Human 1,25-Dihydroxy Vitamin D ELISA Kit

Instruction Manual

PromoKine

Contents

Intended Use

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) is intended for the quantitative determination of 1,25-dihydroxy vitamin D in serum and plasma. This assay is intended for research use only.

Summary and Explanation

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-hydroxy vitamin D, is formed in the liver. The hormone 1,25-dihydroxy vitamin D (D hormone) is formed in a second hydroxylation step in the kidney. The responsible enzyme, the kidney 1α-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-dihydroxy vitamin D normally re-adjusts itself to the demands of metabolism. Deviations from the normal range of 1,25-dihydroxy vitamin D must therefore always be interpreted in the context of the remaining parameters of the calcium metabolism. The serum concentration of 1,25-dihydroxy vitamin 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-dihydroxy vitamin D can be found in metabolic disturbances, caused either by genetic defects of the enzyme 1α -hydroxylase (rare) or kidney malfunctions (more common). Even a slightly impaired kidney function can lead to a decrease of the 1,25-dihydroxy vitamin D concentration.

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

Kit Components

Content	Kit Components	Quantity
PLATE	One holder with precoated strips	12 x 8 wells
WASHBUF	ELISA wash buffer concentrate 10x	100 ml
ETHANOL	Ethanol, ready-to-use	1.5 ml
TRIS-HCL	Tris-HCL buffer, ready-to-use	30 ml
AB	Detection antibody, anti 1,25-(OH)2 vitamin D, ready-to-use	25 ml
STD	Standard incl. NSB, ready-to-use (for range see specification or label)	7 x 2.5 ml
CTRL	Controls, ready-to-use (for range see specification)	2 x 2.5 ml
CONJ	Conjugate, polyclonal peroxidase-labeled antibody, ready-to-use	22 ml
SUB	TMB substrate (Tetramethylbenzidine), ready-to-use	2 x 15 ml
STOP	ELISA stop solution, ready-to-use	15 ml

Material and Equipment Required but not Provided

- Bidistilled water (aqua bidest.)
- 48 Chromabond columns (PK-EL-K2112Se; available on request)
- 48 Silica Cartridges (solid phase extraction cartridges, PK-EL-K2112Sb; available on request)
- Diisopropylether (p.A.) 99.0 %
- Isopropanol (p.A) 99.9 %
- n-Hexan (p.A.) 98.3 %
- Methanol (p.A.) 99.9 %
- 75 x 12 glass tubes (no plastic)

- Extraction rack (PK-EL-K2112SV; available on request)
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Vacuum centrifuge or nitrogen distributor
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm (reference wave length 620 or 690 nm)

Preparation and Storage of Reagents

- To run the assay more than one time, make sure that the reagents are stored at the conditions stated on the label. Prepare just the appropriate amount necessary for the assay. The kit can be used up to 2 times within the expiry date stated on the label.

- The ELISA wash buffer concentrate (WASHBUF) should be diluted with aqua bidest. 1:10 before use (100 ml concentrate + 900 ml aqua bidest.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals have to be redissolved at room temperature or at 37°C using 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 could be stored in a closed flask at 2-8°C for no longer than one month.

- All other test reagents are ready to use. The test reagents are stable until the expiry date (see label of test package) when stored at 2-8°C.

Sample Preparation

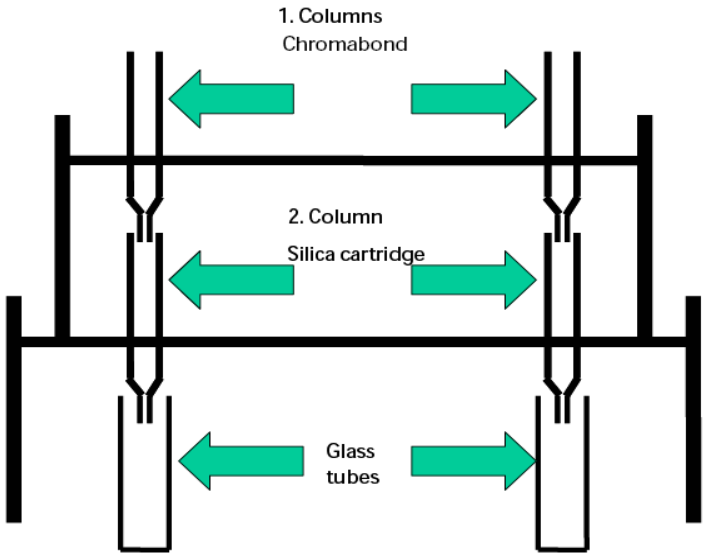
Serum/plasma samples

Fresh collected blood should be centrifuged within one hour. Store samples at -20°C if not assayed on the same day. Lipemic or hemolytic samples may give erroneous results. Samples should be mixed well before assaying. We recommend duplicate analyses for each sample.

We recommend to apply 1000 µl sample per the cartridge. If the sample volume is less than 1000 µl, load an appropriate amount of Tris–HCl buffer in the column, and then the sample (minimum 500 µl) for a total volume of 1,000 µl.

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

Extraction of the samples



- The extraction unit consists of three parts, which were put on top of each other.
- The upper part is used for the Chromabond columns (extraction rack I), the lower part for the silica cartridges (extraction rack II).
- During sample application and the entire washing procedure, the whole unit should be put into a container big enough to collect the extraction solvents (extraction rack I and extraction rack II). After the first extraction step (ether) remove the extraction rack I with the Chromabond columns.
- It is recommended to place the glass-tubes (extraction rack III) directly under the cartridges (extraction rack II) for the last elution step. The tubes can then be used directly for the next step of the assay.

Assay Procedure

Principle of the Test

The assay utilizes of a competitive Enzyme-Immuno-Assay (EIA) technique with a selected monoclonal antibody recognizing 1,25-dihydroxy vitamin D.

Standards, NSB (non-specific binding), controls and patient samples which are assayed for 1,25-dihydroxy vitamin D are incubated after the extraction step with the detection antibody. The pre-incubated solution is then transferred to the microplate coated with 1,25-dihydroxy vitamin D. During this incubation step, 1,25-dihydroxy vitamin D in the sample and a fixed amount of 1,25-dihydroxy vitamin D bound to the microtiter well compete for the binding of the detection antibodies. Then a peroxidase-conjugated anti-mouse antibody is added into each microplate well and a complex of 1,25-dihydroxy vitamin D - detection 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 1,25-dihydroxy 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. 1,25-dihydroxy vitamin D in the samples is determined from this curve.

Extraction

1. Apply 1000 µl of standards, NSB, control and sample (plasma or serum) on the Chromabond columns and incubate for 10 minutes. For sample volumes less than 1000 µl wet the cartridges with Tris–HCl buffer, e.g. pipette 500 µl Tris–HCl buffer in the cartridge and 500µl sample
2. Extract vitamin D from the chromabond columns with 4 x 1 ml diisopropylether (3 min for each elution). The eluate should drip from the chromabond column directly on an untreated and dry silica cartridge. After the extraction the chromabond columns should be removed (extraction rack I)
3. Wash the silica cartridges (extraction rack II) with 5 x 2 ml Isopropanol/Hexane (4/96 v/v)
4. Wash the silica cartridges (extraction rack II) with 3 x 2 ml Isopropanol/Hexane (6/94 v/v)
5. Elute 1,25-dihydroxy vitamin D from the silica cartridges with 2 x 2 ml Isopropanol/Hexane (25/75 v/v). Note: the glass tubes (extraction rack III) should be placed directly under the silica cartridges
6. Evaporate the eluate under a nitrogen stream at 37°C or in a vacuum centrifuge

Pre-Incubation

1. Add 20 µl of ethanol into each glass tube. Immediately after adding ethanol gently vortex each tube to avoid any possible evaporation.
2. Add 450 µl antibody solution into each glass tube. The antibody solution is viscous. Please pipette slowly and carefully. Mix thoroughly.
3. Cover glass tubes with a plastic film and incubate exactly for 1 hour at room temperature.

Test Procedure

- Prior to use in the assay allow all reagents and samples to come to room temperature (18-26°C) and mix well
- Mark the positions of STD/NSB/SAMPLE/CTRL (Standards/non-specific binding/Sample/Control) on a protocol sheet
- Take microtiter strips out of the kit. Store unused strips covered at 2-8°C. Strips are stable until the expiry date stated on the label
- Add 200 µl of STD/NSB/SAMPLE/CTRL in duplicate into respective well. All these solutions are viscous. Please pipette slowly and carefully. We recommend to wet the pipette tip before using it to transfer the pre-incubate.
- Cover the plate tightly and incubate for 18-22 hours at 4 - 8°C*
- Discard the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
- Add 200 µl CONJ (conjugate) into each well
- Cover the plate tightly and incubate for exactly 1 hour at room temperature (18-26°C) on a horizontal mixer
- Discard the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
- Add 200 µl of SUB (substrate) into each well
- Incubate for 20 - 30 minutes at room temperature (18-26°C) in the dark**
- Add 50 µl of STOP (stop solution) into each well, mix thoroughly
- Determine absorption immediately with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference

*As with any competitive immunoassay, consistent incubation times and temperature are essential for accurate plate-to-plate comparisons. Fluctuations in overnight incubation can lead to increased inter-assay CV's. It is therefore recommended to use always the same incubation time, i.e. 20 hours.
**The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

Results

The following algorithms can be used alternatively to calculate the results. We recommend to use the "4-Parameter-algorithm".

1. 4-Parameter-algorithm

   It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator has to be specified with a value smaller than 1 (e. g. 0.01).

2. Point-to-point-calculation

   We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline-algorithm

   We recommend for the optical density a linear ordinate and for the concentration a logarithmic abscissa. When using a logarithmic abscissa, the zero calibrator has to be specified with a value smaller than 1 (e. g. 0.01).

The plausibility of the pairs of values should be examined before 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.

Limitations

Samples with 1,25-dihydroxy vitamin D levels greater than the highest standard value should be re-assayed. Apply instead of 1 ml sample a volume of 500 µl or 750 µl on the pre-buffered Chromabond column. Recalculate the results with the dilution factor.

Quality Control

We recommend the use of commercial control samples for internal quality control if available.

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 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 each laboratory to establish its own norm concentration range.

Performance Characteristics

Precision and Reproducibility

Intra-Assay (n = 20)

Probe	1,25 (OH)2 Vitamin D [pg/ml]	CV [%]
1	55.3	6.69

Inter-Assay (n = 20)

Probe	1,25 (OH)2 Vitamin D [pg/ml]	CV [%]
1	39	9

Sensitivity

The sensitivity was set as B0 + 2SD. The zero-standard was measured 20 times.

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

Cross Reactivity

1,25-(OH)2 Vit D3	100 %
1,25-(OH)2 Vit D2	41 %
Vit D2 & D3	< 0.01 %
24,25-(OH)2 Vit D3	< 0.1 %
25-OH Vit D2	< 0.1 %
25-OH Vit D3	< 0.01 %
Alfacalcidol	< 0.003 %

Linearity

Different volumes of two patient samples were analyzed. The results are shown below:

n= 2

Sample	Dilution	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

Regeneration of the Silica Cartridges

The silica cartridges can used a total of 5 times if regenerated as follows. The regeneration can be performed directly after the extraction. The silica cartridges have to be dry before the next use.

- 2 x 2 ml methanol
- 2 x 2 ml n-hexan
- Dry the columns in the hood

Precautions

- For *in vitro* research use only.
- The quality control guidelines should be followed.
- 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 or thimerosal as bactericides. Sodium azide and thimerosal are toxic. The substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong 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 spill should be wiped out immediately with copious quantities of water.

Technical Hints

- Do not mix different lot numbers of any kit component.
- 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.

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 kit package are for *in vitro* research use only.
- The guidelines for medical laboratories should be followed.
- 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 supplier, may influence the results of the test. PromoCell 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 after receipt of the product. The product shall be send to PromoCell together with a written complaint.

References

- Wildermuth S. et al., 1993; Clinica chimica Acta, 220, 61
- Schilling M. et al., 1987; Clinical Chemistry, 33, 187
- Armbruster F.P. et al., 1990; Ärztl. Lab., 36, 75
- Durham B.W. et al., 1995; Ann. Clin. Biochem., 32, 77
- Hollis B.W., 1996; Calcif. Tissue Int., 58, 4
- Hollis B.W.,1995; Clinical Chemistry, 41, 1313
- Iqbal S.J. et al., 1996; Clinical Chemistry, 42, 112
- Withold W. et al., 1995; Eur. J. Clin. Chem. Clin. Biochem., 33, 15

Ordering Information

Product Name	Product Description	Size	Catalog Number
1,25-Dihydroxy Vitamin D ELISA Kit, human	Human 1,25-(OH)2 Vitamin D ELISA Kit	96 Tests	PK-EL-K2112

For in vitro research use only.
Not for diagnostic or therapeutic procedures.

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