



AccuDiag™
25 – OH Vitamin D (total)
ELISA Kit

Cat# 2065-6



Test	25 OH Vitamin D (total) Elisa
Method	Enzyme Linked Immunosorbent Assay
Detection Range	2.89 130 ng/mL
Sample	25 l serum
Total Time	~ 105 min.
Shelf Life	12 Months from the manufacturing date
Specificity	100%
Sensitivity	>2.89 ng/mL

INTENDED USE

The Diagnostic Automation ,Inc. 25-OH Vitamin D (total) ELISA is an enzyme immunoassay for the quantitative *in vitro diagnostic* measurement of total 25-OH Vitamin D (Vitamin D2 and Vitamin D3) in serum and plasma.

SUMMARY AND EXPLANATION

Vitamin D is a steroid hormone involved in the intestinal absorption of calcium and the regulation of Vitamin D2 (ergocalciferol), have isomeric structures, but D2 is supposed to be less active than D 3 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. D 2 is obtained from plant sources and only represents less than 5% of the total Vitamin D in the body. 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). 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. 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. The concentration of 25-OH D decreases during winter time (reduced sun exposure), with dark skin color and with age. 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 is critically

discussed. 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.

TEST PRINCIPLE

The 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-D3-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 color intensity is inversely proportional to the concentration of 25-OH Vitamin D in the sample.

SPECIMEN COLLECTION AND PREPARATION

Serum or plasma (EDTA-, heparin- or citrate plasma) can be used in this assay. Do not use haemolytic, icteric or lipaemic specimens. Please note: Samples containing sodium azide should not be used in the assay.

Specimen Collection

Serum:

Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.

Plasma:

Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

Specimen Storage and Preparation

Specimens should be capped and may be stored for up to 3 days at 2 °C to 8 °C prior to assaying. Specimens held for a longer time (up to two months) should be frozen only once at -20 °C prior to assay. Thawed samples should be inverted several times prior to testing.

Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with Standard 0 and reassayed as described in Assay Procedure. For the calculation of the concentrations this dilution factor has to be taken into account.

Example:

- a. dilution 1:10: 10 µ L sample + 90 µ L Standard 0 (mix thoroughly)
- b. dilution 1:100 10 dilution a. 1:10 + 90 µ L Standard 0 (mix thoroughly)

MATERIALS AND COMPONENTS

Materials provided with the test kits

- 1. **Microtiterwells:** 12 x 8 (break apart) strips, 96 wells; Wells coated with Vitamin D binding protein (VDBG).
- 2. **Standard (Standard 0-5):** 6 vials, 1 mL, ready to use; Concentrations: 0 – 4 – 10 – 25 – 60 – 130 ng/mL Conversion: 1 ng/mL = 2.5 nmol/L.



The standards are calibrated against the NIST Standard Reference Material (SRM) 2972; Contain non-mercury preservative.

- Control Low & High:** 2 vials, 1 mL each, ready to use; For control values and ranges please refer to vial label or QC-Datasheet. Contain non-mercury preservative.
 - Denaturation Buffer:** 1 vial, 10 mL, ready to use
 - Neutralization Buffer:** 1 vial, 25 mL, ready to use, Contains non-mercury preservative.
 - Enzyme Conjugate:** 1 vial, 7 mL, ready to use, Vitamin D3 conjugated to biotin; Contains non-mercury preservative.
 - Enzyme Complex:** 1 vial, 7 mL, ready to use, Streptavidin-Peroxidase Conjugate Contains non-mercury preservative.
 - Substrate Solution:** 1 vial, 25 mL, ready to use, Tetramethylbenzidine (TMB).
 - Stop Solution:** 1 vial: 14 mL, ready to use, contains 0.5 M H₂SO₄, Avoid contact with the stop solution. It may cause skin irritations and burns.
 - Wash Solution:** 1 vial, 30 mL (40X concentrated), see „Preparation of Reagents“.
 - Cover Foil:** 1 sheet of adhesive cover foil
- Note:** Additional Standard 0 for sample dilution is available upon request.

Materials required but not provided

- Vials for the Vitamin D release step (e.g. rack with 96 vials, 0.65 mL each, 5 pieces;
- Single vials (e.g. single vials, 0.65 mL each, 1000 pieces)
- Incubator 37 °C (98.6 °F)
- A microtiter plate calibrated reader (450 ± 10 nm)
- Calibrated variable precision micropipettes.
- Absorbent paper.
- Distilled or deionized water
- Timer
- Semi logarithmic or linear graph paper or software for data reduction

PREPARATION OF REAGENTS

Bring all reagents and required number of strips to room temperature prior to use.

Wash Solution

Add deionized water to the 40X concentrated Wash Solution. Dilute 30 mL of concentrated *Wash Solution* with 1170 mL deionized water to a final volume of 1200 mL.
The diluted Wash Solution is stable for 2 weeks at room temperature.

Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheet.

TEST PROCEDURE

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

Each run must include a standard curve.
 All standards, samples, and controls should be run in duplicate. All standards, samples, and controls should be run concurrently so that all conditions of testing are the same.

Release Procedure and Pretreatment

- Secure the desired number of appropriate vials or uncoated plates for the Vitamin D release step (not included in the kit).
- Dispense 25 µ L of each Standard, Control and sample with new disposable tips into the vials.
- Dispense 50 µ L Denaturation Buffer into each vial.
- Seal vials and incubate for 30 minutes at 37°C.
- Add 200 µ L of Neutralization Buffer to each vial.
- Add 50 µ L of Enzyme Conjugate to each vial.
- Add 50 µ L of Enzyme Complex to each vial.
- Thoroughly mix for 10 seconds. It is important to have a complete mixing of the solution in this step. Use 200 µ L of this mixed solution for the ELISA.

ELISA Procedure

- Secure the desired number of Microtiter wells in the frame holder.
 - Transfer **200 µL** of the mixed solution of each **Standard, Control and sample** with new disposable tips into the appropriate wells. (E.g. if in Release Procedure and Pretreatment a rack with 96 vials/wells is used, an 8-channel pipette can be used for the transfer.)
 - Seal wells carefully and incubate for 60 minutes at 37°C.
 - Briskly shake out the contents of the wells. Rinse the wells **4 times** with diluted *Wash Solution* (300 µ L per well). Strike the wells sharply on absorbent paper to remove residual droplets.
- Important note:**
 The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
- Add **200 µL** of **Substrate Solution** to each well.
 - Incubate for **15 minutes** at room temperature.
 - Stop the enzymatic reaction by adding **100 µL** of **Stop Solution** to each well.
 - Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader. It is recommended that the wells be read **within 10 minutes** after adding the Stop Solution.

RESULTS

- Calculate the average absorbance values for each set of standards, controls and patient samples.
- Using semi-logarithmic or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
- Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
- Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
- The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as > 130 ng/mL. For the calculation of the concentrations this dilution factor has to be taken into account.

Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Units (450nm)
Standard 0 (0 ng/mL)	2.13
Standard 1 (4 ng/mL)	1.91
Standard 2 (10 ng/mL)	1.63
Standard 3 (25 ng/mL)	1.12
Standard 4 (60 ng/mL)	0.57
Standard 5 (130 ng/mL)	0.24



EXPECTED NORMAL VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

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

	N	Age	Mean Age	Mean Conc.	5.Percentile (ng/mL)	(ng/mL)
Males	77		58	26.1	11.7	46.8
Females	82		55	30.2	14.3	56.9

Samples were collected in the month of September.

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

A review of the literature suggests the following ranges for the classification of 25-OH Vitamin D status.

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

QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.

The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.

It is also recommended to make use of national or international Quality Assessment programs like the international Vitamin D Quality Assessment Scheme (DEQAS) in order to ensure the accuracy of the results. Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.

PERFORMANCE CHARACTERISTICS

Assay Dynamic Range

The range of the assay is between 2.89-130 ng/mL.

Specificity of Antibodies (Cross Reactivity)

The following substances were tested for cross reactivity of the assay:

25-OH Vitamin D ₃ :	100.0%
25-OH Vitamin D ₂ :	74.7%
1,25 (OH) ₂ Vitamin D ₃ :	< 0.1 %
Vitamin D ₃ :	3.6%

Sensitivity

The analytical sensitivity of the DAI ELISA was calculated by subtracting 2 standard deviations from the mean of 20 replicate analyses of the Zero Standard (S0) and was found to be <2.89 ng/mL.

Reproducibility

Intra Assay

The within assay variability is shown below:

Sample	N	Mean (ng/mL)	CV (%)
1	20	25.1	4.4
2	20	43.2	3.0
3	20	93.7	6.6

Inter Assay

The between assay variability is shown below:

Sample	N	Mean (ng/mL)	CV (%)
1	20	23.7	9.9
2	20	47.8	10.7
3	20	64.2	8.6

Recovery

Samples have been spiked by adding 3 solutions with known concentrations in a 1:1 ratio.

The % recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100 (expected value = (endogenous value + added value) / 2; because of a 1:2 dilution of serum with spike material).

	Sample	Sample 2	Sample 3
Concentration [IU/mL]	60.5	36.4	82.6
Average Recovery	105.2	104.1	92.5
Range Recovery [%] From	96.9	92.9	88.2
Range Recovery [%] To	111.3	112.4	94.1

Linearity

	Sample	Sample 2	Sample 3
Concentration [IU/mL]	104.8	69.4	88.7
Average Recovery	114.8	104.6	98.4
Range Recovery [%] From	105.7	85.8	94.7
Range Recovery [%] To	109.2	97.8	103.3

LIMITATIONS OF PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.

Any improper handling of samples or modification of this test might influence the results.

Interfering Substances

Haemoglobin (up to 4 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) have no influence on the assay results.

Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of 25-OH Vitamin D in a sample.

High-Dose-Hook Effect

No hook effect was observed in this test.



Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.

The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DAI.

Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under Reliability. Any laboratory result is only a part of the total clinical picture of a patient.

Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.

The test result itself should never be the sole determinant for deriving any therapeutic consequences.

Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.

Claims submitted due to customer misinterpretation of laboratory results subject to Therapeutic Consequences, are also invalid. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

PRECAUTIONS

1. This kit is for in vitro diagnostic use only. For professional use only.
2. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
3. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
6. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
7. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
8. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
9. Allow the reagents to reach room temperature (21 °C – 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
10. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
11. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
12. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
13. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
14. Do not use reagents beyond expiry date as shown on the kit labels.

15. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
16. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
17. Avoid contact with Stop Solution containing 0.5 M H₂SO₄. It may cause skin irritation and burns.
18. Some reagents contain Proclin 300, BND and/or MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
19. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
20. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
21. For information on hazardous substances included in the kit please refer to Safety Data Sheets. Safety Data Sheets for this product are available upon request directly from DAI.

STORAGE

1. When stored at 2 °C to 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
2. Opened reagents must be stored at 2 °C to 8 °C. Microtiter wells must be stored at 2 °C to 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again.
3. Opened kits retain activity for two months if stored as described above.

REFERENCES

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Diagnostic Automation / Cortez Diagnostics, Inc.
I M M U N O D I A G N O S T I C S

ISO 13485
 ISO 9001



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Date	Cat # 2065-6
Adopted	AccuDiag™- 25-OH Vitamin D (total) ELISA - 2015
EC REP	CEpartner4U, Esdoornlaan 13, 3951DB Maarn. The Netherlands. www.cepartner4u.eu
Revision Date: 2014-04-01	