1,25(OH)$_2$-VIT.D-RIA-CT

KIR1929

For Informational/Research Purposes Only

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

History

Resume of change :

Previous Version : 190619/1	**Current Version :** 191227/1
II. GENERAL INFORMATION **For technical assistance or ordering information contact :** **Tel : +32 (0)10 84.99.11 Fax : +32 (0)10 84.99.91**	***II. GENERAL INFORMATION*** **For technical assistance or ordering information contact :** **Tel : +32 (0)10 84.99.11 Fax : +32 (0)10 84.99.91** **For technical assistance or ordering information in the United States contact :** **Immuno-Biological Laboratories, Inc. (IBL-America)** **Tel : 1-888-523-1246 Fax : 1-763-780-2988 Email : info@ibl-america.com**
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Read entire protocol before use.

1,25(OH)$_2$-VIT.D-RIA-CT

I. INTENDED USE

Radioimmunoassay for the measurement of human 1,25(OH)$_2$-Vitamin D (1,25(OH)$_2$-Vit.D) in serum and plasma.
For Research Use Only. Not for Use in Diagnostic Procedures.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource 1,25(OH)$_2$-VIT.D-RIA-CT Kit

B. Catalog number : KIR1929 : 48 tests

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

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III. BACKGROUND

Biological activities

Vitamin D3 is mainly synthesized in the skin from 7- dehydrocholesterol and is partially from dietary origin. In the liver, Vitamin D3 is hydroxylated on carbon 25 to produce the obligatory intermediate 25-OH-D3. 25-OH-D3 must be metabolized further before it can carry out the functions of Vitamin D on intestine, kidney and bone. This subsequent reaction takes place exclusively in the kidney in the non-pregnant mammal. Thus 25-OH-D3 is further hydroxylated in the 1α-position to produce 1α,25 dihydroxyvitamin D3 (1α,25-(OH)$_2$D3).

In addition to renal tissue, placenta of pregnant women and macrophage cells in case of sarcoidis can also produce some amount of 1α,25-(OH)$_2$D3. 1α,25-(OH)$_2$D3 is the active form of Vitamin D with regard to the known functions whereas 25-OH-D3 and Vitamin D3 itself can be excluded as being physiologically functional. Furthermore since 1α,25-(OH)$_2$D3 is produced in the kidney and has some of its functions in the bone and intestine, it must be considered as a hormone. This hormone stimulates the intestinal absorption of both calcium and phosphorus. It also stimulates bone resorption and mineralization thereby preventing the development of rickets and osteomalacia.

1α,25-(OH)$_2$D3 might also be active in other tissues responsible for Calcium transport (placenta, kidney, mammary gland, ...) and endocrine glands such as parathyroid glands. 1α,25-(OH)$_2$D3 is rapidly metabolized and its lifetime is approximately 2-4 h in plasma. Its main metabolite is calcitroic acid, a C-23 carboxylic derivative essentially without any biological activity. In addition to this pathway, 1α,25-(OH)$_2$D3 undergoes 24-hydroxylation to produce 1,24,25-trihydroxy-Vitamin D3. This compound has less biological activity than its parent and this metabolism is considered as a minor pathway.

The levels of 1α,25-(OH)$_2$D3 in plasma or serum is 100 to 1000 less than that of 25-OH-D3. Due to its low concentrations and the presence of many similar metabolites, the measurement of 1α,25-(OH)$_2$D3 requires extraction and separation either by HPLC or by column chromatography.

IV. PRINCIPLES OF THE METHOD

Only samples and controls, not the calibrators, are extracted with a mix of solvents and applied on cartridges to separate 1,25(OH)$_2$ Vitamin-D from other Vitamin-D metabolites. After elution of samples and controls, the calibrators, samples and controls are incubated in coated tubes. A fixed amount of ^{125}I labelled 1,25(OH)$_2$ Vitamin D competes with the 1,25(OH)$_2$ Vitamin D to be measured present in the sample or in the calibrator for a fixed amount of antibody sites immobilized on the wall of a polystyrene tube. After an overnight incubation at room temperature, an aspiration step terminates the competition reaction. The tubes are then washed with washing solution and aspirated. 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	48 Tests Kit	Colour Code	Reconstitution
Tubes coated with anti 1,25(OH)$_2$-Vitamin D	1 x 48	green	**Ready** for use
Ag | **^{125}I** TRACER: ^{125}Iodine labelled 1,25(OH)2-Vitamin D (HPLC grade) in phosphate buffer with bovine casein and gentamycin.	1 vial lyophilised 75 kBq	red	**Add** 26 ml reconstitution solution
CAL | **N** Calibrators - N = 1 to 5 **(see exact values on vial labels)** in phosphate buffer with bovine casein and gentamycin	5 vials lyophilised	yellow	**Add** 2 ml elution solution
WASH | **SOLN** | **CONC** Wash solution (TRIS-HCl)	1 vial 10 ml	brown	**Dilute** 70 x with distilled water (use a magnetic stirrer).
CONTROL | **N** Controls - N = 1 or 2 in human plasma with gentamycin	2 vials lyophilised	silver	**Add** 2 ml distilled water
REC | **SOLN** Reconstitution Solution: phosphate buffer with bovine casein and azide (<0.1%)	1 vial 30 ml	black	**Ready** for use
ELU | **SOLN** Elution Solution: phosphate buffer with bovine casein, methanol and azide (<0.1%)	1 vial 30 ml	green	**Ready** for use
GEL Bond Elut Silica cartridges	20		**Store** at 18-25°C.

Note : Use elution solution for calibrator 0 and for dilution of samples with values above the highest calibrator (dilute after separation step).

VI. SUPPLIES NOT PROVIDED

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

1 Distilled water
2 Diisopropylether (p.a.)
3 Cyclohexane (p.a.)
4 Ethyl acetate (p.a.)
5 Ethanol absolute (p.a.)
6 Dichloromethane (p.a.)
NB: A DIAsource extraction kit containing all these solvents is available under reference: 3019700. This kit contains quantities of solvents necessary to run 5 x 48 tests of 1,25(OH)$_2$-VIT.D-RIA-CT.
7 Pipettes for delivery of: 200 µl, 500 µl, 1 ml 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 Tube shaker (1200 rpm)
14 5 ml automatic syringe (Cornwall type) for washing
15 Aspiration system (optional)
16 Any gamma counter capable of measuring ^{125}I may be used (minimal yield 70%).

VII. REAGENT PREPARATION

A. **Calibrators**: Reconstitute the calibrators with 2 ml elution solution (**just before the incubation step**).
B. **Controls**: Reconstitute the controls with 2 ml distilled water.
C. **I^{125} 1,25(OH)$_2$-Vitamin.D** : Reconstitute with 26 ml of reconstitution solution.
D. **Working Wash solution**: Prepare an adequate volume of Working Wash solution by adding 69 volumes of distilled water to 1 volume of Wash Solution (70x). 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) according to the number of extractions, as indicated in the table below.
Be careful : the exact proportion of each solvents has to be strictly respected.

Nb of extraction*	Diisopropylether (ml)	Cyclohexane (ml)	Ethyl acetate (ml)
1	1.1	0.9	0.2
8	8.8	7.0	1.8
18	19.8	15.8	4.0

*Specimen samples and controls

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) according to the number of extractions, as indicated in the table below.
Be careful : the exact proportion of each solvents has to be strictly respected.

Nb of extraction*	Diisopropylether (ml)	Cyclohexane (ml)	Ethyl acetate (ml)	Ethanol (µl)
1	0.5	0.4	0.1	11
8	4.4	3.5	0.9	88
18	9.9	7.9	1.98	198

*Specimen samples and controls

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.
- The calibrators and controls are very unstable, use them immediately after reconstitution, freeze immediately in aliquots and keep them at –20°C for 3 months. Avoid subsequent freeze-thaw cycles.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, tracer is stable until expiry date, if kept in the original well-closed vial at 2 to 8°C.
- **Use freshly prepared extraction solvent and washing solvent, do not store them**.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

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IX. SPECIMEN COLLECTION AND PREPARATION

- Serum and plasma 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.
- Serum or plasma (EDTA and heparin) provides similar results.

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.
Use a clean disposable pipette tip for addition of each different reagent and sample in order to avoid cross-contamination. High precision pipettes or automated pipetting equipment will improve the precision.
Respect the incubation times.
Prepare a calibration curve for each run, do not use data from previous runs.

B. Procedure

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

1. Label glass tubes (12x75 mm) for extraction: 2 controls and up to 16 samples.
2. Add 0.5 ml control or sample in the respective tubes.
3. Dispense 2 ml 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 16 samples.
2. Put one "Bond Elut" cartridge in each tube.
3. Apply 1.6 ml of supernatant (2 x 0.8 ml), obtained after extraction step, on cartridge.
4. Then, wash cartridges with 1 ml washing solvent (cfr reagent preparation). ! Be careful never apply vacuum on cartridges, just let solvent draw by gravity.
5. Add 300 µl dichloromethane on each cartridge, let draw by gravity.
6. Add 300 µl of distilled water on each cartridge.
7. Centrifuge each tube for 5 minutes at room temperature (at 800 g).
8. 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.
9. Apply 400 µl elution solution on each cartridge to elute 1,25 $(OH)_2$-Vitamin D and centrifuge 5 minutes at room temperature (at 800 g).
10. **Vortex** the eluted fraction.

Note : After this step, samples must be incubated in coated tubes as soon as possible to avoid degradation.

III. Incubation step :

1. Label coated tubes in duplicate for each calibrator, control and sample. For the determination of total counts, label 2 normal tubes
2. Briefly vortex calibrators (use elution solution as zero calibrator), extracted controls and samples and dispense 150 µl of each into the respective tubes.
3. Dispense 500 µl of ^{125}Iodine labelled 1,25(OH)2-Vitamin D into each tube, including the uncoated tubes for total counts.
4. Shake the tube rack gently by hand to liberate any trapped air bubbles.
5. Incubate overnight at room temperature
6. Aspirate (or decant) the content of each tube (except total counts). Be sure that the plastic tip of the aspirator reaches the bottom of the coated tube in order to remove all the liquid.
7. Wash tubes with 2 ml Working Wash solution (except total counts) and aspirate (or decant). Avoid foaming during the addition of the Working Wash solution.
8. Aspirate (or decant) the content of each tube (except total counts).
9. Wash tubes again with 2 ml Wash solution (except total counts) and aspirate (or decant).
10. After the last washing, let the tubes stand upright for two minutes and aspirate the remaining drop of liquid.
11. Count tubes in a gamma counter for 60 seconds.

XI. CALCULATION OF RESULTS

1. Calculate the mean of duplicate determinations.
2. Calculate the bound radioactivity as a percentage of the binding determined at the zero calibrator point (0) according to the following formula :

$$B/B0\ (\%) = \frac{\text{Counts (Calibrator or sample)}}{\text{Counts (Zero Calibrator)}} \times 100$$

3. Using a 3 cycle semi-logarithmic or logit-log graph paper, plot the (B/B0(%)) values for each calibrator point as a function of the 1,25(OH)2-Vitamin D concentration of each calibrator point. Reject obvious outliers.
4. 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.
5. By interpolation of the sample (B/B0 (%)) values, determine the 1,25(OH)2-Vitamin D concentrations of the samples from the calibration curve.
6. For each assay, the percentage of total tracer bound in the absence of unlabelled 1,25(OH)2-Vitamin D (B0/T) must be checked.

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		cpm	B/Bo (%)
Total count		43937	
Calibrator	0.0 pg/ml	16687	100.0
	6.0 pg/ml	15268	91.5
	20.0 pg/ml	12345	74.0
	63.0 pg/ml	8033	48.1
	230.0 pg/ml	3554	21.3
	430.0 pg/ml	2148	12.9

XIII. PERFORMANCE AND LIMITATIONS

A. Detection limit

The LOB (Limit of Blank) was calculated by measuring the blank several times and was calculated as the mean - 1.65 standard deviations of the distribution of the best values. The LOB was calculated to be 0.55 pg/ml.
The LOD (limit of detection) was calculated as the LOB – 1.65 standard deviations of a low concentration sample tested in 10 different runs. The LOD was calculated to be 2.88 pg/ml.
The LOQ (Limit of Quantitation) was calculated by testing 5 samples of low values 10 times. The LOQ was calculated to be 8.5 pg/ml.

B. Specificity

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

Compound	Cross-Reactivity (%)
1,25$(OH)_2$-Vitamin.D3	100
1,25$(OH)_2$-Vitamin.D2	92.31
25OH-Vitamin-D3	0.001
24,25$(OH)_2$-Vitamin.D3	0.005
25,26$(OH)_2$-Vitamin.D3	0.20

Note: this table shows the cross-reactivity for the anti 1,25(OH)2-Vitamin D

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

C. Precision

INTRA-ASSAY PRECISION INTER-ASSAY PRECISION

Serum	N	<X> ± SD (pg/ml)	CV (%)	Serum	N	<X> ± SD (pg/ml)	CV (%)
A	20	37.9 ± 2.6	6.8	A	10	13.6 ± 1.7	12.7
B	20	98.2 ± 7.3	7.4	B	10	32.3 ± 3.6	11.3

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SD: Standard Deviation; CV: Coefficient of variation

7. D. Accuracy

DILUTION TEST

Sample dilution	Theoritical concent. (pg/ml)	Measured concent. (pg/ml)	Recovery (%)
1/1	70.0	70.0	100%
1/2	35.0	35.7	102%
1/4	17.5	14.5	83%
1/8	8.8	7.8	89%
1/16	4.4	4.6	105%

The sample was diluted with Elution solution.

RECOVERY TEST

Added 1,25(OH)2-Vit.D (pg/ml)	Measured 1,25(OH)2 Vit.D concentrations Total (pg/ml)	Blanked (pg/ml)	Recovery (%)
0.0	22.5		
25.0	46.3	23.8	95.2%
50.0	70.0	47.5	95.0%
100.0	122.7	100.2	100.2%
Added 1,25(OH)2-Vit.D (pg/ml)	**Measured 1,25(OH)2 Vit.D concentrations Total (pg/ml)**	**Blanked (pg/ml)**	**Recovery (%)**
0.0	22.5		
25.0	52.1	29.6	118.4%
50.0	70.4	47.9	95.8%
100.0	112.9	90.4	90.4%

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.

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.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises.

XV. PRECAUTIONS AND WARNINGS

Safety

For *research* use only.

This kit contains ^{125}I (half-life: 60 days) , emitting ionizing X (28 keV) and γ (35.5 keV) radiations. This radioactive product can be transferred to and used only by authorized persons; purchase, storage, use and exchange of radioactive products are subject to the legislation of the end user's country. In no case the product must be administered to humans or animals.

All radioactive handling should be executed in a designated area. away from regular passage. A logbook for receipt and storage of radioactive materials must be kept in the lab. Laboratory equipment and glassware, which could be contaminated with radioactive substances, should be segregated to prevent cross contamination of different radioisotopes.

Any radioactive spills must be cleaned immediately in accordance with the radiation safety procedures. The radioactive waste must be disposed of following the local regulations and guidelines of the authorities holding jurisdiction over the laboratory. Adherence to the basic rules of radiation safety provides adequate protection.

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 reagents (sodium azide as preservative). Azide in this kit may react with lead and copper in the plumbing and in this way form highly explosive metal azides. During the washing step, flush the drain with a large amount of water to prevent azide build-up.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVI. BIBLIOGRAPHY

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XVII. SUMMARY OF THE PROTOCOL

	TOTAL COUNTS µl	CALIBRATORS µl	SAMPLE (S) CONTROLES µl
EXTRACTION			
Calibrators	-	-	-
Samples / Controls	-	-	500
Extraction solvent	-	-	2000
Shaking	1 hour at 1200 rpm		
Centrifugation	5 minutes at 800 g		
SEPARATION			
Supernatant from extraction step	-	-	1600
CARTRIDGE			
Supernatant	1600 µl		
Washing Solvent	1000 µl		
Dichloromethane	300 µl		
Distilled water	300 µl		
Centrifugation	5 minutes at 800 g		
Elution solution	400 µl		
Centrifugation	5 minutes at 800 g		
	Vortex		
INCUBATION			
Calibrators	-	150	-
Extracted samples	-	-	150
Tracer	500	500	500
Incubation	Overnight at 18-25°C.		
Separation	-	Aspirate (or decant)	
Washing Solution	-	2 ml	
Separation	-	Aspirate (or decant)	
Washing Solution	-	2 ml	
Separation	-	Aspirate (or decant)	
Counting	Count tubes for 60 seconds in a gamma counter.		

DIAsource Catalogue Nr :	Revision nr :
KIR1929	191227/1

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