25-OH Vitamin D ELISA Kit

Enzyme Immunoassay for the quantification of 25-hydroxyvitamin D2 and D3 in serum samples

Catalog number: ARG80775

Package: 96 wells

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MANUFACTURED BY:

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INTRODUCTION

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 metabolized into 25-Hydroxyvitamin D3 (25-OH D3) which is the main form of Vitamin D circulating in the body. 25-OH 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 25-OH D3. 25-OH Vitamin D stimulates the intestinal absorption of both calcium and phosphorus and also bone resorption and mineralization.

25-OH 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 metabolized 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 25-OH 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 25-OH 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.

PRINCIPLE OF THE ASSAY

This assay employs the competitive quantitative enzyme immunoassay technique. A specific 25-OH Vitamin D monoclonal antibody has been pre-coated onto a microtiter plate. Standards, controls or samples are pipetted into the wells and 25-OH Vit D in the samples are bound by the immobilized antibodies. After washing, a fixed amount of 25-OH Vitamin D labeled with biotin in presence of HRP compete with unlabeled 25-OH Vit D binding sites on the monoclonal antibodies. After washing away any unbound substances, a substrate solution (TMB) is then added to the wells and color develops in inverse proportion to the amount of 25-OH Vitamin D bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450 nm ±2 nm. The concentration of 25-OH Vitamin D in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	12 X 8 strips	4°C
Standard 0	1 vial (Lyophilized)	4°C
Standard 1-5 (4.62 ng/ml, 12.9 ng/ml, 27.4 ng/ml, 69.8 ng/ml, 129.0 ng/ml)	5 x 1 vial (Lyophilized)	4°C
Control 1 (19.7 ng/ml, acc. range: 12.6-26.8 ng/ml)	1 vials (Lyophilized)	4°C
Control 2 (45.2 ng/ml, acc. range: 31.6-58.8 ng/ml)	1 vials (Lyophilized)	4°C
Incubation Buffer	20 ml (Ready-to-use)	4°C
100X 25-OH Vit. D biotin conjugate	1 vial (0.3 ml)	4°C
Conjugation Buffer	30 ml (Ready-to-use)	4°C
200X HRP-Conjugate	1 vial (0.2ml)	4°C
200X Wash buffer	10 ml	4°C
TMB substrate	12 ml (Ready-to-use)	4°C (Protect from light)
STOP solution	12 ml (Ready-to-use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm (optional reference absorbance at 630 nm)
- Pipettes and pipette tips
- Deionized or distilled water
- Microplate shaker (300-700 rpm)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Standards and controls are stable for one week at 2 to 8°C after reconstitution. For longer storage periods, aliquots should be made and kept at -20°C for maximum 4 months. Avoid repeated freeze/thaw cycles.
- Briefly spin down the 100X 25-OH Vit. D biotin conjugate and HRP-Streptavidin concentrate before use.
- If crystals are observed in the 200X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- It is suggested adding samples, controls and standards within 20 min.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Thoroughly mix all reagents and samples by gentle agitation or swirling.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note:

If the measuring absorbance of samples is higher than the highest standard, the samples could be diluted with Control 1 or a serum sample with a concentration of 25-OH Vitamin D below 25 ng/mL, and above 4.4 ng/mL (limit of quantification of the assay), as measured in this assay. Use Control 1 or this low concentration sample to dilute 2X the out of curve samples. Take the concentration of the Control 1* or the low sample into account when calculating the dilution result.

* Use the concentration of Control 1 measured in the same run as the dilution run, not the mean concentration on the QC data sheet for CONTROL 1! before assay and assay again. For the calculation of the concentrations this dilution factor has to be taken into account.

Calculations:

*Sample value = (Measured value – F1*Measured CONTROL 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 CONTROL 1, and is measured at 70 ng/ml. And the CONTROL 1 is measured in the same run at 20 ng/ml.

- Dilution 4 times, F1 = 0.75; F2 = 0.25
- Sample calculated value = (70 – 0.75*20)/0.25 = 220 ng/ml

REAGENT PREPARATION

- 1X Wash buffer: Dilute 200X Wash buffer into distilled water to yield 1X Wash buffer. Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.
- Standard 0: Reconstitute Standard 0 with 1ml distilled water.
- Standard 1-5: Reconstitute each Standard (1-5) with 1 ml distilled water. Concentration after reconstitution is written on the vial label.
- Controls: Reconstitute each Control (1 and 2) with 1 ml distilled water.
- Working HRP conjugate mixture solution: The working HRP conjugate mixture solution should be only prepared fresh before use. Prepare an adequate volume of working HRP conjugate solution by mixing the 3 reagents in the following sequence: (1) Conjugate buffer, (2) 100X 25-OH Vit. D biotin conjugate, (3) Vortex, (4) Concentrated HRP, (5) Vortex. Prepare an adequate volume of working HRP conjugate solution by mixing concentrated 25-OH Vit. D biotin-conjugate, concentrated HRP and conjugate buffer according to the number of strips used:

  For example, 6 strips (48 wells):

1. Mix 100 µl of Biotin conjugate (100X 25-OH Vit. D biotin-conjugate) with 10 ml of conjugate buffer. Use a vortex to homogenize.
2. Add 200X of 50µl HRP conjugate into the solution from step 1. Use a vortex to homogenize.

Note:

1. The order of addition of those 3 reagents is critical and should be rigorously respected to get reproducible Optical Densities.
2. It is recommended that the working HRP conjugate mixture solution is to be prepared fresh before used, or during the incubation step (ASSAY PROCEDURE step 4) and minimum 1h45 minutes before its use. Do not store the rest of prepared working HRP conjugate mixture solution for next assay.
3. Keep the working HRP conjugate mixture solution at room temperature and avoid direct sunlight or use a brown glass vial for its preparation before it used.

Strips	Conjugate Buf (ml)	Biotin-Conjugate (µl)	HRP-Conjugate (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

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl** of **controls, standards and samples** in duplicate into wells.
3. Add **150 µl** of **Incubation buffer** into all wells.
4. Cover wells and **incubate for 2 hours at RT** on a microplate shaker (~400 rpm).

   (Prepare the Working HRP conjugate solution during the incubation and minimum 1h 45 minutes before its use.)
5. Aspirate each well and wash, repeating the process 2 times for a **total 3 washes**. Wash by filling each well with **1X Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
6. Add **200 µl** of **Working HRP conjugate mixture solution** into each well. **Incubate for 30 mins at RT** on a microplate shaker (~400 rpm).
7. **Wash** as according to step 5.
8. Add **100 µl** of **TMB Reagent** to each well. Incubate for **15 minutes at room temperature** in dark on a microplate shaker (~400 rpm).
9. Add **100 µl** of **Stop Solution** to each well. The color of the solution should change from blue to yellow.

10. Read the OD with a microplate reader at 450 nm immediately. (optional reference absorbance at 630 nm or 650 nm) It is recommended read absorbance within 1 hour after adding Stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using semi-log or linear graph paper or semi-log graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

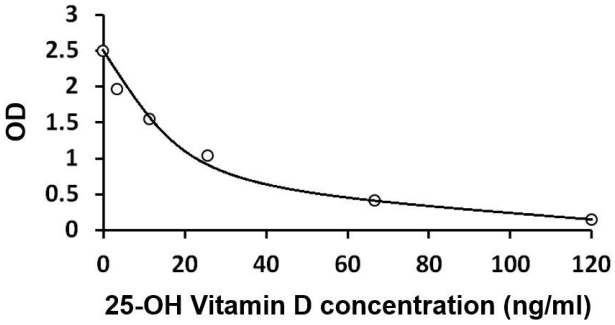
5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for the detail. (https://www.arigobio.com/elisa-analysis)

6. Conversion: 25-OH Vitamin D (ng/ml) x 2.5 = 25-OH Vitamin D (pmol/ml)

7. If the sample has been diluted, for the calculation of the concentrations this dilution factor has to be taken into account.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of total 25-OH Vitamin D ranged from 5.4-132 ng/ml. The mean LoB was 1.69 ng/ml, LoD was 2.81 ng/ml and the LoQ was 4.39 ng/ml.

Intra-assay and Inter-assay precision

The CV value of intra-assay precision was 2.5-7.8% and inter-assay precision was 4.3-9.2%.

Specificity

The cross-reactivity has been tested as below:

Compound (Concentration)	% of cross reaction
25-OH-Vitamin D3 (10 ng/ml)	100
25-OH-Vitamin D2 (10 ng/ml)	86
1,25(OH)2-Vitamin D3 (200 ng/ml)	20
1,25(OH)2-Vitamin D2 (690 ng/ml)	1.9
Vitamin D3 (200 ng/ml)	2.9
Vitamin D2 (200 ng/ml)	1.3
24,25(OH)2-Vitamin D3 (20 ng/ml)	>100
24,26(OH)2-Vitamin D3 (4 ng/ml)	>100
3-epi-25OH-Vitamin D3 (20 µg/ml)	0.1

The effect of potential interfering substances on samples using this 25-OH Vitamin D 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 25-OH Vitamin D Concentration. Our acceptance criteria was to have interference of less than 10%. The tested substances did not affect the performance of this 25-OH Vitamin D ELISA test.

Recovery

92-105%

Linearity

96-106%å