RIASON® 1,25(OH)₂-Vit.D

Radioimmunoassay for the quantitative determination of 1,25(OH)₂-Vitamin D in serum and plasma

Instruction for Use

For in-vitro use only

Product of

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The RIASON® 1,25(OH)$_2$-Vit.D kit is a radioimmunoassay for the \textit{in vitro} quantitative measurement of human 1,25(OH)$_2$-Vitamin D (1,25(OH)$_2$-Vit.D) in serum and plasma. The measurement of circulating 1\alpha,25-(OH)$_2$D$_3$ is indicated in several disorders affecting calcium metabolism such as: sarcoidosis, renal failure, hyper and hypoparathyroidism, rickets, tumor-associated hypercalcemia, Vitamin-resistant dysfunction and treatment with anti-convulsive medication.

\textbf{Intended use}

\textit{For in-vitro use only.}
Summary

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 nonpregnant mammal. Thus 25-OH-D₃ is further hydroxylated in the 1α-position to produce 1α,25 dihydroxyvitamin D₃ (1α,25-(OH)₂D₃).

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)₂D₃. 1α,25-(OH)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ requires extraction and separation either by HPLC or by column chromatography.

Assay principle

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 ¹²⁵I 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.

Warnings and precautions

The RIASON® 1,25(OH)₂ Vit.D kit is for in vitro diagnostic use only and is not for internal use in humans or animals. This product must be used strictly in accordance with the instructions set out in the Package Insert. IASON will not be held responsible for any loss or damage (except as required by statute) caused, arising out of non-compliance with the instructions provided.
CAUTION: this kit contains material of human and/or animal origin. Handle kit reagents as if capable of transmitting an infectious agent. Source material from Human origin which is used in this kit was tested and found negative for HbsAG and HIV as well as for HCV antibodies. However, since there is no diagnostic procedure that excludes these agents with 100 percent certainty all components should be handled as potentially hazardous material. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. Disposal of kit reagents should be in accordance with local regulations.

**Damaged test kit**

In case of serious damage to the test kit or components, the company IASON must be notified in writing at least one week after receiving the kit. Severely damaged single components should not be used for the test run. They must be stored until a final solution has been found. After that they should be disposed in accordance with official regulations.

**Materials provided**

*Allow all reagents 1-8 to reach room temperature before use.*

1. **CT** tubes coated with anti-1,25-(OH)$_2$-Vitamin D; 1 x 48 pieces, green.
2. **CAL1** – **CAL5** Calibrators lyophilized in phosphate buffer with bovine casein and gentamycin, reconstitution with 2 mL **ELU** concentration see QC certificate, yellow.
3. **CO1** **CO2** Controls 2 vials lyophilized; reconstitution with 2 mL aqua dest.; silver; in human plasma with gentamycin; concentration see QC certificate
4. **TRAC** $^{125}$ labelled 1,25-(OH)$_2$ Vitamin D; in phosphate buffer with bovine casein and gentamycin; lyophilized; reconstitution with 26 mL **RECSOL**; approx. 75kBq radioactive, red
5. **ELU** Elution Solution; phosphate buffer with bovine casein, methanol and azide (<0.1%); 1 vial; 30 mL; green; ready for use
6. **WASH** 1 vial; 10 mL; concentrated wash solution; dilute 70 x with distilled water (use a magnetic stirrer)
7. **GEL** Bond Elut Silica cartridges; 20 pieces; ready to use; store at room temperature
8. **RECSOL** Reconstitution solution; 1 vial; 30 mL; ready to use; black

**Materials required but not provided in the kit**

- Distilled water
- Diisopropylether (p.a.)
- Cyclohexane (p.a.)
- Ethyl acetate (p.a.)
- Ethanol absolute (p.a.)
- Dichloromethane (p.a.)
NB: An extraction kit containing all these solvents is available upon request. This kit contains quantities of solvents necessary to run 5 x 48 tests of 1,25(OH)$_2$-VIT.D-RIA-CT.

- Pipettes for delivery of: 200 µL, 500 µL, 1 mL and 2 mL (the use of accurate pipettes with disposable plastic tips is recommended)
- Glass tubes (12 x 75 mm) for extraction and for elution. (closed with a cap for the extraction step)
- Glass tubes (16 x 100 mm) or (12 x 120 mm) or polypropylene tubes (falcon 2097), for the washing of the cartridges.
- Vortex mixer
- Magnetic stirrer
- Centrifuge operating at 800 g.
- Tube shaker (1200 rpm)
- 5 mL automatic syringe (Cornwall type) for washing
- Aspiration system (optional)
- Any gamma counter capable of measuring $^{125}$I may be used (minimal yield 70%).

### Preparation of Reagents, Storage

A. **CAL1-5**: Reconstitute with 2 mL ELU (just before the incubation step).
B. **CO**: Reconstitute with 2 mL distilled water.
C. **TRAC**: Reconstitute with 26 mL of RECSOL solution.
D. **WASH**: Prepare an adequate volume of WASH by adding 69 volumes of distilled water to 1 volume of Wash Solution (70x). Use a magnetic stirrer to homogenize. Discard unused WASH solution at the end of the day.
E. **Extraction solvent** (2 mL for each CO or sample to be tested, are needed):
   - Prepare a fresh solution of diisopropylether, cyclohexane, ethyl acetate, (50, 40, 10 v/v).
F. **Washing Solvent** (1 mL for each CO or sample to be tested, are needed):
   - Prepare a fresh solution of diisopropylether, cyclohexane, ethyl acetate, ethanol absolute (50, 40, 10, 1 v/v).

### Storage and expiration 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 CAL and CO 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 WASH should be used on the same day.
- After its first use, TRAC is stable until expiry date, if kept in the original well closed vial at 2 to 8°C.
- Use freshly prepared Extraction Solution and Washing Solvent, do not store them.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.
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.

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.

Assay procedure

I. Extraction step: Only for CO and samples.

1. Label glass tubes (12 x 75 mm) for extraction: 2 CO and up to 16 samples.
2. Add 0.5 mL CO or sample in the respective tubes.
3. Dispense 2 mL Extraction Solvent in each tube.
4. Close the tubes with a cap and place them 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 CO 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 GEL in each tube.
3. Apply 1.6 mL of supernatant (2 x 0.8 mL), obtained after extraction step, on each cartridge.
4. Then, wash cartridges with 1 mL Washing Solvent. Be careful never apply vacuum on cartridge, 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 ELU 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 CAL, CO and sample. For the determination of total counts, label 2 normal tubes.
2. Briefly vortex CAL (use ELU as zero calibrator), extracted CO and samples and dispense 150 µL of each into the respective tubes.
3. Dispense 500 µL of TRAC 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 WASH (except total counts) and aspirate (or decant). Avoid foaming during the addition of the WASH.
8. Aspirate (or decant) the content of each tube (except total counts).
9. Wash tubes again with 2 mL WASH (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.

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:

\[
\text{B/BO} \% = \frac{\text{Counts (CAL or sample)}}{\text{Counts (zero CAL)}} \times 100
\]

3. Using a 3 cycle semi-logarithmic or logit-log graph paper, plot the (B/BO(\%)) values for each calibrator point as a function of the 1,25(OH)\textsubscript{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/BO (\%)) values, determine the 1,25(OH)\textsubscript{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)\textsubscript{2}-Vitamin D (B0/T) must be checked.
Typical Data

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

<table>
<thead>
<tr>
<th>1,25(OH)2-Vitamin D</th>
<th>cpm</th>
<th>B/BO%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total count</td>
<td>43937</td>
<td></td>
</tr>
<tr>
<td>CAL1 0.0 pg/mL</td>
<td>16687</td>
<td>100.0</td>
</tr>
<tr>
<td>CAL2 6.0 pg/mL</td>
<td>15268</td>
<td>91.5</td>
</tr>
<tr>
<td>CAL3 20.0 pg/mL</td>
<td>12345</td>
<td>74.0</td>
</tr>
<tr>
<td>CAL4 63.0 pg/mL</td>
<td>8033</td>
<td>48.1</td>
</tr>
<tr>
<td>CAL5 230.0 pg/mL</td>
<td>3554</td>
<td>21.3</td>
</tr>
<tr>
<td>CAL6 430.0 pg/mL</td>
<td>2148</td>
<td>12.9</td>
</tr>
</tbody>
</table>

Expected Values

<table>
<thead>
<tr>
<th></th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal subjects</td>
<td>19.6 – 54.3</td>
</tr>
</tbody>
</table>

It is recommended that each laboratory determine a reference range for its own patient population.

Quality control

The regular use of control samples at several analyte levels is advised to ensure day-to-day validity of results. Controls should be tested as unknowns. Quality Control charts should be maintained to follow the assay performance. Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels. The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results. It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results. Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices; gamma counter, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods. After checking the above mentioned items without finding any error contact your distributor or IASON directly.
Test characteristics

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 counts at zero binding, was 1.4 pg/mL.

Specificity

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-Reactivity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)₂-Vitamin D₃</td>
<td>100</td>
</tr>
<tr>
<td>1,25(OH)₂-Vitamin D₂</td>
<td>92.31</td>
</tr>
<tr>
<td>25OH-Vitamin-D₃</td>
<td>0.001</td>
</tr>
<tr>
<td>24,25(OH)₂-Vitamin.D₃</td>
<td>0.005</td>
</tr>
<tr>
<td>25,26(OH)₂-Vitamin.D₃</td>
<td>0.20</td>
</tr>
</tbody>
</table>

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) do not interfere with this assay.

Precision

<table>
<thead>
<tr>
<th>Intra-Assay Precision</th>
<th>Inter-Assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>&lt;X&gt; ± SD [pg/mL]</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
</tbody>
</table>

Linearity

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Theoretical concentration [pg/mL]</th>
<th>Measured concentration [pg/mL]</th>
<th>Recovery [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>70.0</td>
<td>70.0</td>
<td>100</td>
</tr>
<tr>
<td>1/2</td>
<td>35.0</td>
<td>35.7</td>
<td>102</td>
</tr>
<tr>
<td>1/4</td>
<td>17.5</td>
<td>14.5</td>
<td>83</td>
</tr>
<tr>
<td>1/8</td>
<td>8.8</td>
<td>7.8</td>
<td>89</td>
</tr>
<tr>
<td>1/16</td>
<td>4.4</td>
<td>4.6</td>
<td>105</td>
</tr>
</tbody>
</table>

The sample was diluted with ELU.
Recovery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added 1,25(OH)_{2} VitaminD (pg/mL)</th>
<th>Measured 1,25(OH)_{2} Vit.D concentrations</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total [pg/mL]</td>
<td>Blanked [pg/mL]</td>
<td></td>
</tr>
<tr>
<td>Serum 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>46.3</td>
<td>23.8</td>
<td>95</td>
</tr>
<tr>
<td>50</td>
<td>70.0</td>
<td>47.5</td>
<td>95</td>
</tr>
<tr>
<td>100</td>
<td>122.7</td>
<td>100.2</td>
<td>100</td>
</tr>
<tr>
<td>Serum 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>52.1</td>
<td>29.6</td>
<td>118</td>
</tr>
<tr>
<td>50</td>
<td>70.4</td>
<td>47.9</td>
<td>96</td>
</tr>
<tr>
<td>100</td>
<td>112.9</td>
<td>90.4</td>
<td>90</td>
</tr>
</tbody>
</table>

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.

Limitation of use

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice. Any improper handling of samples or modification of this test might influence the results.

Legal aspects

Reliability of results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.

The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact IASON.
**Therapeutic consequences**

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under Reliability of Results. Any laboratory result is only a part of the total clinical picture of a patient. Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived. The test result itself should never be the sole determinant for deriving any therapeutic consequences.

**Liability**

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement. Claims submitted due to customer misinterpretation of laboratory results subject to point Therapeutic Consequences are also invalid. Regardless, in the event of any claim, the manufacturer’s liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

**Useful publications**


Pipetting scheme

I. Extraction Step: only for CO and samples
Label glass tubes for extraction: 2 CO and approx. 16 samples

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Pipetting CO1, CO2, Probe 500µl</td>
</tr>
<tr>
<td>B.</td>
<td>Pipetting Extraction Solution 2000µl</td>
</tr>
<tr>
<td>C.</td>
<td>Incubation Close tubes and incubate for 1 hour on a shaker at 1200 rpm</td>
</tr>
<tr>
<td>D.</td>
<td>Centrifuge Supernatants are needed for the next step of separation</td>
</tr>
</tbody>
</table>

II. Separation Step: only for CO and samples
Label glass tubes for separation: 2 CO and approx. 16 samples

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Put one GEL in each tube</td>
</tr>
<tr>
<td>B.</td>
<td>Pipetting Supernatant from I. Extraction in GEL 2x800µl</td>
</tr>
<tr>
<td>C.</td>
<td>Pipetting Washing Solvent 1000µl Be careful never apply vacuum on cartridges, just let solvent draw by gravity.</td>
</tr>
<tr>
<td>D.</td>
<td>Pipetting Dichloromethane 300µl</td>
</tr>
<tr>
<td>E.</td>
<td>Pipetting aqua dest. 300µl</td>
</tr>
<tr>
<td>F.</td>
<td>Centrifugation 5 minutes at room temperature at 800 g</td>
</tr>
<tr>
<td>G.</td>
<td>New tubes Label glass tubes for elution. After centrifugation transfer cartridges in the corresponding glass tubes.</td>
</tr>
<tr>
<td>H.</td>
<td>Pipetting ELU 400µl</td>
</tr>
<tr>
<td>I.</td>
<td>Centrifugation 5 minutes at room temperature at 800g</td>
</tr>
</tbody>
</table>

III. Incubation Step
Label coated tubes in duplicate for each CAL, CO and sample. For the determination of total counts, label 2 normal tubes.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Pipetting ELU CAL1 – CAL5 extracted CO1 CO2 extracted sample 150µl 150µl 150µl</td>
</tr>
<tr>
<td>2.</td>
<td>Pipetting TRAC 500µl</td>
</tr>
<tr>
<td>3.</td>
<td>Incubation overnight at room temperature (at least 18h)</td>
</tr>
<tr>
<td>4.</td>
<td>Washing Aspirate or decant CT; except total counts Pipette 2 mL WASH, aspirate or decant repeat one more time let the tubes stand upright for 2 minutes and aspirate the remaining drop of liquid</td>
</tr>
<tr>
<td>5.</td>
<td>Reading Count tubes 1 – 2 minutes 125, don’t forget the total counts</td>
</tr>
</tbody>
</table>

Expected Values

| Normal subjects | 19.6 – 54.3 |