New 25-OH Vitamin D total ELISA: a fast and straightforward competitive Elisa for the quantification of 25-OH Vitamin D2/3 (total) in human serum and plasma.

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Introduction

Vitamin D is a steroid hormone involved in the intestinal absorption of calcium and the regulation of calcium homeostasis. The two major forms of Vitamin D, named Vitamin D3 (cholecalciferol) and Vitamin D2 (ergocalciferol), have isomeric structures, but D2 is supposed to be less active than D3 [1].

Physiological Vitamin D3 levels result not only from dietary uptake but can also be produced from a cholesterol precursor, 7-dehydrocholesterol, in the skin during sun exposure. D2 is obtained from plant sources and only represents less than 5% of the total Vitamin D in the body [2]. In the liver, the Vitamin D is hydroxylated to 25-hydroxyvitamin D (25-OH D), the major circulating metabolite of Vitamin D. Vitamin D and 25-OH D enter the circulation bound to Vitamin D binding protein (VDBP). Upon request, a small portion of 25-OH D is further hydroxylated in the kidney to form the biologically active hormone 1,25 dihydroxyvitamin D (1,25-(OH)2 D) [3]. This process is tightly regulated by the concentration of 1,25-(OH)2 D, parathyroid hormone, hypophosphatemia and ionized calcium levels. Concentrations of 1,25-(OH)2 are about 1000-fold lower than that of 25-OH D [4]. Although 1,25-(OH)2 D portrays the biological active form of Vitamin D, it is widely accepted that the measurement of circulating 25-OH D provides better information with respect to patients Vitamin D status and allows its use in diagnosis of hypovitaminosis [5].

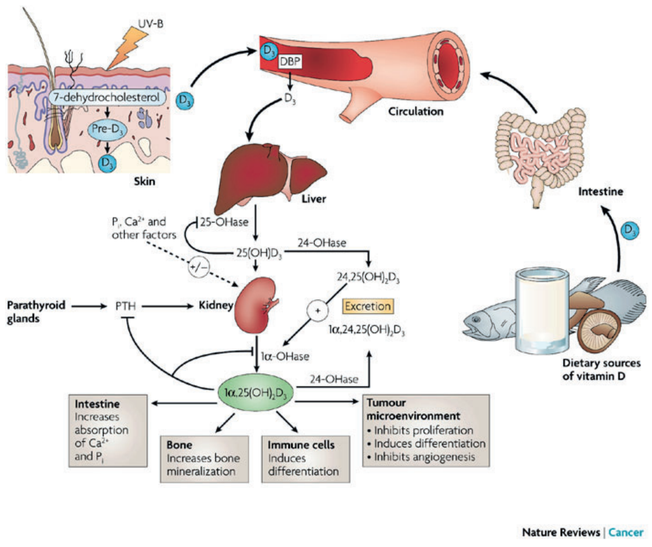
Physiological effects of 1,25-(OH)2 include increase of bone mineralization, induction of immune cell differentiation, and increase of calcium and phosphate. Furthermore, preclinical research indicates that anti-proliferative effects of 1,25-(OH)2 and vitamin D analogues might indicate potential as anticancer agents [6]. The concentration of 25-OH D decreases during winter time (reduced sun exposure), with dark skin colour and with age [7].

Determination of 25-OH D in serum or plasma will support the diagnosis and therapy control of postmenopausal osteoporosis, rickets in children, osteomalacia, renal osteodystrophy, neonatal hypocalcemia and hyperparathyroidism. In addition, the effects of prevailing subclinical Vitamin D deficiency in different European countries are critically discussed [7]. Vitamin D intoxication mostly occurs during a large intake of pharmaceutical preparations of Vitamin D and may lead to hypercalcemia and nephrocalcinosis in susceptible infants.

Here we present a competitive ELISA for the quantification of total 25-OH Vitamin D in human serum and plasma.

Benefits of the assay are ready-to-use reagents, a total assay time < 2 hours, and a very straight forward procedure for release of vitamin D (no precipitation or centrifugation).

Summary Assay Characteristics:	
Sample Volume:	25 µl Serum or Plasma
Incubation Times:	30 min + 60 min + 15 min
Total Assay Time:	approx. 2 hours
Measuring Range:	2.89 – 130 ng/mL
Analytical Sensitivity:	2.89 ng/mL
Mean Intra-Assay Precision:	4.7 %
Mean Inter-Assay Precision:	9.7 %
Mean Recovery:	100.6 %
Mean Linearity:	98.9 %



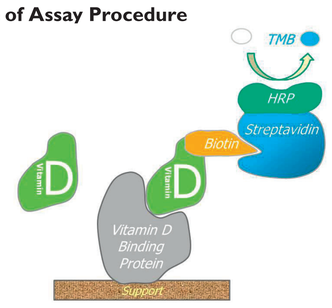
Courtesy of K.K. Deeb et al. Nature Reviews Cancer 7, 2007, 684-700.

Assay Procedure

The **DRG 25-OH Vitamin D total** ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.

In the first step, samples have to be pretreated in separate vials with denaturation buffer to extract the analyte, since most circulating 25-OH Vit D is bound to VDBP in vivo. After neutralization, biotinylated 25-OH Vitamin D (enzyme conjugate) and peroxidase-labeled streptavidin- (enzyme complex) are added. After careful mixing, the solution is transferred to the wells of the microtiter plate. Endogenous 25-OH Vitamin D of a patient sample competes with a 25-OH Vitamin-D_3-biotin conjugate for binding to the VDBG that is immobilized on the plate. Binding of 25-OH Vitamin D-biotin is detected by peroxidase-labeled streptavidin. Incubation is followed by a washing step to remove unbound components. The color reaction is started by addition enzyme substrate and stopped after a defined time. The colour intensity is inversely proportional to the concentration of 25-OH Vitamin D in the sample.

Scheme of Assay Procedure



Test Procedure

Release Procedure and Pretreatment

1. Secure the desired number of appropriate vials or uncoated plates for the Vitamin D release step.
2. Dispense 25 µL of each Standard, Control and sample into the vials.
3. Dispense 50 µL Denaturation Buffer into each vial.
4. Seal vials and incubate for 30 minutes at 37 °C.
5. Add 200 µL of Neutralization Buffer to each vial.
6. Add 50 µL of Enzyme Conjugate to each vial.
7. Add 50 µL of Enzyme Complex to each vial.
8. Thoroughly mix for 10 seconds. Use 200 µL of this mixed solution for the ELISA.

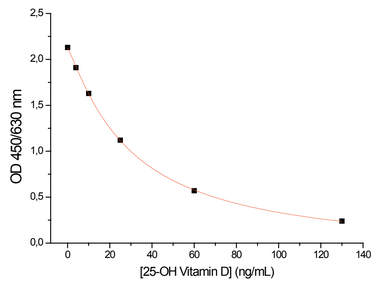
ELISA Procedure

1. Secure the desired number of Microtiter wells in the frame holder.
2. Transfer 200 µL of the mixed solution of each Standard, Control and sample into the appropriate wells.
3. Seal wells carefully and incubate for 60 minutes at 37 °C.
4. Rinse the wells 4 times with diluted Wash Solution (300 µL per well).
5. Add 200 µL of Substrate Solution to each well.
6. Incubate for 15 minutes at room temperature.
7. Stop the enzymatic reaction by adding 100 µL of Stop Solution to each well.
8. Determine the absorbance (OD) of each well at 450 ± 10 nm with a microtiter plate reader.

Results

The Elisa allows the quantitative determination of 25-OH vitamin D (total) covering a measuring range form 4 - 130 ng/mL.

Standard Curve



Analytical Sensitivity: The analytical sensitivity of the assay is 2.89 ng/ml.

Intra-Assay Precision

The test shows good reproducibility with a mean intra-assay precision of 4.7% (mean of 20 repeated measurements of 3 different samples).

Sample	n	Mean Concentration (ng/mL)	CV (%)
1	20	25.1	4.4
2	20	43.2	3.0
3	20	93.7	6.6
Mean	60		4.7

Inter-Assay Precision

The mean inter-assay precision is 9.7% (average of 40 repeated measurements of 3 different samples by two observers on 20 days with 2 different lots).

Sample	n	Mean Concentration (ng/mL)	CV (%)
1	40	23.7	9.9
2	40	47.8	10.7
3	40	64.2	8.6
Mean	120		9.7

Linearity of Dilution

Mean linearity (determined by 1:2 to 1:16 dilution of 3 samples with high 25-OH Vitamin D concentration in Zero Standard) was 98.9% (n=12; range from 85.8-102.8%).

	Sample 1	Sample 2	Sample 3
Concentration (ng/mL)	104.8	69.4	88.7
Average Recovery (%)	107.5	90.8	98.4
Min Recovery (%)	105.7	85.8	94.7
Max Recovery (%)	109.2	97.8	103.3
Mean Recovery	98.9		

Recovery

Mean recovery after spiking (determined by addition of S1-S5 to 3 sera with defined concentration of 25-OH Vitamin D) was 100.6% (n=12; range from 88.2 – 105.2%).

	Sample 1	Sample 2	Sample 3
Concentration (ng/mL)	36.4	60.5	82.7
Average Recovery (%)	104.1	105.2	92.5
Min Recovery (%)	92.9	96.9	88.2
Max Recovery (%)	112.4	111.3	94.1
Mean Recovery (%)	100.6		

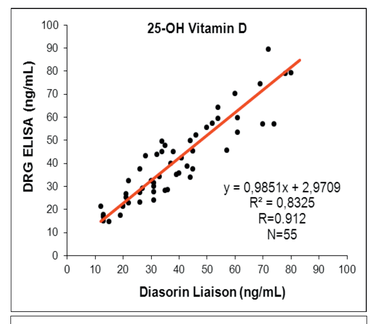
Cross reactivity

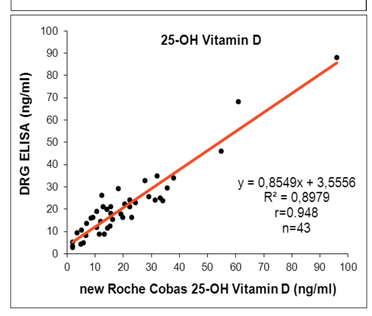
Cross reactivity was estimated by comparison of the concentration yielding 50% inhibition:
25-OH Vitamin D_2: 74.7%
Vitamin D_3: 3.8 %
Vitamin D_2: 3.3 %
1,25-Dihydroxy Vitamin D_3: 0.9%

Hemoglobin (up to 1 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) had no influence on the assay results.

Method Comparison

The activity of the 25-OH Vitamin D total ELISA was determined by comparison with the Diasorin Liaison (r=0.912; n=55) and the new Roche Cobas total 25-OH Vitamin D RIA (r=0.948; n=43).





Normal values

In a study conducted with apparently normal healthy Caucasian adolescents and adults, using the DRG 25-OH Vitamin D ELISA the following values were observed:

Population	Valid N	Age (Years)	Mean Age	Mean Conc. (ng/mL)	Median (ng/mL)	5th Percentile (ng/mL)	95th Percentile (ng/mL)
Males	77	14 - 79	58	26.1	23.7	11.7	46.8
Females	82	17 - 79	55	30.2	25.2	14.3	56.9

Samples were collected in Germany during the month of September.

64.1% (102/159) of the samples showed 25-OH Vitamin D concentrations below 30 ng/mL, and 31.6% (50/159) below 20 ng/mL.

A review of the literature suggests the following ranges for the classification of 25-OH Vitamin D status [3]:

Vitamin D status	25-OH Vitamin D (ng/mL)	25-OH Vitamin D (nmol/L)
Deficiency	< 10	< 25
Insufficiency	10 – 29	25 – 72.5
Sufficiency	30 – 100	75 – 250
Toxicity	> 100	> 250

Conclusions:

- The quantification of 25-OH Vitamin D from serum and plasma with DRG´s sandwich ELISA will provide standardized and reliable results within 2 hours.
- A good correlation was found for 25-OH Vitamin D between the DRG ELISA and the Diasorin Liaison as well as the new Roche Cobas total 25-OH Vitamin D RIA.
- The DRG 25-OH Vitamin D total ELISA is easy to perform without need of extraction or centrifugation
- All reagents are ready-to-use and can be stored at 2-8 °C for one year.

References:

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