25-Hydroxyvitamin D Total ELISA

INSTRUCTION FOR USE
REF: GDB 7119/96T

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1. INSTRUCTION FOR USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of 25-hydroxyvitamin D2 and D3 (25OH-D2 and 25OH-D3) in serum and plasma.

2. SUMMARY AND EXPLANATION

Vitamin D is the generic term used to designate Vitamin D2 or ergocalciferol and Vitamin D3 or cholecalciferol.
Humans naturally produce Vitamin D3 when the skin is exposed to ultraviolet sun rays.
In the liver mainly, Vitamin D3 is metabolised into 25-Hydroxyvitamin D3 (25OH D3) which is the main form of Vitamin D circulating in the body.
25OH D3 is a precursor for other Vitamin D metabolites and has also a limited activity by itself.
The most active derivative is 1,25-hydroxyvitamin D3, produced in the kidney (or placenta) by 1-hydroxylation of 25OH D3.
25OH Vitamin D stimulates the intestinal absorption of both calcium and phosphorus and also bone resorption and mineralisation.
25OH Vitamin D might also be active in other tissues responsible for calcium transport (placenta, kidney, mammary gland …) and endocrine gland (parathyroid glands, beta cells…).
Vitamin D3 and Vitamin D2 are also available by ingestion through food or dietary supplementation.
As Vitamin D2 is metabolised in a similar way to Vitamin D3, both contribute to the overall Vitamin D status of an individual.
It is the reason why it is very important to measure both forms of 25OH Vitamin D equally for a correct diagnosis of Vitamin D deficiency, insufficiency or intoxication.
Vitamin D deficiency is an important risk factor for rickets, osteomalacia, senile osteoporosis, cancer and pregnancy outcomes.
The measurement of both 25OH Vitamin D forms is also required to determine the cause of abnormal serum calcium concentrations in patients.
Vitamin D intoxication has been shown to cause kidney and tissue damages.

3. PRINCIPLE OF THE TEST

The Global Diagnostics B 25OH Vitamin D Total ELISA is a solid phase Enzyme Linked Immunosorbent Assay performed on microtiterplates. During a first 1 hour incubation step, at room temperature, total 25OH Vitamin D (D_2 and D_3) present in calibrators, controls and samples is dissociated from binding serum proteins to fix on binding sites of a specific monoclonal antibody. After 1 washing step, a fixed amount of 25OH Vitamin D-labelled with biotin in presence of horseradish peroxidase (HRP), compete with unlabelled 25OH Vitamin D_2 and 25OH Vitamin D_3 present on the binding sites of the specific monoclonal antibody. After a 15 minutes incubation at room temperature, the microtiterplate is washed to stop the competition reaction. The Chromogenic solution (TMB) is added and incubated for 15 minutes. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is inversely proportional to the total 25OH Vitamin D (D_2 and D_3) concentration.
A calibration curve is plotted and the total 25OH Vitamin D (D_2 and D_3) concentrations of the samples are determined by dose interpolation from the calibration curve.

4. REAGENTS

Reagents	GDB 7119	Name indicated on vial	Colour Code	Reconstitution
1 Microtiterplate with 96 Mab anti 25OH Vit. D2 and D3	12x8 strips		blue	**Ready** for use
Calibrator 0: biological matrix with gentamycin and proclin	1 vial lyophilised	**CAL** | **0**	yellow	**Add** 1 ml distilled water
Calibrators 1-5 (see exact values on vial labels) in horse serum with gentamycin and proclin	5 vials lyophilised	**CAL** | **N**	yellow	**Add** 1 ml distilled water
Incubation Buffer with casein and proclin	1 vial	**INC** | **BUF**	green	**Ready** for use
Controls - N = 2 Contains human plasma and proclin	2 vials lyophilised	**CTL** | **N**	silver	**Add** 1 ml distilled water
25OH Vit D Concentrated Conjugate	1 vial 0.3 ml	**CONJ** | **CONC**	blue	**Dilute** 100 x with conjugate buffer
Wash solution (TRIS-HCl)	1 vial 10 ml	**WASH** | **SOLN** | **CONC**	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer).
Concentrated HRP	1 vial 0.2 ml	**HRP** | **CONC**	yellow	**Dilute** 200 x with conjugate buffer
Conjugate Buffer with casein and proclin	1 vial 30 ml	**CONJ** | **BUF**	red	**Ready** for use
Chromogen Solution (Tetramethylbenzydine)	1 vial 12 ml	**CHROM** | **TMB**	orange	**Ready** for use
1M HCL Stopping Solution	1 vial 12 ml	**STOP** | **SOLN**		**Ready** for use

Note :Use Calibrator 0 for dilution of samples with values above the highest calibrator.
No international reference material is available

5. MATERIALS NOT PROVIDED

The following material is required but not provided in the kit:
- Distilled water
- Pipettes for delivery of: 25 µl,75 µl, 100µl and 1 ml (the use of accurate
- pipettes with disposable plastic tips is recommended)
- Vortex mixer
- Magnetic stirrer
- Plate shaker (400 rpm)
- Washer for microtiterplates
- Microtiterplate reader capable of reading at 450 nm and 650 (bichromatic reading)

6. PRECAUTIONS

For *in vitro* diagnostic use only.
The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.
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.
Avoid any skin contact with all reagents, Stop Solution contains HCl. In case of contact, wash thoroughly with water.
Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

7. STORAGE CONDITIONS

Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the label, if kept at 2 to 8°C.
After reconstitution, calibrators and controls are stable for eight weeks at 2 to 8°C. For longer storage periods, aliquots should be made and kept at –20°C for maximum 4 months. Avoid subsequent freeze-thaw cycles.
Freshly prepared Working Wash solution should be used on the same day.
Alterations in physical appearance of kit reagents may indicate instability or deterioration

8. SPECIMEN COLLECTION AND HANDLING

This kit is suitable for serum samples.
Serum samples must be kept at 2-8°C.
If the test is not run within 24 hrs, **sampling and storage at -20°C is recommended.**
Avoid subsequent freeze-thaw cycles.
Serum and heparinized plasma provide similar results.
Y(Heparin plasma) = 0.95 x (serum) + 2.8 ng/ml r = 0.97 n = 19

9. ASSAY PROCEDURE

9.1 Preparation of reagents

Calibrator 0 : Reconstitute the calibrator 0 with 1 ml distilled water
Calibrators 1 - 5 : Reconstitute the calibrators 1-5 with 1 ml distilled water
Controls: Reconstitute the controls with 1 ml distilled water.
Working HRP conjugate solution :

! The working HRP conjugate solution is to be prepared during the incubation and minimum 45 minutes before its use.

Prepare an adequate volume of working HRP conjugate solution by mixing the 3 reagents in the following sequence: (1) Conjugate buffer, (2) Concentrated conjugate, (3) Vortex, (4) Concentrated HRP, (5) Vortex. The order of addition of those 3 reagents is critical and should be rigorously respected to get reproducible Optical Densities.
Prepare the solution according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 60 µl of concentrated conjugate and 30 µl of concentrated HRP to 6 ml of conjugate buffer.
Use a vortex to homogenize.
Until its use keep the working HRP conjugate at room temperature and avoid direct sunlight or use a brown glass vial for its preparation.
The preparation of working HRP conjugate is not stable and must be discarded if not used.
Working Wash solution : Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.
Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.

For the dispensing of the Chromogenic Solution and the Stop Solution avoid pipettes with metal parts.
High precision pipettes or automated pipetting equipment will improve the precision.
Respect the incubation times.
To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section 12.5: Time Delay.
Prepare a calibration curve for each run, do not use data from previous runs.
Dispense the Chromogenic Solution within 15 minutes following the washing of the microtiterplate.
During incubation with Chromogenic Solution, avoid direct sunlight on the microtiterplate

Nb of strips	Volume of Concentrated Conjugate (µl)	Volume of Concentrated HRP (µl)	Volume of Conjugate Buffer (ml)
1	10	5	1
2	20	10	2
3	30	15	3
4	40	20	4
5	50	25	5
6	60	30	6
7	70	35	7
8	80	40	8
9	90	45	9
10	100	50	10
11	110	55	11
12	120	60	12

9.2. ASSAY Procedure

1. 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.
2. Secure the strips into the holding frame.
3. Pipette 25 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 75 µl of Incubation Buffer into all the wells.
5. Incubate for 1 hour at room temperature, on a plate shaker (400 rpm)
   Prepare the Working HRP conjugate solution during the incubation and minimum 45 minutes before its use.
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   - dispensing 0.35 ml of Wash Solution into each well
   - aspirating the content of each well
8. Pipette 100 µl of the working HRP conjugate solution into each well
   Incubate the microtiterplate for 15 minutes at room temperature, on a plate shaker (400 rpm)
9. Aspirate the liquid from each well.
10. Wash the plate 3 times by:
    - dispensing 0.35 ml of Wash Solution into each well
    - aspirating the content of each well
11. Pipette 100 µl of the Chromogenic solution into each well within 15 minutes following the washing step.
12. Incubate the microtiterplate for 15 minutes at room temperature, on a plate shaker (400 rpm), avoid direct sunlight.
13. Pipette 100 µl of Stop Solution into each well.
14. Read the absorbances within 1h at 450 nm(filter 630 nm or 650 nm)

10. RESULTS

Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
Calculate the mean of duplicate determinations.
We recommend the use of computer assisted methods to construct the calibration curve. 4-parameter logistic function curve fitting is the preferred method. Reject obvious outliers.
By interpolation of the sample OD values, determine the 25OH Vitamin D concentrations of the samples from the calibration curve.

TYPICAL DATA
The following data are for illustration only and should never be used instead of the real time calibration curve.

Calibrator	OD Units
0 ng/ml	2.75
4.9 ng/ml	2.58
10.5 ng/ml	2.11
27.0 ng/ml	1.47
60.0 ng/ml	0.63
105.0 ng/ml	0.26

Note : 1 ng/ml = 2.5 pmol/ml

EXPECTED RESULTS
Dietary intake, race, season and age are known to affect the normal levels of 25OH.Vit.D3.
Each laboratory should establish its own range based on their local population.

Recent literature has suggested the following ranges for the classification of 25 OH Vitamin D status: deficiency: 0-10ng/ml, insufficiency: 10-29 ng/ml, sufficiency: 30-100 ng/ml, potential toxicity:>100 ng/ml

11. QUALITY CONTROL

If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
It is good practise to check visually the curve fit selected by the computer.

12. PERFORMANCE CHARACTERISTICS

12.1. Detection Limit

The LOB (Limit of blank) was calculated by measuring the blank several times and was calculated as the mean – 1.65 standard deviation of the distribution of the test values. The LOB was calculated to be 2.16 ng/ml.
The LOD (Limit of detection) was calculated as the LOB – 1.645 standard deviation of a low concentration sample tested in 10 different run. The LOD was calculated to be 4.12 ng/ml.
The LOQ (Limit of quantitation) was calculated by testing 5 samples of low values, 10 times. The LOQ was calculated to be 8.3 ng/ml.

12.2. Specificity

Cross reactivity of the 25OH Vitamin D Total ELISA 90'assay was determined by testing sera with spiked and unspiked cross reactants. The results are summarized in the following table:

Compound and Concentration	Cross reactivity
25OH-Vitamin D_3 at 25-50 ng/mL	100%
25OH-Vitamin D_2 at 25-50 ng/mL	79.8%
1,25$(OH)_2$-Vitamin D_3 at 200 ng/mL	13.3%
1,25$(OH)_2$-Vitamin D_2 at 2 µg/mL	0.3%
Vitamin D_3 at 200 ng/mL	1.7%
Vitamin D_2 at 200 ng/mL	1.7%
24,25$(OH)_2$-Vitamin D_3 at 20 ng/mL	>100%
3-epi-25OH-Vitamin D_3 at 20 µg/mL	0.1%

The assay performance is not affected by hemolysis (2.5 - 5 g/L hemoglobin tested), bilirubinemia (0.5 - 1 g/L bilirubin tested) or triglycerides (0.6 - 5 g/L tested).
Ascorbic acid (Vitamin C) (0.01 - 1g/L tested) and bilirubin conjugate (0.5 - 1g/L tested) don't interfere with this assay.

12.3. Precision

INTRA-ASSAY				INTER-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**	**Sample**	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	24	21.8 ± 0.8	3.6	A	16	17.7 ± 1.1	6.4
B	22	47.7 ± 4.1	8.6	B	16	38.5 ± 3.0	7.7

SD : Standard Deviation; CV: Coefficient of variation

12.4. Accuracy

RECOVERY TEST	
Added 25OH-Vit.D_3 (ng/ml)	**Recovery**
0	100%
12.8	102%
Added 25OH-Vit.D_2 (ng/ml)	**Recovery**
0	100%
11.2	97%

DILUTION TEST			
Sample dilution	**Theoretical concent. (ng/ml)**	**Measured concent. (ng/ml)**	**Recovery**
1/1	74.9	74.9	100%
1/2	37.4	37.9	101%
1/4	18.7	19.2	103%
1/8	9.3	10.2	110%
1/1	56.3	56.3	100%
1/2	28.1	28.7	102%
1/4	14.0	14.8	106%
1/8	7.0	5.8	83%

12.5. Time delay between last calibrator and sample dispensing
As shown hereafter, assay results remain accurate even when incubation buffer is dispensed 10, 15 and 20 minutes after the sample has been added in the coated wells.

TIME DELAY				
	0' (ng/ml)	10' (ng/ml)	15' (ng/ml)	20' (ng/ml)
Sample 1	18.0	20.1	21.2	23.0
Sample 2	40.1	41.4	37.6	41.5

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