

1,25-(OH)₂-Vitamin D ELISA

*For the in vitro determination of 1,25-(OH)₂-vitamin D
in plasma and serum*

Valid from 2021-08-04

REF KR2112

Σ
48

+2°C
+8°C

RUO



Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany

Tel.: +49 6251 70190-0

Fax: + 49 6251 70190-363

e.mail: info@immundiagnostik.com

www.immundiagnostik.com

Table of Contents

1. INTENDED USE	2
2. INTRODUCTION	2
3. MATERIAL SUPPLIED	2
4. MATERIAL REQUIRED BUT NOT SUPPLIED	3
5. STORAGE AND PREPARATION OF REAGENTS	3
6. STORAGE AND PREPARATION OF SAMPLES	4
<i>Sample storage</i>	4
<i>Sample preparation</i>	4
<i>Preparation of sample extraction</i>	5
7. ASSAY PROCEDURE	6
<i>Principle of the test</i>	6
<i>Sample extraction</i>	6
<i>Pre-incubation</i>	7
<i>Test procedure</i>	7
8. RESULTS	9
9. LIMITATIONS	9
10. QUALITY CONTROL	9
<i>Reference range (plasma or serum)</i>	10
11. PERFORMANCE CHARACTERISTICS	10
<i>Precision and reproducibility</i>	10
<i>Analytical Sensitivity</i>	10
12. REGENERATION OF THE SILICA CARTRIDGES	10
13. PRECAUTIONS	11
14. TECHNICAL HINTS	11
15. GENERAL NOTES ON THE TEST AND TEST PROCEDURE	12
16. REFERENCES	13
<i>General literature</i>	13
<i>Literature using Immundiagnostik 1,25-(OH)₂-vitamin D ELISA</i>	13

1. INTENDED USE

This ELISA is intended for the quantitative determination of 1,25-dihydroxyvitamin D in serum and plasma. For research use only. Not for use in diagnostic procedures.

2. INTRODUCTION

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. The hormone 1,25-dihydroxyvitamin D (D hormone) is formed in a second hydroxylation step 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. 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.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KR2112	PLATE	Microtiter plate, pre-coated	12 x 8 wells
KR0001.C.100	WASHBUF	Wash buffer concentrate, 10x	1 x 100 ml
KR2112	ETHANOL	Ethanol, ready-to-use	1 x 1.5 ml
KR2112	TRIS-HCL	Tris-HCl buffer, ready-to-use	1 x 30 ml
KR2112	AB	Detection antibody, anti 1,25-(OH) ₂ -vitamin D, ready-to-use	1 x 25 ml
KR2112	STD 1-6	Standards, ready-to-use (see specification or label for range)	6 x 2.5 ml
KR2112	CTRL A	Controls, ready-to-use (see specification for range)	1 x 2.5 ml
KR2112	CTRL B	Controls, ready-to-use (see specification for range)	1 x 2.5 ml
KR2112	CONJ	Conjugate, polyclonal peroxidase-labelled antibody, ready-to-use	1 x 24 ml

Cat. No.	Label	Kit components	Quantity
KR0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	2 x 15 ml
KR0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml
KR2112	FOL	Foil to cover the microtiter plate	2 x 1

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultrapure water*
- 48 Chromabond columns (catalog no.: Se2112)
- 48 Silica Cartridges (solid phase extraction cartridges, catalog no.: Sb2221)
- Diisopropylether (p.A.) 99.0 %
- Isopropanol (p.A.) 99.9 %
- n-Hexan (p.A.) 98.3 %
- Methanol (p.A.) 99.9 %
- 75 x 12 mm glass tubes (no plastic)
- Extraction rack (catalog no.: K2221 sv), containing three plastic stands and one nitrogen distributor
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Horizontal microtiter plate shaker
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 g
- Vortex
- Vacuum centrifuge or nitrogen distributor
- Microtiter plate reader at 450 nm (reference wave length 620 or 690 nm)

* Immundiagnostik AG recommends the use of ultrapure 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).

5. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** The kit can be used up to 2 times within the expiry date 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 (100 ml WASHBUF +

900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37°C. 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 1 month**.

- Care must be taken when **preparing the solvent mixtures** because of the volume contraction:
 1. First, measure the solvents in separate measuring cylinders, then mix in a storage bottle.
 2. Use glass storage bottles and glass measuring cylinders; plastic vessels are not suitable.
- **Microtiter strips:** After opening the sealed aluminium packaging, unused strips have to be covered with adhesive foil and stored in the closed aluminium packaging together with desiccant at 2–8°C. We recommend not to assemble wells of different microtiter plates for analysis, even if they are of the same batch. Opened microtiter plates are exposed to different conditions than sealed ones.
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8°C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample storage

Freshly collected blood should be centrifuged **within one hour**. Vitamin D is an inert substance. The serum samples can be stored at room temperature. However, **serum** storage at **2–8°C** is recommended if the analysis is performed within 72 h after collection. Otherwise, the serum samples should be stored at **-20°C** until analysis.

Avoid repeated freeze-thaw cycles.

Serum samples can be shipped at **2–8°C** (e.g. with coolpacks) and remain stable for up to 3 days. If the serum samples are not analysed within this time period, it is recommended to freeze and store them at -20°C.

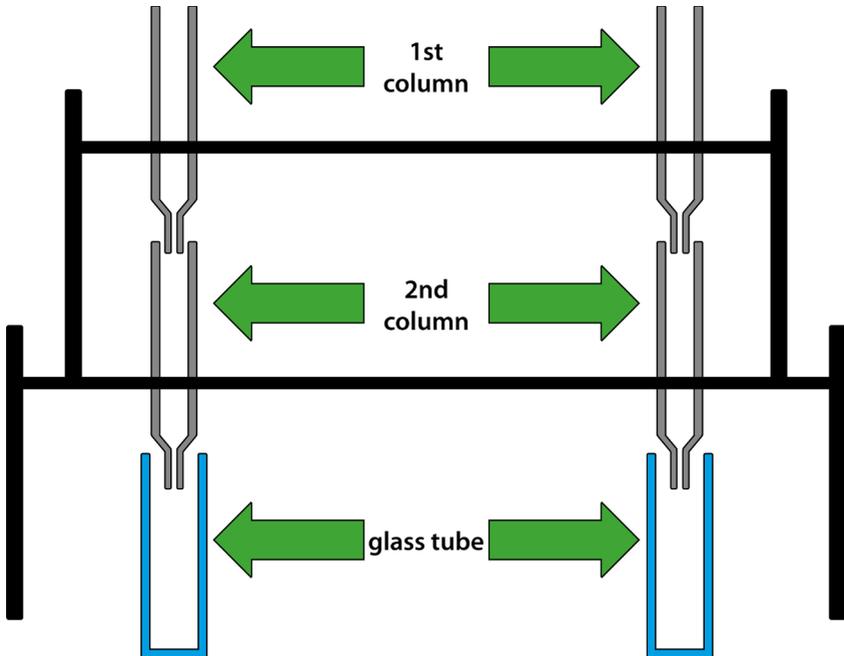
Sample preparation

Lipemic or haemolytic samples may give erroneous results. Samples should be mixed well before assaying. We recommend to carry out the tests in duplicate.

We recommend to apply **1000 µl sample** per cartridge. If the sample volume is less than 1000 µl, load an appropriate amount of Tris-HCl buffer into the column, then

add the sample (minimum 500 µl) for a total volume of 1000 µl. To do so, first apply 500 µl of Tris-HCl to the column, allow it to be completely absorbed, and then pipet 500 µl of sample. The obtained results have to be multiplied by the dilution factor used to get the actual concentrations.

Preparation of sample extraction



The extraction unit consists of three parts, which are put on top of each other.

The upper part is used for the chromabond columns (extraction rackRI), the lower part for the silica cartridges (extraction rackRII).

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 rackRI and extraction rackRII). After the first extraction step (ether), remove the extraction rackRI with the chromabond columns.

It is recommended to place the glass tubes (extraction rackRIII) directly under the cartridges (extraction rackRII) for the last elution step. The tubes can then be used directly for the next step of the assay.

7. ASSAY PROCEDURE

Principle of the test

This assay utilises a competitive enzyme immunoassay (EIA) technique with a selected monoclonal antibody recognizing 1,25-dihydroxyvitamin D.

Standards, controls and 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-dihydroxyvitamin D. During this incubation step, 1,25-dihydroxyvitamin D in the sample and a fixed amount of 1,25-dihydroxyvitamin 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-dihydroxyvitamin 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-dihydroxyvitamin 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-dihydroxyvitamin D in the samples is determined from this curve.

Sample extraction

1.	Apply 1000 µl of standards, 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 500 µl sample (results in factor 2 for the sample evaluation).
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).

During steps 3 and 4, ensure that the columns never run dry for longer than 5 min.

3.	Wash the silica cartridges (extraction rack II) with 5 x 2 ml isopropanol/hexane (4/96 v/v).
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4.	Wash the silica cartridges (extraction rack II) with 3 x 2 ml isopropanol/hexane (6/94 v/v) .
5.	After step 4, place the extraction rack unit II with the silica cartridges on the extraction rack unit III with the glass tubes. Note: The glass tubes (extraction rack unit III) should be placed directly under the silica cartridges.
6.	Elute 1,25-dihydroxy vitamin D from the silica cartridges with 2 x 2 ml isopropanol/hexane (25/75 v/v) .
7.	Evaporate the eluate under a nitrogen stream at 37 °C or in a vacuum centrifuge.
8.	Before starting the pre-incubations, allow the glass tubes to cool down to room temperature.

Pre-incubation

1.	Add 20 µl of ethanol into each glass tube. Immediately after adding ethanol, gently vortex each tube ~ 1 s to avoid any possible evaporation.
2.	Add 450 µl antibody solution into each glass tube. Mix thoroughly for at least 10 s.
3.	Cover the glass tubes with a plastic film and incubate for exactly 1 hour at room temperature.

Test procedure

Bring all **reagents and samples to room temperature** (18–26 °C) and mix well.

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 and together with the desiccant bag in the closed aluminium packaging at 2–8 °C. Strips are stable until expiry date stated on the label.

We recommend to carry out the tests in duplicate.

1.	Add 200 µl of standards/controls/samples in duplicate into respective well. All these solutions are viscous; pipet slowly and carefully. We recommend to rinse the inside of the pipet tip with the pre-incubate before pipetting it. The transfer of the samples to the microtiter plate should not take longer than 20 minutes.
2.	Cover the plate tightly and incubate for 18–22 hours at 6–10 °C* .
3.	Discard the content of each well and wash 5 times with 250 µl wash buffer . After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
4.	Add 200 µl conjugate (CONJ) into each well.
5.	Cover the plate tightly and incubate for exactly 1 hour at room temperature (18–26 °C) on a horizontal shaker**.
6.	Discard the content of each well and wash 5 times with 250 µl wash buffer . After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
7.	Add 200 µl substrate (SUB) into each well. Attention: Mind the time needed for adding the substrate. It should not take longer than 2 min. Adding the stop solution in the next step should span the same time to ensure an identical incubation time with substrate for all samples.
8.	Incubate for 15–25 min*** at room temperature (18–26 °C) in the dark .
9.	Add 50 µl stop solution (STOP) into each well, mix thoroughly. Keep the same pipetting order and time as in step 7.
10.	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 CVs. It is therefore recommended to use always the same incubation time, i.e. 20 hours.

** We recommend shaking the strips at 550 rpm with an orbit of 2 mm.

*** The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using 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 **standard** must be specified with a value less than 1 (e.g. 0.001).

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

The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the programme used, the duplicate values should be evaluated manually.

9. LIMITATIONS

Samples with 1,25-dihydroxyvitamin 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 to the pre-buffered chromabond column. Recalculate the results with the appropriate dilution factor, e.g.:

250 µl Tris-HCl + 750 µl sample results in factor 1.3

500 µl Tris-HCl + 500 µl sample results in factor 2

Samples with concentrations lower than the analytical sensitivity cannot be quantified clearly.

10. QUALITY CONTROL

Immundiagnostik AG recommends 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 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 range (plasma or serum)

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS*Precision and reproducibility***Intra-Assay (n = 20)**

Sample	1,25-dihydroxy-vitamin D [pg/ml]	CV [%]
1	55,3	6,69

Inter-Assay (n = 20)

Sample	1,25-dihydroxy-vitamin D [pg/ml]	CV [%]
1	39	9

Analytical Sensitivity

The sensitivity was set as $B_0 - 2SD$. The zero-standard was measured 20 times.

Sample	1,25-dihydroxyvitamin D Mittelwert [OD]	Standard deviation (SD)	Detection limit [pg/ml]
1	1,201	0,025	4,8

12. REGENERATION OF THE SILICA CARTRIDGES

The silica cartridges (lower columns) must be regenerated directly after sample extraction as follows:

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

Please note the following hints:

- Before next use, the silica cartridges have to be dry.
- Up to 5 regeneration cycles are possible.
- Use equally often regenerated columns in the same run.
- Store dried columns in plastic bags with drying agent at room temperature.

13. PRECAUTIONS

- All reagents in the kit package are for *research* use only.
- Human materials used in kit components were 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.
- Kit reagents contain sodium azide or ProClin as bactericides. Sodium azide and ProClin are toxic. Substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The 10x Wash buffer concentrate (WASHBUF) contains surfactants which may cause severe eye irritation in case of eye contact



Warning: Causes serious eye irritation

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical Advice/attention.

- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still should 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 up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

14. 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.
- Control samples should be analysed with each run.
- Reagents should not be used beyond the expiration date stated 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.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to the enclosed manual.

15. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The guidelines for 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 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 sent to Immundiagnostik AG along with a written complaint.

16. REFERENCES

General literature

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Literature using Immundiagnostik 1,25-(OH)₂-vitamin D ELISA

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Used symbols:

	Temperature limitation		Catalogue number
	For research use only		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by
	Attention		Consult instructions for use
	Consult specification data sheet		

Immundiagnostik AG

Stubenwald-Allee 8a
64625 Bensheim, Germany

Tel.: +49 6251 70190-0

Fax: +49 6251 70190-363

info@immundiagnostik.com

www.immundiagnostik.com

