BIOHIT HealthCare

Innovating for Health

BIOHIT TOTAL 25OH VITAMIN D

ELISA kit for the measurement of 25-hydroxyvitamin D2 and D3 in plasma and serum

INSTRUCTIONS FOR USE

REF 602 310.02 IVD CE

For *in vitro* diagnostic use

Store at 2-8 °C Upon Receipt

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IVD	For *in vitro* diagnostic use	CAL 0	Calibrator 0
REF	Catalogue number	CAL 1-5	Calibrators 1-5
LOT	Batch code	CONTROL N	Controls
Σ 96	96 tests	INC BUF	Incubation Buffer
	Protect from sunlight	CONJ BUF	Conjugate Buffer
	Use by	CONJ CONC	25OHD Conjugate Concentrate
+8°C +2	Store at 2-8°C	HRP CONC	SA-HRP Concentrate
2	Do not re-use	WASH 200x	Wash Solution (200x concentrate)
i	Consult instructions for use	SUBS	Substrate Solution
LYO	Lyophilized	STOP	Stop Solution

INSTRUCTIONS FOR USE **English**

Note! Other languages available at www.biohithealthcare.com.

BIOHIT Total 25OH Vitamin D Elisa kit Cat. No. 602 310.02

CONTENTS

1. INTENDED USE

The BIOHIT Total 25OH Vitamin D ELISA is a quantitative immunoenzymatic assay for the *in vitro* determination of 25-hydroxyvitamin D2 and D3 (25OHD2 and 25OHD3) in serum and plasma to aid in the diagnosis of Vitamin D deficiency, insufficiency or intoxication.

2. BACKGROUND

Vitamin D is the common name for a group of fat-soluble steroid prohormones extremely important in multiple aspects of human health. The two forms relevant to humans are vitamin D2, ergocalciferol, and Vitamin D3, cholecalciferol. Vitamin D2 and D3 are both naturally present in certain foods, as well as added to many food stuffs. Vitamin D supplementation by dietary supplements is recommended in many countries, especially during pregnancy, and when exposure to sun is limited either due to climatic or cultural reasons. (1-3)

The main circulating form of vitamin D3 in the body is the 25-hydroxylated form (25OHD3, also known as calcidiol). Vitamin D3 is metabolized into 25-hydroxyvitamin D3 in the liver, and recently it has been found that 25OHD3 is also available directly from certain animal-based foods. 25OHD3 has limited activity by itself, and acts as the precursor for other vitamin D metabolites. In the kidney, 25OHD3 is converted to 1,25-dihydroxyvitamin D3, or calcitriol, the most active of the vitamin D3 forms. Vitamin D2 is metabolized essentially along the same routes as vitamin D3, and both D2 and D3 contribute to the overall vitamin D status. To correctly diagnose vitamin D deficiency, insufficiency or intoxication, it is thus very important to measure both the D2 and D3 forms of vitamin D. The most reliable way to determine the vitamin D status of an individual is to measure the level of 25OHD in blood: 25OHD has a relatively long half-life of 15 days in circulation and the 25OHD level reflects vitamin D obtained both by ingestion of food and dietary supplements as well as vitamin D produced in the skin. (1-9)

25OH Vitamin D stimulates the intestinal absorption of both calcium and phosphorus, and affects bone resorption and mineralization. Vitamin D deficiency can cause weakening of the bones: it is an important risk factor for rickets, osteomalacia and senile osteoporosis. The measurement of both 25OH Vitamin D2 and D3 is also necessary for determining the cause for abnormal serum calcium concentrations. Too high levels of vitamin D (vitamin D intoxication) can lead to kidney and tissue damages. Vitamin D has been found to have several other roles in the body, including modulation of cell growth, neuromuscular and immune function, and reduction of inflammation. An association between cognitive impairment, dementia, and vitamin D deficiency has also been confirmed. Recent literature has also presented emerging evidence of the multiple roles of Vitamin D may in cancer, diabetes and pregnancy outcomes. (1-6, 10-16)

3. PRINCIPLE OF THE TEST

The BIOHIT Total 25OH Vitamin D ELISA assay is a microplate-based competitive enzyme linked immunosorbent assay (ELISA). First, sample, controls and calibrators are added to the wells of the microtiter plate, immediately followed by incubation buffer. During the 2h incubation step at room temperature, 25OHD is dissociated from serum/plasma proteins and is bound to the 25OHD-specific monoclonal antibody coated onto the microtiter well. The wells are washed to remove unbound components. A pre-mixed solution of a biotin-labeled 25OHD derivative and streptavidin-labeled horse-radish peroxidase (HRP) is then added to the well. During the following incubation, the labeled 25OHD and the un-labeled 25OHD

bound to the antibody compete with each other for antibody binding sites. The wells are then washed to remove unbound material, and a chromogenic substrate (Tetramethylbenzidine, TMB) is added. After incubation for 15 min, the reaction is stopped by addition of Stop solution, and the intensity of the color developed is measured. The concentration of 25OHD is inversely proportional to the color generated and can be calculated by dose interpolation from the calibration curve.

4. WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use.

CAUTION: Handle plasma samples as a potentially biohazardous material.
All samples should be regarded as potentially contaminated and treated as if they were infectious. Please refer to the U.S. Department of Health and Human Services (Bethesda, MD., USA) publication Biosafety in Microbiological and Biomedical Laboratories, 1999, 4th ed. (CDC/NIH) and No. (CDC) 88-8395 on reports of laboratory safety procedures on different diseases, or any other local or national regulation.

This kit contains reagents manufactured from human blood components. The source materials provided in the kit have been tested for the presence of antibodies to hepatitis B and C as well as antibodies to HIV, and found to be negative. However, as no test method can offer absolute assurance that these pathogens are absent; all recommended precautions in the handling of a blood derivative should be observed.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Always use protective gloves when handling patient samples. Use a safety pipetting device for all pipetting. Never pipette by mouth. Read all instructions prior to performing this assay. Components containing ProClin may cause an allergic skin reaction (see Safety Data Sheet). Dispose of solutions containing Proclin according to local waste management legislation. Stop Solution contains HCl (see Safety Data Sheet). In case of contact, wash thoroughly with water. Incubation buffer contains PFOA, Perfluorooctanoic acid (see Safety Data Sheet). In case of contact, wash thoroughly with soap and water.

5. TRACEABILITY OF VALUES

The test is calibrated against the ID-LC/MS-MS Reference Measurement Procedure (Ghent method) (17-18) as approved by the Vitamin D Standardization Program (VDSP) established by the National Institutes of Health (NIH) Office of Dietary Supplements (ODS).

6. KIT CONTENTS

The reagents are sufficient for 96 wells. Reagents of different kit lots should not be mixed.

6.1 Microplate

Contents: 12 x 8 strips (with break-apart wells) in frame coated with 25OH Vitamin D2 and D3 specific monoclonal antibodies. Packed with desiccant in a re-sealable pouch.
Preparation: Ready for use. The microplate or individual strips should not be removed from the foil pouch until equilibrated to room temperature (20-25°C).

Stability: Stable until expiry date. Discard the strips after use. Unused strips should be returned to the foil pouch, sealed, and stored at 2-8°C.

6.2 Calibrator 0

Contents: 1 vial containing lyophilized biological material with gentamycin and Proclin as preservatives.
Preparation: Reconstitute the Calibrator by adding 2 ml of distilled water.
Stability: Lyophilizate is stable until expiry date. After reconstitution, calibrator 0 is stable for eight weeks at 2-8 °C. For longer storage periods, aliquots should be made and kept at –20°C for maximum 4 months. Avoid subsequent freeze-thaw cycles.

6.3 Calibrators 1-5

Contents: 5 vials containing lyophilized calibrators 1-5 (exact values printed on vial labels) in horse serum with gentamycin and Proclin as preservatives. The calibrators have lot-specific 25OHD values. The exact 25OHD concentration of the calibrators is indicated on the vials.
Preparation: Reconstitute the calibrators by adding 1 ml of distilled water into each vial.
Stability: Stable until expiry date. After reconstitution, the calibrators are stable for eight weeks at 2-8 °C. For longer storage periods, aliquots should be made and kept at –20°C for maximum 4 months. Avoid subsequent freeze-thaw cycles.

6.4 Controls

Contents: 2 vials containing lyophilized controls (n=2) in human plasma with Proclin as preservative.
Note: Control 1 (or a low serum sample with a concentration above 4.4 ng/ml but below 25 ng/ml) should be used for dilution of serum samples with values above the highest calibrator. For plasma samples with values above the highest calibrator use Control 1 for dilution.
Preparation: Reconstitute by adding 1 ml distilled water to each vial.
Stability: Stable until expiry date. After reconstitution, the controls are stable for eight weeks at 2-8 °C. For longer storage periods, aliquots should be made and kept at –20°C for maximum 4 months. Avoid subsequent freeze-thaw cycles.

6.5 Incubation buffer

Contents: 1 bottle (20 ml) of Incubation Buffer with casein and Proclin as preservatives.
Preparation: Ready for use.
Stability: Stable until expiry date.

6.6 Wash Buffer Concentrate

Contents: 1 bottle (10 ml) of Wash Buffer concentrate (200x, TRIS-HCl).
Preparation: Dilute 1: 200 with distilled water (e.g. 5 ml Wash Buffer concentrate + 995 ml of water). Mix using a magnetic stirrer. Use a clean plastic container to prepare the Wash Solution. To avoid microbial growth, it is recommended that Working Wash solution should be freshly prepared and used on the same day.
Stability: Stable until expiry date.

6.7 Conjugate Buffer

Contents: 1 bottle (30 ml) of Conjugate Buffer with casein and Proclin as preservatives.
Preparation: Ready for use. To prepare Conjugate Working Solution, add 25OHD Conjugate Concentrate and SA-HRP Concentrate to Conjugate Buffer as described below in the Dilution Scheme.
Stability: Stable until expiry date.

6.8 25OHD Conjugate Concentrate

Contents: 1 vial (0.3 ml) of Concentrated 25OH Vitamin D Conjugate.
Preparation: For the preparation of Conjugate Working Solution, dilute 1:100 with conjugate buffer, as described in the Dilution Scheme below.
Stability: Concentrate is stable until expiry date.

6.9 SA-HRP Concentrate

Contents: 1 vial (0.2 ml) of concentrated Streptavidin-HRP.
Preparation: For the preparation of Conjugate Working Solution, dilute 1:200 with conjugate buffer, as described in Dilution Scheme below.
Stability: Concentrate is stable until expiry date.

Preparation of Conjugate Working Solution. The solution must be prepared at least 1 h 45 min before its use! It recommended that the Conjugate Working Solution is prepared during the first incubation step.

Prepare an adequate volume of working HRP conjugate solution by mixing the 3 reagents in the following sequence: (1) Conjugate buffer, (2) 25OHD Conjugate Concentrate, (3) Vortex, (4) SA-HRP Concentrate, (5) Vortex. **The order of addition of those 3 reagents is critical and should be strictly respected to get reproducible Optical Densities.** Store the Conjugate Working Solution at RT until use. Avoid direct sunlight. The Working Solution is not stable for elongated times, and must be discarded if not used during the same working day.

Dilution Scheme (200 µl per well)

Strips	Conjugate Buffer (ml) CONJ | BUF	25 OHD Conjugate Concentrate (µl) CONJ | CONC	SA-HRP Concentrate (µl) HRP | CONC	Total volume (ml)
1	3	30	15	3.045
2	5	50	25	5.075
3	6	60	30	6.090
4	8	80	40	8.120
5	9	90	45	9.135
6	10	100	50	10.150
7	12	120	60	12.180
8	14	140	70	14.210
9	16	160	80	16.240
10	18	180	90	18.270
11	20	200	100	20.300
12	22	220	110	22.330

6.10 Substrate Solution

Contents: 1 bottle (13 ml) of tetramethylbenzidine (TMB) in aqueous solution.
Preparation: Ready for use.
Stability: Stable until expiry date. Avoid exposure to direct light.

6.11 Stop Solution

Contents: 1 bottle (13 ml) of HCl 1M.
Preparation: Ready for use. Stop Solution contains HCl (see Safety Data Sheet). In case of contact, wash thoroughly with water.
Stability: Stable until expiry date.

6.12 Instructions for Use

Instructions for use in English inserted into each kit.
Other languages available at www.biohithealthcare.com.

7. MATERIALS REQUIRED BUT NOT PROVIDED

Distilled or deionized water, micropipettes and disposable tips to accurately deliver 50-1000 µl, pipettes to accurately deliver 1-10 ml, 8-channel pipette delivering 100 and 200 µl µl, graduated cylinder (e.g. 1000 ml), magnetic stirrer, vortex mixer, plate shaker, microplate washer, timer, vertical measurement principle microplate reader for reading at 450 nm (vs. 630 or 650 nm, bi-chromatic reading is recommended), plastic blood collection tube for serum or EDTA plasma, container for ice-water bath.

8. STORAGE AND STABILITY

Store the BIOHIT 25OH Vitamin D kit refrigerated (2-8°C). When stored at these temperatures, the kit is stable until the expiration date printed on the box label and the label of each individual kit component. Do not freeze or expose the kit to high temperatures or store at above 8°C when not in use.

Do not use reagents after the expiration date printed on the label. Do not use reagents from kits with different lot numbers or substitute reagents from kits of other manufacturers. Use only distilled or deionized water. The components of the kit are provided at precise concentrations. Further dilution or other alterations of the reagents may cause incorrect results. See section 6 for stability of individual components after reconstitution.

Alterations in physical appearance of kit reagents may indicate instability or deterioration. The substrate solution should be colorless or pale blue. Any other color indicates deterioration of the substrate solution.

9. SPECIMEN COLLECTION AND HANDLING

The sample matrix for the BIOHIT Total 25OH Vitamin D kit is serum or EDTA plasma.

Plasma Preparation:
After drawing the blood sample into an EDTA tube without additives, the tubes are mixed immediately by turning them upside down 5-6 times. Plasma is separated by centrifugation no later than 2h after sampling (e.g. StatSpin® Express 2, centrifugation for 2 minutes at 4440 x g; please refer to centrifuge manufacturer's instructions for plasma separation). Transfer the plasma into a clean plastic tube.

Serum Preparation:
After drawing the blood sample into a serum tube without additives, allow the specimen to fully clot by leaving it undisturbed and vertically positioned in a tube rack at room temperature for 30 min. Remove the clot by centrifuging (e.g. Stat Spin 180s, centrifugation for 10 min 2000×*g*, please refer to tube and centrifuge manufacturers' instructions for serum separation). Centrifugation should take place within one hour of collection. Serum is transferred to a clean plastic tube.

Sample Storage:
After separation of the serum/plasma, the sample can be stored for 4 days in a refrigerator at 2-8°C and temporarily (<24 h) at room temperature (RT). If not to be used within four days, storage at –20 °C is recommended. For prolonged storage, –70 °C should be used. Mix the samples thoroughly after thawing. Avoid repeated freezing and thawing of the samples. Grossly hemolyzed, lipemic or turbid specimens should be discarded.

Serum samples suspected of containing concentrations above the highest calibrator should be diluted in Control 1 or in a serum sample with a low 25OH concentration of >4.4 ng/ml (limit of quantification of the assay) and <25 ng/ml, as measured in this assay. Use Control 1 or the low sample to dilute 2X the high sample. Take the concentration of the Control 1* or the low serum sample into account (depending on which one was used) when calculating the dilution result.

Similarly, plasma samples suspected of containing concentrations above the highest calibrator should be diluted in Control 1. Do not use a low concentration serum sample to dilute plasma samples.

*) Use the concentration of Control 1 measured in the same run as the dilution run, not the mean concentration on the Control 1 label!

Calculations:

Sample value = (Measured value – F1*Measured control 1) / F2

with the following values for F1 and F2:
- Sample diluted 2 times, F1 = 0.5; F2 = 0.5
- Sample diluted 4 times, F1 =0.75; F2 = 0.25
- Sample diluted 8 times, F1 = 0.875; F2 = 0.125

Example:

A sample with concentration above the highest calibrator is diluted 4 times with Control 1, and is measured to have a concentration of 70 ng/ml. Control 1 is measured in the same run to have concentration 20 ng/ml. Dilution 4 times, F1 = 0.75; F2 = 0.25.

Calculated value for the sample = (70 – 0.75*20)/0.25 = 220 ng/ml.

10. TEST PROCEDURE

10.1 Preliminary Preparations

Allow all reagents and the microplate to reach room temperature (20-25°C). Reconstitute the lyophilized calibrators and controls with distilled water as described in section 6. Dilute the washing buffer concentrate 1 to 200 (e.g. 5 ml + 995 ml) with distilled or deionized water. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C. Secure the strips into the holding frame.

Frozen samples should be thawed fast in a room temperature water bath with occasional mixing. Once they are almost thawed, place them in a crushed ice bath. **Read the complete assay procedure before starting. It is recommended that all calibrators and the control are applied on the plate as duplicates. It is necessary to use calibrators and controls in each test run**.

Mix all reagents and samples well before use by gentle agitation or swirling. Note! All incubations may be performed at 20-30°C (= ambient temperature), do not exceed the specified temperature.

10.2 Test Protocol

STEP 1: SAMPLE
Pipette 50 µl of each Calibrator, Control and Sample into the microtiter plate wells.

Note 1: Serum samples expected to contain 25OHD above the concentration of the highest calibrator should be diluted in Control 1 or a low serum sample (serum sample concentration above 4.4 ng/ml but below 25 ng/ml). Accordingly, plasma samples should be diluted in Control 1 (not in low serum sample).

Note 2: To avoid drift between result levels due to longer pipetting times, the time between pipetting of the first calibrator and the last sample should be no longer than 20 min.

STEP2: INCUBATION BUFFER
Pipette 150 µl of Incubation Buffer into the wells. Incubate for 2 hours at ambient temperature, on a plate shaker (300-700 rpm).

Note! Prepare the Conjugate Working Solution during the incubation and minimum 1h 45 minutes before its use.

STEP 3: WASHING
Wash the microplate strips 3 times by dispensing 350 µl of Wash Solution into each well and aspirating the content of each well.

STEP 4: CONJUGATE WORKING SOLUTION
Pipette 200 µl of the Conjugate Working Solution into each well. Incubate the microplate for 30 minutes at ambient temperature, on a plate shaker (300-700 rpm).

STEP 5: WASHING
Wash the microplate strips 3 times by dispensing 350 µl of Wash Solution into each well and aspirating the content of each well.

STEP 6: SUBSTRATE
Pipette 100 µl of the Substrate Solution (TMB) into each well. Incubate the microplate for 15 minutes at ambient temperature, on a plate shaker (300 to 700 rpm). Take care to avoid direct sunlight. Dispense the Substrate Solution within 15 minutes following the washing of the microplate.

STEP 7: REACTION STOP
Pipette 100 µl of the Stop Solution with an 8-channel pipette into the microplate wells.

STEP 8: MEASURING OF RESULTS BY VERTICAL MEASUREMENT PRINCIPLE
Measure the absorbance of microplate wells at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section 11.

10.3 Automated method

The BIOHIT Total 25OH Vitamin D ELISA has been designed with automation in mind. As soon as test specific protocols have been created and validated for use, running the BIOHIT Total 25OHD assay with a walk-away open ELISA automate saves resources, and is easy and user-friendly, e.g. by avoiding pipetting-induced disorders such as repetitive strain injuries (RSI).

11. RESULTS

11.1 Quality Control Values

Good Laboratory Practice requires the use of appropriate controls to establish that all the reagents and protocols are performing as designated. The Biohit Total 25 OH Vitamin D kit is provided with two lot-specific controls. Quality control charts within the lot should be maintained to follow the performance of the control. Alternatively, appropriate statistical methods may be used for analyzing internal laboratory control values, which should fall within the appropriate confidence intervals employed in each laboratory. The expected control results must be obtained so that the results can be accepted. It is recommendable to visually check the curve fit selected by the computer.

11.2 Calculation of results and typical data

TYPICAL DATA:

The following data represent typical values obtained for the calibrators. The values are for illustration only, and should not be substituted for the real-time data obtained for each run.

25OH Vitamin D ELISA		OD (450 nm)
Calibrator concentration	0 ng/ml	2.96
	6.0 ng/ml	2.56
	13.0 ng/ml	2.03
	33.0 ng/ml	1.29
	69.0 ng/ml	0.68
	138 ng/ml	0.23

Note: 1 ng/ml = 2.5 pmol/ml

CALCULATION OF RESULTS:

Prepare a calibration curve for each run separately. Do not use data from previous runs.

Calculate the mean OD values of the duplicate determinations. For each calibrator, control and sample, calculate the relative signal (%) by dividing the signal (A) by the signal obtained from the 0-calibrator (A0) and by multiplying by 100.

$$\frac{A}{A0}(\%) = \frac{OD\ (calibrator, control\ or\ sample)}{OD\ (calibrator\ 0)} x100$$

Plot the mean relative signal (A/A0, %) of the calibrators vs. their respective concentrations (indicated on the vials). The absorbance readings are converted to 25OHD concentrations by interpolating unknowns from the best-fit curve of the calibrators. The use of computer-assisted methods is recommended, in which case a 4-parameter logistic function curve fitting should be employed. A typical calibration curve is shown in Figure 1.

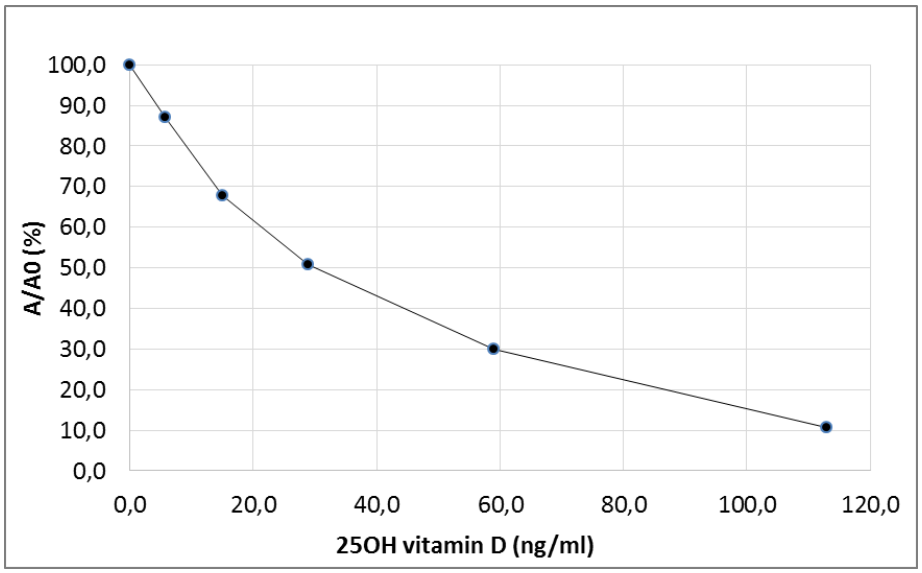

Figure 1. Example of a typical calibration curve.

11.3 Interpretation of Results and Biological reference intervals

The Clinical Practice Guideline from the Endocrine Society defines Vitamin D deficiency as a 25OHD level below 20 ng/ml and vitamin D insufficiency as 25OHD 21–29 ng/ml (2). Serum concentrations consistently above 150 or 200 ng/ml are considered potentially toxic (1, 10, 20) based on which a wider safety limit of 100 ng/ml has been suggested (20). However, adverse health effects have possibly been linked to much lower concentrations of 25OHD, and it has been suggested that concentrations above 50-60 ng/ml should be avoided (2). Factors such as dietary intake, demographics and season are known to affect the normal levels of 25OH Vitamin D (1, 6, 21). Each laboratory should establish its own range based on their local population and possible recommendations by national health authorities.

There is still some debate regarding the optimal levels of vitamin D in serum (1-2, 4, 6, 21). A suggestion, based on the Endocrine Society guideline (2), and recent literature (21-23) for defining 25OH vitamin D levels in serum is presented below.

Level	ng/ml
Deficiency	<20 ng/ml
Insufficiency	21-29 ng/ml
Sufficiency	>30 ng/l
Potential toxicity	>100 ng/ml

Note: 1 ng/ml = 2.5 nmol/l

12. LIMITATIONS OF THE PROCEDURE

As with any diagnostic procedure, the Biohit 25OH Vitamin D test results must be interpreted together with the patient's clinical presentation and any other information available to the physician.

13. ANALYTICAL PERFORMANCE CHARACTERISTICS

All performance tests were carried out at room temperature (20-25 °C). All samples were analyzed with duplicate microplate wells.

13.1 Detection Limit and Quantitation Limit

The Limit of Blank (LoB), Limit of Detection (LoD), and the Limit of Quantitation (LoQ) were determined in accordance with the CLSI guideline EP17-A, with proportions of false positive (α) less than 5% and false negatives (β) less than 5% (14). The LoB was found to be 1.7 ng/ml and the LoD 2.8 ng/ml.

LoQ was determined by testing 5 low value samples in 14 different runs. The LoQ was calculated to be 4.4 ng/ml with a CV% between measurements of ≤ 20.0.

The LoD was also assayed by a second method using twenty 0-calibrators along with another set of calibrators. The detection limit, defined as the apparent concentration of two standard deviations below the average OD at zero binding, was found to be 1.5 ng/ml.

13.2 Reportable Range

The reportable range for the Biohit 25OH Vitamin D assay is 4.4-123 ng/ml.

13.3 Precision

The precision of the assay was determined by running samples in 20 runs (during at least 20 days) using 3 different kit lots.

For the Repeatability (Intra-assay) precision, the range for means was from 5.5 ng/ml to 81.2 ng/ml, the standard deviations from 0.4 to 2 ng/ml, and the CV% from 2.5 to 7.8 %.

For the Within-Laboratory (Inter-assay) precision, the range for the standard deviations was from 1.2 ng/ml to 7.8 ng/ml, and the %CV from 4.7% to 9.2%.

Repeatability Precision				**Within-laboratory Precision**			
Sample	N	[X] ± SD (ng/ml)	CV (%)	Sample	N	[X] ± SD (ng/ml)	C.V. (%)
S1	24	5.5 ± 0.4	7.8	S4	39	17.7 ± 1.3	7.4
S2	35	27.4 ± 1.6	5.7	S5	10	26.3 ± 1.2	4.7
S3	24	81.2 ± 2.0	2.5	S6	21	85.4 ± 7.8	9.2

SD: Standard Deviation, CV: Coefficient of variation

13.4 Analytical Specificity

The 25OH Vitamin D assay was evaluated for cross reactions by other vitamin D forms. Cross-reactivity of the BIOHIT Total 25OH Vitamin D ELISA assay was determined by testing sera spiked with cross-reactants. The percentages of cross reaction for each substance, estimated by comparison of the concentration yielding a 50 % inhibition, are summarized in the following table, respectively:

Compound and Concentration	% Cross-reaction
25OH-Vitamin D3	100
25OH-Vitamin D2	84
Vitamin D3	<0.2
Vitamin D2	<0.2
1,25$(OH)_2$-Vitamin D3	50
1,25$(OH)_2$-Vitamin D2	<0.2
24,25$(OH)_2$-Vitamin D3	>100
25,26$(OH)_2$-Vitamin D3	>100
3-epi-25OH-Vitamin D3	<0.2

13.5 Interference

The Biohit 25 OH Vitamin D test was evaluated for interference at three different 25OHD serum levels (25 OHD at 6-43 ng/ml). The bias caused by hemoglobin, un-conjugated bilirubin or triglycerides at interferent concentrations of 5 mg/ml, 1.0 mg/ml and 5 mg/ml, respectively, was found to be less than 10%. This was considered as a non-significant interference. Grossly hemolyzed, lipemic, or turbid specimens should be avoided.

Interference with ascorbic acid (Vitamin C, 1 mg/ml tested), bilirubin conjugate (1 mg/ml tested), and biotin (40 µg/ml tested) was similarly tested, and found to be less than 10%. Inhibition by Zemplar (50 ng/ml) was tested at 25OHD concentrations of 18 ng/ml and 34 ng/ml, and similarly found to be <10 %, which was considered a non-significant interference.

13.6 Recovery and Linearity

Recovery was assessed by spiking a low 25OHD concentration sample with different levels of 25OH Vitamin D2 or D3. The results are summarized in the table below:

RECOVERY TEST	
Added 25OH Vitamin D3 (ng/ml)	Recovery (%)
0	100
31.3	96
53.9	92
Added 25OH Vitamin D2 (ng/ml)	Recovery (%)
0	100
22.9	105
38.4	95

For analysis of linearity of dilutions, a high sample was tested at equidistant dilutions, according to the dilution protocol in Chapter 9. A linear regression analysis was performed. The results are summarized in the following table:

Sample Dilution	Diluted with	Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	Slope	Y-intercept	R^2	Recovery (%)
1/1	-	101.8	101.8	1.02	-1.91	>0.98	100
1/2	Control 1 measured concentration 27.1 ng/ml	64.4	62.9				98
1/4		45.7	52.0				114
1/8		36.4	34.8				96
1/16		31.7	33.6				106

The test shows that the dilutions series are linear from 33.6 ng/ml to 101.8 ng/ml.

13.7 Method Comparison

The performance of the BIOHIT Total 25OH Vitamin D ELISA test was determined by conducting a correlation study using a total of 127 samples. The samples were tested on both the BIOHIT Total 25OH Vitamin D ELISA test and a commercially available 25OH Vitamin D CLIA (chemiluminescent immunoassay) test. The correlation coefficient between the two methods was 0.94, with a slope of 0.976 and a y-intercept of 1.94. The results are displayed in Figure 2, below.

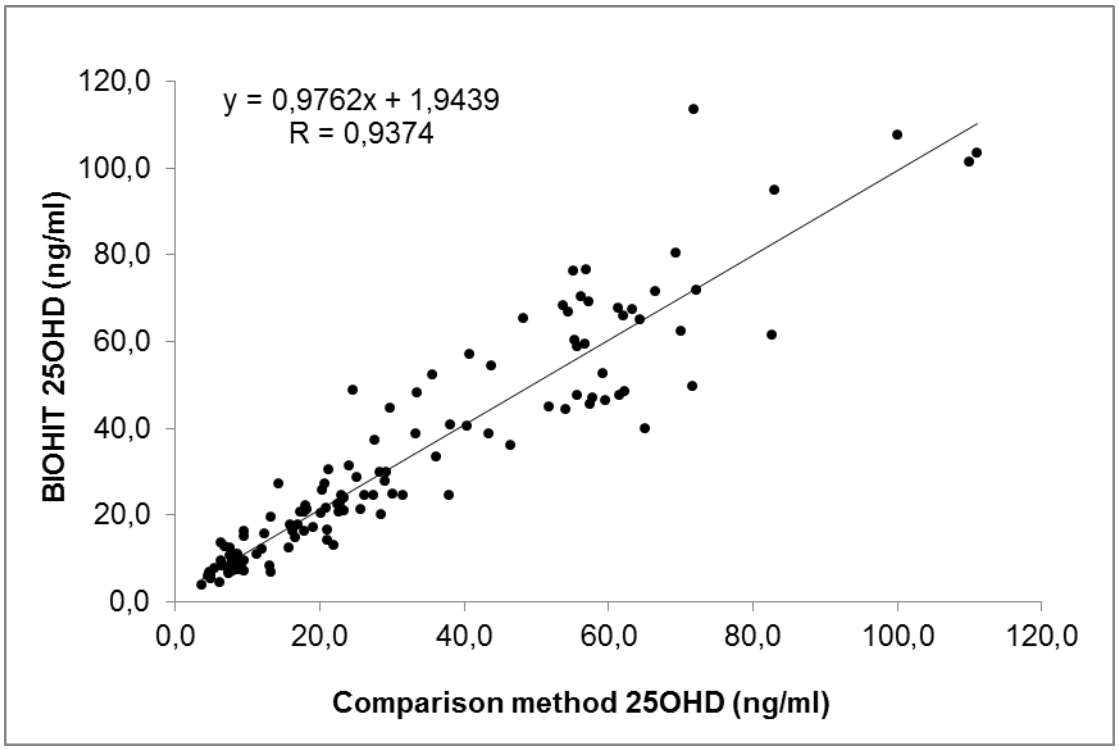

Figure 2. Correlation of the Biohit 25OH Vitamin D assay to a commercially available CLIA-based method.

14. BIBLIOGRAPHY

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15. DATE OF ISSUE

BIOHIT Total 25OH Vitamin D kit insert.
Version 5.0, August 2018.

16. WARRANTY

The Manufacturer shall remedy all defects discovered in any Product (the “Defective Product”) that result from unsuitable materials or negligent workmanship and which prevent the mechanical functioning or intended use of the Products including, but not limited to, the functions specified in the Manufacturer’s specifications for the Products. ANY WARRANTY WILL, HOWEVER, BE DEEMED VOID IF FAULT IS FOUND TO HAVE BEEN CAUSED BY MALTREATMENT, MISUSE, ACCIDENTIAL DAMAGE, INCORRECT STORAGE OR USE OF THE PRODUCTS FOR OPERATIONS OUTSIDE THEIR SPECIFIED LIMITATIONS OR OUTSIDE THEIR SPECIFICATIONS, CONTRARY TO THE INSTRUCTIONS GIVEN IN THE INSTRUCTION MANUAL.
The period of this warranty for the Distributor is defined in the instruction manual of the Products and will commence from the date the relevant Product is shipped by the Manufacturer. In case of interpretation disputes the English text applies.
This Biohit diagnostic kit has been manufactured according to ISO 9001/ISO 13485 quality management protocols and has passed all relevant Quality Assurance procedures related to this product.

17. ORDERING INFORMATION

BIOHIT Total 25OH Vitamin D Elisa kit
Cat. No. 602 310.02

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18. SHORT OUTLINE OF THE PROCEDURE

Allow all the reagents to reach ambient temperature

Remember to mix all the reagents and samples well just before pipetting
*

After mixing, pipette 50 µl of the patient samples,
calibrators and controls into the wells
*

Add 150 µl of Incubation buffer
*

Incubate for 2h at ambient temperature with shaking

During the Incubation, prepare the Conjugate Working Solution
(min. 1h 45 min before use)
*

Wash the wells 3 times with 350 µl of the diluted Washing Solution
*

Pipette 200 µl of the Conjugate Working Solution into the wells
*

Incubate for 30 min at ambient temperature with shaking
*

Wash the wells 3 times with 350 µl of Wash Solution
*

Pipette 100 µl of the mixed Substrate Solution into the wells
*

Incubate for 15 min at ambient temperature with shaking
*

Pipette 100 µl of the mixed Stop Solution into the wells
*

Measure at **450 nm** (vs. 630 or 650 nm)