Human 25-OH Vitamin D (direct) ELISA Kit

PromoKine

Instruction Manual

Cat.No. PK-EL-K2109

Contents

Intended Use

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

Summary and Explanation of the Test

Vitamin D_3 is photochemically synthetised from its precursor 7-dehydrocholesterol in the skin under the influence of ultraviolet light (UV-B). Vitamin D_2 originates from plant food and is resorbed via the small intestine. Vitamin D_2 and D_3 are metabolised in the same way.

In the blood, vitamin D is bound to the vitamin D binding protein (VDBP) and transported to the liver, where it is metabolised to 25-hydroxyvitamin D (25(OH)-vitamin D). The 25-hydroxylation is dependend from the substrate available. 25-hydroxyvitamin D has some biological activity left and is the most abundant D metabolite in the circulation. Due to its high affinity to the binding protein VDBP, it constitutes the storage form of vitamin D. Therefore, the serum concentration of 25-hydroxyvitamin D is the best indicator for the vitamin D status.

In the kidneys, 25-hydroxyvitamin D is further metabolised to 1,25-dihydroxyvitamin D, whic is the most active vitamin D metabolite and acts as a hormone (D hormone). It regulates calcium resorption from the intestine, bone mineralisation, osteoblast differenciation and bone matrix synthesis. Furthermore, the neuromuscular function is influenced by the D hormone.

A secondary parathyroid hormone increase and increased bone resorption is already caused by a slight vitamin D deficiency of 20 – 29 ng/ml (50 – 74 nmol/l) due to the resulting reduced calcium resorption.

In the German general population aged > 50 years, vitamin D status is significantly correlated with bone density (Scharla et al., 1996). Therefore, vitamin D deficiency is one of the most important risk factors for senile osteoporosis. The early detection of a vitamin D deficiency allows for an effective prevention of fractures by vitamin D supplementation. Severe vitamin D deficiency (< 20 ng/ml / < 50 nmol/l) results in rickets (in children) or osteomalacia (in adults),

which are both characterised by a disturbed bone formation and defective matrix mineralisation (Scharla, 1997). An excess of vitamin D (drug overdose) leads to an hypercalcemia syndrome.

Material Supplied

Content	Kit Components	Quantity
PLATE	One holder with precoated strips (with 25-hydroxyvitamin D antigen linked to the inner surface of the polystyrene wells)	12 x 8 wells
WASHBUF	ELISA wash concentrate 10x (phosphate buffered saline containing Triton X-100 and ProClin 300)	2 x 100 mL
RELREAG	Releasing reagent (lyophilised phosphate bufferd saline containing a vitamin D binding protein inhibitor)	2 x 16 ml
AB	Anti 25(OH)-vitamin D antibody, ready to use (phosphate buffered saline containing monoclonal mouse antibodies, stabilisers and preservative)	1 x 18 mL
STD	Standards, ready to use (buffered human serum containing 25-hydroxyvitamin D and 0.09 % sodium azide)	6 x 300 µL
CTRL-A CTRL-B	Controls, ready for use (see specification for range) (buffered human serum containing 25-hydroxyvitamin D and 0.09 % sodium azide)	1 x 300 µL 1 x 300 µL
CONJ	Conjugate, peroxidase labeled, ready to use (phosphate buffered saline containing anti mouse-HRP, stabilisers and preservative)	1 x 24 mL
SUB	TMB substrate (aqueous formulation of tetramethylbenzidine and hydrogen peroxide)	2 x 15 mL
STOP	ELISA stop solution, ready to use (sulfuric acid)	1 x 15 mL
FOL	Foil to cover the microtiter plate	3 x 1

Material Required but not Supplied

- Ultrapure water*
- 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 8–10 °C

* We recommend the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

Preparation and Storage of Reagents

- Bring all reagents and samples to room temperature (20–28°C) and mix well.
- The test kit is designed for 96 single determinations. 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.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultrapure water **1:10** before use (100ml WASHBUF + 900ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. The crystals must be redissolved at room temperature or in a water bath at 37°C before dilution of the buffer solution. The **WASHBUF** is stable at **2–8°C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8°C for 2 weeks.**

- Warm the **releasing reagent (RELREAG)** to 37°C in a water bath at least 30min before use. 16ml releasing reagent are enough to perform 48 single determinations, for 96 determinations both vials of releasing reagent are needed.
- Bring **AB** (antibody) at room temperature (20 – 28°C) 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: Microtiter strips:** Once the vacuum-sealed aluminum bag has been opened, all unused strips must be covered with the foil supplied and put back into the aluminium bag. Close the aluminium bag and store it at 2–8 ° C. Strips handled in such a way can be used within 4 weeks.

Specimen Collection and Preparation Procedure

a) Fresh collected blood should be centrifuged within one hour. Vitamin D is an inert substance (Wielders & Wijnberg, 2009). 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 analysed. Avoid repeated freeze-thaw cycles.

b) **Serum** samples can be shipped at 4-8 °C (for example with Coolpacks) and remain stable for up to 3 days.

c) Serum is the preferred sample matrix; **whole blood is not suitable.**

d) Indicated **incubation times and temperatures** must be strictly observed. The room temperature should be checked with a thermometer. A room temperature < 22°C can result in low values. **Attention: We advise not to use a heatable incubator.**

e) Mix samples well before use.

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-VDBP-complex.
Standards, controls and patient samples which are assayed for 25(OH)-vitamin D are prediluted with the releasing reagent and transferred to the microplate coated with 25(OH)-vitamin D. After an incubation to release the 25(OH) vitamin D, an anti-25(OH)-vitamin D antibody is added. During an 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

Bring all **reagents and samples to room temperature** (20–28°C) and mix well. **For this purpose, open the kit and remove the individual components needed.** Before use, gently mix reagents and samples avoiding foaming.

Mark the positions of standards/controls/samples on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips covered with the foil supplied together with the desiccant bag in the closed aluminium packaging at 2–8°C. Strips treated in this way can be used within 4 weeks.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact PromoCell.

We recommend to carry out the tests in duplicate.

Predilution of standards, controls and samples

1. Prepare the amount of polypropylene reaction tubes needed.
2. Pipet **20 µl of standards (STD/controls (CTRL A and CTRL B)/samples** respectively, into the corresponding tube.
3. Add **300 µl of releasing reagent (RELREAG)** into each tube. The transfer of the diluted samples to the microtiter plate has to be done within 5–10 minutes.

Instead of dilution in polypropylene reaction tubes, you can also dilute the samples in Deep Well® DSX dilution tubes (available as single tubes or 8 well strips). This offers the additional advantage that you can transfer the diluted samples with a multichannel pipet directly to the microtiter stripes used for the test.

Test procedure

1. Add quickly each **20 µl prepared standards/controls/samples** into the respective wells.
2. Cover the strips with the enclosed foil (FOL)and incubate **for 60 min at room temperature*** (20–28°C).
3. Add **150 µl anti 25(OH)-vitamin D antibody** (AB) into each well.
4. Cover the plate again tightly with the enclosed foil (FOL) and incubate **for 45min at room temperature*** (20–28°C).
5. Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper. For TECAN and Dynex instruments a programming protocol can be requested.
6. Add **200 µl conjugate** CONJ into each well.
7. Cover the plate again tightly with the enclosed foil (FOL) and incubate **for 45 min at room temperature*** (20–28°C).
8. Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.

9. Add **200 µl substrate** (SUB) into each well.
10. **Incubate for 10–15 minutes at room temperature*** (20–28°C) **in the dark.**
11. Add **50 µl stop solution** (STOP) into each well.
12. Determine **absorption** immediately with an ELISA reader at **450 nm**. If the highest extinction of the standards is above the range of the photometer, absorption must be measured immediately at **405 nm** and the obtained results used for evaluation. If possible, the extinctions from each measurement should be compared with extinctions obtained at a reference wavelength, e.g. 595nm, 620nm, 630nm, 650nm and 690nm can be used.

* **The optimal ambient temperature range is 22–25 °C.** All other temperatures result in strong deviations from the optical densities described in the QC data sheet.

Results

We recommend the 4-parameter algorithm for result calculation.

4-parameter algorithm

It is recommended a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.001).
The usage of the 4-parameter algorithm is strongly recommended. If it is not possible to use the 4-parameter algorithm for result calculation, it is possible to switch to a point-to-point calculation.

Point-to-point calculation

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

Measurement range

Samples with concentrations above the measurement range (> 525nmol/l) can be further diluted and re-assayed. Please consider this higher dilution when calculating the results.

Samples with 25(OH)-vitamin D concentrations higher than the highest standard should be diluted e.g. 1+1 with standard 1 (= 0nmol/l) (e.g. 50µl sample + 50µl standard 1) and re-assayed.

Samples with concentrations lower than the measurement range (= LoQ 20.4nmol/l) cannot be clearly quantified.

Whole blood is not suitable as a sample.

Biotin interference

Samples containing a biotin concentration of < 100 ng/ml show a change of the results of ≤ 25%. Higher concentrations of biotin can lead to falsely low results. Patients taking > 5 mg biotin per day should wait at least 24 hours after taking biotin to have their samples collected. Results of patients taking biotin supplements or receiving a high-dose biotin therapy should generally be interpreted along with the total clinical picture.

Quality Control

We recommend the use of external controls for internal quality control, if possible.
Control samples should be analysed 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.

Reference ranges for 25(OH) vitamin D_3

Information from ASBMR 2011

Deficiency (seriously deficient)	< 20 ng/ml or < 50 nmol/l
Insufficiency (deficient)	20–29 ng/ml or 50–74 nmol/l
Sufficiency (adequately supplied)	> 30 ng/ml or > 75 nmol/l

Source: Medscape (https://emedicine.medscape.com/article/2088694-overview)

Conversion factor

1 ng/ml = 2.5 nmol/l

1 nmol/l = 0.4 ng/ml

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, VDBP) and serve only as orientation.

Literature references

The following literature references for the vitamin D reference values can be found in the references on page 28: Grant et al., Soldin et al., Visser et al., Wicherts et al.

Performance Characteristics

Analytical sensitivity

The following values have been estimated based on the concentrations of the standard curve without considering possibly used sample dilution factors

Limit of blank, LoB	5.2 nmol/l	2.1 ng/ml
Limit of detection, LoD	11.9 nmol/l	4.8 ng/ml
Limit of quantitation, LoQ	20.4 nmol/l	8.2 ng/ml
Measuring range	20.4 – 525 nmol/l	8.2 – 210 ng/ml

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 20% CV.

NIST standard reference material (SRM) 972 traceable.

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefor, 25(OH)-vitamin D_3 spikes with known concentrations were added to 3 different serum samples. All samples were measured in triplicates on three days. The table shows the average expected values and obtained values.

Sample [nmol/l]	Spike [nmol/l]	Obtained [nmol/l]	Expected [nmol/l]	Recovery [%]
31.1	11	38.8	40.8	95.1
	22	46.9	50.2	93.4
	30	61.3	59.8	102.6
	60	91.4	88.2	103.6
	120	150.1	145.1	103.5
41.5	11	46.6	50.4	92.5
	22	56.4	59.3	95.1
	30	74.8	69.4	107.8
	60	107.1	97.3	110.1
	120	173.9	153.2	113.5
31.4	11	39.1	40.8	95.9
	22	43.1	50.2	85.8
	30	57.0	59.8	95.4
	60	86.8	88.2	98.3
	120	163.2	145.1	112.5

Accuracy – Precision

Repeatability (Intra-Assay); n=60

The repeatability was assessed according to CLSI guideline EP5-A2 with 2 serum samples under **constant** parameters (same operator, measurement system, day and kit lot).

Sample	Mean value [nmol/l]	CV [%]
1	96.8	9.1
2	37.2	12.5

Reproducibility (Inter-Assay); n=20

The reproducibility was assessed according to CLSI guideline EP5-A2 with 3 serum samples under **varying** parameters (different operators, measurement systems, days and kit lots).

Sample	Mean value [nmol/l]	CV [%]
1	40.8	13.7
2	106.3	10.8
3	83.6	8.2

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A with a serial dilution of 3 different serum samples on 3 days with 3 kit lots in quadruplicates. For this, the samples were diluted with standard 1 (0 nmol/l) und used in the test. The table shows the average expected values and obtained values.

For 25(OH)-vitamin D_3 in serum, the method has been demonstrated to be linear from 19.4 – 126.7, showing a non-linear behaviour of less than ±20% in this interval for concentrations greater than the limit of quantitation.

Sample	Dilution	Expected [nmol/l]	Obtained [nmol/l]	Recovery [%]
A	undiluted	115.6	115.6	100.0
	75 %	86.7	84.5	97.4
	50 %	57.8	55.9	96.6
	22 %	28.9	33.2	114.9
B	undiluted	121.2	121.2	100.0
	75 %	90.9	84.4	92.8
	50 %	60.6	64.0	105.6
	22 %	30.3	33.9	111.9
C	undiluted	109.9	109.9	100.0
	75 %	82.5	79.1	96.0
	50 %	55.0	54.4	99.0
	22 %	27.5	24.6	89.4

Analytical 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 D_3. The specificity is calculated in percent, based on the cross-reactivity of these compounds with the anti-25(OH)-vitamin D_3 antibody compared to the 25(OH)-vitamin D_3 antigen.

Substance tested	Concentration added [nmol/l]	Concentration obtained [nmol/l]	Cross reactivity [%]
25(OH)-vitamin D_3 (Calcidiol)	25.0	25.0	99.9
25(OH)-vitamin D_3 (Ergocalcidiol)	25.0	20.4	81.6
Vitamin D_3 (Ergocalciferol)	500.0	-0.4	-0.1
Vitamin D_3 (Cholecalciferol)	500.0	2.4	0.5

Analytical specificity

The potentially interfering substances listed below were added in the corresponding concentrations. There was no influence on the measuring signal observed.

Substances tested	**Concentration added [µg/ml]**	**Result**
Hemoglobin	300	No influence
Bilirubin	100	No influence
Triglycerides	4500	No influence
Vitamin C	100	No influence
Biotin	0.1	No influence

Precautions

- For *in vitro* research 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 or Proclin as bactericides. Sodium azide and Proclin 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. Do not breath vapour and avoid inhalation.

Technical Hints

- **Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis, even if they are of the same batch as wells from already opened microtiter plates are exposed to different conditions than sealed ones.**
- 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.
- Quality control guidelines 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 producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to PromoCell along with a written complaint.

References

1. Chapuy, M. C. et al. Healthy elderly French women living at home have secondary hyperparathyroidism and high bone turnover in winter. EPIDOS Study Group. The Journal of clinical endocrinology and metabolism 81, 1129–33 (1996).

2. Scharla, S. H. Prevalence of subclinical vitamin D deficiency in different European countries. Osteoporosis international 8 Suppl 2, S7–12 (1998).

3. Grant, W. B. & Holick, M. F. Benefits and requirements of vitamin D for optimal health: a review. Alternative medicine review : a journal of clinical therapeutic 10, 94–111 (2005).

4. Visser, M., Deeg, D. J. H., Puts, M. T. E., Seidell, J. C. & Lips, P. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. The American journal of clinical nutrition 84, 616–22; quiz 671–2 (2006).

5. Wicherts, I. S. et al. Vitamin D status predicts physical performance and its decline in older persons. The Journal of clinical endocrinology and metabolism 92, 2058–65 (2007).

6. Wielders, J. P. M. & Wijnberg, F. A. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. Clinical chemistry 55, 1584–5 (2009).

7. Soldin OP, Sharma H, Husted L, Soldin SJ. (2009) Pediatric reference intervals for aldosterone, 17alpha-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D_3 using tandem mass spectrometry. Clin Biochem. Jun;42(9):823-7.

Ordering Information

Product Name	Product Description	Size	Catalog Number
25-OH Vitamin D (direct) ELISA Kit, human	Human 25-OH Vitamin D (direct) ELISA Kit	96 Tests	PK-EL-K2109

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

PromoCell GmbH
Sickingenstr. 63/65
69126 Heidelberg
Germany

North America
Phone: 1 – 866 – 251 – 2860 (toll free)
Fax: 1 – 866 – 827 – 9219 (toll free)

Deutschland
Telefon: 0800 – 776 66 23 (gebührenfrei)
Fax: 0800 – 100 83 06 (gebührenfrei)

France
Téléphone: 0800 90 93 32 (ligne verte)
Téléfax: 0800 90 27 36 (ligne verte)

United Kingdom
Phone: 0800 – 96 03 33 (toll free)
Fax: 0800 – 169 85 54 (toll free)

Other Countries
Phone: +49 6221 – 649 34 0
Fax: +49 6221 – 649 34 40

Email: info@promokine.info