（**FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !**）

Catalog No: E-EL-0015

Product size: 96T/48T/24T/96T*5

Elabscience® 25-HVD3(25-Hydroxy Vitamin D3) ELISA Kit

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Tel**:** 1-832-243-6086
Fax: 1-832-243-6017
Email**:** techsupport@elabscience.com
Website**:** www.elabscience.com

Please refer to specific expiry date from label outside of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Intended use

This ELISA kit applies to the in vitro quantitative determination of 25-HVD3 concentrations in serum, plasma and other biological fluids.

Character

Item	
Sensitivity	1.88 ng/mL
Detection Range	3.13-200 ng/mL
Specificity	This kit recognizes 25-HVD3 in samples. No significant cross-reactivity or interference between 25-HVD3 and analogues was observed
Repeatability	Coefficient of variation is < 10%

Test principle

This ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with 25-HVD3. During the reaction, 25-HVD3 in samples or Standard competes with a fixed amount of 25-HVD3 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 25-HVD3. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of 25-HVD3 in the samples is then determined by comparing the OD of the samples to the standard curve.

Kit components & Storage

An unopened kit can be stored at 2-8℃ for 1 month. If the kit is not supposed to be used within 1 month, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips 24T: 8 wells ×3 strips 96T*5: 5 plates, 96T	-20℃, 12 months
Reference Standard	96T: 2 vials 48T/24T: 1 vial 96T*5: 10 vials	
Concentrated Biotinylated Detection Ab(100×)	96T: 1 vial, 120 μL 48T/24T: 1 vial, 60 μL 96T*5: 5 vials, 120 μL	
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 μL 48T/24T: 1 vial, 60 μL 96T*5: 5 vials, 120 μL	-20℃(Protect from light), 12 months
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL 96T*5: 5 vials, 20 mL	2-8℃, 12 months
Biotinylated Detection Ab Diluent	96T/48T/24T: 1 vial, 14 mL 96T*5: 5 vials, 14 mL	
HRP Conjugate Diluent	96T/48T/24T: 1 vial, 14 mL 96T*5: 5 vials, 14 mL	
Concentrated Wash Buffer(25×)	96T/48T/24T: 1 vial, 30 mL 96T*5: 5 vials, 30 mL	
Substrate Reagent	96T/48T/24T: 1 vial, 10 mL 96T*5: 5 vials, 10 mL	2-8℃(Protect from light)
Stop Solution	96T/48T/24T: 1 vial, 10 mL 96T*5: 5 vials, 10 mL	2-8℃
Plate Sealer	96T/48T/24T: 5 pieces 96T*5: 25 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

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 accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Microplate reader with 450 nm wavelength filter
High-precision transfer pipette, EP tubes and disposable pipette tips
Incubator capable of maintaining 37℃
Deionized or distilled water
Absorbent paper
Loading slot

Sample collection

Serum: Allow samples to clot for 1 hour at room temperature or overnight at 2-8℃before centrifugation for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

Plasma: Collect plasma using EDTA-Na_2 as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8℃ within 30 min of collection. Collect the supernatant to carry out the assay.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolyzed blood may affect the results, so the tissues should be minced into 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 (mL) volume=1:9) with a glass homogenizer on ice. To further break down 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 5-10 min at 5000×g at 2-8℃ to get the supernatant.

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times or use an ultrasonic cell disrupter until the cells are fully lysed. Centrifuge for 10 min at 1500×g at 2-8℃. Remove the cell fragments, collect the supernatant to carry out the assay.

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

Recommended reagents for sample preparation: 10×EDTA Anticoagulant (Cat No. E-EL-SR003), PMSF Protease Inhibitor (Cat No. E-EL-SR002), 0.25% Trypsin Solution (Cat No. E-EL-SR001).

Note

■ Note for kit

1) For research use only. Not for use in diagnostic procedures.
2) Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
3) A freshly opened ELISA plate may appear a water-like substance, which is normal and will not have any impact on the experimental results. Return the unused wells to the foil pouch and store according to the conditions suggested in the above table.
4) Do not reuse the reconstituted standard, biotinylated detection Ab working solution, HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
5) The microplate reader should be able to be installed with a filter that can detect the wave length at 450 ±2 nm. The optical density should be within 0-3.5. Follow the Instructions of the Microplate Reader for set-up and preheat it for 15 min before OD measurement.
6) Do not mix or substitute reagents with those from other lots or sources.
7) Change pipette tips in between adding of each standard level, between sample adding and between reagent adding. Also, use separate reservoirs for each reagent.
8) The kit should not be used beyond the expiration date on the kit label.

■ Note for sample

1) Tubes for blood collection should be disposable and be non-endotoxin. Samples with high hemolysis or much lipid are not suitable for ELISA assay.
2) Samples should be assayed within 7 days when stored at 2-8℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen samples should be slowly thawed and centrifuged to remove precipitates.
3) 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.
4) If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
5) If a lysis buffer is used to prepare tissue homogenates or cell lysates, there is a possibility of causing a deviation due to the introduced chemical substance.
6) Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

Dilution method

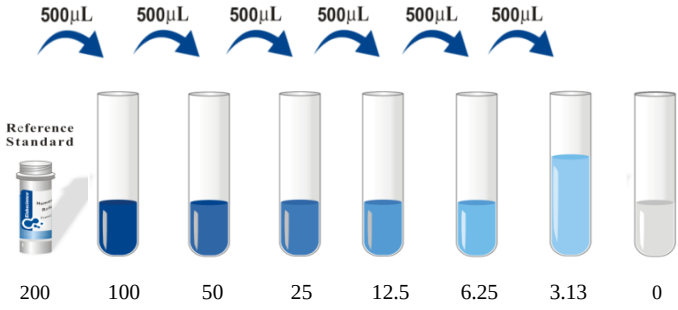
Please predict the concentration range of samples in advance, and determine the dilution ratio through preliminary experiments or technical support recommendations.

If your test sample needs dilution, please refer to the dilution method as follows:
For 100 fold dilution: One-step dilution. Add 5 μL sample to 495 μL sample diluent to yield 100 fold dilution.
For 1000 fold dilution: Two-step dilution. Add 5 μL sample to 95 μL sample diluent to yield 20 fold dilution, then add 5 μL 20 fold diluted sample to 245 μL sample diluent, after this, the neat sample has been diluted at 1000 fold successfully.
For 100000 fold dilution: Three-step dilution. Add 5 μL sample to 195 μL sample diluent to yield 40 fold dilution, then add 5 μL 40 fold diluted sample to 245 μL sample diluent to yield 50 fold dilution, and finally add 5 μL 2000 fold diluted sample to 245 μL sample diluent, after this, the neat sample has been diluted at 100000 fold successfully.

Reagent preparation

1. Bring all reagents to room temperature (18-25℃) before use. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃ 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 1mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 200 ng/mL (or add 1 mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). 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 working solution to the first tube and mix up to produce a 100 ng/mL working solution. Pipette 500 μL of the solution from the former tube into the latter one according to this step. The illustration on the next page is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube. Gradient diluted standard working solution should be prepared just before use.

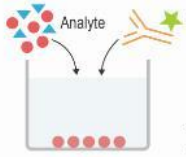


4. **Biotinylated Detection Ab working solution**: Calculate the required amount before the experiment (50 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99). The working solution should be prepared just before use.
5. **HRP Conjugate working solution:** HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99). The working solution should be prepared just before use.

Assay procedure

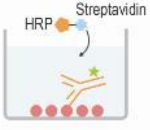
1. Determine wells for **diluted standard**, **blank** and **sample**. Add 50 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Immediately add 50 μL of **Biotinylated Detection Ab working solution** to each well. Cover the plate with the sealer provided in the kit. Incubate for 45 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Decant the solution from each well, add 350 μL of **wash buffer** to each well. Soak for 1 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. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
3. Add 100 μL of **HRP Conjugate working solution** to each well. Cover the plate with a new sealer. Incubate for 30 min at 37 ℃.
4. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 2.
5. Add 90 μL of **Substrate Reagent** to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37 ℃. 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. Preheat the Microplate Reader for about 15 min before OD measurement.
6. Add 50 μL of **Stop Solution** to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
7. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Assay Procedure Summary

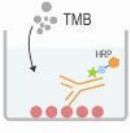


1. Add 50μL standard or sample to the wells, immediately add 50μL Biotinylated Detection Ab working solution to each well. Incubate for 45 min at 37°C

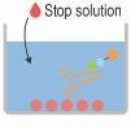
2. Aspirate and wash the plate for 3 times



3. Add 100μL HRP conjugate working solution. Incubate for 30 min at 37°C. Aspirate and wash the plate for 5 times



4. Add 90μL Substrate Reagent. Incubate for 15 min at 37°C



5. Add 50μL Stop Solution

6. Read the plate at 450nm immediately. Calculation of the results

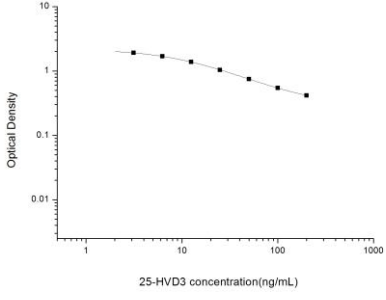
Calculation of results

Average the duplicate readings for each standard and samples. Plot a four parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the OD of the sample under the lowest limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

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

ng/mL	OD	Standard Curve
200	0.418	
100	0.543	
50	0.749	
25	1.04	
12.5	1.378	
6.25	1.687	
3.13	1.915	
0	2.228	

Performance

- **Precision**

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level 25-HVD3 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level 25-HVD3 were tested on 3 different plates, 20 replicates in each plate, respectively.

	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
CV (%)	6.57	5.22	3.27	5.73	4.41	4.75

■ **Recovery**

The recovery of 25-HVD3 spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=8)	87-100	92
EDTA plasma (n=8)	86-99	92
Cell culture media(n=8)	95-112	102

■ **Linearity**

Samples were spiked with high concentrations of 25-HVD3 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

Troubleshooting

If the results are not good enough, please take pictures and save the experimental data in time. Keep the used plate and remaining reagents. Then contact our technical support to solve the problem. Meanwhile, you could also refer to the following materials

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing.
	Wells are not completely aspirated	Completely aspirate wells in between steps.
Low signal	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB, rapid coloring.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Open the Microplate Reader ahead to pre-heat.
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution is not added	Stop solution should be added to each well before measurement.

Declaration

1. Limited by current conditions and scientific technology, we can't conduct 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. This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.
3. The final experimental results will be closely related to the validity of products, operational skills of the operators, the experimental environments and so on. We are only responsible for the kit itself, but not for the samples consumed during the assay. The users should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
4. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions.
5. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
6. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
7. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment, and so on. Intra-assay variance among kits from different batches might arise from the above reasons too.
8. Kits from different manufacturers or other methods for testing the same analyte could bring out inconsistent results, since we haven’t compared our products with those from other manufacturers.
9. The kit is designed for research use only, we will not be responsible for any issues if the kit is applied in clinical diagnosis or any other related procedures.