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IMMUNODIAGNOSTICS AND CHEMISTRY KITS

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Vit D125

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1. INTENDED USE

The assay is intended for the quantitative determination of **1,25 (OH)₂ Vitamin D** in plasma and serum. For research use only. Not for use in diagnostic procedures.

2. SUMMARY AND EXPLANATION OF THE TEST

Vitamin D is either produced in the skin (under the influence of UV light) or taken up from nourishment. The storage type of vitamin D, namely 25-hydroxyvitamin D is formed in the liver. In a second hydroxylation step the hormone 1,25-dihydroxyvitamin D (D hormone) is formed in the kidney. The responsible enzyme, the kidney 1 α -hydroxylase, is subjected to a rigid control through hormones (especially parathyroid hormone) and its activity is influenced by the serum concentrations of calcium and phosphate.

The serum concentration of 1,25-dihydroxyvitamin D normally re-adjusts itself to the demands of metabolism. Deviations from the normal range of 1,25-dihydroxyvitamin D must therefore always be interpreted in the context of the remaining parameters of the calcium metabolism. The serum concentration of 1,25-dihydroxyvitamin D decreases only in seldom cases of vitamin D deficiency. For the diagnosis of vitamin D deficiency the precursor metabolite, 25-hydroxyvitamin D should be measured.

The reason for a non-physiological deficiency of 1,25-dihydroxyvitamin D can be found in metabolic disturbances, caused either by genetic defects of the enzyme 1 α -hydroxylase (rare) or kidney malfunctions (more common). Even a slightly impaired kidney function can lead to a decrease of the 1,25-dihydroxyvitamin D concentration (Rickers et al. 1985).

Since 1,25-dihydroxyvitamin D has important functions in calcium metabolism as well as supplementing secretion of parathyroid hormone from the parathyroid glands, increasing kidney malfunctioning leads to development of renal osteopathy, which is characterized by osteomalacia and Osteitis fibrosa.

Treatment of renal osteopathy consists of the administration of 1,25-dihydroxyvitamin D (Calcitriol) or the prohormone 1 α -hydroxyvitamin D. In renal tubules malfunctions decreased or relatively low levels of 1,25-dihydroxyvitamin D (e.g. diabetes insipidus, Fanconi-Syndrom) are found. A non-physiological over-production of 1,25-dihydroxyvitamin D arises in granulomatosis (e.g. sarcoidosis), where extra-renal synthesis of 1,25-dihydroxyvitamin D occurs. This can lead to hypercalcaemia. Also in idiopathic hypercalciuria a relatively high level of 1,25-dihydroxyvitamin D is found. Increased concentrations of 1,25-dihydroxyvitamin D can be seen in case of non functional vitamin D receptors (rare), during calcium

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deficient nutrition, as well as a result from overproduction of parathyroid hormone (primary hyperthyroidism).

Indications

- Defect of kidney functions
Chronic kidney failure
Haemodialysis-Following kidney transplants
- Renal osteopathy
- Osteomalacia from various types of vitamin D metabolism disturbances
- Kidney tubules function disturbances (diabetes insipidus, Fanconi-Syndrom)
- Monitoring of therapy with active vitamin D metabolites
- Ideopathic hypercalciuria
- Hypercalcaemia

3. PRINCIPLE OF THE TEST

In this assay, the biological active Vitamin D metabolite $1,25\text{-(OH)}_2\text{D}_3$, has to be extracted with two separate extraction columns. This procedure is necessary to separate the $1,25\text{-(OH)}_2$ Vit D from other Vitamin D metabolites especially from the 25-(OH) Vit D and the $24,25\text{-(OH)}_2$ Vit D.

This assay is a competitive enzyme immuno assay for the measurement of $1,25\text{ (OH)}_2$ Vit D. It is based on the competition of $1,25\text{ (OH)}_2$ Vit D present in the sample with labeled $1,25\text{ (OH)}_2$ Vit D tracer for the binding site of the vitamin D specific antibody.

After evaporation, samples, calibrators, NSB and control are dissolved in ethanol, antibody is added followed by an incubation of one hour. After this pre-incubation, the samples have to be transferred to the microtiterplate. $1,25\text{ (OH)}_2$ Vit D present in the sample competes with the tracer for the specific binding site of the specific antibody. Hence, with increasing concentrations of $1,25\text{ (OH)}_2$ Vit D in the sample, the amount of antibody, immobilized to the well via the tracer, is decreased. After a washing step to remove unbound components, $1,25\text{ Vit D}$ is indirectly measured with a host specific peroxidase labeled antibody using TMB (tetramethylbenzidine) as enzyme substrate. The reaction is terminated by adding a stopping solution. The blue color converts to yellow. The intensity of the yellow color is indirectly proportional to the concentration of $1,25\text{ (OH)}_2$ Vit D in the sample. A dose response curve of the absorbance

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unit vs. concentration is generated using the results obtained from the calibrators. Concentrations of 1,25 (OH)₂ Vit D, present in the patient samples, are determined directly from this curve.

4. MATERIAL SUPPLIED

Catalogue No.	Components	Quantity
K 2112MTP	one holder with precoated strips	96
K 2112WP	ELISA wash concentrate 10x	1 x 100 ml
K 2112TR	Tris-HCl Buffer	1 x 30 ml
K 2112E	Ethanol, ready to use	1 x 1.5 ml
K 2112A1	1. Antibody, (Mouse -anti 1,25-(OH) ₂ Vitamin D), ready-to-use	1 x 25 ml
K 2112ST	Calibrator and NSB, ready-to-use (0; 5.1; 12.5; 32; 80; 200 pg/ml)	7 x 2.5 ml
K 2112KO	Controls, ready-to-use	2 x 2.5 ml
K 2112K	Conjugate, ready-to-use	1 x 22 ml
K 2112TMB	TMB Substrate (Tetramethylbenzidine), ready-to-use	2 x 15 ml
K 2112AC	ELISA stop solution, ready-to-use	1 x 7 ml

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K 2112AC	ELISA stop solution, ready-to-use	1 x 7 ml

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- 48 Extrelut or Chromabond columns (Merck, Darmstadt/Macherey-Nagel, Düren)
- 48 Silica-cartridges
- Diisopropylether (p.A.)
- Isopropanol (p.A.)
- Hexane (p.A.)
- Methanol (p.A.)
- Deionized water
- 75 x 12 mm glass tubes
- Precision pipettors calibrated to deliver 20-1000 μ l
- A multi-channel dispenser or repeating dispenser
- Vortex-Mixer
- Refrigerator
- Microplate reader 450 nm
- Speedvac or nitrogen stream unit

6. PREPARATION AND STORAGE OF REAGENTS

- To use the kit components several times, make sure that the reagents are carefully stored. **Just prepare the appropriate amount necessary for the assay.**
- Reagents with a volume less than 100 μ l should be shortly centrifuged before use.
- The **ELISA wash buffer concentrate** should be diluted with deionized water **1:10** before use (add 900 ml deionized water to 100 ml concentrate). Crystals could occur due to high salt concentration. The crystals have to be re-suspended **before dilution of the buffer solutions** using a water bath (37°C). The buffer concentrates are stable at **2-8°C** until the expiry date stated on the label. Diluted buffer solutions could be stored at **2-8 °C** for 1 month.
- All other kit components are ready to use and stable at **2-8 °C** until the expiry date stated on the label.

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7. PRECAUTIONS

- For research use only. Not for use in diagnostic procedures.
- The calibrators and controls contain 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.
- Stop Solution consists of diluted Sulfuric Acid. This is 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. Do not breath vapor and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

Plasma/Serum

If the samples are not used within 24 h, the samples should be stored at -20°C. We recommend pipetting 1000 µl of the sample's volume in the cartridges. In case of fewer amounts of sample volume (min.500µl) make the cartridges wet with Tris – HCL buffer. (The total capacity of the cartridges is 1000 µl)

To calculate the actual concentration each result should be multiplied with the respective dilution factor.

9. ASSAY PROCEDURE

Procedural notes

- Do not interchange different lot numbers of any kit component within the same assay.
- The quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results.

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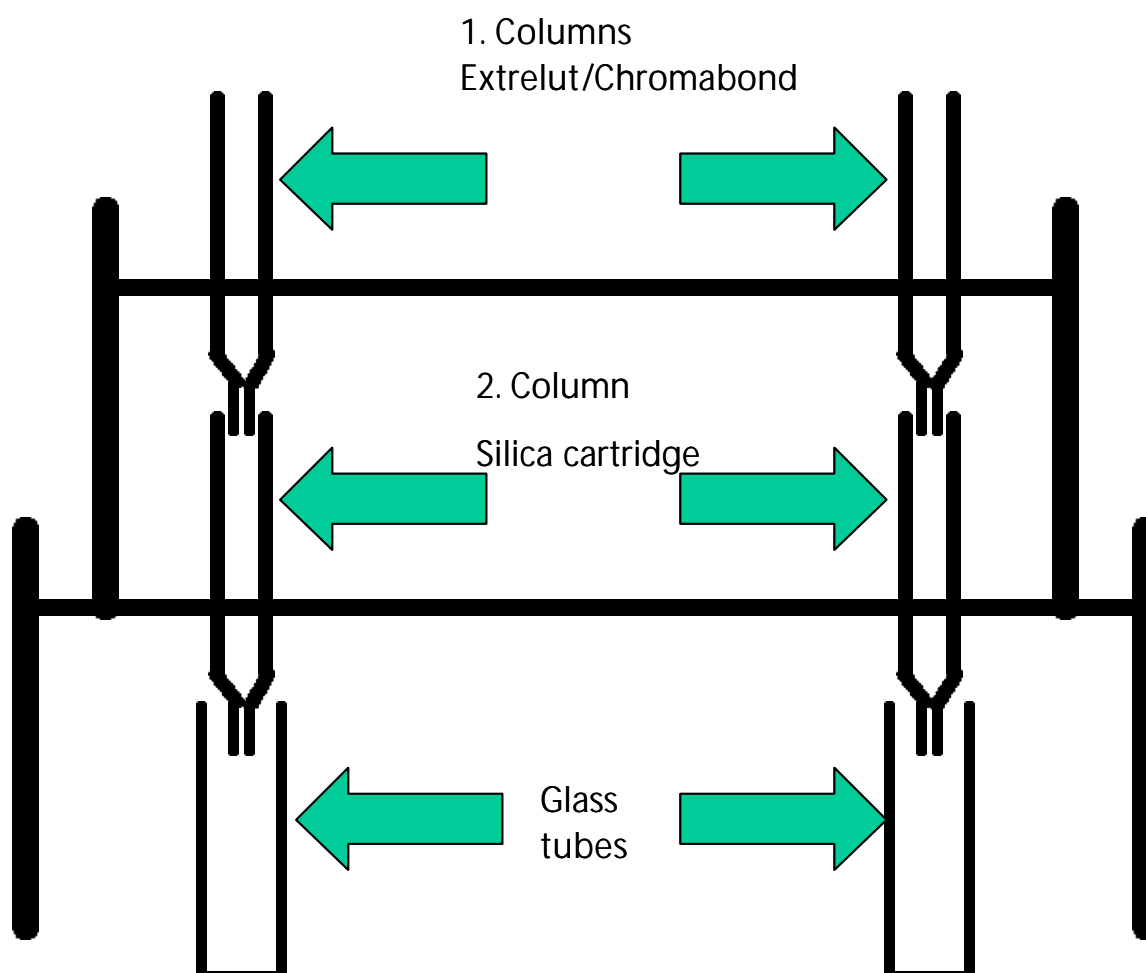
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Extraction unit

Scheme of the extraction unit:

The extraction unit consists of two parts, which were put on top of each other. The upper part is used for the Extrelut or Chromabond columns, the lower part for the silica cartridges. During sample application and the entire washing procedure, the whole unit should be put into a container big enough to collect the extraction solvents. For the last elution step it is recommended to place the glass-tubes directly under the cartridges. The tubes could then be used directly for the next step of the assay.

Extraction unit

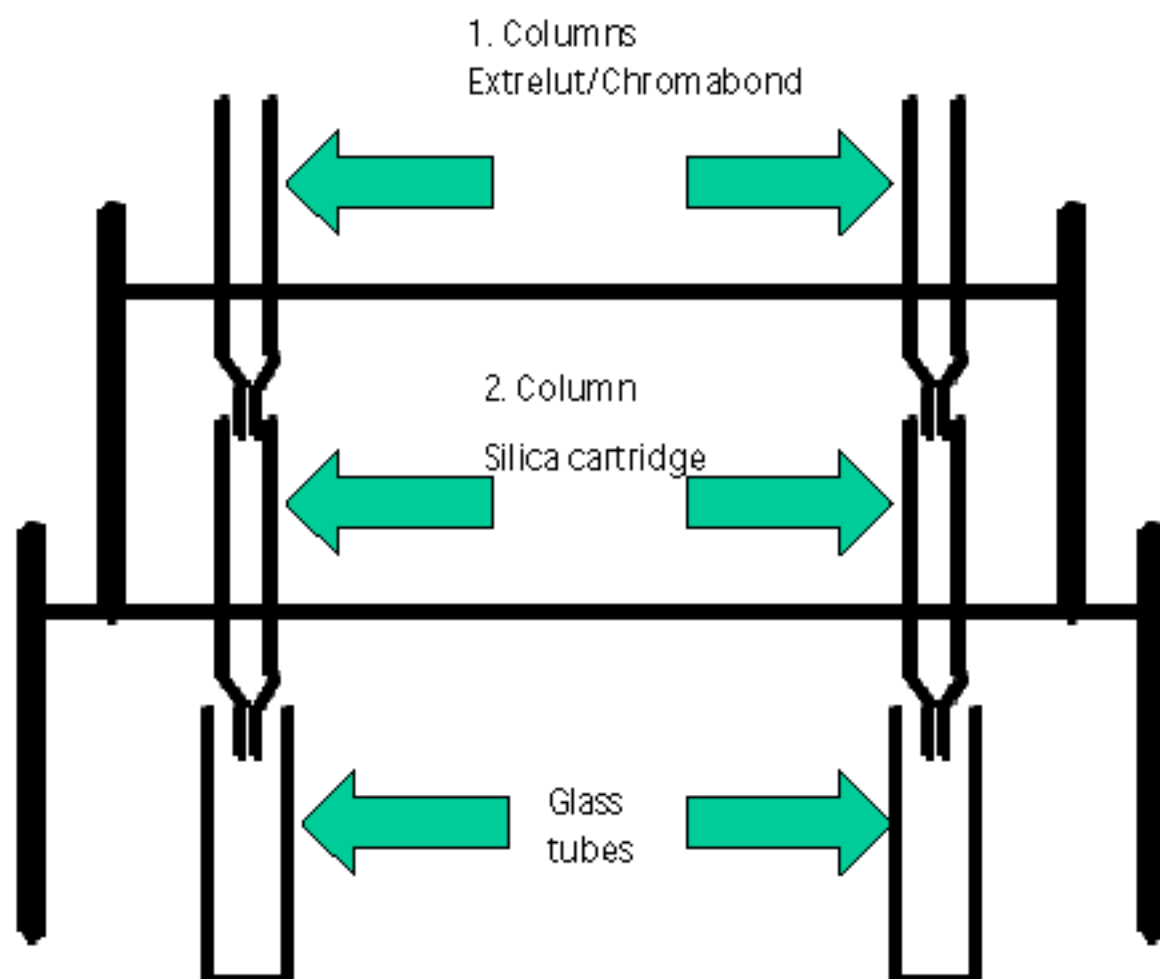


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Extraction unit



Extraction

1. **1000 µl** calibrators, NSB, control and sample (plasma or serum) are applied on the 1. column (Extrelut or Chromabond). To get fully absorbed by the column-material this procedure takes about 10 minutes (For sample volumes less than 1000 µl make the cartridges wet with Tris-HCL buffer e.g. pipette 250µl Tris-HCL buffer in the cartridge + 750µl sample.)
2. The Vitamin D is extracted from the columns with **4 x 1 ml** diisopropylether (3 min for each elution). The eluate is applied directly on a untreated and dry silica cartridge. **(After the extraction the columns should be removed.)**
3. Afterwards the cartridge is washed with **5 x 2 ml Isopropanol/Hexane (4/96 v/v)**
4. Afterwards the cartridge is washed with **3 x 2 ml Isopropanol/Hexane (6/94 v/v).**
5. **2 x 2 ml Isopropanol/Hexane (25/75 v/v)** is used to elute 1,25-(OH)₂ D₃ from the column. (Note: the glass tubes should be placed directly under the columns.)
6. The eluate is evaporated under a nitrogen stream at 37 °C or in a speed vac. The evaporated samples could be stored at -20°C for 24 h.

Pipetting scheme

Pre-incubation

8. Add **20 µl** of Ethanol into each glass tube. Mix shortly.
9. Add **450 µl** antibody solution into each glass tube. Mix thoroughly.
10. Cover glass tubes with plastic film and incubate for **1 h** at room temperature.

ELISA procedure

11. Add **200 µl** of pre-incubated samples into the respective wells. Carry out the test in duplicates.
12. Cover strips with plastic film and incubate for **18-22 h** at 4°C.
13. Discard contents of the wells and wash **5 x with 300 µl** wash buffer.

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2. The Vitamin D is extracted from the columns with **4 x 1 ml** diisopropylether (3 min for each elution). The eluate is applied directly on a untreated and dry silica cartridge. **(After the extraction the columns should be removed.)**
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14. Add **200 μ l** conjugate into each well. Shake plate gently.
 15. Cover strips with plastic film and incubate for **1 h** at room temperature on a plate shaker.
 16. Discard contents of the wells and wash **5 x with 300 μ l** wash buffer.
 17. Add **200 μ l** substrate into each well. Shake plate gently.
 18. Incubate for **20 –30 minutes** at room temperature in the dark.
 19. Add **50 μ l** stop solution into each well. Shake plate gently.
 20. Measure the extinction of the samples at **450 nm** against 620 nm as reference directly after adding the stop solution and mixing.

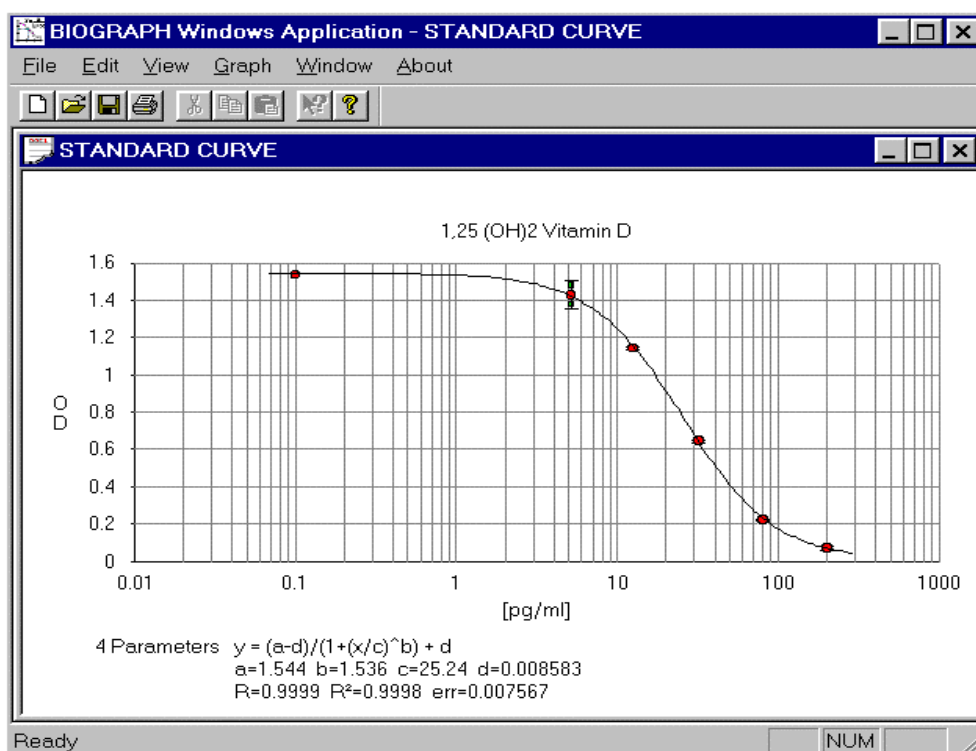
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10. RESULTS

A calibration curve is constructed from the calibrators. Commercially available software can be used as well as graph paper. Results of the samples are read from this calibration curve.

THE CALIBRATION CURVE IS NOT LINEAR, therefore a spline- or 4PL algorithmus is recommended.

Typical calibration curve



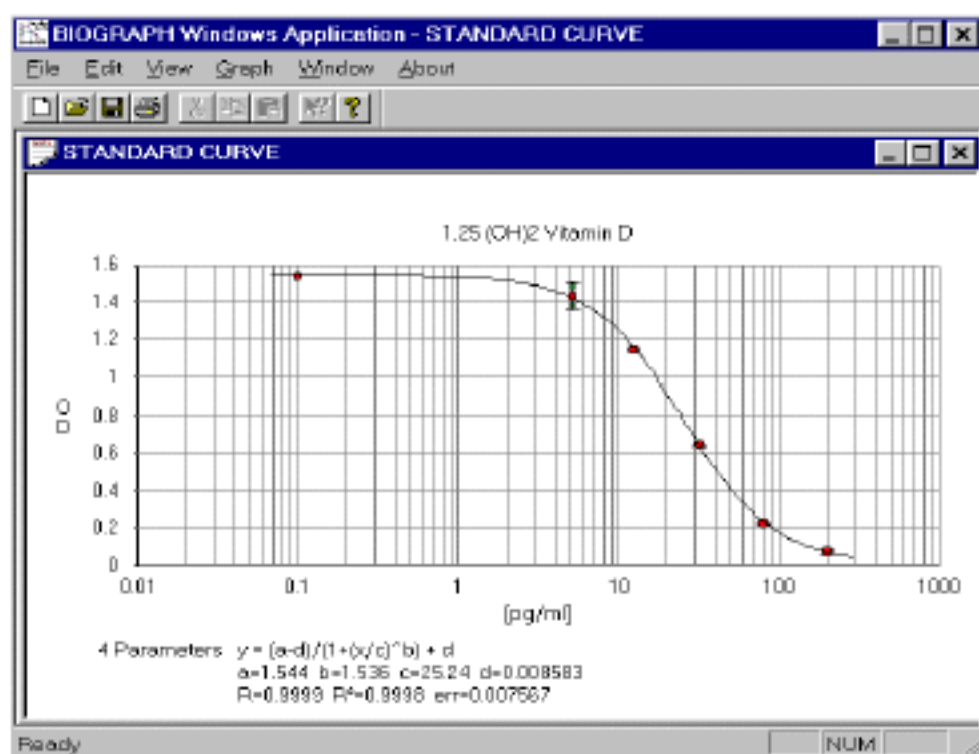
Calibrator	B/BO mean [%]	Concentration [pg/ml]	CV [%]
NSB			
1	100	0	2.5
2	89	5.1	1.8
3	76	12.5	10.5
4	57	32.0	2.7
5	30	80.0	2.2
6	7	200.0	19.9

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Typical calibration curve



Calibrator	B/BO mean [%]	Concentration [pg/ml]	CV [%]
NSB			
1	100	0	2.5
2	89	5.1	1.8
3	76	12.5	10.5
4	57	32.0	2.7
5	30	80.0	2.2
6	7	200.0	19.9

11. LIMITATIONS

Samples with 1,25 (OH)₂ Vitamin D levels greater than the highest calibrator, should be re-assayed. Use instead of 1 ml sample a volume of 500 µl or 750 µl. Recalculate the results with the dilution factor.

12. QUALITY CONTROL

Control samples or serum pools should be analyzed with each run of calibrators and patient samples. Results, generated from the analysis of 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.

Expected values

Normal range (Plasma or Serum):

Healthy adults (age 20-50) : 17 - 53 pg/ml
Children up to 12: ca 40% higher values
Pregnant women (8.-42. week): ca. 60% higher values
Persons older than 70: ca. 40% lower values

The normal range is independent of the season.

We recommend all laboratories establish their own normal range.

Precision and reproducibility

The precision (intra-assay variation) of the 1,25 (OH) Vitamin D ELISA test was calculated from 21 replicate determinations of one sample.

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Intra-Assay CV (n= 21)

Sample	1,25-(OH) ₂ Vit D mean [pg/ml]	Intra-Assay CV [%]
1	55.3	6.6

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Normal range (Plasma or Serum):

Healthy adults (age 20-50):	17 - 53 pg/ml
Children up to 12:	ca 40% higher values
Pregnant women (8.-42. week):	ca. 60% higher values
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Intra-Assay CV (n= 21)

Sample	1,25-(OH) ₂ Vit D mean [pg/ml]	Intra-Assay CV [%]
1	55.3	6.6

The total precision (inter-assay variation) of the 1,25(OH)₂ Vitamin D ELISA test was calculated from data on 1 sample obtained in 20 different assays by one technician on two different lots of reagents over a period of three months.

Inter-Assay CV (n= 20)

Sample	1,25(OH) ₂ VitD mean [pg/ml]	Inter-Assay CV [%]
1	39	9

Sample dilution

Sample	Volume [μl]	Expected [pg/ml]	Measured [pg/ml]
A	1000	21.2	21.2
	750	15.9	15.9
	500	10.6	11.2
	250	5.3	8.5
B	1000	29.6	29.6
	750	22.2	27.3
	500	14.8	16.9
	250	7.4	7.2

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Sensitivity

The detection limit was defined as $B_0 - 2SD$

n= 11

Sample	1,25 (OH) ₂ Vit D Mean value [OD]	Standard variation	Detection limit [pg/ml]
1	1.201	0.025	4.8

Cross reactivity

1,25-(OH) ₂ Vit D ₃	100 %
Vit D ₂ & D ₃	< 0.01 %
24,25-(OH) ₂ Vit D ₃	< 0.1 %
25-OH Vit D ₂	< 0.1 %
25-OH Vit D ₃	< 0.01 %
Alfacalcidol	< 0.003 %

13. REGENERATION OF THE SILICA CARTRIDGES

- **2 x 2 ml** Methanol
- **2 x 2 ml** n-Hexan
- Dry the columns in the hood

14. LITERATURE

1. Wildermuth S. et al.: Clinica chimica Acta, 220, 1993, S. 61
2. Schilling M. et al.: Clinical Chemistry, 33, 1987
3. Armbruster F.P. et al.: Ärztl. Lab., 36, 1990, S. 75
4. Durham B.W. et al.: Ann. Clin. Biochem., 32, 1995, S. 77
5. Hollis B.W.: Calcif. Tissue Int., 58, 1996, S.4
6. Hollis B.W.: Clinical Chemistry, 41, 1995, S.1313
7. Iqbal S.J.et al.: Clinical Chemistry, 42, 1996, S.112
8. Withold W. et al.: Eur. J. Clin. Chem., Clin. Biochem., 33, 1995

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15. 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 which are made of human serum are tested for Australia antigen and HIV and found to be negative. However, since no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as recommended for any potentially infectious human serum or blood specimen. The normal precautions for laboratory working should be observed.
- Reagents of the test package contain sodium azide as a bactericide. Contact with skin or mucous membranes has to be avoided.
- The reagents should not be used after the date of expiry (see label on the test package).
- Single components with different lot numbers should not be mixed or exchanged.
- The guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the results of the test. Immundiagnostik and their distributors can therefore not be held responsible for any damage resulting from this.

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