Diagnostics Biochem Canada Inc.
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25-HYDROXYVITAMIN D [25(OH)D] ELISA

EU: CE IVD CAN: IVD USA: For Research Use Only. Not For Use in Diagnostic Procedures.

REF: CAN-VD-510

Version: 7.1 (COMB)
Effective: October 17, 2023

INTENDED USE

For the quantitative determination of 25-hydroxyvitamin D [25(OH)D] in human serum and plasma by an enzyme immunoassay.

PRINCIPLE OF THE TEST

This kit measures the total concentration of both 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 (25(OH)D). The results are expressed in ng/mL.

DBC's immunoassay of 25(OH)D is a sequential competitive assay that uses two incubations, with a total assay incubation time of less than two hours. During the first incubation, unlabelled 25(OH)D (present in the standards, controls, serum and plasma samples) is dissociated from binding proteins such as vitamin D binding protein and binds to the anti-25(OH)D antibody immobilized on the microplate wells. A washing step is performed next. During the next incubation, the complex of 25(OH)D-biotin conjugate and streptavidin-HRP conjugate competes with antibody-bound 25(OH)D for antibody binding sites. The washing and decanting procedures remove any unbound materials. The TMB substrate is added next which reacts with HRP to form a coloured product. The intensity of the colour is proportional to the amount of immobilized HRP. Stopping solution is added next which stops the colour development reaction. The optical density of each well is measured in a microplate reader. The absorbance values are inversely proportional to the concentration of 25(OH)D in the sample. A set of calibrators is used to plot a standard curve from which the concentrations of 25(OH)D in the samples and controls can be directly read.

CLINICAL APPLICATIONS

Vitamin D concentration in blood should be measured regularly to ensure that satisfactory physiological levels are maintained year round (see references). Vitamin D is assimilated from food sources (both vitamin D2 and vitamin D3) or produced in the skin by sun exposure (vitamin D3). The body stores both vitamin D2 and vitamin D3 mainly in the form of 25-hydroxyvitamin D2 or 25-hydroxyvitamin D3 respectively. Therefore, the best approach to assess the physiological levels of vitamin D is to analyze the total concentration of both hydroxylated forms [25(OH)D].

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Each laboratory is suggested to establish its own internal QC materials and procedure for assessing the reliability of results.
3. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
4. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
5. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
6. All kit reagents and specimens should be at room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and serum and plasma specimens.
7. Avoid exposing kit reagents, serum and plasma specimens to light.
8. A calibrator curve must be established for every run. The kit controls should be included in every run and fall within established confidence limits.
9. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
10. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
11. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the presence of a blue colour, in which case it should not be used.
12. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
13. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
14. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges. The performance of this assay is markedly influenced by the correct execution of the washing procedure.
15. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. This kit is specifically designed and validated for the determination of 25(OH)D in human serum and plasma. The kit is not validated for the determination of 25(OH)D in other specimens of human or animal origin.
2. The 25(OH)D level depends on multiple factors. Therefore, only carefully prepared serum or plasma samples are suitable for this test. Do not use grossly haemolysed, lipaemic, icteric serum or plasma, and any sample that was not handled properly according to the instructions.
3. Bacterial contamination, prolonged exposure to light or high temperatures, damage during transportation, repeated freeze and thaw cycles may affect the assay results.
4. The interpretation of the results should recognise the conditions that can affect vitamin D levels, such as medications, food supplements or extreme exposure to sun light or UV rays.
5. Modification of the test procedure, exchange of reagents from different lots, use of reagents after their expiry date, exposure of reagents to intense light or improper transportation of the product can negatively affect the results and the validity of the test.
6. The results obtained with this kit should not be used as the sole basis for clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient's background including the frequency of exposure to animal products. If false results are suspected, such as unusually elevated values, samples should be tested using an alternative method.
7. Any sample result greater than 160 ng/mL should not be diluted further. Rather, the sample should be reported as >160 ng/mL.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

All reagents in this kit should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen. Human material used in the preparation of this product has been tested and found negative for the presence of HIV I / II, Hepatitis B surface antigen, HCV (NAT), HIV-1 (NAT) and RPR by FDA approved methods. Notwithstanding, the reagents should be handled as if capable of transmitting infections and in accordance with good laboratory practices.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

A minimum of 0.05 mL of serum or plasma is required per duplicate determination. Appropriate sample collection is essential for the accurate determination of 25(OH)D.

Serum: Collect 4–5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date.

Plasma: Collect 4–5 mL of blood into EDTA plasma tubes. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Single and multi-channel pipettes and disposable tips
2. Distilled or deionized water
3. Disposable glass test tubes or glass bottles
4. Microplate absorbance reader equipped with a 450 nm filter

REAGENTS PROVIDED

1. Anti-25(OH)D Antibody Coated Break-Apart Well Microplate — Ready To Use

Contents: One 96-well antibody coated microplate in a resealable pouch with desiccant.
Storage: Refrigerate at 2–8°C.
Stability: 12 months or as indicated on label.

2. 25(OH)D-Biotin Conjugate Concentrate — Requires Preparation X100

Contents: One glass bottle containing 25-hydroxyvitamin D-Biotin conjugate in a stabilizer with a non-mercury preservative.
Volume: 1.0 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: See preparation of working conjugate solution section.

3. Streptavidin-HRP Conjugate Concentrate — Requires Preparation X100

Contents: One plastic bottle containing Streptavidin-Horse Radish Peroxidase (HRP) conjugate concentrate in a stabilizer with a non-mercury preservative.
Volume: 0.3 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label.
Preparation: See preparation of working conjugate solution section.

4. 25(OH)D Calibrators — Ready To Use

Contents: Six glass bottles containing 25-hydroxyvitamin D in human plasma with a non-mercury preservative. Calibrators are traceable to NIST SRM 972 and to concentrations determined by UV spectrophotometric analysis using a molar extinction coefficient of 18,300 M-1cm-1 at 264 nm.
Calibrator concentrations*: 0, 10, 20, 40, 80 and 160 ng/mL.
* Approximate value. Please refer to bottle labels for exact concentrations.
Volume: Calibrators A–F: 1 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label.

5. Controls — Ready To Use

Contents: Two glass bottles containing 25(OH)D in human plasma with a non-mercury preservative. Refer to bottle labels for acceptable ranges. Controls are traceable to NIST SRM 972 and to concentrations determined by UV spectrophotometric analysis using a molar extinction coefficient of 18,300 M-1cm-1 at 264 nm.
Volume: Controls Low and High: 1 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label.

6. Incubation Buffer — Ready To Use

Contents: One bottle containing a buffer with a blue dye and non-mercury preservative.
Volume: 20 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

7. Assay Buffer — Ready To Use

Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 20 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

8. Wash Buffer Concentrate — Requires Preparation X10

Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

9. TMB Substrate — Ready To Use

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in buffer.
Volume: 16 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

10. Stopping Solution — Ready To Use

Contents: One bottle containing 1M sulfuric acid.
Volume: 6 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

PREPARATION OF WORKING CONJUGATE SOLUTION

1. To a disposable glass test tube or glass bottle (**do not use plastic containers**), first add the required amount of assay buffer and then to this add a 1:100 volume of 25(OH)D-Biotin Conjugate Concentrate and a 1:100 volume of Streptavidin-HRP Conjugate Concentrate.

   For example, if the whole plate is to be used, to 16 mL of assay buffer in a glass test tube or bottle, add 0.16 mL of the 25(OH)D-Biotin Conjugate Concentrate and 0.16 mL of the Streptavidin-HRP Conjugate Concentrate.

*Note: It is very important to add the assay buffer to the glass tube or bottle **first** and then add the conjugate concentrates to the assay buffer. Failure to prepare the working conjugate solution in this order can lead to decreased OD values.*

2. Mix the working conjugate thoroughly and store in a dark place until it is used in step 7 of the assay procedure.

*Note: It is essential to prepare the working conjugate solution **before** the assay procedure is started. The working conjugate solution is stable for up to four hours, therefore it can be prepared between 0 and 120 minutes before starting the assay.*

ASSAY PROCEDURE

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. After all kit components have reached room temperature mix gently by inversion. Prepare working solutions of the conjugate (see preparation of working conjugate solution section) and wash buffer (see wash buffer concentrate under reagents provided section).
2. Remove the required number of microplate strips and assemble into a plate frame. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 25 µL of each calibrator, control and serum or plasma sample into correspondingly labelled wells in duplicate.
4. Pipette 150 µL of the incubation buffer into each well (the use of a multichannel pipette is recommended). Tap the microplate gently by hand for 10 seconds to mix the contents in the wells.
5. Incubate the microplate for 60 minutes at room temperature in a dark place (no shaking).
6. Wash the wells 3 times each time with 300 µL/well of diluted wash buffer. After washing tap the plate firmly against absorbent paper to remove any residual liquid (the use of an automatic strip washer is strongly recommended). The performance of this assay is markedly influenced by the correct execution of the washing procedure.
7. Pipette 150 µL of the working conjugate solution into each well (the use of a multichannel pipette is recommended). Tap the microplate gently by hand for 10 seconds to mix the contents in the wells.
8. Incubate the microplate for 30 minutes at room temperature in a dark place (no shaking).
9. Wash the wells 3 times using the same procedure as in step 6.
10. Pipette 150 µl of the TMB substrate into each well (the use of a multichannel pipette is recommended). Tap the microplate gently by hand for 10 seconds to mix the contents in the wells.
11. Incubate the microplate for 10–15 minutes at room temperature in a dark place (no shaking).
12. Add 50 µL of stopping solution to each well and mix thoroughly by gently tapping the plate by hand for 10 seconds to mix the contents in the wells.
13. Measure the absorbance at 450 nm in all wells with a microplate reader within 0–20 minutes after addition of the stopping solution.

CALCULATIONS

Using immunoassay software, choose either a 4-parameter or 5-parameter curve fitting method for calculating results.

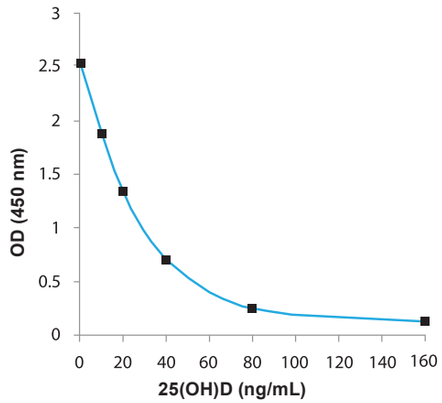
TYPICAL TABULATED DATA

Sample data only. **Do not** use to calculate results.

Calibrator	25(OH)D (ng/mL)	Mean OD (450 nm)	Binding (%)
A	0	2.556	100
B	10	2.207	86
C	20	1.906	75
D	40	1.475	58
E	80	0.711	28
F	160	0.253	10

TYPICAL CALIBRATOR CURVE

Sample curve only. **Do not** use to calculate results.



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The limit of detection (LoD) was determined from the analysis of 64 samples of the blank and a low value sample and it was calculated as follows:

$LoD = \mu_B + 1.645\sigma_B + 1.645\sigma S$,

where σB and σS are the standard deviation of the blank and low value sample and μ_B is the mean value of the blank.

LoD = 5.5 ng/mL of 25(OH)D

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity using the Abraham method with 25(OH)D3 cross reacting at 100%:

Antigen	% Cross Reactivity
25 (OH)D3	100
25 (OH)D2	100
1,25 $(OH)_2$D3	8.3
3-epi-25 (OH)D3	66
Vitamin D2	< 1.0
Vitamin D3	< 1.0

INTERFERENCE

Interference testing was performed according to the CLSI guideline EP7-A2. Serum samples with varying levels of 25(OH)D were spiked with potential interfering substances at recommended levels and analyzed. Results were compared to the same serum samples with no extra substances added to calculate the % interference. The following substances were tested and did not show significant interference in the assay up to concentrations more elevated than the highest occurring levels: hemoglobin up to 7.5 mg/mL; bilirubin conjugated and free up to 200 µg/mL; triglycerides up to 5.5 mg/mL; cholesterol up to 2.6 mg/mL; ascorbic acid up to 10 mg/mL, biotin up to 40 µg/mL and caffeine up to 10 µg/mL.

PRECISION

The precision study followed EP5-A3 and used a nested components-of-variance design with 21 testing days, two runs per testing day, and two replicate measurements per run (a 21 x 2 x 2 design) for each sample. Data was analyzed with a two-way nested ANOVA and summarized in the table below:

Sample	Mean (ng/mL)	Repeatability SD	Repeatability CV %	Within Lab SD	Within Lab CV %
1	21.87	1.09	5.0%	1.77	8.1%
2	36.57	1.01	2.8%	3.17	8.7%
3	45.01	1.07	2.4%	4.45	9.9%
4	60.25	2.82	4.7%	6.21	10.3%

COMPARATIVE STUDIES

The DBC 25(OH)D ELISA kit (y) was compared to a higher level test (LC-MS/MS) (x). The comparison of 40 serum samples yielded the following linear regression results:

y = 0.93x – 4.68, r = 0.96

REFERENCE VALUES (SERUM/PLASMA)

As for all clinical assays each laboratory should collect data and establish their own range of reference values. Data presented here are from samples collected in Florida (USA) from putatively healthy Black, White and Hispanic individuals of both genders and between 20 and 60 years old. Population reference ranges for 25(OH)D vary widely depending on age, ethnic background, geographic location and season. Population-based ranges correlate poorly with serum 25(OH)D concentrations that are associated with biologically and clinically relevant vitamin D effects and are therefore of limited clinical value.

N	25(OH)D Mean (ng/mL)	25(OH)D Median (ng/mL)	25(OH)D Range (2.5^{th}–97.5^{th} percentile) (ng/mL)
120	24.6	23.5	12.6–42.3

Results from the NHANES III study (1) yielded a mean of 30 ng/mL among 15,390 individuals.

CLINICAL DECISION VALUES

The Institute of Medicine at Washington DC (2) concluded that the levels of vitamin D can be associated with health conditions as in the following table:

25(OH)D, ng/mL	Health Status
< 12	Vitamin D deficiency leading to rickets in infants and children and osteomalacia in adults.
12–20	Generally considered inadequate for bone and overall health in healthy individuals.
≥ 20	Generally considered adequate for bone and overall health in healthy individuals.
> 60	Emerging evidence links potential adverse effects to such high levels.

Another source reports the following threshold levels:

25(OH)D, ng/mL	Health Status
< 10	Severe deficiency. Could be associated with osteomalacia or rickets.
10–19	Mild to moderate deficiency. May be associated with increased risk of osteoporosis or secondary hyperparathyroidism.
20–50	Optimum levels in the healthy population; patients with bone disease may benefit from higher levels within this range.
51–80	Increased risk of hypercalciuria. Sustained levels > 50 ng/mL 25OH-VitD along with prolonged calcium supplementation may lead to hypercalciuria and decreased renal function.
> 80	Toxicity possible. 80 ng/mL is the lowest reported level associated with toxicity in patients without primary hyperparathyroidism who have normal renal function. Most patients with toxicity have levels > 150 ng/mL. Patients with renal failure can have very high 25(OH)D levels without any signs of toxicity, as renal conversion to the active hormone 1,25(OH)D is impaired or absent.

These reference ranges represent clinical decision values that apply to males and females of all ages, rather than population-based reference values.

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