



1,25(OH)₂ Vitamin D
ELISA
KAP1921

Read entire protocol before use.

1,25(OH)₂ Vitamin D ELISA

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of 1,25(OH)₂ Vitamin D in serum.

II. GENERAL INFORMATION

- A. **Proprietary name :** DIAsource 1,25(OH)₂ Vitamin D ELISA
- B. **Catalog number :** KAP1921 : 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

A. Biological activities

Vitamin D is mainly synthesized in the skin from 7-dehydrocholesterol and is partially from dietary and supplementation origin. In the liver, Vitamin D is hydroxylated on carbon 25 to produce the intermediate 25OH Vitamin D. 25OH Vitamin D is further metabolized before it can carry out the functions of Vitamin D on intestine, kidneys, bone and other organs and tissues. This subsequent reaction takes place in the kidneys and in other tissues. Thus 25OH Vitamin D is further hydroxylated in the 1 α -position to produce 1 α ,25-dihydroxyvitamin D (1,25(OH)₂ Vitamin D). In addition to the above-mentioned tissues, placenta of pregnant women and macrophage cells in case of sarcoidosis can also produce some amount of 1,25(OH)₂ Vitamin D.

1,25(OH)₂ Vitamin D is the active form of Vitamin D with regard to the known functions whereas 25OH Vitamin D and Vitamin D itself can be excluded as being physiologically functional. 1,25(OH)₂ Vitamin D stimulates the intestinal absorption of both calcium and phosphorus. It also stimulates bone resorption and mineralization thereby preventing the development of rickets and osteomalacia.

1,25(OH)₂ Vitamin D is also active in other tissues responsible for Calcium transport (placenta, kidney, mammary gland,...) and endocrine glands such as parathyroid glands. 1,25(OH)₂ Vitamin D is rapidly metabolized and its half-life is approximately 12h in plasma. Its main metabolite is calcitroic acid, a C-23 carboxylic derivative, essentially without any biological activity. In addition to this pathway, 1,25(OH)₂ Vitamin D undergoes 24-hydroxylation to produce 1,24,25-trihydroxyvitamin D. This compound has less biological activity than its parent and this metabolic route is considered as a minor pathway.

The levels of 1,25(OH)₂ Vitamin D in plasma or serum is 100 to 1000 less than that of 25OH Vitamin D. Due to its low concentrations and the presence of many similar metabolites, the measurement of 1,25(OH)₂ Vitamin D requires extraction and separation by chromatography.

B. Clinical application

The measurement of circulating 1,25(OH)₂ Vitamin D is indicated in several disorders affecting calcium metabolism such as : phosphate diabetes, sarcoidosis, renal failure, hyper and hypo-parathyroidism, rickets, tumor-associated hypercalcemia, hypercalciuria, Vitamin-resistant dysfunction and treatment with anti-convulsive medication.

IV. PRINCIPLES OF THE METHOD

Only samples and controls, not the calibrators, are extracted with a mixture of solvents and applied on cartridges to separate 1,25(OH)₂ Vitamin D from the other Vitamin D metabolites. After elution of the 1,25(OH)₂ Vitamin D from the samples and controls cartridges, the calibrators, eluted samples and eluted controls are incubated directly in microtiterplate coated with anti-1,25(OH)₂ Vitamin D antibodies.


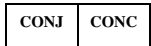
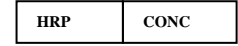
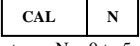

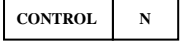
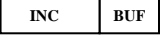
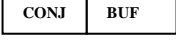

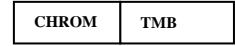
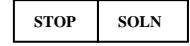
After an overnight incubation at 4°C, the microtiter plate is washed and the working conjugate solution is added and incubated for 1 hour at 4°C.



The microtiterplate is then washed to stop the competition reaction. The Chromogenic solution (TMB) is added and incubated for 15 minutes at room temperature. The reaction is stopped with the addition of Stop Solution and the microtiterplate is read at the appropriate wavelength.

The amount of 1,25(OH)₂ Vitamin D is determined colourimetrically by measuring the absorbance, which is inversely proportional to the 1,25(OH)₂ Vitamin D concentration.

A calibration curve is plotted and the 1,25(OH)₂ Vitamin D 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 breakable wells coated with anti-1,25(OH) ₂ Vitamin D antibodies	96 wells	blue	Ready for use
 1,25(OH) ₂ Vitamin D Concentrated Conjugate	1 vial 1 mL	blue	Dilute 40 x with conjugate buffer
 Concentrated HRP	1 vial 0.2 mL	yellow	Dilute 200 x with conjugate buffer
 Calibrators - N = 0 to 5 (see exact values on vial labels) in phosphate buffer with bovine casein and gentamycin	6 vials lyophilised	yellow	Add 1mL distilled water
 Wash solution (TRIS-HCl)	1 vial 10 mL	brown	Dilute 200 x with distilled water (use a magnetic stirrer)
 Controls - N = 1 or 2 in human plasma with gentamycin	2 vials lyophilised	silver	Add 2mL distilled water
 Incubation Buffer with proclin	1 vial 20 mL	green	Ready for use
 Conjugate Buffer with casein and proclin	1 vial 30 mL	red	Ready for use
 Elution Solution: contains methanol	1 vial 25 mL	white	Ready for use
 Chromogenic solution TMB (Tetramethylbenzidine)	1 vial 25 mL	brown	Ready for use
 Stop solution HCl 1.5 N	1 vial 12 mL		Ready for use

	4		
Adhesive Strips			
	42		
Silica cartridges			

Note : Use Calibrator 0 for dilution of samples with values above the highest calibrator (dilute before extraction step).

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

- 1 Distilled water
- 2 Diisopropylether (“for analysis”; GC purity ≥ 99%)
- 3 Cyclohexane (“for analysis”; GC purity ≥ 99.5 %)
- 4 Ethyl acetate (“for analysis”; GC purity ≥ 99.5 %)
- 5 Ethanol absolute (“for analysis”; GC purity ≥ 99.9 %)
- 6 Dichloromethane (“for analysis”; GC purity ≥ 99.8 %)
- 7 Pipettes for delivery of: 50µL, 100µL, 150µL, 200 µL, 1mL and 2 mL (the use of accurate pipettes with disposable plastic tips is recommended)
- 8 Glass tubes (12 x 75 mm) for extraction and for elution (closed with a cap for the extraction step).
- 9 Glass tubes (16 x 100 mm) or (12 x 120 mm), or polypropylene tubes (falcon 2097), for the washing of the cartridges.
- 10 Vortex mixer
- 11 Magnetic stirrer
- 12 Centrifuge operating at 800 g
- 13 Microtiterplate reader capable of reading at 450 nm and 650 (bichromatic reading)

VII. REAGENT PREPARATION

A. **Calibrators:** Reconstitute the calibrators with 1mL distilled water (**just before incubation step**).

B. **Controls:** Reconstitute the controls with 2mL distilled water.

C. **Working HRP conjugate solution:**
! The working HRP conjugate solution is to be prepared absolutely one hour before adding the solution in the plate !

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): 250µL of concentrated conjugate and 50µL of concentrated HRP to 10mL 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	75	15	3
2	125	25	5
3	150	30	6
4	200	40	8
5	225	45	9
6	250	50	10
7	300	60	12
8	350	70	14
9	400	80	16
10	450	90	18
11	500	100	20
12	550	110	22

D. **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.

E. **Extraction solvent:** 2 ml for each control or sample to be tested are needed. **Prepare a fresh solution** of diisopropylether, cyclohexane and ethyl acetate: 50/40/10 volume/volume).

Be careful : exact proportion of volume have to be strictly respected.

- F Washing solvent** : 1 ml for each control or sample to be tested is needed.
Prepare a fresh solution of diisopropylether, cyclohexane, ethyl acetate and ethanol absolute (50/40/10/1 volume/volume).

Be careful : exact proportion of volume have to be strictly respected .

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; **except the cartridges which must be stored at room temperature.**
- After their reconstitution, calibrators and controls are stable for one week at 2-8°C. For longer storage periods, aliquots should be made and kept at -20°C for 2 months maximum. Avoid subsequent freeze-thaw cycles.
- Freshly prepared Working Wash solution should be used on the same day.
- After its use, discard working HRP conjugate.
- **Use freshly prepared extraction solvent and washing solvent, do not store them.**
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- The kit is suitable for serum samples.
- Serum samples must be kept at 2-8°C.
- If the test is not run within 24 hrs, storage in aliquots, at -20°C is recommended.
- Avoid subsequent freeze-thaw cycles.
- After thawing, the samples should be vortexed and centrifuged.

X. PROCEDURE

I. Extraction step : ! Only for controls and samples !

1. Label glass tubes (12x75 mm) for extraction: 2 controls and up to 40 samples.
2. Add 0.5mL control or sample in the respective tubes.
3. Dispense 2mL extraction solvent in each tube.
4. Tubes are closed with a cap and placed on a shaker for 1 hour at 1200 rpm.
5. Centrifuge each tube for 5 minutes at room temperature (at 800 g).
6. Supernatants are needed for the next step of separation.

II. Separation step : ! Only for controls and samples !

1. Label glass tubes (16 x 100 mm) or (12 x 120 mm), or polypropylene tubes (falcon 2097), for washing cartridges: 2 controls and up to 40 samples.
2. Put one silica cartridge in each tube.
3. Apply 1.6mL of supernatant (2 x 0.8mL), obtained after extraction step, on cartridge. Let draw by gravity.
4. Wash cartridges with 1mL washing solvent (cf: reagent preparation).
! Be careful: never apply vacuum on cartridges, just let solvent draw by gravity.
5. Add 500µL dichloromethane on each cartridge, let draw by gravity.
6. Add 500µL of distilled water on each cartridge and centrifuge each tube for 5 minutes at room temperature (at 800 g).
7. Label glass tubes (12 x 75 mm) for elution of 1,25(OH)₂ Vitamin D. After centrifugation, transfer cartridges in the corresponding glass tubes.
8. Apply 300µL elution solution on each cartridge to elute 1,25(OH)₂ Vitamin D and centrifuge for 5 minutes at room temperature (at 800 g).
9. **Vortex** the eluted fraction.

Note : After this step, samples must be incubated in coated microtiterplate immediately to avoid degradation.

III. Incubation step :

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. Vortex briefly reconstituted calibrators, extracted controls and extracted samples.
4. Pipette 150µL of Incubation Buffer into all wells.
5. Pipette 50µL of each Calibrator (not extracted), eluted controls and eluted samples into the appropriate wells.
6. Incubate for 18±2 hours, at 2-8°C. Cover the plate with a lid or a sealing film.

Prepare the Working HRP conjugate solution 60 min +/- 15 min before washing the wells after the overnight incubation.

7. Aspirate the liquid from each well.
Wash the plate 3 times by:
 - dispensing 0.35mL of Wash Solution into each well and
 - aspirating the content of each well
8. Pipette 200µL of Working HRP conjugate solution into each well.
9. Incubate for 1 hour at 4°C. Cover the plate with a lid or a sealing film.
10. Aspirate the liquid from each well.
Wash the plate 3 times by:

- dispensing 0.35 ml of Wash Solution into each well and
- aspirating the content of each well

11. Pipette 200µL of the Chromogenic solution into each well within 15 minutes following the washing step.
12. Incubate the microtiterplate for 15 minutes at room temperature, avoid direct sunlight.
13. Pipette 100µL Stop Solution into each well.
14. Read absorbances at 450 nm (reference filter 630nm 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 1,25(OH)₂ 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.

1,25(OH) ₂ Vitamin D ELISA	OD units
Calibrator :	
0 pg/mL	3.06
7.5 pg/mL	2.54
25 pg/mL	1.96
62.5 pg/mL	1.32
170 pg/mL	0.60
322.5 pg/mL	0.30

Note : 1 pg/mL = 2.4 pmol/L

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 1.7pg/mL.

B. Specificity

Cross reactivity of the 1,25(OH)₂ Vitamin D 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-Reactivity (%)
1,25(OH) ₂ -Vitamin.D3 at 200 pg/mL	114
1,25(OH) ₂ -Vitamin.D2 at 200 pg/mL	108
25OH-Vitamin-D3 at 1µg /mL	0.004
25OH-Vitamin-D2 at 1µg /mL	0.0003
24,25(OH) ₂ -Vitamin.D3 at 200 ng/mL	0.03
25,26(OH) ₂ -Vitamin.D3 at 400ng/mL	0.02

The effect of potential interfering substances on samples using the DIAsource 1,25(OH)₂ Vitamin D ELISA test was evaluated. Different levels of Hemoglobin, Bilirubin (conjugated and unconjugated), Triglyceride and Vitamin C in serum samples were tested on samples with different 1,25(OH)₂ Vitamin D Concentration. Our acceptance criteria was to have interference of less than 15%. The tested substances did not affect the performance of the DIAsource 1,25(OH)₂ Vitamin D ELISA.

Substance	1,25(OH) ₂ VitaminD (pg/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	31.7	250	5.0%
		500	
	186.5	250	
		500	
Bilirubin Conjugated	31.7	50	-12.3%
	186.5	50	
Bilirubin Unconjugated	31.7	50	-0.4%
		100	
	186.5	50	
		100	
Triglyceride	31.7	50	-1.0%
		100	
		250	
	186.5	50	
		100	
		250	
Vitamin C	31.7	100	4.9%
		1000	
	186.5	100	
		1000	

C. Precision

INTRA-ASSAY PRECISION

INTER-ASSAY PRECISION

Sample	N	<X> ± SD (pg/mL)	CV (%)	Sample	N	<X> ± SD (pg/mL)	CV (%)
A	13	36.5 ± 4.1	11.3	A	8	35.4 ± 4.2	11.9
B	13	237.7 ± 12.2	5.1	B	8	201.2 ± 16.0	8.0

SD: Standard Deviation; CV: Coefficient of variation

D. Accuracy

The sample was diluted with Calibrator 0, before extraction step.

DILUTION TEST

Sample Dilution	Theoretical Concentration (pg/mL)	Measured Concentration (pg/mL)	Slope	Y-Intercept	R	Recovery (%)
1/1	118.9	118.9	0.99	1.12	0.99	100
1/2	59.4	60.7				102
1/4	29.7	29.3				99
1/8	14.8	16.6				112

Conversion factor :

From pg/mL to pmol/L : x 2.4

From pmol/L to pg/mL : x 0.42

To the best of our knowledge, no international reference material exists for this parameter.

RECOVERY TEST

Added 1,25(OH) ₂ -Vitamin D (pg/mL)	Recovered 1,25(OH) ₂ -Vitamin D (pg/mL)	Recovered (%)
52.4	54.1	103
104.7	111.1	106
157.1	155.8	99

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. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values.

The observed ranges are based on 2.5% to 97.5% percentiles.

Population	Range (pg/ml)	Median	SD	n
Normal subjects	5.3 – 91.6	32.6	23.4	118

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

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	CALIBRATORS μL	SAMPLE(S) CONTROLS μL
<u>EXTRACTION</u> Calibrators Samples / Controls Extraction solvent	- - -	- 500 2000
Shaking Centrifugation	1 hour at 1200 rpm 5 minutes at 800 g	
<u>SEPARATION</u> Supernatant from extraction step	-	1600
<u>CARTRIDGE</u> Supernatant Washing Solvent Dichloromethane Distilled water Centrifugation Elution solution Centrifugation	1600μL 1000μL 500μL 500μL 5 minutes at 800 g 300μL 5 minutes at 800 g Vortex	
<u>INCUBATION STEP</u> <u>In microtiterplate</u> Incubation Buffer Calibrators Extracted samples	150μL 50μL -	150μL - 50μL
	Cover the plate with a lid or sealing film Incubate 18 ± 2 h (overnight) at 4°C (2-8°C) Prepare working HRP solution 1 hour before next step Aspirate the contents of each well Wash 3 times with 350μL of Wash Solution and aspirate	
Working HRP Conjugate	200μL	200μL
Cover the plate with a lid or sealing film and Incubate for 1 hour at 4°C (2-8°C). Aspirate the contents of each well. Wash 3 times with 350 μl of Wash Solution and aspirate.		
TMB	200μL	200μL
Incubate for 15 min at room temperature (18°C to 25°C).		
Stop Solution	100μL	100μL
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

DIAsource Catalogue Nr : KAP1921	P.I. Number : 1700551/en	Revision nr : 141002/1
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	Used symbols
	Consult instructions for use
	Storage temperature
	Use by
LOT	Batch code
REF	Catalogue number
CONTROL	Control
I V D	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	Immunoabsorbent
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
µT	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
PREC AGENT	Precipitating Agent
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
STRIP	Dot Strip
SUB	Substrate
EXTR SOLN CONC	Extraction Buffer Concentrate
CART	Cartridge
SAV HRP	Streptavidin HRP
PIPETTE	Pipette
WASH SOLN	Wash buffer