EPITOPE DIAGNOSTICS, INC.

EDI™ Total 25-OH Vitamin D EIA Kit

Enzyme Immunoassay (EIA) for the Quantitative Measurement of Total 25-OH Vitamin D2/3 Level in Serum or Plasma

REF KT-815 CE IVD 96

INTENDED USE

This test kit is intended for use in the quantitative determination of total 25-OH Vitamin D (Vitamin D_2 and Vitamin D_3) in human serum and plasma.

For In-vitro Diagnostic Use Only

SUMMARY OF PHYSIOLOGY

The group of compounds referred to as Vitamin D, are actually fat soluble steroidal pre-hormones. The main forms which occur in the body are Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol). The active form of these molecules is Dihydroxyvitamin D_3 (1, 25(OH) $_2$ D_3). Vitamin D_3 is formed in the skin by photolysis of 7-dehydrocholesterol by ultraviolet radiation from the 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 the 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. An abnormally high level (> 200 ng/mL) of Vitamin D leads to Vitamin D toxicity and may cause hypercalcaemia.

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 D_2 and 25-OH Vitamin D_3 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 D_2, D_3 antibody. A buffer designed to release Vitamin D from binding proteins is then added to the wells. After the first incubation period, unbound material is washed away and 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 is 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.

REAGENTS: PREPARATION AND STORAGE

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

1. Vitamin D Antibody Coated Microplate (30850)
Microplate coated with anti-Vitamin D2/D3 antibody.
Qty: 1 x 96 well microplate
Storage: 2 – 8°C
Preparation: Ready to use

2. HRP-Streptavidin (30870)
HRP labeled streptavidin in a stabilized protein matrix.
Qty: 1 x 11.5 mL
Storage: 2 – 8°C
Preparation: Ready to use

3. Biotinylated Vitamin D Analogue (30869)
Biotin-Vitamin D analogue in a stabilized buffer matrix with preservative
Qty: 1 x 11.5 mL
Storage: 2 – 8°C
Preparation: Ready to use

4. Vitamin D Assay Buffer (30871)
Buffered matrix to release Vitamin D from its binding proteins.
Qty: 1 x 15.0 mL
Storage: 2 – 8°C
Preparation: Ready to use

5. ELISA Wash Concentrate (10010)
Surfactant in a phosphate buffered saline with non-azide preservative
Qty: 1 x 30.0 mL
Storage: 2 – 8°C
Preparation: 30X Concentrated, should be diluted with 870 mL distilled water and mixed well before use.

6. ELISA HRP Substrate (10020)
Tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.
Qty: 1 x 12.0 mL
Storage: 2 – 8°C
Preparation: Ready to use

7. ELISA Stop Solution (10030)
0.5 M sulfuric acid
Qty: 1 x 12.0 mL
Storage: 2 – 8°C
Preparation: Ready to use

8. Vitamin D Calibrators Levels 0 to 5 (30880 - 30885)
Liquid 25-OH Vitamin D_3 in bovine serum albumin-based matrix with non-azide preservative. Refer to vials for exact concentration.
Qty: 6 x 0.5 mL
Storage: 2 – 8°C
Preparation: Ready to use

9. Vitamin D Controls (30886, 30887)
Liquid 25-OH Vitamin D_3 in bovine serum albumin-based matrix with non-azide preservative. Refer to vials for exact concentration.
Qty: 2 x 0.5 mL
Storage: 2 – 8°C
Preparation: Ready to use

SAFETY PRECAUTIONS

The reagents must be used in a professional laboratory environment and are for in vitro diagnostic use. Source material which contains reagents of bovine serum albumin 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 were potentially infectious. Avoid contact with reagents containing hydrogen peroxide. 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. Exercise Good Laboratory Practices.

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. ELISA plate shaker.
8. Calibrated timer

SPECIMEN COLLECTION & STORAGE

Serum, EDTA-plasma, and citrate plasma samples were validated 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 stored at – 15ºC. Avoid more than three freeze-thaw cycles of specimen. Animal serum Total 25-OH-Vitamin D from bovine/calf, goat, horse, chicken, mouse, and equine can be detected using this kit.

ASSAY PROCEDURE

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.

3. Assay Procedure

1. Place a sufficient number of microwell strips (30850) in a holder to run calibrators (30880 - 30885), controls (30886, 30887), and samples in duplicate.
2. Test Configuration

Row	Strip 1	Strip 2	Strip 3
A	Calibrator level 0	Calibrator level 4	Sample 1
B	Calibrator level 0	Calibrator level 4	Sample 1
C	Calibrator level 1	Calibrator level 5	Sample 2
D	Calibrator level 1	Calibrator level 5	Sample 2
E	Calibrator level 2	Control 1	Sample 3
F	Calibrator level 2	Control 1	Sample 3
G	Calibrator level 3	Control 2	Sample 4
H	Calibrator level 3	Control 2	Sample 4

3. Add **25 µL** of calibrators (30880 - 30885), controls (30886, 30887), and samples into the designated microwells.
4. Add **100 µL** of assay buffer (30871) to each microwell.
5. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 170 or 450 rpm** for **60 minutes.**
6. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
7. Add **100 µL** of Biotinylated Vitamin D Analogue (30869) to each microwell.
8. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 170 or 450 rpm** for **30 minutes.**
9. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used
10. Add **100 µL** of HRP-Streptavidin (30870) to each microwell.
11. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 170 or 450 rpm** for **20 minutes.**
12. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution (10010) into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
13. Add **100 µL** of substrate (10020) into each microwell. Mix by gently tapping the plate.
14. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** for **20 minutes.**
15. Remove the aluminum foil and plate sealer. Add **100 µL** of Stop Solution (10030) into each of the wells. Mix by gently tapping the plate.
16. Read the absorbance at **450 nm** with a **4-parameter curve** within **10 minutes** with a microplate reader.

PROCEDURAL NOTES

1. Vitamin D is sensitive to heat and light. It is important to avoid direct exposure to these conditions.
2. 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.
3. Keep light sensitive reagents in the original amber bottles.
4. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
5. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
6. Incubation times or temperatures other than those stated in this insert may affect the results.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. It is important to seal the plate properly during incubation periods to avoid evaporation.

INTERPRETION OF RESULTS

1. It is recommended to use a 4-parameter calibration curve fitting.
2. Calculate the average absorbance for each pair of duplicate test results.
3. 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.

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

LIMITATIONS 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 150 ng/mL, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100. 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 controls to spike into the samples.

QUALITY CONTROL

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

EXPECTED VALUES

Dietary intake, race, season, and age are known to affect the 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.

Recent literature has suggested the following ranges for the classification of Total 25-OH Vitamin D status

Level	Concentration (ng/mL)
Severe Deficiency	<10
Insufficient	10 – 24
Optimal	25 – 100
Potential Toxicity	>100

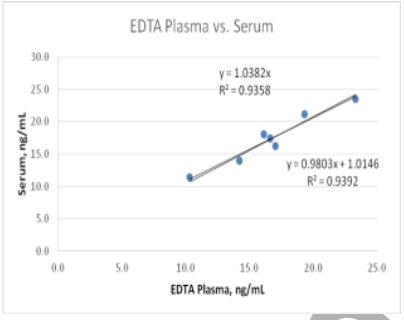
The Endocrine Society Clinical Practice Guideline (2011) has suggested a higher target level of at least 30 ng/mL.

Level	Concentration (ng/mL)
Deficiency	<20
Insufficient	20 - 29
Sufficiency	30 – 100

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

	Concentration (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.

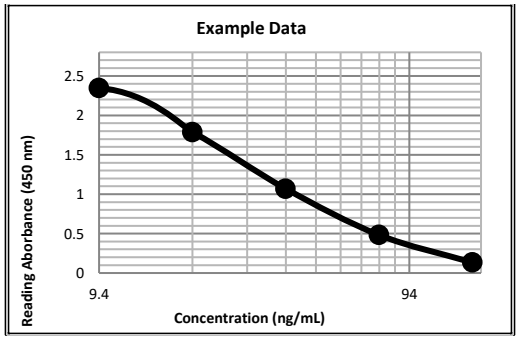


EXAMPLE DATA

A typical absorbance data and the resulting standard curve from are represented.

Note: This curve should not be used in lieu of calibrator curve run with each assay.

Well ID	Reading Absorbance (450 nm)		
	Readings	Average	B/B0
Calibrator Level 0: 0 ng/mL	3.159	3.159	100%
	3.195		
Calibrator Level 1: 9.4 ng/mL	2.729	2.726	86%
	2.717		
Calibrator Level 2: 18.8 ng/mL	2.181	2.152	68%
	2.123		
Calibrator Level 3: 37.5 ng/mL	1.219	1.258	40%
	1.297		
Calibrator Level 4: 75 ng/mL	0.468	0.454	14%
	0.440		
Calibrator Level 5: 150 ng/mL	0.159	0.158	5%
	0.156		



PERFORMANCE CHARACTERISTICS

Sensitivity

The Limit of Blank (LoB) was calculated by measuring the Calibrator zero in 16 replicates and calculating the 95th percentile of the distribution of the test values.The LoB was calculated to be 1.000ng/mL.

The Limit of Detection (LoD) was calculated by measuring the Calibrator 0, 1, and a low sample and calculating the 95th percentile of the distribution of the test values. The LoD was calculated to be 4.781ng/mL.

The Limit of Quantitation (LoQ) was calculated to be 8.558ng/mL

Hook Effect

The hook effect was validated using a high concentrated 25-OH-D3 stock. The assay showed no hook effect at a concentration of 19.65µg/mL (19,650 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 as follows:

Compound and Concentration	Cross reaction (%)
25OH Vitamin D3 at 10ng/mL	100
25OH Vitamin D2 at 10ng/mL	100
1,25(OH)2 Vitamin D3 at 200 ng/mL	20
1,25(OH)2 Vitamin D2 at 690 ng/mL	1.9
Vitamin D3 at 200ng/mL	2.9
Vitamin D2 at 200ng/mL	1.3
24,25(OH)2 Vitamin D3 at 20 ng/mL	>100
24,26(OH)2 Vitamin D3 at 4 ng/mL	>100
3-epi 25OH Vitamin D3 at 20 ng/mL	0.1

Reproducibility and Precision

The intra-assay precision was validated by measuring three samples in sixteen (16) replicate determinations. The inter-assay precision was validated by measuring three samples in twelve (12) different assays in duplicate. The results are as follows:

	Intra-Assay			Inter-Assay		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
Mean (ng/mL)	94.6	50.2	26.2	22.2	60.5	48.6
Standard Deviation	1.4	2.8	2.4	1.7	4.6	2.5
CV (%)	1.4	5.5	9.0	7.5	7.6	5.2

Linearity

Three (3) calibrators were diluted with standard matrix and tested. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator A	-	135.1	-
80%	117.9	108.1	109%
60%	94.2	81.1	116%
40	58.0	54.0	107%
20%	28.5	27.0	105%
Calibrator B	-	120.6	-
80%	102.4	96.5	106%
60%	73.9	72.4	102%
40	46.3	48.3	96%
20%	24.9	24.1	104%
Calibrator C	-	87.2	-
80%	68.7	69.8	98%
60%	49.0	52.3	94%
40	34.1	34.9	98%
20%	17.9	17.4	102%

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample A	-	146.8	-
80%	124.7	117.4	106%
60%	98.4	88.1	112%
40	60.7	58.7	103%
20%	27.4	29.3	93%
Sample B	-	129.6	-
80%	109.6	103.7	106%
60%	77.1	77.8	99%
40	47.9	51.9	92%
20%	22.0	25.9	85%
Sample C	-	91.5	-
80%	74.4	73.2	102%
60%	50.3	54.9	92%
40	30.8	36.6	84%
20%	16.5	18.3	90%

Spike Recovery

Calibrators were spiked with each other in equal volume and assayed. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator Level 1	9.4	-	-
+ Calibrator Level 3: *37.5 ng/mL*	26.4	23.45	113%
+ Calibrator Level 4: *75.0 ng/mL*	41.7	42.2	99%
+ Calibrator Level 5: *150.0 ng/mL*	85.3	79.7	107%
Calibrator Level 2	18.8		-
+ Calibrator Level 3: *37.5 ng/mL*	29.836	28.15	106%
+ Calibrator Level 4: *75.0 ng/mL*	48.15	46.9	103%
+ Calibrator Level 5: *150.0 ng/mL*	93.95	84.4	111%
Calibrator Level 3	37.5	-	-
+ Calibrator Level 4: *75.0 ng/mL*	54.931	56.25	98%
+ Calibrator Level 5: *150.0 ng/mL*	106.491	93.75	114%
Calibrator Level 4	75	-	-
+ Calibrator Level 5: *150.0 ng/mL*	128.5	112.5	114

Six (6) serum/plasma samples were spiked with each other in equal volume and tested. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	65.5	-	-
+ Sample A *67.8*	66.7	66.6	100%
+ Sample B *68.8*	76.1	66.6	114%
+ Sample C *20.0*	44.5	42.7	104%
Sample 2	43.7	-	-
+ Sample A *67.8*	58.9	55.8	106%
+ Sample B *68.8*	54.9	55.8	98%
+ Sample C *20.0*	30.1	31.9	94%
Sample 3	19.1	-	-
+ Sample A *67.8*	39.6	43.5	91%
+ Sample B *68.8*	39.4	43.4	91%
+ Sample C *20.0*	19.6	19.6	100%

Interference

Interference was tested by spiking (95%) serum and plasma samples with (5%) concentrations of hemoglobin, lipid, and bilirubin. The results are as follows:

	Results (ng/mL)	Bias (%)	Amount (mg/mL)
Test Control	13.3	-	-
Bilirubin	13.0	-2%	10
	12.9	-3%	2
	13.4	1%	0.4
Test Control	18.7	-	-
Hemoglobin	20.4	9%	10
	18.4	-2%	2
	17.7	-5%	0.4
Lipids	18.5	-1%	200
	18.2	-3%	40
	15.8	-16%	8
Test Control	38.6	-	-
Bilirubin	41.1	6%	10
	39.8	3%	2
	37.6	-3%	0.4
Test Control	63.1	-	-
Hemoglobin	64.5	2%	10
	67.1	6%	2
	69.3	10%	0.4
Lipids	64.2	2%	200
	59.4	-6%	40

	52.9	-16%	8

Assay Delay

The assay delay was tested using real human samples. The samples were added after the calibrators in different times. The results are as follows:

Samples	Concentration (ng/mL)	Bias (%)
Sample 1	18.516	-
After 5 min	20.414	10%
After 15 min	18.094	-2%
After 25 min	19.481	5%
Sample 2	65.839	-
After 5 min	68.271	4%
After 15 min	57.089	-13%
After 25 min	62.023	-6%

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.

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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.
This product is developed and manufactured by

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Please visit our website at www.epitopediagnostics.com to learn more about our products and services.

EC	REP

MDSS GmbH
Schiffgraben 41,
30175 Hannover, Germany

GLOSSARY OF SYMBOLS (EN 980/ISO 15223)

IVD In Vitro Diagnostic Device	RUO For Research Use Only	LOT Lot Number
REF Catalog Number		

Read instructions before use

Number of Tests

Store at

Use by

Keep away from heat and direct sun light

Manufacturer

EC REP
Authorized Representative in Europe

European Conformity

SHORT ASSAY PROCEDURE

1. Add **25 µL** of calibrators, controls, and samples into the designated microwells.
2. Add **100 µL** of assay buffer into each microwell.
3. Mix, cover, and incubate at **room temperature (20-25 ºC)** with **shaking 350 rpm – 450 rpm** for **60 minutes**.
4. Wash each well five times.
5. Add **100 µL** of biotinylated analogue.
6. Mix, cover, and incubate at **room temperature (20-25 ºC)** with **shaking 350 rpm – 450 rpm** for **30 minutes**.
7. Wash each well five times.
8. Add **100 µL** of HRP-streptavidin.
9. Mix, cover, and incubate at **room temperature (20-25 ºC)** with **shaking 350 rpm – 450 rpm** for **20 minutes**.
10. Add **100 µL** of substrate to each well.
11. Cover and incubate at **room temperature (20-25 ºC)** for **20 minutes**.
12. Add **100 µL** of the stop solution to each well.
13. Read the absorbance at **450nm**.