TRUSTwell®

Vitamin-D (Total) ELISA Kit

REF

AE8010

IVD

- 96-well ELISA kit for the quantitative determination of Vitamin D levels in human serum
- Store at 2-8°C upon receipt

INTENDED USE

The TRUSTwell Vitamin-D (Total) ELISA is a solid-phase enzyme-linked immuno-sorbent assay for the quantitative determination of Vitamin D (Vit D) level in human serum. It is intended to be used by trained professionals only.

INTRODUCTION

Vitamin-D is a fat-soluble steroid hormone that is ingested through diet and synthesized in the skin, liver and kidneys after exposure to sunlight[1]. Two main forms of Vitamin-D are found in the diet, Vitamin D3 (cholecalciferol) and Vitamin D2 (ergocalciferol). Vitamin D3, however, is more effective at regulating Vitamin-D levels than is Vitamin D2.

Vitamin-D plays an important role regulation of calcium and phosphorus in the human body. It has been shown to be important for maintenance of healthy bones and teeth, and for support of immune, nervous, endocrine, and cardiovascular system health[1,2].

Vitamin-D deficiency is a global health issue and is one of the most common nutrient deficiencies. It is more prevalent in females than males, and varies according to ethnicity and eating habits[1,3-5]. Vitamin-D deficiency has been associated with rickets, osteomalacia, and osteoporosis, as well as fetal development, immune system function, neurological disorder such as multiple sclerosis, cancer, and many other pathologies[1-4]. Therefore, accurate measurement of Vitamin-D levels in patients is very important for the diagnosis of Vitamin-D deficiency and for the monitoring of deficient patients in order to achieve optimal levels.

For Vitamin-D to become “active”, it goes through two conversion steps. In the liver, it is first converted to 25-hydroxy-vitamin D (25(OH)D), which is the “storage” and major circulating form of Vitamin-D in the body. It is next converted to the “active” form of Vitamin-D, 1, 25-hydroxy calcitriol (1,25(OH)2D), in the kidneys. 1,25(OH)2D has a short half-life, however, making detection less accurate. Because 25(OH)D is the major circulating form and stays constant in the body for longer, it is the most accurate and therefore most common biomarker of Vitamin-D status[2,5]. The sum of 25(OH)D in serum is referred to as total 25(OH) Vitamin-D.

TEST PRINCIPLE

The TRUSTwell Vitamin-D (Total) ELISA is a solid-phase enzyme-linked immuno-sorbent assay for the quantitative determination of Vitamin-D level in human serum.

The TRUSTwell Vitamin-D (Total) ELISA is comprised of five key components:

1) Solid microwells pre-coated with anti-Vit-D antibody
2) Vit D Calibrators
3) Vit D Assay Controls comprised of human serum with the Vit D concentration on the label. Contains preservative. Store at 2-8°C.
4) Vit D Releasing Agent comprised of Vitamin D binding protein releasing agents,
5) Vit D Enzyme Reagent comprised of 25-OH Vitamin D3 analog-HRP conjugate in a protein stabilizing matrix (Vit-D-HRP).

During the assay, the calibrator, control, or test specimen and Vit D Enzyme Concentrate mixture in the releasing agent are added to the microwell coated with Vit D antibody. During the 45-minute incubation, the Vit D in the patient specimen competes with the HRP-Vit-D to bind to the plate coated antibody. Excess conjugate and serum protein are then removed by washing. In the next step, the TMB single substrate is added. The presence of the HRP-Vit-D is shown by the development of a blue color. The reaction is then terminated with the Stop Solution and absorbance is determined using a spectrophotometer at 450/620-690 nm. The color development is indirectly proportional to the concentration of Vit D in the calibrators, controls, or specimens.

A Dose Response Curve (DRC) can be generated to determine the concentration of unknown by plotting the calibrator Vit D concentrations on the x-axis and the OD values on the y-axis. By interpolating the absorbance, the concentrations for unknown specimens can be determined.

MATERIALS AND REAGENTS

Materials and reagents provided with the kit

Item	Description	Quantity	Catalog
1	Anti-Vit D Ab Coated Microwells	8 wells x 12 strips	AE8010W
2	Vit D Calibrators: C1 (0 ng/mL)	0.4 mL	AE8010C1
3	C2 (5 ng/mL)	0.4 mL	AE8010C2
4	C3 (25 ng/mL)	0.4 mL	AE8010C3
5	C4 (50 ng/mL)	0.4 mL	AE8010C4
6	C5 (85 ng/mL)	0.4 mL	AE8010C5
7	C6 (150 ng/mL)	0.4 mL	AE8010C6
8	Vit D Assay Control A	0.4 mL	AE8010ACA
9	Vit D Assay Control B	0.4 mL	AE8010ACB
10	Vit D Releasing Agent (RA)	12 mL	AE8010RA
11	Vit D Enzyme (Concentrate-51x)	0.6 mL	AE8010H
12	Wash Buffer (Concentrate-40x)	20 mL	AWE3002
13	TMB Substrate	12 mL	ATME2000
14	Stop Solution	6 mL	ASE1000
15	Instructions for Use	1	PI-AE8010 AE0001ES
16	Working Sheet	2 No’s	
17	Sealent	2 No’s	
18	Desiccant	4 No’s	

*The Assay Control and calibrator values are lot-specific. Please see vial labels for exact concentration ranges.

Materials and reagents required but not provided in the kit

1. Micropipettes capable of delivering 10 µL, 50 µL, 100 µL, and 1 mL
2. 1.5 mL and 10 mL micro tube
3. Microplate reader with a bandwidth of 10 nm or less and an optical density range of 0-2.5 or greater at 450 nm wavelength is acceptable
4. Microplate washer
5. Vortex mixer or equivalentr
6. Absorbent paper for blotting the microwells
7. Graph paper
8. Timer
9. Distilled or de-ionized water

STORAGE AND STABILITY

All reagents except the concentrated wash buffer are ready to use as supplied. Store all components at 2-8°C. Do not freeze. Avoid strong light. Ensure that the reagents are brought to room temperature before opening. Reseal the microwells after removing the desired number of wells. Place unused wells in the resealable plastic bag provided and return to 2-8°C. All reagents are stable through the expiration date printed on the label if not opened. Once opened, the kit is stable for 60 days at 2-8°C, or until the labeled expiration date, whichever is earlier.

SPECIMEN COLLECTION AND PREPARATION

- Serum specimens should be prepared from whole blood obtained by acceptable venipuncture technique.
- This kit is designed for use with serum specimens without additives only.
- If a specimen is not tested immediately, refrigerate at 2-8°C for up to 5 days or frozen (-20°C) for up to 30 days. Avoid multiple freeze-thaw cycles. If a specimen is to be shipped, pack in compliance with federal regulations covering the transportation of etiologic agents.
- Specimens containing precipitants may give inconsistent test results. Clarify such specimens by centrifugation prior to performing the assay.
- Do not use specimens demonstrating gross lipemia, gross hemolysis or turbidity. Do not use specimens containing sodium azide

PREPARATION OF THE REAGENTS

1) Bring all reagents to room temperature (22-28°C).
   Preparation of working enzyme solution:
2) Dilute the Vit D Enzyme Concentrate (51x) in the releasing agent in 1:51 ratio. For example, for 48 wells add 0.12 mL of the Vit D Enzyme Concentrate to 6 mL of the releasing agent.
   Volume of releasing agent (RA) = Number of wells x 0.1 = ... mL
   Volume of Vit D Enzyme Concentrate (51x) = RA x 0.02 = ... mL
3) **Preparation of working wash buffer:**
   If precipitants are visible, warm up the wash buffer (40x concentrate) at 37°C. Dilute the concentrated wash buffer 40-fold with water as follows:

Plate	Plate	40x wash buffer	Final volume
Full plate	195 mL	5 mL	200 mL
Half plate	97.5 mL	2.5 mL	100 mL
Quarter plate	48.75 mL	1.25 mL	50 mL

4. Mix each reagent before adding it to the test wells.
5. Determine the number of strips needed and mark on the ELISA working sheet with the appropriate information. Calibrators should be run in duplicate to ensure accuracy.

ASSAY PROCEDURE

1. Remove the desired number of microwells and secure them in the microplate frame. Place un-used microwells into pouch, seal and store at 2-8°C.
2. Add 10 µL of Vit D Calibrators, controls and patient specimens into the assigned wells, respectively.
3. Add 100 µL of working enzyme solution (refer to Preparation of Reagents section for preparation of working enzyme reagent solution) into all wells.
4. Mix the microplate gently for 20-30 seconds, and then cover the plate with a microplate sealer.
5. Incubate the wells at room temperature (22-28°C) for 45 minutes.
6. Wash Step (can be performed manually or with automated washing):
   Manual washing: Carefully remove the incubation mixture by disposing the solution into a waste container. Fill each well with 350 µL working wash buffer and shake gently for 20-30 seconds. Discard the wash solution completely. Repeat 2 more times. After completing the last wash step, tap the plate on absorbent paper to remove residual liquid.
   Automated washing: Automatic plate washer must be calibrated to ensure efficient washing. Fill each well with 350 µL working wash buffer and soak for 20-30 seconds. Aspirate all wells completely. Repeat 2 more times.
   TMB substrate solution must be colorless when used; if the solution turns blue, it must be replaced
7. Add 100 µL of TMB Substrate into all wells. Do not shake the microplate. Incubate at room temperature (22-28°C) in the dark for 15 minutes.
8. Stop the reaction by adding 50 µL of Stop Solution into all wells. Gently mix for 15-20 seconds. Pipette the Stop Solution in the same sequence as substrate addition. It is important to make sure that all the blue color changes completely to a yellow color.
9. Set the microplate reader wavelength at 450 nm. Measure the absorbance (OD) of each well within 15 minutes after adding Stop Solution. A filter of 620-690 nm can be used as a reference wavelength to optimize the assay result.

Flow chart of assay procedure

1.	Secure strips in microwell frame		Number of strips
2.	Add calibrators, controls or specimens, respectively		10 µL
3.	Add working enzyme		100 µL
4.	Mix gently Incubate		20-30 seconds 22-28°C, 45 minutes
5.	Wash: manual or automatic		3 times
6.	Add TMB Substrate (Do not Shake) Incubate in dark		100 µL 22-28°C, 15 minutes
7.	Add Stop Solution Mix Gently		50 µL 15-20 seconds
8.	Read the result		450/620-690 nm within 15 minutes

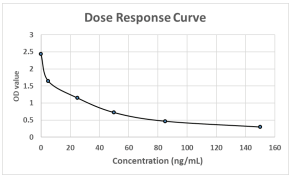
RESULTS CALCULATION

1. Calculate the mean absorbance value (A450/620-690) for each set of calibrators.
2. Construct a curve by plotting the mean value (or subtracted mean value) obtained for each calibrator against its concentration on graph paper with absorbance values on the vertical Y axis, and concentrations on the horizontal X axis.
3. Use the absorbance values (or subtracted absorbance values) for each specimen to determine the corresponding concentration of Vitamin D in ng/mL from the curve.
   Alternatively, if software is used, calculate the concentration of Vitamin D following software menu.

INTERPRETATION

1. Results of a typical standard run are shown below:

Calibrator ID	Average Value (ng/mL)	Abs Value (OD)
C1	0	2.438
C2	5	1.643
C3	25	1.148
C4	50	0.721
C5	85	0.466
C6	150	0.297
PatientID	Concentration	ODValue
1	22.2	1.218



2. The above data and figure are for example purposes only, and should not be used to calculate your result. Test was interpolated from the dose response curve (DRC)
   In order for the assay results to be considered valid the following criteria must be met:
   The absorbance (OD) of calibrator 0 ng/mL should be ≥ 1.
   The assays controls should be within the specified range on the vial labels, if not, reject the test results.

EXPECTED NORMAL VALUE

It is recommended that each laboratory establish its own normal ranges based on a representative sampling of the local population as it varies by stages of life, by race, and ethnicity. The following values for Vitamin D obtained from published literature[6] can be used as initial guideline ranges only:

Vit-D Status[6]	Range (ng/mL)
Deficiency	< 12
Inadequate	12 to <20
Adequate	⩾ 20
Potential adverse effects, particularly > 60 ng/mL	>50
Vit D Intoxication	> 150

NORMAL REFERENCE

1) Analytical Sensitivity

The analytical sensitivity of the test was determined by running 20 replicates of calibrator C1. The mean and standard deviation (SD) of the "0" calibrator reading was taken and the analytical sensitivity was interpolated from the DRC. The analytical sensitivity at 2SD was determined to be 1.20 ng/mL.

2) Specificity

TRUSTwell Vitamin-D (Total) ELISA was evaluated by spiking of following related metabolites into a human serum matrix. The cross reactivity was calculated by deriving a ratio between the concentration of each tested metabolite to the concentration of Vitamin D needed to displace the same amount of conjugate.

Substance
25-OH Vitamin D3
25-OH Vitamin D2
1,25-OH Vitamin D3
Vitamin D2
Vitamin D3

Vit-D ELISA showed higher specificity towards 25-OH-vitamin D3 and 25-OH-vitamin D2. It does not react with non-hydroxylated vitamin D2 and Vitamin D3. Vit-D ELISA also showing higher reactivity with 1-25-OH vit-D3 (active form). However, it has short half life measured in hours and does not affect the diagnostic outcome of the assay.

3) Precision

Intra-assay precision: Twenty replicates of each of three pooled human serum controls (low, medium, and high concentrations) were tested in the same assay. The mean, SD, and coefficient of variation (CV) were determined. The results are showed in the following table:

Sample	N	Mean (ng/mL)	SD	CV
Low	20	3.93	0.18	4.7
Medium	20	28.77	0.72	2.5
High	20	72.16	3.6	5.1

Inter-Assay Precision: Three human serum pooled controls (low, middle, and high concentrations) were assayed in duplicates. The mean, SD, and CV were determined. The results are shown in the following table:

Sample	N	Mean (ng/mL)	SD	CV
Low	20	3.93	0.16	4.3
Medium	20	28.76	0.44	1.6
High	20	72.82	2.45	3.3

QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to monitor assay performance. Any material used should be assayed repeatedly to establish mean values and acceptable ranges to ensure proper performance.

WARNINGS AND PRECAUTIONS

For in Vitro Diagnostic Use

1. This Instructions for Use must be read completely before performing the test. Failure to follow the insert gives inaccurate test results.
2. Do not use expired kits.
3. Bring all reagents to room temperature (22-28°C) before use.
4. Do not use the components of any other type of test kit as a substitute for the components in this kit. Do not reuse the used microwells to test new samples.
5. Do not use serum derived from hemolyzed blood specimens for testing.
6. Do not ingest the reagents. Avoid contact with eyes, skin and mouth. Wear protective clothing and disposable gloves while handling the kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
7. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
8. Follow the US CDC Universal Precautions for prevention of transmission of HIV, HBV and other blood-borne pathogens.
9. Dispose of all specimens and materials used to perform the test as bio-hazardous waste.
10. Prior to the first incubation and after adding Stop Solution, gently rock the microwells to ensure thorough mixing. Avoid the formation of air bubbles which results in inaccurate absorbance values. Avoid splashing liquid while rocking or shaking the wells.
11. Do not allow the microwells to dry between the end of the washing operation and the reagent distribution.
12. The enzyme-substrate reaction is very sensitive to metal ions. Thus, do not allow any metal elements to come into contact with the conjugate or TMB Substrate.
13. The enzyme-substrate is temperature dependent. Ensure that the room temperature for incubation falls between 22-28˚C.
14. The TMB substrate must be colorless. The appearance of color indicates that the reagent cannot be used and must be replaced. The TMB Substrate must be stored in the dark.
15. Use a new dispensing tip for each specimen. Never use the specimen container to distribute conjugate and TMB Substrate. Always use new pipette tip for pipetting TMB substrate.
16. The wash procedure is critical. Wells must be aspirated completely before adding the Wash buffer or liquid reagents. Automatic washers must be validated with the test kit prior to use. Insufficient washing will result in poor precision and falsely elevated absorbance values.
17. Microplate reader must be calibrated per manufacturer's instruction to ensure accurate determination of absorbance. Non-calibrated reader often leads to invalid test results.
18. Avoid exposure to strong light during color development.

LIMITATIONS OF TEST

1. The Assay Procedure and the Interpretation of Results must be followed closely when testing the level of Vitamin D in serum from individual subjects. Failure to follow the procedure may give inaccurate results.
2. The test is limited to the quantitative determination Vitamin D in human serum. The use of other specimen types has not been validated.
3. Any interpretation or use of this test result must also integrate other clinical findings as well as on the professional judgment of health care providers.
4. A clinical diagnosis should not be based on the results of a single test and should only be made by the physician after all clinical and laboratory findings have been evaluated.

REFERENCES

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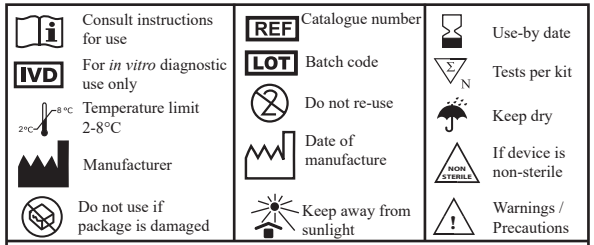

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