DiaSource®

IVD

CE

25OH Vitamin D Total ELISA

KAP1971

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History

Summary of change:

Previous Version:	Current Version:
200224-1	**220722**
Old DIAsource logo 	New DIAsource logo 
Immunoenzymetric assay for the in vitro quantitative measurement of 25-hydroxyvitamin D2 and D3 (25OH-D2 and 25OH-D3) in serum.	Immunoenzymetric assay for the *in vitro* quantitative measurement of 25-hydroxyvitamin D2 and D3 (25OH-D2 and 25OH-D3) in serum and plasma.
V. REAGENTS PROVIDED	**V. REAGENTS PROVIDED**
	Removal of the column relative to color code.
INC / BUF Incubation Buffer with casein and proclin — 1 vial 20 ml — green — Ready for use	INC / BUF Incubation Buffer with casein and proclin — 1 vial 30 ml — Ready for use
CHROM / TMB Chromogenic solution TMB (Tetramethylbenzydine) — 1 vial 12 ml — orange — Ready for use	CHROM / TMB Chromogenic solution TMB (Tetramethylbenzydine) — 1 vial 13 ml — Ready for use
STOP / SOLN Stop solution HCl 1M — 1 vial 12 ml — Ready for use	STOP / SOLN Stop solution 0.2M H_2SO_4 — 1 vial 13 ml — Ready for use
Note: For dilution of samples having concentrations of 25OH Vitamin D above the highest calibrator concentration, use Control 1 or a serum sample with a concentration of 25OH below 25ng/mL, and above 4.4ng/mL (limit of quantification of the assay), as measured in this assay. Use Ctrl 1 or this sample to dilute 2X the out of curve samples. Take the concentration of the Ctrl 1* or the low sample into account when calculating the dilution result. * Use the concentration of Ctrl 1 measured in the same run as the dilution run, not the mean concentration on the Ctrl 1 label! **Calculations:** Sample value = (Measured value – F1*Measured Ctrl 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 out of the calibration curve is diluted 4 times with Ctrl 1, and is measured at 70ng/mL. Ctrl 1 is measured in the same run at 20ng/mL. Dilution 4 times, F1 = 0.75; F2 = 0.25 Sample calculated value = (70 – 0.75*20) / 0.25 = 220ng/mL No international reference material is available	**Note**: Use Calibrator 0 for dilution of samples with values above the highest calibrator. No international reference material is available.
VII. D. REAGENT PREPARATION Prepare the solution according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 100 µl of concentrated conjugate and 50 µl of concentrated HRP to 10 ml of conjugate buffer.	**VII. D. REAGENT PREPARATION** Prepare the solution according to the number of used strips, as indicated in the below table: for example for 6 strips (48 wells): 130 µl of concentrated conjugate and 65 µl of concentrated HRP to 13 ml of conjugate buffer.

Nb. of strips	Volume of Conjugate Buffer (ml)	Volume of Concentrated Conjugate (µl)	Volume of Concentrated HRP(µl)
1	3	30	15

Nb of strips	Volume of Conjugate Buffer (ml)	Volume of Concentrated Conjugate (µl)	Volume of Concentrated HRP (µl)
1	3	30	15

2	5	50	25
3	6	60	30
4	8	80	40
5	9	90	45
6	10	100	50
7	12	120	60
8	14	140	70
9	16	160	80
10	18	180	90
11	20	200	100
12	22	220	110

2	5	50	25
3	7	70	35
4	9	90	45
5	11	110	55
6	13	130	65
7	15	150	75
8	17	170	85
9	19	190	95
10	21	210	105
11	23	230	115
12	25	250	125

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.

IX. SPECIMEN COLLECTION AND PREPARATION

- This kit is suitable for serum and heparinized plasma samples.
- Serum and heparinized plasma 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) = 0.9922 x (serum) + 0.2129 ng/ml, R^2 = 0.9944, n = 10

X. PROCEDURE

B. Procedure

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.
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 (400 rpm)

X. PROCEDURE

B. Procedure

3. Pipette 25 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 250 µl of Incubation Buffer into all the wells.
8. Pipette 250 µl of the working HRP conjugate solution into each well Incubate the microtiterplate for 30 minutes at room temperature, on a plate shaker (400 rpm)

XII. TYPICAL DATA

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

25OH-ELISA		OD units
Calibrator	0 ng/ml	2.66
	5.3 ng/ml	2.39
	15.0 ng/ml	1.83
	25.7 ng/ml	1.46
	54.3 ng/ml	0.81
	133 ng/ml	0.21

XII. TYPICAL DATA

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

25OH-ELISA		OD units
Calibrator	0 ng/ml	2.79
	3.44 ng/ml	2.56
	16.28 ng/ml	1.93
	32.39 ng/ml	1.20
	63.54 ng/ml	0.49
	122.76 ng/ml	0.16

XIII. PERFORMANCE AND LIMITATIONS

A. Limits of Detection

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.
The LoB was calculated by measuring the blank several times and calculating the 95th percentile of the distribution of the test values. The LoB was calculated to be 1.69ng/ml.
The LoD was calculated as described in the guideline. The LoD was calculated to be 2.81ng/ml.
The LoQ was calculated by testing 5 samples of low value 14 times in different test. The LoQ was calculated to be 4.39ng/ml with CV of 20%.

XIII. PERFORMANCE AND LIMITATIONS

A. Limits of Detection

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 2.07 ng/ml.

The LOD (Limit of detection) was calculated as the LOB - 1.65 standard deviation of a low concentration sample tested in 10 different run. The LOD was calculated to be 3.26 ng/ml.
The LOQ (Limit of quantitation) was calculated by testing 5 samples of low values, 10 times. The LOQ was calculated to be 3.35 ng/ml.

B. Specificity

Compound and Concentration	% Cross reaction
25OH-Vitamin D_3 at 10 ng/mL	100
25OH-Vitamin D_2 at 10 ng/mL	86
1,25$(OH)_2$-Vitamin D_3 at 200 ng/mL	20
1,25$(OH)_2$-Vitamin D_2 at 690 ng/mL	1.9
Vitamin D_3 at 200 ng/mL	2.9
Vitamin D_2 at 200 ng/mL	1.3
24,25$(OH)_2$-Vitamin D_3 at 20 ng/mL	>100
25,26$(OH)_2$-Vitamin D_3 at 4 ng/mL	>100
3-epi-25OH-Vitamin D_3 at 20 µg/mL	0.1

The effect of potential interfering substances on samples using the DIAsoure 25 OH Vitamin D Total ELISA test was evaluated. Different levels of Hemoglobin, Triglyceride, Vitamin C, Bilirubin Conjugate and Unconjugated and Zemplar in serum samples were tested on samples with different 25OH Vitamin D Concentration. Our acceptance criteria was to have interference of less than 10%. The tested substances did not affect the performance of the DIAsoure 25 OH Vitamin D Total ELISA test.

Substance	25OH Vitamin D (ng/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	7.6	250	-0.6%
		500	
	29.3	250	
		500	
	42.5	250	
		500	
Bilirubin Conjugated	6.0	50	-3.4%
		100	
	21.6	50	
		100	
	38.6	50	
		100	
Bilirubin Unconjugated	7.6	50	2.5%
		100	
	29.3	50	
		100	
	42.5	50	
		100	
Triglyceride	7.6	7.5	-4.3%
		125	
		250	
		500	
	29.3	7.5	
		125	
		250	
		500	
	42.5	7.5	
		125	
		250	
		500	
Vitamin C	6.0	1	2.5%
		10	
		100	
	21.6	1	
		10	
		100	
	38.6	1	
		10	
		100	

B. Specificity

Compound and Concentration	% Cross reaction
25OH-Vitamin D_3 at 10 ng/mL	92
25OH-Vitamin D_2 at 10 ng/mL	91
1,25$(OH)_2$-Vitamin D_3 at 200 ng/mL	3.10
1,25$(OH)_2$-Vitamin D_2 at 667 ng/mL	0.35
Vitamin D_3 at 200 ng/mL	0.17
Vitamin D_2 at 200 ng/mL	0.22
3-epi-25OH-Vitamin D_3 at 20 µg/mL	0.91

The effect of potential interfering substances on samples using the DIAsoure 25 OH Vitamin D Total ELISA test was evaluated. Different levels of Hemoglobin, Triglyceride, Vitamin C, Bilirubin Conjugate and Unconjugated in serum samples were tested on samples with different 25OH Vitamin D Concentration. Our acceptance criteria was to have interference of less than 10%. The tested substances did not affect the performance of the DIAsoure 25 OH Vitamin D Total ELISA test.

Substance	25OH Vitamin D (ng/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	21.87	250	-3.7%
		500	
	37.36	250	
		500	
Bilirubin Conjugated	21.87	50	2.5%
		100	
	37.36	50	
		100	
Bilirubin Unconjugated	21.87	50	-0.2%
		100	
	37.36	50	
		100	
Triglyceride	21.87	6.25	-2.1%
		125	
		250	
		500	
	37.36	6.25	
		125	
		250	
		500	
Vitamin C	21.87	1	2.9%
		10	
		100	
	37.36	1	
		10	
		100	
Biotin	21.87	0.2	2.5%
		2	
		4	
	37.66	0.2	
		2	
		4	

Biotin	8.7	0.2	4.7%
		2	
		4	
	19.8	0.2	
		2	
		4	
	36.1	0.2	
		2	
		4	
Zemplar	17.6	0.0013	-4.4%
		0.0025	
		0.0050	
	33.5	0.0013	
		0.0025	
		0.0050	

C. Precision

INTRA-ASSAY				INTER-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**	**Sample**	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	24	5.5 ± 0.4	7.8	A	39	17.7 ± 1.3	7.4
B	35	27.4 ± 1.6	5.7	B	10	26.3 ± 1.2	4.7
C	35	43.0 ± 1.2	2.7	C	10	42.1 ± 1.8	4.3
D	24	81.2 ± 2.0	2.5	D	21	85.4 ± 7.8	9.2

SD: Standard Deviation, CV: Coefficient of variation

E. Accuracy

RECOVERY TEST	
Added 25OH-Vit.D_3 (ng/ml)	**Recovery (%)**
0	100
25	96
50	92
Added 25OH-Vit.D_2 (ng/ml)	**Recovery (%)**
0	100
25	105
50	95

DILUTION TEST

A sample with a concentration known to be distributed throughout the measurable range was tested at equidistant dilutions, according to the dilution protocol in chapter V, to determine the linear range of the assay. A linear regression analysis was performed. The results are summarized in the following table:

Sample Dilution		Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	Slope	Y-Intercept	R^2	Recovery (%)
1/1	with a Ctrl 1 measured at 27.1ng/mL	101.8	101.8	1.02	-1.91	>0.98	100
1/2		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 linear range of the assay was found to be 33.6 ng/mL to 101.8 ng/mL.

C. Precision

INTRA-ASSAY				INTER-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**	**Sample**	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	24	20.40 ± 0.69	3.4	A	10	12.62 ± 0.79	6.3
B	24	33.32 ± 0.96	2.9	B	10	20,99 ± 0.69	3.3
				C	10	33.22 ± 1.30	3.9
				D	10	70.25 ± 2.42	3.4

SD: Standard Deviation, CV: Coefficient of variation

E. Accuracy

RECOVERY TEST	
Added 25OH-Vit.D_3 (ng/ml)	**Recovery (%)**
5	90
10	92
25	85
50	71
Added 25OH-Vit.D_2 (ng/ml)	**Recovery (%)**
5	88
10	91
25	88
50	83

DILUTION TEST

DILUTION TEST:			
Sample dilution	**Theoretical concentration (ng/ml)**	**Measured concentration (ng/ml)**	**Recovery (%)**
1/1	-	65.20	-
1/2	32.60	29.34	90.0
1/4	16.30	15.01	92.1
1/8	8.15	8.91	109.3

Samples were diluted with the zero calibrator.

The linear range of the assay was found to be 8.91 ng/mL to 65.20 ng/ml

XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5)	50	-
Controls, Samples	-	50
Incubation Buffer	150	150
Incubate for 2 hours at room temperature with continuous shaking at 400 rpm. Prepare the working HRP conjugate during the incubation and minimum 1h 45 minutes before its use. **The sequence of preparation is critical, see VII. Reagent Preparation** Aspirate the contents of each well. Wash 3 times with 350 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 350 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 15 min at room temperature with continuous shaking at 400 rpm.		
Stop Solution	100	100
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5)	25	-
Controls, Samples	-	25
Incubation Buffer	250	250
Incubate for 2 hours at room temperature with continuous shaking at 400 rpm. Prepare the working HRP conjugate during the incubation and minimum 1h 45 minutes before its use. The sequence of preparation is critical, see VII. Reagent Preparation Aspirate the contents of each well. Wash 3 times with 350 µl of Wash Solution and aspirate.		
Working HRP Conjugate	250	250
Incubate for 30 minutes at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 350 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 15 min at room temperature with continuous shaking at 400 rpm.		
Stop Solution	100	100
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

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US: For in-vitro diagnostic use only.

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 and plasma.

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.

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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.

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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 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 30 minutes incubation at room temperature, the microtiterplate is washed to stop the competition reaction. The Chromogene 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 Tests Kit	Reconstitution
Microtiterplate (96 breakable wells) with anti 25OH Vit. D2 and D3 (monoclonal antibodies)	96 wells	Ready for use
CAL 0 Calibrator 0: biological matrix with gentamycin and proclin	1 vial lyophilised	Add 1 ml distilled water
CAL N Calibrators 1-5 (see exact values on vial labels) in horse serum with gentamycin and proclin	5 vials lyophilised	Add 1 ml distilled water
CONTROL N Controls N = 2 in human serum with proclin	2 vials lyophilised	Add 1 ml distilled water
INC BUF Incubation Buffer with casein and proclin	1 vial 30 ml	Ready for use
CONJ CONC 25OH Vit D Concentrated Conjugate	1 vial 0.3 ml	Dilute 100 x with conjugate buffer
CONJ BUF Conjugate Buffer with casein and proclin	1 vial 30 ml	Ready for use
HRP CONC Concentrated HRP	1 vial 0.2 ml	Dilute 200 x with conjugate buffer
WASH SOLN CONC Wash solution (TRIS-HCl)	1 vial 10 ml	Dilute 200 x with distilled water (use a magnetic stirrer).
CHROM TMB Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 13 ml	Ready for use
STOP SOLN Stop solution 0.2M H_2SO_4	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: 25 µl, 250 µl and 1 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Plate shaker (400 rpm)
6. Washer for microtiterplates
7. Microtiterplate reader capable of reading at 450 nm and 650 nm (bichromatic reading)

VII. REAGENT PREPARATION

A. Calibrator 0: Reconstitute the Calibrator 0 with 1 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 during the incubation and minimum 1h45 minutes before its use (cf X.B.5).
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: example for 6 strips (48 wells): 130 µl of concentrated conjugate and 65 µl of concentrated HRP to 13 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.

Nb.of strips	Volume of Conjugate Buffer (ml)	Volume of Concentrated Conjugate (µl)	Volume of Concentrated HRP (µl)
1	3	30	15
2	5	50	25
3	7	70	35
4	9	90	45
5	11	110	55
6	13	130	65
7	15	150	75
8	17	170	85
9	19	190	95
10	21	210	105
11	23	230	115
12	25	250	125

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 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.

IX. SPECIMEN COLLECTION AND PREPARATION

- This kit is suitable for serum and heparinized plasma samples.
- Serum and heparinized plasma 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) = 0.9922 x (serum) + 0.2129 ng/ml, r2 = 0.9944, n = 10

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 25 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 250 µl of Incubation Buffer into all the wells.
5. Incubate for 2 hours at room temperature, on a plate shaker (400 rpm) Prepare the Working HRP conjugate solution during the incubation and minimum 1h 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 250 µl of the working HRP conjugate solution into each well Incubate the microtiterplate for 30 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 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. 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.
4. By interpolation of the sample OD 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-ELISA		OD units
Calibrator	0 ng/ml	2.79
	3.44 ng/ml	2.56
	16.28 ng/ml	1.93
	32.39 ng/ml	1.20
	63.54 ng/ml	0.49
	122.76 ng/ml	0.16

Note: 1 ng/ml = 2.5 pmol/ml

XIII. PERFORMANCE AND LIMITATIONS

A. Limits of Detection

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 2.07 ng/ml.
The LOD (Limit of detection) was calculated as the LOB - 1.65 standard deviation of a low concentration sample tested in 10 different run. The LOD was calculated to be 3.26 ng/ml.
The LOQ (Limit of quantitation) was calculated by testing 5 samples of low values, 10 times. The LOQ was calculated to be 3.35 ng/ml

B. Specificity

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

Compound and Concentration	% Cross reaction
25OH-Vitamin D_3 at 10 ng/mL	92
25OH-Vitamin D_2 at 10 ng/mL	91
1,25$(OH)_2$-Vitamin D_3 at 200 ng/mL	3.10
1,25$(OH)_2$-Vitamin D_2 at 667 ng/mL	0.35
Vitamin D_3 at 200 ng/mL	0.17
3-epi-25OH-Vitamin D_3 at 20 µg/mL	0.91

The effect of potential interfering substances on samples using the DIAsoure 25 OH Vitamin D Total ELISA test was evaluated. Different levels of Hemoglobin, Triglyceride, Vitamin C, Bilirubin Conjugate and Unconjugated in serum samples were tested on samples with different 25OH Vitamin D Concentration. Our acceptance criteria was to have interference of less than 10%. The tested substances did not affect the performance of the DIAsoure 25 OH Vitamin D Total ELISA test.

Substance	25OH Vitamin D (ng/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	21.87	250	-3.7%
		500	
	37.36	250	
		500	
Bilirubin Conjugated	21.87	50	2.5%
		100	
	37.36	50	
		100	
Bilirubin Unconjugated	21.87	50	-0.2%
		100	
	37.36	50	
		100	
Triglyceride	21.87	6.25	-2.1%
		125	
		250	
		500	
	37.36	6.25	
		125	
		250	
		500	
Vitamin C	21.87	1	2.9%
		10	
		100	
	37.36	1	
		10	
		100	
Biotin	21.87	0.2	2.5%
		2	
		4	
	37.36	0.2	
		2	
		4	

C Precision

The assay precision was calculated by running samples for a span of at least 20 days on 3 different lots. The results are summarized in the table below:

INTRA-ASSAY				INTER-ASSAY			
Sample	N	<X> ± SD (ng/ml)	C.V. (%)	Sample	N	<X> ± SD (ng/ml)	C.V. (%)
A	24	20.40 ± 0.69	3.4	A	10	12.62 ± 0.79	6.3
B	24	33.32 ± 0.96	2.9	B	10	20.99 ± 0.69	3.3
				C	10	33.22 ± 1.30	3.9
				D	10	70.25 ± 2.42	3.4

SD: Standard Deviation, CV: Coefficient of variation

D. Reproducibility

The reproducibility of the assay was done by testing three samples in duplicate for five days, twice a day, at three sites with two technicians per site. The mean results are summarized in the table below:

Sample	n	ng/mL		Within-Run	Between-Run	Between-Day	Between-Tech	Between-Site	Total
1	57	25.5	SD	0.22	0.61	0.98	1.54	2.21	2.59
			CV	0.3%	0.9%	3.8%	6.0%	8.7%	10.2%
2	57	52.9	SD	0.64	1.57	1.11	2.28	4.29	5.19
			CV	0.9%	2.3%	2.1%	4.3%	8.1%	9.8%
3	59	124.9	SD	1.00	1.74	1.84	3.39	4.98	6.25
			CV	1.4%	2.5%	1.5%	2.7%	4.0%	5.0%

E. Accuracy

Recovery was assessed by adding different levels of 25OH Vitamin D to samples. The results are summarized in the table below:

RECOVERY TEST	
Added 25OH-Vit.D_3 (ng/ml)	**Recovery (%)**
5	90
10	92
25	85
50	71
Added 25OH-Vit.D_2 (ng/ml)	**Recovery (%)**
5	88
10	91
25	88
50	83

DILUTION TEST:			
Sample dilution	**Theoretical concentration (ng/ml)**	**Measured concentration (ng/ml)**	**Recovery (%)**
1/1	-	65.20	-
1/2	32.60	29.34	90.0
1/4	16.30	15.01	92.1
1/8	8.15	8.91	109.3

Samples were diluted with the zero calibrator.

The linear range of the assay was found to be 8.91 ng/mL to 65.20 ng/ml.

F. Time delay

Time delay test between the last Calibrator and sample dispensing results is shown in the following table.

TIME DELAY			
	0 min (ng/ml)	10 min (ng/ml)	20 min (ng/ml)
Sample 1	27.9	30.5	30.2
Sample 2	49.5	47.5	49.0

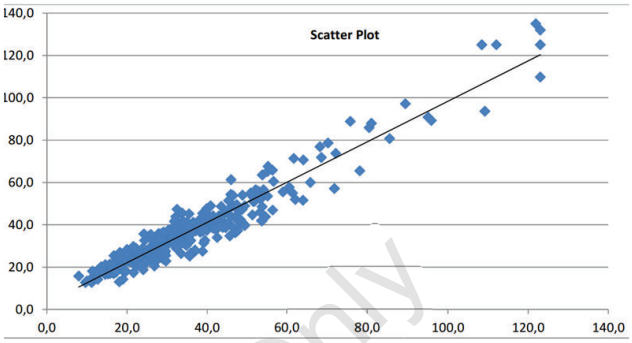
Assay results remain accurate even when incubation buffer is dispensed 10 and 20 minutes after the Calibrator has been added in the coated wells.

G. Limitations of the test

1. The test is an aid in the diagnosis and is to be used in conjunction with clinical findings.
2. The performance of this assay has not been established in a pediatric population.
3. Samples suspected of containing concentrations above the highest calibrator should be assayed in dilution.
4. Hemolysed samples should not be used.

H. Method comparison

The performance of the DIAsource 25OH Vitamin D Total ELISA test was determined by conducting a correlation study tested at three different sites using a total of 356 samples. The samples were tested on both the DIAsource 25OH Vitamin D Total ELISA test and a commercially available 25OH Vitamin D ELISA test. The results ranged from 8.0ng/ml to 123.0ng/ml, the correlation coefficient between the two methods was 0.917, with the 95% confidence interval of 87.6% to
93.6%, a slope of 0.954 and the y-intercept of 3.05. The following graph summarizes the results:



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:

Level	ng/mL
Deficient	<10
Insufficient	10-29
Sufficient	30-100
Potential Toxicity	>100

Reference ranges have been established based on 150 apparently healthy individuals. The individual patient serum samples used were obtained from a certified commercial source and were collected from an FDA Licensed Donor Center with informed consent. 50 samples were from Northern US (Pennsylvania), 50 samples were from Central US (Tennessee), and 50 samples were from Southern US (Florida). Samples were collected in the winter months (January - March), were between the ages of 21-92 years old and included both light skin and dark skin population. The donors from which samples were collected were not taking vitamin D supplements, had no family history of parathyroid, or calcium regulatory disease, had no history or Kidney, Liver, Parathyroid, Calcium related disease or bariatric surgery, and were not taking any medications known to affect absorption or catabolism of Vitamin D. The following table is the summary or results:

	Florida	Tennessee	Pennsylvania	Overall
Highest Conc. (ng/mL)	88.6	71.4	54.6	88.6
Lowest Conc. (ng/mL)	6.1	4.9	5.9	4.9
Median Conc. (ng/mL)	20.8	17.2	14.3	17.3

Only Central 95% (2.5% - 97.5%) of the results observed were used.

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 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 H_2SO_4. 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.

For more information, refer to the MSDS.

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5) Controls, Samples Incubation Buffer	25 - 250	- 25 250
Incubate for 2 hours at room temperature with continuous shaking at 400 rpm. Prepare the working HRP conjugate during the incubation and minimum 1h 45 minutes before its use. **The sequence of preparation is critical, see VII. Reagent Preparation** Aspirate the contents of each well. Wash 3 times with 350 µl of Wash Solution and aspirate.		
Working HRP Conjugate	250	250
Incubate for 30 minutes at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 350 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 15 min at room temperature with continuous shaking at 400 rpm.		
Stop Solution	100	100
Read on a microtiterplate reader Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

Other translations of this Instruction for Use can be downloaded from our website: https://www.diasource-diagnostics.com/

DIAsource Catalogue Nr : KAP1971	Revision nr : 220722

Revision date : 22/07/2022