standard preparation

- Add 0.5 ml precipitation reagent (PREC) to 0.5 ml serum, calibrator (CAL) or controls (CTRL1, CTRL2).
Attention - Always work with acetonitril in a fume hood!
- Vortex, incubate for 10 min at 4 °C and then centrifuge with 10.000 g for 3 minutes.
- Pipette supernatant on the prepared cartridges and let it soak in.
- Rinse with 3 ml washing solution (WASHSOL).
- Elute with 4 ml elution solution (ELUSOL). **For optimal recovery, the elution must be performed for minimum 60 seconds.**
- Evaporate the elute with nitrogen or with a vacuum centrifuge.
Attention - Always work with acetonitril in a fume hood!
- Dissolve evaporated sample in 150 µl running mobile phase (MOPHA) and mix well. Incubate for **10 min** at 2-8°C and centrifuge for **10 min** at 10.000 g. The prepared sample is stable for min. 6 days at 2-8°C.
- Inject **100 µl** of the supernatant into the HPLC system.

Chromatographic conditions

Column material	: Silica column, 4 µm
Column dimension	: 125 mm x 4 mm
Temperature	: 30 °C
UV-Detector	: 264 nm
Flowing	: 0,75 ml / min
Injection volume	: 100 µl

Running time / chromatogram : ca. 20 min.

For the determination of retention time a run with the ethanolic standards are recommended before each test. For this reason 100 µl of ethanolic standards are evaporated under nitrogen or in the vacuum centrifuge, resuspended in mobile phase and injected into the HPLC-system.

Notice: The mobile phase (MOPHA) can be recirculated. The mobile phase (MOPHA) should be renewed after analysis of 100 samples.

It is recommended that a guard column is used to extend column life.

10. TREATMENT OF THE COLUMN

The HPLC column is filled with silica (normal-phase). **Do not use water** in the system, because water damages silica columns. After the analysis, the column should be stored in HPLC-eluent.

Before use, the system should be equilibrated with 50 ml eluent: Run first 20 ml without column, and then the remaining 30 ml with integrated column.

11. RESULTS

Calculation

$$\text{Concentration sample} = \frac{\text{Peak height sample} \times \text{Concentration of the calibrator}}{\text{Peak height calibrator}}$$

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

Conversion factors:

for 25-OH-Vitamin D₃

1 ng/ml = 2.5 nmol/l

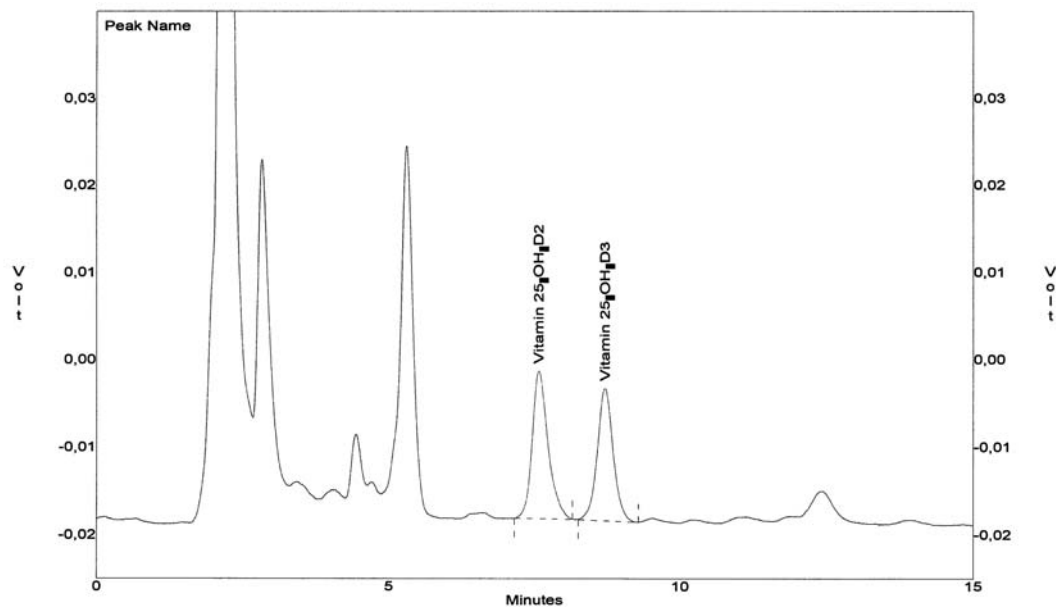
1 nmol/l = 0.4 ng/ml

for 25-OH-Vitamin D₂

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

Expected values

Normal ranges for 25-OH-Vitamin D

Information from ASBMR 2006

Deficiency (seriously deficient)	< 12 ng/ml	resp. < 30 nmol/l
Insufficiency (deficient)	12 - 30 ng/ml	resp. 30 - 75 nmol/l
Sufficiency (adequately supplied)	> 30 ng/ml	resp. > 75 nmol/l

Society of Osteology SACHSEN E. V.

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

Note

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

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

Literature references

Visser M, Deeg DJ, Puts MT, Seidell JC, Lips P (2006) Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. *Am J Clin Nutr.* Sep;84(3):616-22; quiz 671-2

Grant WB, Holick MF. Benefits and requirements of vitamin D for optimal health: a review. (2005) *Altern Med Rev.* Jun;10(2):94-111. Review

Wicherts IS, van Schoor NM, Boeke AJ, Visser M, Deeg DJ, Smit J, Knol DL, Lips P. (2007) Vitamin D status predicts physical performance and its decline in older persons. *J Clin Endocrinol Metab.* Jun;92(6):2058-65

It is recommended that each laboratory should establish its own normal range. Above mentioned values are only for orientation and may vary from other published data.

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.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Inter-Assay CV:	25-OH Vitamin D ₃	4,58 % (54 nmol/l)	[n = 11]
	25-OH Vitamin D ₂	9,07 % (104 nmol/l)	[n = 11]
Intra-Assay CV:	25-OH Vitamin D ₃	5,08 % (52 nmol/l)	[n = 11]
	25-OH Vitamin D ₂	8,39 % (96 nmol/l)	[n = 11]

Linearity	25-OH Vitamin D ₃	Up to 1250 nmol/l
	25-OH Vitamin D ₂	

Detection limit	25-OH Vitamin D ₃	6,2 nmol/l
	25-OH Vitamin D ₂	5,9 nmol/l

Recovery	25-OH Vitamin D ₃	94,82 %
	25-OH Vitamin D ₂	96,65 %

15. DISPOSAL

The mobile phase (MOPHA), ethanolic standard (STD), precipitation solution (PREC), washing solution (WASHSOL), elution solution (ELUSOL) and regeneration solution (REGSOL) 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
Baseline is drifting	Detector lamp did not reach working temperature yet	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

- Merke et al. (1986) *Med Wochenschr* Vol. 9:345-349.
- Reichel et al. (1989) *New Engl J Med* Vol 320:980-991.
- Schmidt-Gayk et al. (1991) *Klin Lab* Vol 37:219.
- Visser M et al. (2006) *Am J Clin Nutr* 84(3):616-22; quiz 671-2
- Grant WB et al. (2005) *Altern Med Rev* 10(2):94-111
- Wicherts IS et al, (2007) *J Clin Endocrinol Metab* 92(6):2058-65

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the test package are for *in-vitro* diagnostic 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 wrong use.

Used symbols:

Temperature limitation



Catalogue Number



In Vitro Diagnostic Medical Device



Contains sufficient for <n> tests



Manufacturer



Use by



Lot number