



EAGLE
BIOSCIENCES

25-OH Vitamin D ELISA Assay Kit

Catalog Number: VID31-K01 (96 Wells)

For Research Use Only (RUO). Not for use in clinical, diagnostic or therapeutic procedures.

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INTRODUCTION

The Eagle Biosciences 25-OH Vitamin D ELISA assay kit is designed for the serological determination of the vitamin D concentration in the human organism types of vitamin D that are differentiated are vitamin D2 (ergocalciferol) that is contained in plant food (mushrooms, avocado) and vitamin D3 (cholecalciferol) that is produced from 7-dehydrocholesterol in the skin under ultra-violet irradiation or found in animal food or products (sea fish, egg yolk, butter). These two forms of vitamin D, which are not yet biologically active, are bound by a protein called VDBP (vitamin D binding protein) in the bloodstream, then metabolised in the liver and converted into 25-OH vitamin D2 (calcidiol) and 25-OH vitamin D3 (calcitriol), respectively, which are storage forms of the vitamin with little activity. In contrast to other commercially available tests, the Eagle Biosciences 25-OH Vitamin D ELISA assay kit uses a newly designed monoclonal antibody which is specific for both vitamin D2 and vitamin D3 at 100% specificity. This is necessary because sometimes vitamin D2 instead of D3 is used in therapy.

ASSAY PRINCIPLE

The new 25-OH Vitamin D ELISA assay test kit is designed for the determination of 25-OH Vitamin D in human serum or plasma samples. In the first analysis step, the calibrators and patient samples are diluted with biotin-labelled 25-OH vitamin D and added to microplate wells coated with monoclonal anti-25-OH Vitamin D antibodies. During the incubation an unknown amount of 25-OH Vitamin D in the patient sample and a known amount of biotin-labelled 25-OH vitamin D compete for the antibody binding sites in the microplate wells plate. Unbound 25-OH vitamin D is removed by washing. For the detection of bound biotin-labelled 25-OH vitamin D, a second incubation is performed using peroxidase-labelled streptavidin. In a third incubation using the peroxidase substrate tetramethylbenzidine (TMB) the bound peroxidase promotes a colour reaction. The colour intensity is inversely proportional to the 25-OH vitamin D concentration.

SAFETY PRECAUTIONS

- The 25-OH Vitamin D ELISA assay kit is for research use only and not to be used in clinical, therapeutic or diagnostic procedures.
- Some reagents of the 25-OH Vitamin D ELISA assay kit contain sodium azide as preservative (<0.1%). Avoid skin contact.
- All reagents of human origin used in this 25-OH Vitamin D ELISA assay kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.



REAGENTS PROVIDED AND PREPARATION

- On arrival, store the 25-OH Vitamin D ELISA assay kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.
- Do not use components beyond the expiration date shown on the kit labels.

1. **MT-Strips** 12 strips
8 wells per strip, single break apart
coated with monoclonal antibody (vitamin D2 and D3)

2. **Standards 1 – 6** 6 vials
1 ml each, colored red-brown; To be diluted 1:26 in working strength biotin.

Standard	1	2	3	4	5	6
ng/mL	0	4	10	25	60	120

3. **Control 1 + 2** 2 vials
1 ml each, colored red-brown, To be diluted 1:26 in working strength biotin.
values for the Control are given on the vial label

4. **Biotin** 1 vial
1.2 ml, 100 x concentrated; colored blue

5. **Sample Buffer** 1 vial
100 ml, colored yellow

6. **Enzyme Conjugate** 1 vial
12 ml, colored blue, ready for use

7. **Substrate** 1 vial
12 ml tetramethyl benzidine (TMB)/ H₂O₂, ready for use

8. **Wash Buffer** 1 vial
100 ml, 10 x concentrated

9. **Stop Solution** 1 vial
12 ml, ready for use
0.5M sulphuric acid

Additional materials and equipment required but not provided:

- Pipettes for 10 µl, 100 µl, 200 µl, 1 ml, and 4.5 ml
- Pure water
- Microtiter plate reader (450 nm/ 620 nm)



SPECIMAN COLLECTION AND STORAGE

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at 2°C to 8°C for up to 14 days. Severely haemolytic or lipemic serum samples should not be used.

Please note: Diluted samples should only be used for one test run and subsequently discarded. Always use fresh samples and calibrator dilutions for every test run. Always pipette samples and calibrators first, then add the working strength biotin within 5 minutes to the dilution tubes, particularly if the test is performed manually or if large sample series (> 20 samples) are analysed in order to avoid any drift effects.

Performance: The calibrators/controls and patient samples for analysis are diluted 1:26 in working strength biotin.

- Example:
 - Add 20 µl of sample (calibrators, controls, samples) to suitable dilution tubes
 - Add 0.5 ml diluted biotin to all tubes within 5 minutes and mix thoroughly (vortex).
 - Incubate the mixture for at least 10 minutes at room temperature (+18°C to +25°C).
 - The samples can subsequently be pipetted into the reagent wells according to the pipetting scheme.

TEST PROCEDURE

Preparation of Reagents

Note: All reagents of the 25-OH Vitamin D ELISA assay kit must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

Coated wells: Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag). Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

Calibrators and controls: The reagents must be mixed thoroughly before use. Calibrators and controls are to be diluted 1:26 in working strength biotin prior to use, see point 5 on page 7.

Biotin: The biotin is a 100x concentrate. Mix thoroughly before diluting. The required volume should be removed with a clean pipette tip and diluted in sample buffer (1 part biotin plus 99 parts sample buffer). Example: 1 ml biotin concentrate plus 99 ml sample buffer. The working-strength biotin is stable for 2 weeks when stored at +2 °C to +8 °C. For longer storage freeze at -20 °C.

Sample buffer: It can be used for sample dilution after adding the biotin concentrate.



Enzyme conjugate: Ready for use. The enzyme conjugate must be mixed thoroughly before use.

Wash buffer: The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water). The working-strength wash buffer is stable until the expiry date when stored at +2 °C to +8 °C and handled properly.

Chromogen/substrate solution: Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

Stop solution: Ready for use.

Storage and stability: The 25-OH Vitamin D ELISA assay kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The controls and calibrators contain serum of animal origin. Therefore, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact!

ASSAY PROCEDURE

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips to reach room temperature (at least 30 min), remove the number of strip wells required and fit them firmly into the frame provided. Controls must always be included in each assay run.

1. Pipette each 200 µl of prediluted samples into each well to be used in the assay
2. Pipette each 200 µl of prediluted Standards 1 - 6, prediluted Control 1 and Control 2 into the appropriate wells.
3. Incubate at room temperature (+18°C to +25°C) for 2 hours.
4. After the 2-hour incubation, aspirate or discard the samples from the wells, add 300 µl of Wash Buffer and aspirate or discard again. Repeat washing with each 300 µl Wash Buffer two more times for a total of three washings. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
5. Pipette 100 µl of Enzyme Conjugate into each well and incubate for 30 min at room temperature (+18°C to +25°C).
6. After the 30 minute incubation, aspirate or discard the reagent from the wells, add 300 µl of Wash Buffer and aspirate or discard again. Repeat washing with each 300 µl Wash Buffer

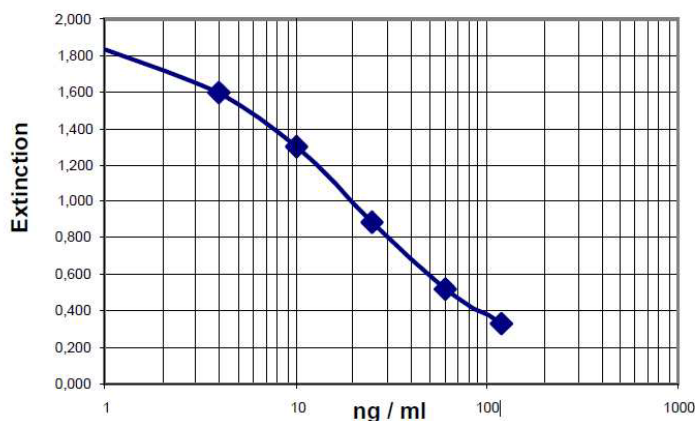


- two more times for a total of three washings. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
7. Pipette 100 μ l of diluted chromogen/ substrate solution into each well and incubate for 15 minutes at room temperature without shaking (protect from direct sunlight!).
 8. Stop the substrate reaction by addition of 100 μ l of Stop Solution to each well (this will cause the blue colour to turn yellow).
 9. Photometric measurement of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

CALCULATION

Quantitative: The standard curve from which the 25-OH vitamin D concentrations in the serum samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 6 calibration sera against the corresponding units (linear/log). Use "cubic spline" or "4-PL" plotting for calculation of the standard curve by computer. For correct logarithmic representation it might be necessary to set the concentration of calibrator 1 from 0 to e.g. 0.1 ng/ml. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of concentrations in samples.

Typical Standard Curve



If the extinction of a sample lies below the value of calibrator 6 (120 ng/ml), the result should be given as ">120 ng/ml". It is recommended that the sample be re-tested at a dilution of 1:2 with calibrator 1 before following the test instructions. The result in ng/ml read from the calibration curve for this sample must then be multiplied by a factor of 2.

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.



CHARACTERISTICS

Calibration

As there is no international standard for 25-OH Vitamin D, the standards and controls are calibrated gravimetrically using UV-Vis (264nm) verified stock standards and compared with NIST standards (National Institute of Standards and Technology, USA), DEQAS (Vitamin D External Quality Assessment Scheme, UK) quality assessment data and in-house quality control sera. For every group of tests performed, the values of the concentrations must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

Antibodies

The reagent wells of the 25-OH Vitamin D ELISA assay kit are coated with monoclonal antibodies which identify specifically 25-OH vitamin D3 and 25-OH vitamin D2.

Detection limit

The lower detection limit of the 25-OH Vitamin D ELISA assay kit is defined as the mean value of an analyte-free sample minus three times the standard deviation and is the smallest detectable 25-OH Vitamin D concentration. The detection limit of 25-OH Vitamin D ELISA is 1.6 ng/ml.

Cross reactivity

This 25-OH Vitamin D ELISA assay kit detects 25-OH Vitamin D2 and D3 specifically. Cross reactions with other metabolites are given in the following table.

	Cross reactivity (%)
25-OH Vitamin D3	100%
25-OH Vitamin D2	100%
Vitamin D3 (cholecalciferol)	< 0.03%
Vitamin D2 (ergocalciferol)	< 0.05%
24,25-OH Vitamin D3	< 0.3%

Interference

Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 5 mg/ml for hemoglobin, 5 mg/ml for triglycerides and 0.2 mg/ml for bilirubin in this ELISA.

Reproducibility

The reproducibility of the 25-OH Vitamin D ELISA assay kit was investigated by determining the intra- and interassay coefficients of variation using 3 sera from different areas of the calibration curve. The intra-assay CVs are based on 40 measurements for each serum and the inter-assay CVs on four measurements performed in six different test runs.



Intra-assay variation, n = 40		
Serum	Mean value (ng/ml)	CV (%)
1	10.8	4.9
2	24.6	6.9
3	64.1	3.2

Inter-assay variation, n = 4 x 10		
Serum	Mean value (ng/ml)	CV (%)
4	16.6	7.8
5	43.5	7.0
6	67.8	8.6

Linearity

The linearity of the 25-OH Vitamin D ELISA assay kit was investigated by diluting three samples with calibrator 1 and determining the concordance. The average concordance amounted to 98% (85-117%).

Sample	Dilution	Measured Value (ng/ml)	Expected Value (ng/ml)	Concordance (%)
1	native	70.7		
	1:2	37.2	35.4	105
	1:4	19.7	17.7	111
	1:8	9.4	8.8	106
	1:16	4.6	4.5	103
2	native	86.4		
	1:2	45.7	43.2	106
	1:4	23	21.6	106
	1:8	11.5	10.8	106
	1:16	5.4	5.4	99
3	native	100.2		
	1:2	51.6	50.1	103
	1:4	28.8	25.1	115
	1:8	13.6	12.5	109
	1:16	7.1	6.3	112

Reference Range

359 plasma samples from apparently healthy blood donors in the age range of 13 to 99 years old were investigated using the ELISA. The mean 25-OH vitamin D concentration was 20.9 ng/ml with a 5-95% percentile range of 8.2 to 37.4 ng/ml.

25-OH vitamin D concentration	Amount (n = 359)	ng/ml
Very severe vitamin D deficiency	4	<5
Severe vitamin D deficiency	29	5-10
Vitamin D deficiency	147	10-20
Suboptimal vitamin D provision	126	20-30
Optimal vitamin D level	49	30-50
Upper norm	4	50-70
Overdose, but not toxic	0	70-150
Vitamin D intoxication	0	>150
Mean Value		20.9
5 – 95 % percentile		8.2 – 37.4
2.5 – 97.5 % percentile		6.9 – 44.4



Every laboratory should use their own normal values established under specific ambient conditions.

Calculation

25-OH Vitamin D3 (ng/mL) x 2.5 = 25-OH Vitamin D3 (nmol/L)

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- 5) Hollis Editorial: The Determination of circulating 25-Hydroxyvitamin D: No Easy Task BW J *Clin Endocrinol Metab*, July 2004, 89(7):3149–3151
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Pipetting Scheme

	B0	Standards	Positive Controls	Patients
*Standard 1 μl	200			
*Standards 2 - 6 μl		200		
*Control 1 - 2 μl			200	
*Patient sample diluted in biotin/ sample buffer μl				200

Cover and incubate for 2 hours at RT

Aspirate / discard and wash once with each 300 μl Wash Buffer

Enzyme Conjugate μl	100	100	100	100
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Incubate for 30 min at RT

Aspirate / discard and wash once with each 300 μl Wash Buffer

TMB-Substrate μl	100	100	100	100
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Incubate for 15 minutes at RT in the dark

Stop Solution μl	100	100	100	100
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Slightly shaking on an ELISA plate shaker to ensure homogeneous distribution of the solution

Reading of absorbance at 450 nm/ 620nm

* prediluted 1:26 in biotin/sample buffer



Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.