



User's Manual

Human 25 (OH) D3 ELISA kit



DEIA-BJ574-1



96T



This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This ELISA kit can be applied to the in vitro quantitative determination of 25-hydroxy Vitamin D3 concentrations in serum, plasma and other biological fluids. This kit is for research use only and not to be used in diagnostic procedures.

Principles of Testing

This ELISA kit uses Competitive-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with 25-hydroxy Vitamin D3. During the reaction, 25-hydroxy Vitamin D3 in the sample or standard competes with a fixed amount of 25-hydroxy Vitamin D3 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 25-hydroxy Vitamin D3. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then the Substrate Reagent is added to each well. The enzyme-substrate reaction is terminated by adding Stop Solution and the color change can be measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The concentration of 25-hydroxy Vitamin D3 in samples can be calculated by comparing the OD of the samples with the standard curve.

Reagents And Materials Provided

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution. The volume of reagents in partial shipments is a little more than the volume marked on the label, please use in measuring instead of directly pouring.

1. Micro ELISA Plate (Dismountable), 8 wells \times 12 strips, -20°C, 6 months
2. Reference Standard, 2 vials, -20°C, 6 months
3. Concentrated Biotinylated Detection Ab (100 \times), 1 vial, 120 μ L, -20°C, 6 months
4. Concentrated HRP Conjugate (100 \times), 1 vial, 120 μ L, -20°C (shading light), 6 months
5. Reference Standard & Sample Diluent, 1 vial, 20 mL, 4°C, 6 months
6. Biotinylated Detection Ab Diluent, 1 vial, 14 mL, 4°C, 6 months
7. HRP Conjugate Diluent, 1 vial, 14 mL, 4°C, 6 months
8. Concentrated Wash Buffer (25 \times), 1 vial, 30 mL, 4°C, 6 months
9. Substrate Reagent, 1 vial, 10 mL, 4°C (**shading light**)
10. Stop Solution, 1 vial, 10 mL, 4°C
11. Plate Sealer, 5 pieces
12. Product Description, 1 copy
13. Certificate of Analysis, 1 copy

Materials Required But Not Supplied

1. Microplate reader with 450 nm wavelength filter

2. High-precision transferpettor, EP tubes and disposable pipette tips
3. 37°C Incubator
4. Deionized or distilled water
5. Absorbent paper
6. Loading slot for Wash Buffer

Storage

The unopened kit can be stored at 4°C for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions since the kit is received.

Specimen Collection And Preparation

1. Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 min at 1000×g. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable, and non-endotoxin.

2. Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8°C within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysis samples are not suitable for ELISA assay!

3. Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells by trypsin. Collect the cell suspension into the centrifugal tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells for 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 µL of pre-cooled PBS to keep the cells resuspended. Repeat the freeze-thaw process for several times until the cells are lysed fully. Centrifuge for 10 min at 1500×g at 4°C. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

4. Tissue homogenates: It is recommended to get detailed references from other literatures before detecting different tissue types. For general information, hemolysis blood may affect the result, so the tissues should be minced to small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight(g): PBS volume(mL) = 1:9) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×g to get the supernatant.

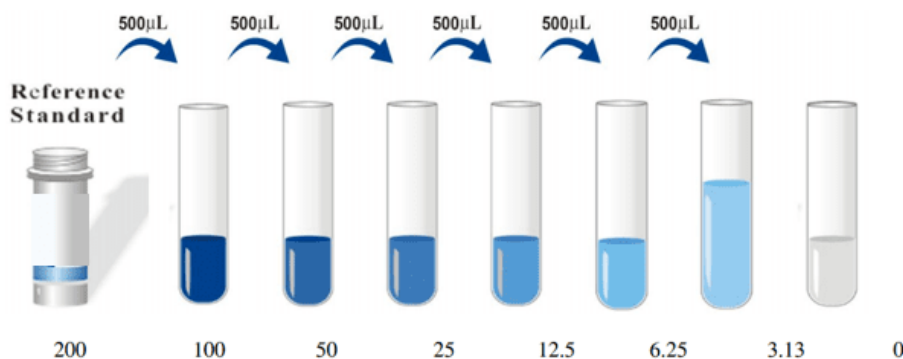
5. Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2-8°C. Collect the supernatant to carry out the assay.

Note for sample:

1. Samples should be assayed within 7 days when stored at 4°C, otherwise samples must be divided and stored at -20°C (≤1 month) or -80°C (≤3 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If lysis buffer is used to prepare tissue homogenate or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.

Reagent Preparation

1. Bring all reagents to room temperature (18-25°C) before use. Preheat the Microplate reader for 15 min before OD measurement.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with deionized or distilled water to prepare 750 mL Wash Buffer. **Note: if crystals have formed in the concentrate, warm it in 40°C water bath and mix it gently until the crystals have completely dissolved.**
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard & Sample Diluent, let it stand for 10 min and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 200 ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 200, 100, 50, 25, 12.5, 6.25, 3.13, 0 ng/mL. Dilution method: Take 7 EP tubes, add 500 µL of Reference Standard & Sample Diluent to each tube. Pipette 500 µL of the 200 ng/mL stock solution to the first tube and mix up to produce a 100 ng/mL stock solution. Pipette 500 µL of the solution from former tube to the latter one in order according to this step. The illustration below is for reference. **Note: the last tube is regarded as blank. Don't pipette solution to it from the former tube.**



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before experiment (50 µL/well). In actual preparation, more account of 100-200 µL should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before experiment (100 µL/well). In actual preparation, more account of 100-200 µL should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

Assay Procedure

1. Add Standard working solution of different concentrations to the first two columns: Each concentration of the solution is added into two wells side by side (50 µL for each well). Immediately add 50 µL of Biotinylated Detection Ab working solution to each well. Cover the plate with sealer provided in the kit. Incubate for 45 min at 37°C. **Note: solutions should be added to the bottom of micro ELISA plate well, avoid touching the inside wall and foaming as possible.**

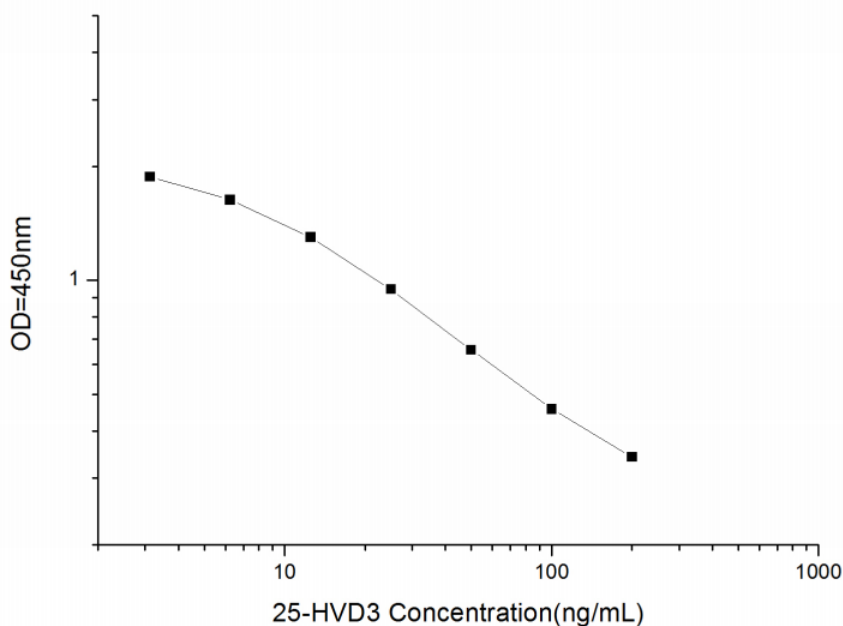
2. Aspirate or decant the solution from each well, add 350 μ L of wash buffer to each well. Soak for 1-2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. **Note: a microplate washer can be used in this step and other wash steps.**
3. Add 100 μ L of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
4. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
5. Add 90 μ L of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. **Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min.**
6. Add 50 μ L of Stop Solution to each well. **Note: the order to add stop solution should be the same as the substrate solution.**
7. Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

Calculation

Average the duplicate readings for each standard and samples. Plot a four-parameter logistic curve on log-log graphpaper, with standard concentration on the x-axis and OD values on the y-axis. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the lower limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor.

Typical Standard Curve

As the OD values of the standard curve may vary according to the conditions of actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish standard curve for each test. Typical standard curve and data below is provided for reference only.



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level 25-hydroxy Vitamin D3 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level 25-hydroxy Vitamin D3 were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	9.44	28.15	88.19	9.08	27.46	85.89
Standard deviation	0.62	1.47	2.88	0.52	1.21	4.08
C V (%)	6.57	5.22	3.27	5.73	4.41	4.75

Specificity

This kit recognizes 25-hydroxy Vitamin D3 in samples. No significant cross-reactivity or interference between 25-hydroxy Vitamin D3 and analogues was observed.

Linearity

Samples were spiked with high concentrations of 25-hydroxy Vitamin D3 and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)	Cell culture media(n=5)
1:2	Range (%)	93-105	97-112	97-111
	Average (%)	98	102	103
1:4	Range (%)	91-101	99-113	99-112
	Average (%)	96	105	105
1:8	Range (%)	90-103	93-110	97-111
	Average (%)	96	100	105
1:16	Range (%)	92-104	97-111	92-108
	Average (%)	98	103	100

Recovery

The recovery of 25-hydroxy Vitamin D3 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	87-100	92
EDTA plasma (n=5)	86-99	92
Cell culture media (n=5)	95-112	102

Precautions

1. Please wear lab coats and latex gloves for protection. Please perform the experiment following the national security columns of biological laboratories, especially detecting samples of blood or other body fluid.
2. The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
3. Do not reuse the diluted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solution should be stored back according to the storage condition in the above table.
4. Do not mix or use components from other lots (except for washing buffer and stop solution).
5. Change pipette tips between adding of each standard level, between sample adding, and between reagent adding. Also, use separate reservoirs for each reagent.

Limitations

1. Limited by current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operation skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions in the description!
4. Incorrect results may occur because of wrong operations during the reagents preparation and loading, as well as incorrect parameter setting of Micro-plate reader. Please read the instruction carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some unexpected reasons such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from above reasons, too.
7. Valid period: 6 months.