Epitope Diagnostics,Inc.

EDI™ Total 25-OH Vitamin D EIA Kit

Enzyme Immunoassay (EIA) for the Quantitative Measurement of Total 25-OH Vitamin $D_{2/3}$ Level in Serum or Plasma

REF **KT 715** EU: IVD CE Σ 96 +2 +8 °C

I. INTENDED USE

This test kit is intended for use in the quantitative determination of total 25-OH Vitamin D (Vitamin D2 and Vitamin D3) in human serum and plasma. This kit is for in vitro diagnostic use only.

II. PHYSIOLOGY

The group of compounds referred to as Vitamin D, are actually fat soluble steroidal prehormones. The main forms which occur in the body are Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol). The active form of these molecules is Dihydroxyvitamin D3 (1, 25$(OH)_2$ D_3). Vitamin D3 is formed in the skin by photolysis of 7-dehydrocholesterol by ultraviolet radiation from sunlight. It is transported in blood circulation bound to proteins to the liver where it is hydroxylated. Further hydroxylation occurs in the kidneys to produce the most active form. Vitamin D levels are highest in newborns and decrease exponentially throughout life. Sufficient circulating levels of vitamin D are necessary for healthy bone maintenance and cell metabolism. Recent studies have shown that it may also lower incidents of certain cancers. Insufficient levels of Vitamin D can result in osteoporosis and bone fracture in the elderly, secondary hyperparathyroidism, abnormal cell metabolism and even increased incidents of cancer. Severe deficiency may lead to rickets in children and osteomalacia in adults. Disease associated with Vitamin D deficiency may also include: impaired immunity, increased autoimmunity, myopathy, diabetes mellitus, and an increased risk of colon, breast, and prostate cancers. Abnormally high levels (> 200 ng/ml) of Vitamin D leads to Vitamin D toxicity and may cause hypercalcaemia.

III. ASSAY PRINCIPLE

This EIA kit is designed, developed and produced for the quantitative measurement of total 25-OH Vitamin $D_{2/3}$ in serum utilizing the competitive immunoassay technique. This assay utilizes a monoclonal antibody that binds to both 25-OH Vitamin D2 and 25-OH Vitamin D3 equally.

Assay calibrators, controls and test samples are added directly to wells of a microtiter plate that is coated with specific anti-25-OH Vitamin D2, D3 antibody. A buffer designed to release Vitamin D from binding proteins is then added to the wells. After the first incubation period, biotinylated Vitamin D analogue is added to the wells and binds to remaining antibody sites. After the second incubation period, unbound biotin-D is washed away and horseradish peroxidase (HRP) conjugated streptavidin is added to each well. During the third incubation step, an immune complex of well coated "vitamin D antibody – vitamin D, biotin D and HRP conjugated streptavidin" is formed. The unbound matrix are removed in the subsequent washing steps. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction, which is terminated with an acidic reagent (ELISA stop solution). The absorbance is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is inversely proportional to the amount of total 25-OH Vitamin $D_{2/3}$ in the test sample. A calibration curve is generated by plotting the absorbance versus the respective Vitamin D concentration for each calibrator on a 4-parameter or point to point curve fitting. The concentration of total 25-OH Vitamin $D_{2/3}$ in test samples is determined directly from this calibration curve.

IV. REAGENTS: Preparation and Storage

This test kit must be stored at 2 – 8 °C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.

1. **Vitamin D Antibody Coated Microplate (Cat. No. 30850)**
One microplate with twelve by eight strips (96 wells total) coated with anti-Vitamin D2/D3 antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.

2. **HRP - Streptavidin (Cat. No. 30870)**
One bottle containing **11.5 mL** ready to use HRP labeled streptavidin in a stabilized protein matrix. This reagent should be stored in 2-8ºC and is stable until the expiration date on the kit box.

3. **Biotinylated Vitamin D Analogue (Cat. No. 30879)**
One bottle containing **3 mL** of ready to use biotin-Vitamin D analogue in a stabilized buffer matrix with preservative. This reagent should be stored in 2-8ºC and is stable until the expiration date on the kit box.

4. **Vitamin D Assay Buffer (Cat. No. 30888)**
One bottle containing **15 ml** of a buffered matrix. This buffer is ready to use and it releases Vitamin D from its binding proteins. This reagent may be stored in room temperature and/or 2-8ºC and is stable until the expiration date on the kit box.

5. **ELISA Wash Concentrate (Cat. No. 10010)**
One bottle containing **30 mL** of 30-fold concentrate. Before use, the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

6. **ELISA HRP Substrate (Cat. No. 10020)**
One bottle containing **15 mL** of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.

7. **ELISA Stop Solution (Cat. No. 10030)**

One bottle containing **15 mL** of stop solution. This reagent may be stored at 2-8ºC or room temperature and is stable until the expiration date on the kit box.

8. Vitamin D Calibrators 0 to 5 (Cat. No. 30880 - 30885)
Six vials contain **0.5 mL** each of liquid 25-OH Vitamin D3 in a bovine serum albumin-based matrix with a non-azide preservative. **Refer to the vial for exact concentration.** This should be stored at 2-8°C or after the first use.

9. Vitamin D Controls (Cat. No. 30889, 30890)
Two vials each contain **0.5 mL** of liquid Vitamin D3 in a human serum -based matrix with a non-azide preservative. **Refer to vials for concentration range for each control.** Both controls should be stored at 2-8°C after the first use.

V. SAFETY PRECAUTIONS

The reagents must be used in professional laboratory. Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 25 µl, 100 µl, 500 µL, etc.
2. Disposable pipette tips suitable for above volume dispensing.
3. Aluminum foil.
4. Plastic microtiter well cover or polyethylene film.
5. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
6. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
7. Oven or water bath with adjustable temperature to 37°C.

VII. SPECIMEN COLLECTION

Both serum and EDTA-plasma can be used with this test kit. Only 50 µL total (25 µL each) of human EDTA-plasma or serum is required for the 25-OH Vitamin D measurement in duplicate. No special preparation of individual is necessary prior to specimen collection. Collect whole blood with Vacutainer and separate the serum or plasma from cells according to manufacturer's instruction. Serum and plasma samples can be stored at room temperature for 3 days. For longer term storage, sample can be kept at – 15ºC. Avoid more than three freeze-thaw cycles of specimen.

VIII. ASSAY PREPARATION

1. Reagent Preparation

(1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
(2) ELISA Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.

IX. ASSAY PROCEDURE

Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	Cal 0	Cal 4	SAMPLE 1
B	Cal 0	Cal 4	SAMPLE 1
C	Cal 1	Cal 5	SAMPLE 2
D	Cal 1	Cal 5	SAMPLE 2
E	Cal 2	Control 1	SAMPLE 3
F	Cal 2	Control 1	SAMPLE 3
G	Cal 3	Control 2	
H	Cal 3	Control 2	

(1) Add **20 µl** of calibrators, controls and test samples into the designated microwells.
(2) Add **100 µl Vitamin D Assay Buffer** to each well.
(3) Cover plate with aluminum foil or other material to protect from light and incubate at room temperature for **30 minutes, static.**
Note: Don't spill the liquid from each well! ***THERE IS NO WASH STEP AFTER THE 1ST INCUBATION***
(4) Carefully take off the cover foil and add **25 µl** of **Biotinylated Vitamin D Analogue** to each well.
(5) Seal the plate wells securely, cover with foil or other material to protect from light, and incubate at room temperature for **1 hour, static.**
(6) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
(7) Add 1**00 µL** of **Streptavidin-HRP** into each of the wells.
(8) Seal the plate wells securely, cover with foil or other material to protect from light, and incubate at room temperature for **30 minutes, static.**
(9) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
(10) Add **100 µl TMB** reagent to each of the wells.
(11) Cover plate with aluminum foil, and incubate at room temperature for **20 minutes, static.**
(12) Immediately add **100 µL of ELISA Stop Solution** into each of the wells. Mix gently.
(13) Read the absorbance at 450 nm within 10 min.

X. PROCEDURAL NOTES

1. It is recommended that all calibrators and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results. It is recommended to add external controls to each assay.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.

XI. INTERPRETATION OF RESULTS

It is recommended to use a 4-parameter calibration curve fitting. However, a point-to-point curve fitting can also be used.

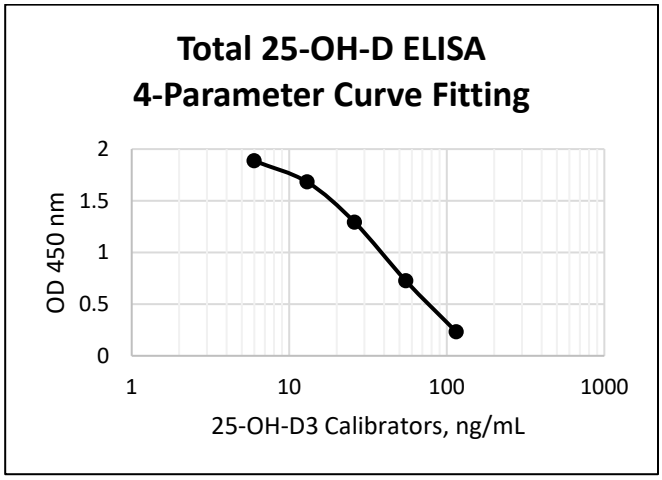
1. Calculate the average absorbance for each pair of duplicate test results.
2. The calibration curve is generated by the corrected absorbance of all calibration levels on the ordinate against the calibrator concentration. Appropriate computer assisted data reduction programs should be used for the calculation of results.

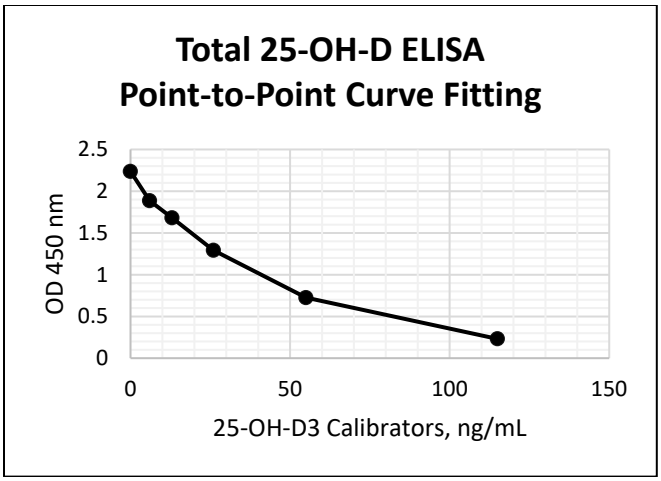
The total 25 OH vitamin D concentrations for the test samples are read directly from the calibration curve using their respective average absorbance.

XII. EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting calibration curve from this 25-OH Vitamin D EIA is represented. **This curve should not be used in lieu of calibration curve generated with each assay.**

Well I.D	OD 450 nm Absorbance		B/B_0
	Readings	Average	
Cal-0: 0 ng/mL	2.177	2.237	100%
	2.298		
Cal-1: 6 ng/mL	1.855	1.886	84.3%
	1.918		
Cal-2: 13 ng/mL	1.684	1.681	75.1%
	1.679		
Cal-3: 26 ng/mL	1.286	1.292	57.8%
	1.299		
Cal-4: 55 ng/mL	0.747	0.725	32.4%
	0.702		
Cal-5: 115 ng/mL	0.233	0.231	10.3%
	0.229		





XIII. EXPECTED VALUE

Dietary intake, race, season and age are known to affect the normal levels of Total 25-OH Vitamin D.

The following data is provided for guidance only. It is important for each laboratory to establish its own reference ranges, which may better represent its typical population and region.

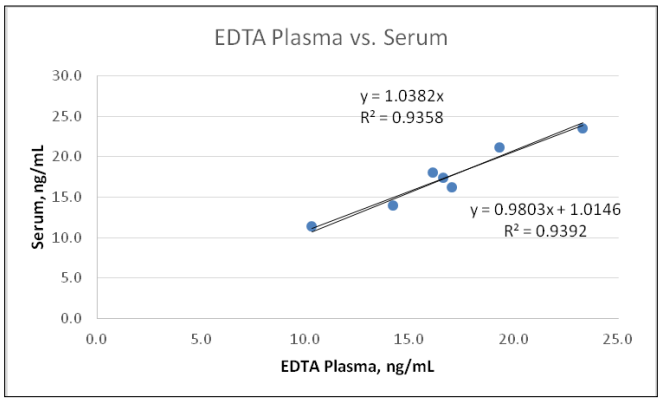
It is suggested the following ranges for the classification of Total 25-OH Vitamin D status:

Level	ng/mL
Deficiency	<20
Optimal	20 - 70
Overdose	>70

We have validated the above reference range with 56 apparently healthy individuals. Donors that were not taking Vitamin D supplements from which samples were collected and tested. Patient EDTA plasma and serum were used to obtain the summarized data below.

	ng/mL
Mean	32.4
Highest	74.6
Lowest	12.6

Donor serum and EDTA plasma paired samples were correlated using this kit. The result yielded an excellent slope and correlation.



XIV. LIMITATION OF THE PROCEDURE

1. This assay requires serum or plasma sample for testing.
2. Serum or plasma samples from different species may show different matrix background.
3. For sample values greater than 115 ng/mL, it is recommended to re-assay samples with dilution (i.e. 1:5 or 1:10. The best dilution matrix is vitamin D free human serum.
4. Cell culture or tissue culture samples should be validated with total binding and other performance specifications before being used.
5. Severely hemolyzed samples, icteric or lipaemic sample should not be used
6. If Spike Recovery is desired, use kit controls to spike into the samples.
7. If highly sensitive assay is desired, extending the first incubation is recommended; Calibrator 1 may also be diluted with calibrator 0.

XV. QUALITY CONTROL

The performance of the EDI Total 25-OH Vitamin D EIA Kit was determined by a correlation study test using an FDA approved 25-OH Vitamin D ELISA test kit. To assure the validity of the results each assay should include adequate controls with known 25-OH Vitamin D levels. We recommend that all assays include the laboratory's own Vitamin D controls in addition to those provided with this kit.

XVI. PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity (LLOD) of this Total 25-OH Vitamin D EIA as determined by the 2 times calibrator deviation below the mean of B_0 on 10 duplicate determinations of zero calibrator (B_0) is approximately 5.0 ng/mL.

Specificity

Cross reactivity of this Total 25-OH Vitamin D ELISA kit 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 10ng/mL	100
25OH Vitamin D_2 at 10ng/mL	100
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 200ng/mL	2.9
Vitamin D_2 at 200ng/mL	1.3
24,25$(OH)_2$ Vitamin D_3 at 20 ng/mL	>100
24,26$(OH)_2$ Vitamin D_3 at 4 ng/mL	>100
3-epi 25OH Vitamin D_3 at 20 ng/mL	0.1

Animal serum Total 25-OH Vitamin D from bovine/calf, goat, horse, chicken, mouse, and equine can be detected using this kit.

Precision

The intra-assay precision was validated by measuring three samples in eight replicate determinations. The concentrations are 16.5 ng/mL, 34.4 ng/mL and 63.9 ng/mL with CV% of 8.3%, 5.8% and 5.1% respectively.

The inter-assay precision was validated by measuring three samples in 11 different assays. The concentrations are 32.6 ng/mL and 64.5 ng/mL with CV% of 6.0% and 4.6% respectively.

Linearity

Two calibrators were diluted and tested. The results of dilution recovery value are summarized as follows:

#	DILUTION	OBSERVED VALUE (ng/mL)	EXPECTED VALUE (ng/mL)	RECOVERY %
1	100% Calibrator 5	-	115	-
	80% Calibrator 5	85.0	92	92
	60% Calibrator 5	63.2	69	92
	40% Calibrator 5	39.9	46	87
	20% Calibrator 5	20.2	23	88
2	100% Calibrator 3	-	26	-
	80% Calibrator 3	21.1	20.8	101
	60% Calibrator 3	16.6	15.6	106
	40% Calibrator 3	10.7	10.4	103
	20% Calibrator 3	5.3	5.2	102

Spike Recovery

Test calibrators were spiked each other in equal volume and assayed. The results indicate below:

#	Spiked Sample	OBSERVED VALUE (ng/mL)	EXPECTED VALUE (ng/mL)	RECOVERY (%)
1	Cal 1 + Cal 2	10.4	9.5	109
2	Cal 1 + Cal 3	17.1	16.0	107
3	Cal 1 + Cal 4	29.5	30.5	98
4	Cal 2 + Cal 3	19.7	19.5	101
5	Cal 2 + Cal 4	29.8	34.0	88
6	Cal 3 + Cal 4	38.3	40.5	95

Note: Cal 1: 6 ng/mL; Cal 2: 13 ng/mL; Cal 3: 26 ng/mL; Cal 4: 55 ng/mL.

XVII. WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

XVIII. REFERENCES

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6. Holick MF, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an endocrine society clinical practice guideline. J. Clin Endocrinol Metabol 2011;96:1911-1930

TECHNICAL ASSISTANCE AND CUSTOMER SERVICE
For technical assistance or place an order, please contact Epitope Diagnostics, Inc. at (858) 693-7877 or fax to (858) 693-7678.
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Manufacturer	No. of tests
Catalog Number	Keep away from heat and direct sun light
Concentrate	Store at
In Vitro Diagnostic Device	Use by
Read instructions before use	Lot No.
Authorized Representative In Europe	

KT-715: Total 25-OH Vitamin D EIA: Condensed Assay Protocol

1. 20 µl calibrators, controls and unknown samples
+
100 µL Assay Buffer

↓ *Incubate @RT for 30 min, static*
NO WASH STEP

2. 25 µl Biotin - Vitamin D

↓ *Incubate @RT for 60 min static*
Wash 5 x

3. 100 µl Conjugate Streptavidin

↓ *Incubate @RT for 30 min static*
Wash 5x

4. 100 µl HRP Substrate

↓ *Incubate @RT for 20 min static*

5. 100 µl Stop Solution *Immediately*

↓

6. Read absorbance at 450 nm (4-parameter or point-to-point) *within 10 minutes*