25-HYDROXYVITAMIN D [25(OH)D] ELISA

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 immunoassay that uses two incubations, with a total assay incubation time of less than two hours at room temperature. During the initial 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 bound to the anti-25(OH)D antibody immobilized on the microplate wells. After washing, during the next step, 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 decontaining procedures remove any unbound materials. The MB substrate is added next which reacts with HRP to form a coloured product. The intensity of the colour is proportional to the amount of anti-25(OH)D antibody bound to the MB substrate. The result is expressed as the concentration of 25(OH)D in the samples.

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 any sample that has been or is expected to be used in the preparation of this product has been tested and found negative for the presence of HIV-1 and HIV-2, HBsAg, HCV, VHB (HbsAg) 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 serum or plasma vacutainer tube. Invert the tube gently but thoroughly before use. 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.

- 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. Plasma samples should be stored in a stabilizer with a non-mercury preservative.

- Stability: 12 months in unopened vials or as indicated on label.

- Controls
- Standard: Read-To Use
- Test: Read-To Use
- Calibration: Read-To Use
- Confirm: Read-To Use

- Calibration

- Stabilizer: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stabilizer: 12 months in unopened vials or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months in unopened vials or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.

- Controls
- Concentration: 0, 10, 20, 40, 80 and 160 ng/mL
- Appropriate Value: Please 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 13,800 M-1 cm-1 at 284 nm.

- Stability: 12 months or as indicated on label.
ASSAY PROCEDURE

1. After all kit components have reached room temperature, 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 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). Mix the contents gently by inversion. Prepare working solutions of the conjugate (see preparation of working conjugate solution) and wash buffer (see wash buffer concentrate under reagents provided section).

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, read within 0–20 minutes after addition of the stopping solution.

CALCULATIONS

Using immunosassay 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.

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

TYPICAL CALIBRATOR CURVE

Sample curve only. Do not use to calculate results.

SPECIFICITY (CROSS ACTIVITY)

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

<table>
<thead>
<tr>
<th>Antigen</th>
<th>% Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 (OH)D3</td>
<td>100</td>
</tr>
<tr>
<td>25 (OH)D2</td>
<td>100</td>
</tr>
<tr>
<td>1,25 (OH)D3</td>
<td>8.3</td>
</tr>
<tr>
<td>3-epi-25 (OH)D3</td>
<td>66</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>&lt; 1.0</td>
</tr>
</tbody>
</table>

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; bilirubin 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:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (ng/mL)</th>
<th>SD</th>
<th>Repeatability %CV</th>
<th>Reproducibility %CV</th>
<th>Within Lab SD Mean %CV</th>
<th>Within Lab CV Mean %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.87</td>
<td>1.09</td>
<td>5.0%</td>
<td>1.77</td>
<td>8.1%</td>
<td>10%</td>
</tr>
<tr>
<td>2</td>
<td>36.57</td>
<td>1.01</td>
<td>2.8%</td>
<td>3.17</td>
<td>8.7%</td>
<td>9.9%</td>
</tr>
<tr>
<td>3</td>
<td>45.01</td>
<td>1.07</td>
<td>2.4%</td>
<td>4.45</td>
<td>9.9%</td>
<td>9.9%</td>
</tr>
<tr>
<td>4</td>
<td>60.25</td>
<td>2.82</td>
<td>4.7%</td>
<td>6.21</td>
<td>10.3%</td>
<td>10.3%</td>
</tr>
</tbody>
</table>

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:

\[ \text{LoD} = \mu_B + 1.645\sigma_S \]

where \( \mu_B \) and \( \sigma_S \) are the standard deviation of the blank and low value sample and \( \mu_B \) is the mean value of the blank.

\[ \text{LoD} = 5.5 \text{ng/mL of 25(OH)D} \]
COMPARATIVE STUDIES

The 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:

\[ y = 0.93x - 4.68, \quad r = 0.96 \]

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 geographic location and season. Population-based ranges and between 20 and 60 years old. Population reference ranges here are from samples collected in Florida (USA) from putatively healthy 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:

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

Another source reports the following threshold levels:

<table>
<thead>
<tr>
<th>25(OH)D, ng/mL</th>
<th>Health Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10</td>
<td>Severe deficiency. Could be associated with osteomalacia or rickets.</td>
</tr>
<tr>
<td>10–19</td>
<td>Mild to moderate deficiency. May be associated with increased risk of osteoporosis or secondary hyperparathyroidism.</td>
</tr>
<tr>
<td>20–50</td>
<td>Optimum levels in the healthy population; patients with bone disease may benefit from higher levels within this range.</td>
</tr>
<tr>
<td>51–80</td>
<td>Increased risk of hypercalcemia. Sustained levels &gt; 50 ng/mL 25OH-ViD along with prolonged calcium supplementation may lead to hypercalcemia and decreased renal function.</td>
</tr>
<tr>
<td>&gt; 80</td>
<td>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 &gt; 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.</td>
</tr>
</tbody>
</table>

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

REFERENCES


SYMBOLS

European Conformity
Contains sufficient for <20 tests
In vitro diagnostic device
Legal Manufacturer
Consult Instructions for use
In贮存
Use by
Catalogue Number
Authorized Representative
Dilute 1: # before use
Manufacturer
Before use
Use Only (USA)
Authorized

Diagnostics Biochem Canada Inc.
384 Neptune Crescent
London, Ontario, Canada N6M 1A1
T: (519) 681-8731 F: (519) 681-8734
e-mail: dbc@dbc-labs.com
www.dbc-labs.com
An ISO 13485 Registered Company