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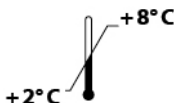
25(OH)-Vitamin D *direct* ELISA Kit

*For the determination of 25(OH)-Vitamin D
in human serum*

Valid from 16.10.2009

REF

IMM-K 2106



IVD



EU patent #EP1097132

Australian patent # 763458

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1. INTENDED USE

The Immundiagnostik ELISA is intended for the quantitative determination of the 25-OH-Vitamin D in serum and fresh plasma. For *in vitro* diagnostic use only.

2. SUMMARY AND EXPLANATION OF THE TEST

Vitamin D is a steroid hormone involved in the intestinal absorption of calcium and the regulation of calcium homeostasis. There are two different forms of vitamin D, named D₃ and D₂, which are very similar in structure. The D₂ is a synthetic product, which is predominantly absorbed by fortified food.

Physiological vitamin D₃ levels result not only from dietary uptake but can also be produced from a cholesterol precursor, 7-dehydrocholesterol, in the skin during sun exposure. In the liver, the vitamin is hydroxylated to 25-hydroxyvitamin D (25(OH)-vitamin D), the major circulating metabolite of vitamin D. Although 1,25-(OH)₂ vitamin D portrays the biological active form of vitamin D, which is synthesized in the kidney, it is widely accepted that the measurement of circulating 25(OH)-vitamin D provides better information with respect to patients vitamin D status and allows its use in diagnose hypovitaminosis (1,2).

Preanalytical stability of 25(OH)-vitamin D₃ in human blood or serum at room temperature: solid as a rock (Wielders and Wijnberg, 2009).

The concentration of 25(OH)-vitamin D decreases with age and a deficiency is common among elderly persons.

Clinical applications of 25(OH)-vitamin D measurements are the diagnosis and therapy control of postmenopausal osteoporosis, rickets, osteomalacia, renal osteodystrophy, pregnancy, neonatal hypocalcemia and hyperparathyroidism. In addition, a prevalence of subclinical vitamin D deficiency has been discussed in different European countries.

Vitamin D intoxication mostly occurs during a large intake of pharmaceutical preparations of Vitamin D and may lead to hypercalcemia, hypercalcuria and nephrocalcinosis in susceptible infants.

3. MATERIAL SUPPLIED

Catalogue No.	Content	Kit Components	Quantity
K 2106MTP	PLATE	One holder with precoated strips	12 x 8 wells
K 2106WP	WASHBUF	ELISA wash concentrate 20x	50 mL
K 2106RS	RECSOL	Reconstitution solution	20 mL
K 2106RR	RELREAG	Releasing reagent	1 vial
K 2106AK	AB	Anti 25-OH-Vitamin D antibody ready to use	18 mL
K 2106CAL	CAL	Calibrator, ready to use	1 vial 300 µL
K 2106KO	CTRL	Controls, ready for use (see specification for range)	2 vials 300 µL each
K 2106K	CONJ	Conjugate, peroxidase labeled, ready to use	22 mL
K 2106TMB	SUB	TMB substrate (Tetramethylbenzidine)	2 x 15 mL
K 2106SD	SAMDIL	Sample Dilution Buffer	35 mL
K 2106AC	STOP	ELISA stop solution, ready to use	1 x 15 mL

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Bidistilled water (aqua bidest.)
- Deep freezer -20 °C
- Precision pipettors calibrated to deliver 10–1000 µl
- Horizontal microtiter plate shaker
- Multi-channel dispenser or repeating dispenser
- Vortex-Mixer
- Water bath or heating block
- Standard laboratory glass or plastic vials, cups, etc. (one time products)
- Microtiter plate reader 450 nm (reference wave length 620 or 690 nm)
- Refrigerator with **defined 8–10 °C**

5. PREPARATION AND STORAGE OF REAGENTS

- The test kit is designed for 48 duplicate determinations. If single values only are desired, additional amounts of the components should be bought. A reduction of the sample or buffer volumes results in erroneous values.
- To run assay more than once, ensure that reagents are stored at conditions stated on the label. The kit can be used up to 2 times within the expiry date stated on the label.
- The **WASHBUF** (wash buffer concentrate) should be diluted with aqua bidest. **1:20** before use (50 ml WASHBUF + 950 ml aqua bidest.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or at 37°C in a water bath before dilution of the buffer solutions. The **buffer concentrate** is stable at **2-8°C** until the expiry date stated on the label. **Diluted buffer solution** can be stored in a closed flask at **2-8°C for two weeks**.
- **Pre-heat RECSOL (reconstitution solution) for 10 min at 37°C in a water bath before use.**
- Reconstitute **RELREAG** (releasing reagent) in **16 ml RECSOL** (reconstitution solution), mix gently (do not vortex), and leave at room temperature for 5 min. After use, aliquot, freeze remaining releasing reagent and store at -20 °C. Frozen RELREAG can be defrosted and used only once. Pre-heat frozen releasing reagent to 37° C before use (e.g. incubate for **10 minutes at 37° C in a waterbath**). Subsequently, it can be used right away.
- Bring **AB** (antibody) at room temperature at least one hour before use.
- All other test reagents are ready for use. The test reagents are stable up to the date of expiry (see label of test package) when stored at **2 -8 °C**. **Note:** 1. Unused strips are covered and stored at 2-8° C. The covered strips should be used within 4 weeks. 2. The remaining RELREAG is stored at -20 °C.

6. SPECIMEN COLLECTION AND PREPARATION

1. Fresh collected blood should be centrifuged within one hour. Vitamin D is an inert substance. However, serum storage at 2-8°C is recommended when the analysis is performed within 24 h after collection. Otherwise, the serum samples must be stored at -20°C until analyzed. Avoid repeated freeze-thaw cycles.
2. Serum samples can be shipped at 4-8 °C (for example with Coolpacks) and remain stable for up to 3 days.
3. **Serum** is the preferred sample matrix; whole blood is not suitable.
4. Indicated incubation times and temperatures must be strictly observed.
5. Mix samples well before use.

7. ASSAY PROCEDURE

Principle of the test

The assay utilizes of a competitive ELISA technique with a selected monoclonal antibody recognizing 25(OH)-vitamin D. For a reliable determination of 25(OH)-vitamin D, it is necessary to release it from the 25(OH)-vitamin D-DBP-complex.

Standards, controls and patient samples which are assayed for 25(OH)-vitamin D are incubated with the releasing reagent. The pre-incubated solutions are then transferred to the microplate coated with 25(OH)-vitamin D, and an anti-25(OH)-vitamin D antibody is added. During an over night incubation step, 25(OH)-vitamin D in the sample and a fixed amount of 25(OH)-vitamin D bound to the microtiter well compete for the binding of the antibody. Then a peroxidase-conjugated antibody is added into each microplate well. A complex of 25(OH)-vitamin D - anti-25(OH)-vitamin D antibody – peroxidase conjugate is formed. Tetramethylbenzidine (TMB) is used as a peroxidase substrate. Finally, an acidic stop solution is added to terminate the reaction, whereby the color changes from blue to yellow. The intensity of the yellow color is inversely proportional to the concentration of 25(OH)-vitamin D. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standard. 25(OH)-vitamin D in the samples is determined from this curve.

Test procedure

1.	Prior to use in the assay allow all reagents and samples to come to room temperature (18 - 26 °C) and mix gentle, avoid foam formation
2.	Mark the positions of CTRLA (control A) / CTRLB (control B) / CAL (Calibrator) / SAMPLE on a protocol sheet
3.	Reconstitute RELREAG (Releasing reagent) (see P. 5., Page 19)
4.	Label V-tubes (e.g. 1.5 ml Eppendorf-tubes)
5.	Pipette 30 µl of CTRLA (control A) / CTRLB (control B) / CAL (Calibrator) / SAMPLE into the corresponding tube
6.	Add 300 µl of RELREAG (releasing reagent) into each tube, vortex shortly
7.	Incubate for 1 hour at 37 °C in a water bath or heating block (do not use an incubator)
8.	Open tubes carefully and add 600 µL SAMDIL (sample dilution buffer). Close the tubes and vortex carefully.

9.	Take microtiter strips out of the kit. Unused strips are covered and stored at 2-8° C. The covered strips should be used within 4 weeks
10.	Transfer 50 µl of CTRLA (control A) / CTRLB (control B) / CAL (Calibrator) / SAMPLE from the V-tubes in duplicate to respective well
11.	Add 150 µl of AB (anti 25(OH)-Vitamin D antibody) into each well
12.	Cover the plate tightly and incubate over night (min. 18 – max. 22 hours) at 8-10 °C in the dark
13.	Aspirate and wash the wells 5x with 250 µl of diluted wash buffer. The use of 8-channel pipette is recommended. Remove remaining wash buffer by hitting the plate against paper towel after the last wash For TECAN and Dynex instruments a programming protocol can be requested from Immundiagnostik AG
14.	Add 200 µl CONJ (Conjugate) into each well
15.	Cover the plate tightly and incubate for 1 hour at room temperature while shaking
16.	Aspirate and wash the wells 5x with 250 µl of diluted wash buffer. The use of 8-channel pipette is recommended. Remove remaining wash buffer by hitting the plate against paper towel after the last wash
17.	Add 200 µl of SUB (Substrate) into each well
18.	Incubate for 10 - 15 minutes at room temperature (18-26°C) in the dark
19.	Add 50 µl of STOP (Stop solution) into each well
20.	Determine the absorption with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. If no reference wave length is available, read only at 450 nm. If the obtained extinctions exceed the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm (690 nm) as a reference

8. RESULTS

For evaluation of the test results, a single-point calibration via a standard curve described by a 4 parameter curve fit is used. The four parameters A, B, C and D as well as the OD value of the calibrator (CAL) are given on the QC certificate of the kit. Results of the samples are read from the constructed calibration curve.

Note: The parameters A, B, C, D and CAL must be entered exactly into the evaluation program. Even small deviations of the values can cause significant interference with the evaluation of the results.

The plausibility of the pairs of values should always be examined after the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually by the operator.

9. LIMITATIONS

Samples, which can not be evaluated by the one-point calibration due to high 25(OH)-vitamin D concentrations, should be diluted maximally 1+1 with ready-prepared 1x wash buffer (e. g. 50 µl sample + 50 µl 1x wash buffer) and re-assayed.

Whole blood is not suitable as a sample.

10. QUALITY CONTROL

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Expected values

Normal ranges for 25(OH)-Vitamin D₃

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

Conversion factor

1 ng/ml = 2.5 nmol/l

1 nmol/l = 0.4 ng/ml

Society of Osteology **SACHSEN E. V.**

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

Reference intervals for 25(OH)-vitamin D₃ (ng/ml)***Males and females**

Age	n	2.5%	97.5%
0 to < 3 months	131	5	42
3 to < 6 months	135	9	60
6 months to < 1 year	147	18	58
1 to < 3 years	394	15	54
3 to < 10 years	619	14	46
10 to < 13 years	286	11	50
13 to < 15 years	275	10	44
15 to < 18 years	390	8	45
> 18 years	421	8	56

*All seasons

(Soldin et al., 2009)

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

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11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 20)		
Sample	25(OH)-Vitamin D [nmol/L]	CV [%]
1	72.3	7.0

Inter-Assay (n = 20)		
Sample	25(OH)-Vitamin D [nmol/L]	CV [%]
1	84.0	7.0

Specificity

The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity to 25(OH)-vitamin D₃. The specificity is calculated in per cent, based on the cross-reactivity of these compounds with the anti-25(OH)-vitamin D₃ antibody compared to the 25(OH)-vitamin D₃ antigen:

25(OH)-Vitamin D ₃	100.0 %
25(OH)-Vitamin D ₂	67.8 %
24, 25(OH)-Vitamin D ₃	≥ 100.0 %
Vitamin D ₂ (Ergocalciferol)	0.3 %

Limit of Blank (LoB) = 1.04 ng/ml resp. 2.6 nmol/l

Limit of Detection (LoD) = 1.28 ng/ml resp. 3.2 nmol/l

Limit of Quantitation (LoQ) = 4.80 ng/ml resp. 12 nmol/l

Working range = 4.80 - 96 ng/ml resp. 12 - 240 nmol/l

The evaluation is performed according to the CLSI-Guideline:EP-17-A.

Day-to-Day Variation

Four samples were measured every day within 21 days. The results in nmol/l are shown below:

Sample	Vitamin D mean value [nmol/l]	VC [%]
1	29.8	14.6
2	45.5	13.1
3	70.8	7.3
4	104.3	7.5

Dilution Recovery

To two serum samples were diluted with ready-prepared 1x wash buffer. The samples were analyzed and the results shown below:

Sample	Dilution	Observed [nmol/l]	Expected [nmol/l]	Recovery [%]
A	undiluted	54.5	54.5	
	90%	50.1	49.1	102
	80%	43.4	43.6	100
	70%	36.4	38.2	95
	60%	31.2	32.7	95
	50%	27.4	27.3	101
B	undiluted	77.0	77.0	
	90%	68.0	69.3	98
	80%	59.5	61.6	97
	70%	54.8	53.9	101
	60%	44.6	46.2	97
	50%	31.5	38.5	82

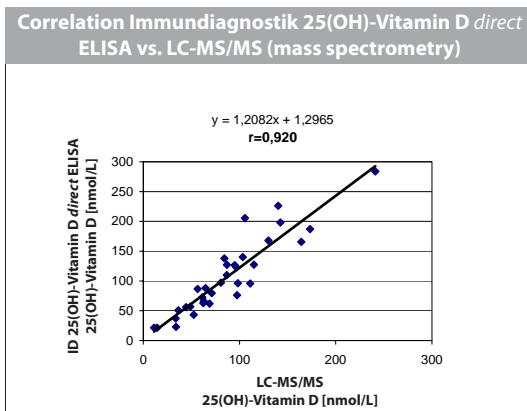
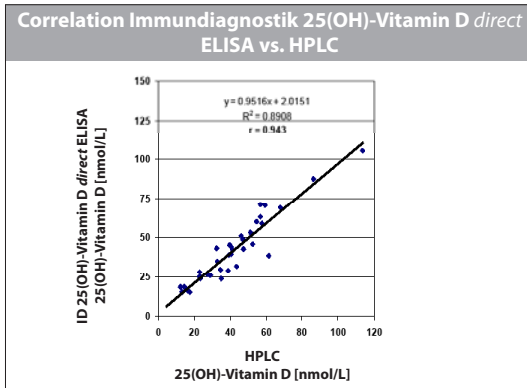
Spiking Recovery

To three serum samples different standard amounts were added. The samples were analyzed and the results shown below:

Sample	Spike [nmol/L]	Target Value [nmol/L]	Obtained Value [nmol/L]	Recovery [%]
6.4	0			
	18.75	25.15	25.80	102.6
	37.50	43.90	35.70	81.3
	75.00	81.40	66.50	81.7
	150.00	156.40	126.90	81.1
	225.00	231.40	204.70	88.5
33.9	0			
	18.75	52.65	49.30	93.6
	37.50	71.40	73.50	102.9
	75.00	108.90	104.80	96.2
	150.00	183.90	185.70	101.0
	225.00	258.90	256.80	99.2
17.2	0			
	18.75	35.95	34.30	95.4
	37.50	54.70	48.00	87.8
	75.00	92.20	81.20	88.1
	150.00	167.20	151.80	90.8
	225.00	242.20	240.80	99.4

Correlation data

Excellent correlation with *direct* ELISA and LC-MS/MS results.



12. PRECAUTIONS

- For *in vitro* diagnostic use only.
- The quality control guidelines should be observed.
- 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.

- Reagents of the kit package contain sodium azide and thimerosal as bactericides. Sodium azide and thimerosal are toxic. The substrates for the enzymatic color reactions are described to be also toxic and carcinogenic. Contact with skin or mucous membranes has to be avoided.
- Stop solution consists of sulfuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water.

13. 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 shown on the kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- The assay should always be performed according the enclosed manual.
- 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.

14. 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.
- All reagents in the kit package are for in vitro diagnostic use only.
- The guidelines for medical laboratories 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.
- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days of receipt of the product. The product shall be send to Immundiagnostik AG together with the complaint in writing.

15. REFERENCES

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Used symbols:



Temperature limitation



Catalogue Number



In Vitro Diagnostic Medical Device



Contains sufficient for <n> tests



Manufacturer



Use by



Lot number

Li StarFish distribuisce: