25OH Vitamin D Total ELISA

KAP1971

DIAsource ImmunoAssays S.A. - Rue du Bosquet, 2 - B-1348 Louvain-la-Neuve - Belgium

CE

en

Read entire protocol before use.

25OH Vitamin D Total ELISA

I. INTENDED USE

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

II. GENERAL INFORMATION

A. Proprietary name : DIAsource 25OH Vitamin D Total ELISA Kit

B. Catalog number : KAP1971 : 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue du Bosquet, 2 B-1348 Louvain-la Neuve, Belgium.

For technical assistance or ordering information contact :
Tel : +32 (0)10 84 99 11 Fax : +32 (0)10 84 99 90

III. CLINICAL BACKGROUND

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 osteoporisis, 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.

.

IV. PRINCIPLES OF THE METHOD

The DIAsource 25OH Vitamin D Total ELISA is a solid phase Enzyme Linked Immunosorbent Assay performed on microtiterplates. During a first 2 hours 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 peroxidise (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 30 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.

V. REAGENTS PROVIDED

Reagents	96 Test Kit	Colour Code	Reconstitution
Microtiterplate with 96 Mab anti 25OH Vit. D2 and D3	96 wells	blue	**Ready** for use
CAL **0** Calibrator 0: biological matrix with gentamycin and proclin	1 vial lyophilised	yellow	**Add** 2 ml distilled water
CAL **N** Calibrators 1-5 in horse serum with gentamycin and proclin	5 vials lyophilised	yellow	**Add** 1 ml distilled water
CONTROL **N** Controls N = 2 in human serum with thymol	2 vials lyophilised	silver	**Add** 1 ml distilled water
INC **BUF** Incubation Buffer with casein and proclin	1 vial 20 ml	green	**Ready** for use
CONJ **CONC** 25OH Vit D Concentrated Conjugate	1 vial 0.4 ml	blue	**Dilute** 100 x with conjugate buffer
HRP **CONC** Concentrated HRP	1 vial 0.2 ml	yellow	**Dilute** 200 x with conjugate buffer
CONJ **BUF** Conjugate Buffer with casein and proclin	1 vial 30 ml	red	**Ready** for use
WASH **SOLN** **CONC** Wash solution (TRIS-HCl)	1 vial 10 ml	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer).
CHROM **TMB** Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 13 ml	brown	**Ready** for use
STOP **SOLN** Stop solution HCl 1.5 N	1 vial 13 ml		**Ready** for use

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

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:
1. Distilled water
2. Pipettes for delivery of: 50 µl,150 µl, 200µl and 1 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Plate shaker (300 to 700 rpm)
6. Washer for microtiterplates
7. Microtiterplate reader capable of reading at 450 nm and 650 (bichromatic reading)

VII. REAGENT PREPARATION

A. Calibrator 0 : Reconstitute the calibrator 0 with 2 ml distilled water

B. Calibrators 1 - 5 : Reconstitute the calibrators 1-5 with 1 ml distilled water

C. Controls: Reconstitute the controls with 1 ml distilled water.

D. Working HRP conjugate solution :

! The working HRP conjugate solution is to be prepared absolutely in the 15 minutes just after the first 2 hours incubation step is started.(cf X.B.5)

Prepare an adequate volume of working HRP conjugate solution by mixing concentrated conjugate, concentrated HRP and conjugate buffer according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 100µl of concentrated conjugate and 50 µl of concentrated HRP to 10 ml of conjugate buffer.
Use a vortex to homogenize.
Keep the working HRP conjugate at room temperature and avoid direct sunlight or use a brown glass vial for its preparation.

Nb of strips	Volume of Concentrated Conjugate (µl)	Volume of Concentrated HRP (µl)	Volume of Conjugate Buffer (ml)
1	30	15	3
2	50	25	5
3	60	30	6
4	80	40	8
5	90	45	9
6	100	50	10
7	120	60	12
8	140	70	14
9	160	80	16
10	180	90	18
11	200	100	20
12	220	110	22

E. 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.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- 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 one week at 2 to 8°C. For longer storage periods, aliquots should be made and kept at –20°C for maximum 3 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.

IX. SPECIMEN COLLECTION AND PREPARATION

- 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.

X. PROCEDURE

A. Handling notes

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 XIII paragraph E (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.

B. 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 50 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 150 µl of Incubation Buffer into all the wells.
5. Incubate for 2 hours at room temperature, on a plate shaker (300 to 700 rpm) Prepare the Working HRP conjugate solution once the incubation is started(within 15 minutes)
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   - § dispensing 0.4 ml of Wash Solution into each well
   - § aspirating the content of each well
8. Pipette 200 µl of the working HRP conjugate solution into each well Incubate the microtiterplate for 30 minutes at room temperature, on a plate shaker (300 to 700 rpm)
9. Aspirate the liquid from each well.
10. Wash the plate 3 times by:
    - § dispensing 0.4 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 (300 to 700 rpm), avoid direct sunlight.
13. Pipette 100 µl of Stop Solution into each well.
14. Read the absorbances at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. Calculate for each calibrator, control and sample:

$$B/B0(\%) = \frac{OD\ (Calibrator, Control\ or\ Sample)}{OD\ (Zero\ Calibrator)} x100$$

4. Using either linear-linear of semi-logarithmic graph paper, plot the (B/B0(%)) values for each calibrator point as a function of the 25OH Vitamin D concentration of each calibrator point. Reject obvious outliers.
5. Computer assisted methods can also be used to construct the calibration curve. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.
6. By interpolation of the sample (B/B0 (%)) values, determine the 25OH Vitamin D concentrations of the samples from the calibration curve

XII. TYPICAL DATA

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

25OH-EASIA		OD units
Calibrator		
	0 ng/ml	2.54
	10 ng/ml	1.71
	25 ng/ml	1.27
	55 ng/ml	0.61
	100 ng/ml	0.23
	180 ng/ml	0.09

Note : 1 ng/ml = 2.5 pmol/ml

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations below the average OD at zero binding, was 1.5 ng/ml..

B. Specificity

The percentage of cross reaction estimated by comparison of the concentration yielding a 50 % inhibition are respectively :

Compound	Cross-Reactivity (%)
25OH-Vitamin D_3	100%
25OH-Vitamin D_2	83%
1,25$(OH)_2$-Vitamin.D_3	50%
1,25$(OH)_2$-Vitamin.D_2	< 0.2%
Vitamin D3	< 0.2%
Vitamin D2	< 0.2%
24,25$(OH)_2$-Vitamin.D_3	≥100%
25,26(OH)2- Vitamin D3	≥100%
3-epi-25 hydroxy vitamin D3	<0.2%

The assay performance is not affected by hemolysis (5 g/L hemoglobin tested), bilirubinemia (0.5 g/L bilirubin tested) or triglycerides (5 g/L tested).

C. Precision

INTRA-ASSAY				INTER-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**	**Sample**	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	35	27.4 ± 1.5	5.5	A	10	26.3 ± 1.3	4.9
B	35	43.0 ± 1.2	2.7	B	10	42.0 ± 1.9	4.5

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST	
Added 25OH-Vit.D_3 (ng/ml)	**Recovery (%)**
0	100
25	95
50	92
Added 25OH-Vit.D_2 (ng/ml)	**Recovery (%)**
0	100
25	105
50	95

DILUTION TEST			
Sample dilution	**Theoretical concent. (ng/ml)**	**Measured concent. (ng/ml)**	**Recovery (%)**
1/1	66.2		
1/2	33.1	34.5	104
1/4	16.5	15.5	93
1/8	8.2	8.2	99
1/16	4.1	4.4	106
1/32	2.1	2.2	106
1/1	62.0		
1/2	31.0	38.3	123
1/4	15.5	15.8	102
1/8	7.7	7.5	97
1/16	3.8	4.0	103

E. Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when incubation buffer is dispensed 10 and 20 minutes after the calibrator has been added in the coated wells.

TIME DELAY			
	0' (ng/ml)	10' (ng/ml)	20' (ng/ml)
Sample 1	27.9	30.5	30.2
Sample 2	49.5	47.5	49

XIV. INTERNAL 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.

XV. EXPECTED VALUES

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: 0-10 ng/mL; Insufficiency: 10-30 ng/mL; Sufficiency: 30 to 150 ng/mL; Toxicity: >150 ng/mL.

XVI. PRECAUTIONS AND WARNINGS

Safety

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.

XVII. BIBLIOGRAPHY

1. ZERWEKH J.E. (2008)
Blood biomarkers of Vitamin D status.
Am. J. Clin. Nutr., 87(suppl):1087S-91S.

2. HOLICK M.F. (2006)
Resurrection of Vitamin D deficiency and rickets.
J. Clin. Invest., 116:2062-2072.

3. HEANEY R.P. (2000)
Vitamin D: how much do we need, and how much is too much.
Osteoporos. Int., 11:553-555.

4. DAWSON-HUGHES B., HEANEY R.P., HOLICK M.F., LIPS P., MEUNIER P.J. (1997)
Prevalence of Vitamin D insufficiency in an adult normal population.
Osteoporos. Int., 7:439-443.

5. BISCHOFF-FERRARI H.A., GIOVANNUCCI E., WILLETT W.C., DIETRICH T., DAWSON-HUGHES B. (2006)
Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes.
Am. J. Clin. Nutr., 84:18-28.

6. HOLICK M.F(2004)
Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers and cardiovascular disease.
Am. J. Clin. Nutr., 80:16788S-1688S.

7. HEANEY R.P. (2010)
Defining deficiency of vitamin D .
Clinical Laboratory International , October 2010, vol.34 : 16-19.

8. HOLICK M.F. (2007)
Vitamin D deficiency.
N. Engl. J. Med., 357:266-281.

9. TAHA N. M. , VIETH R.(2010)
The problem of an optimal target level for 25-Hydroxyvitamin D, the test for vitamin D nutritional status .
Clinical Laboratory International , November 2010, vol.34 : 28-30

XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	**SAMPLE(S) CONTROLS (µl)**
Calibrators (0-6) Controls, Samples Incubation Buffer	50 - 150	- 50 150
Incubate for 2 hours at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Working HRP Conjugate	200	200
Incubate for 30 minutes at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 15 min at room temperature with continuous shaking.		
Stop Solution	100	100
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

DIAsource Catalogue Nr : KAP 1971	P.I. Number : 1700547/en	Revision nr : 120419/1

	Used symbols
	Consult instructions for use
	Storage temperature
	Use by
LOT	Batch code
REF	Catalogue number
CONTROL	Control
IVD	In vitro diagnostic medical device
	Manufacturer
	Contains sufficient for <n> tests
WASH SOLN CONC	Wash solution concentrated
CAL 0	Zero calibrator
CAL N	Calibrator #
CONTROL N	Control #
Ag 125I	Tracer
Ab 125I	Tracer
Ag 125I CONC	Tracer concentrated
Ab 125I CONC	Tracer concentrated
	Tubes
INC BUF	Incubation buffer
ACETONITRILE	Acetonitrile
SERUM	Serum
DIL SPE	Specimen diluent
DIL BUF	Dilution buffer
ANTISERUM	Antiserum
IMMUNOADSORBENT	Immunoadsorbent
DIL CAL	Calibrator diluent
REC SOLN	Reconstitution solution
PEG	Polyethylene glycol
EXTR SOLN	Extraction solution
ELU SOLN	Elution solution
GEL	Bond Elut Silica cartridges
PRE SOLN	Pre-treatment solution
NEUTR SOLN	Neutralization solution
TRACEUR BUF	Tracer buffer
	Microtiterplate
Ab HRP	HRP Conjugate
Ag HRP	HRP Conjugate
Ab HRP CONC	HRP Conjugate concentrate
Ag HRP CONC	HRP Conjugate concentrate
CONJ BUF	Conjugate buffer
CHROM TMB CONC	Chromogenic TMB concentrate
CHROM TMB	Chromogenic TMB solution
SUB BUF	Substrate buffer
STOP SOLN	Stop solution
INC SER	Incubation serum
BUF	Buffer
Ab AP	AP Conjugate
SUB PNPP	Substrate PNPP
BIOT CONJ CONC	Biotin conjugate concentrate
AVID HRP CONC	Avidine HRP concentrate
ASS BUF	Assay buffer
Ab BIOT	Biotin conjugate
Ab	Specific Antibody
SAV HRP CONC	Streptavidin HRP concentrate
NSB	Non-specific binding
2nd Ab	2nd Antibody
ACID BUF	Acidification Buffer
DIST	Distributor
TRAY	Incubation trays
PMSF	PMSF solution
	Protect from light