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ab213966
25(OH) Vitamin D ELISA kit

For the quantitative determination of 25(OH) Vitamin D3 and 25(OH) Vitamin D2 and was tested in human plasma and serum samples.

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

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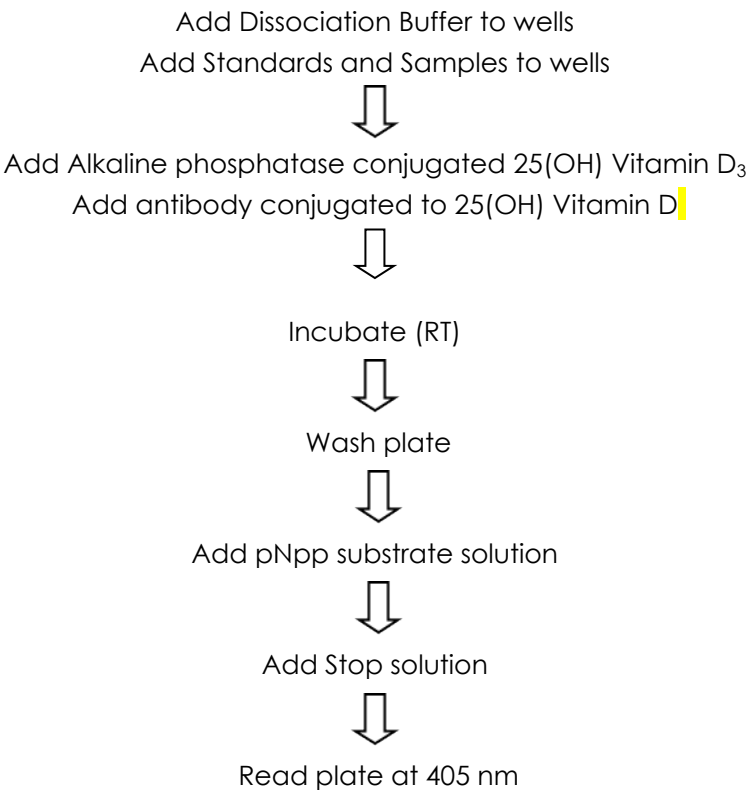
1. Overview

The 25(OH) Vitamin D Enzyme-Linked Immunosorbent Assay (ELISA) kit (ab213966) is a complete kit for the quantitative determination of 25(OH) Vitamin D_3 and 25(OH) Vitamin D_2 in human plasma and serum samples. It is recommended that you read the entire kit insert before proceeding with the assay.

Recent research efforts have shown that Vitamin D levels affect various disease states and are being linked with numerous indicators of well-being in humans. These include bone diseases such as osteoporosis and arthritis, but also additional disease including hypertension, diabetes, cancer and heart disease to name a few. Our Vitamin D ELISA kit offers an alternative to labor intensive and/or costly methods of testing for Vitamin D levels in human plasma and serum.

The transformation to the active form of Vitamin D begins with 7-dehydrocholesterol being acted upon by UV rays from the sun to form parent Vitamin D_3. Alternatively, Vitamin D can be ingested as parent Vitamin D_2 from various food sources, native or fortified. These parent compounds are transported to the liver and undergo hydroxylation to 25(OH) Vitamin D. This metabolite is then transported to the kidney where it undergoes a second hydroxylation to 1,25$(OH)_2$Vitamin D, the biologically active form of Vitamin D. It is important to note that levels of Vitamin D metabolites increase proportionately with increased uptake of parent Vitamin D. This combined with the greater half-life and stability of 25(OH) Vitamin D in circulation versus the active form (25 days versus 8 hours) are the reasons that the detection of the 25(OH) Vitamin D metabolite is used as the indicator for total Vitamin D concentration.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All ELISA kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store ELISA kit at -20°C immediately upon receipt

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- ELISA kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition (Before prep)	Storage Condition (After prep)
Dissociation Buffer	10 mL	-20°C	RT
25(OH) Vitamin D Standard 1 (1010 ng/mL)	40 µL	-20°C	-20 °C
25(OH) Vitamin D Standard 2 (279 ng/mL)	40 µL	-20°C	-20 °C
25(OH) Vitamin D Standard 3 (71.6 ng/mL)	40 µL	-20°C	-20 °C
25(OH) Vitamin D Standard 4 (24.4 ng/mL)	40 µL	-20°C	-20 °C
25(OH) Vitamin D Standard 5 (4.8 ng/mL)	40 µL	-20°C	-20 °C
25(OH) Vitamin D Standard 6 (0.5 ng/mL)	40 µL	-20°C	-20 °C
Sample Diluent	1.8 mL	-20°C	-20°C
25(OH) Vitamin D Conjugate (100X)	50 µL	-20°C	-20°C
Donkey anti-Sheep IgG coated microplate (12x 8 well strips)	96 wells	-20°C	+2-8°C
25(OH) Vitamin D Antibody	5 mL	-20°C	-20°C
25(OH) Vitamin D3 Conjugate Diluent	6 mL	-20°C	+2-8°C
Wash Buffer 4 Concentrate (20X)	20 mL	-20°C	RT
pNpp Substrate	20 mL	-20°C	-20°C
Stop Solution	5 mL	-20°C	-20°C
Plate Sealer	3 each	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the ELISA kit, but will be required to successfully perform this assay:

- Deionized or distilled water
- Precision pipettes for volumes between 5 µL and 1,000 µL
- Repeater or multichannel pipette for dispensing 50 µL and 200 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Absorbent paper for blotting
- Microplate reader capable of reading a 405 nm
- Software for calculating sample values from optical density readings utilizing a four parameter logistic curve fit.

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- This ELISA kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 Dissociation Buffer:

10 mL Ready to use. Store at -20°C.

9.2 25(OH) Vitamin D_3 Standards

6 vials of 40 µL. Ready to use. Store at -20°C.
(0.5, 4.8, 24.4, 71.6, 279, 1010 ng/mL)

9.3 Sample Diluent:

1.8 mL. Ready to use. Store at -20°C.

9.4 25(OH) Vitamin D3 Conjugate, 100X

50 µL. Store stock at - 20°C. Dilute 50 µL of the thawed 100X conjugate stock with 5 mL of the thawed 25(OH) Vitamin D_3 Conjugate Diluent, mix thoroughly.

ΔNote: The 1X Conjugate cannot be stored for later use and should be used in the assay within 1 hour of dilution/preparation.

9.5 Donkey anti-Sheep IgG Microtiter Plate:

One plate (break-apart strips) of 96 wells. Ready to use. Store at -20°C.

9.6 25(OH) Vitamin D Antibody:

5 mL. Ready to use. Store at -20°C.

9.7 25(OH) Vitamin D_3 Conjugate Diluent

6 mL. Ready to use. Store at -20°C.

9.8 Wash Buffer 4 Concentrate, 20X

20 mL. Store conc. at -20°C. Dilute 20 mL of the Wash Buffer 4 concentrate with 380 mL of deionized water.

ΔNote: Concentrated Wash Buffer 4 may precipitate during shipping. If a precipitate forms, allow this to come to room temperature and agitate until precipitate is fully dissolved.

9.9 pNpp Substrate

20 mL. Ready to use. Store at -20°C.

9.10 Stop Solution

5 mL. Ready to use. Store at -20°C.

9.11 Plate Sealer

3 each. Ready to use. Store at -20°C.

10. Sample Preparation

- The ELISA kit is suitable for the measurement of 25(OH) Vitamin D in human serum and plasma samples. This kit is not species specific. However, samples containing sheep IgG will interfere in the assay due to the donkey anti-sheep IgG coated plate. Prior to assay, frozen samples should be brought to room temperature and centrifuged, if necessary, to isolate residual debris.
- Accurate measurements of 25(OH) Vitamin D in serum and plasma will be obtained by the 1:10 dilution of samples with Dissociation Buffer as described in the assay procedure. If further dilution of samples is needed, use of the sample diluent is highly recommended. The 1:10 dilution ensures that 25(OH) Vitamin D levels will be within the boundaries of the standard curve concentrations.

  ΔNote: Standards and Sample Diluent are prepared from human-derived serum and should be handled accordingly. Materials have been tested and found negative for anti-Human Immunodeficiency Virus (HIV 1 and 2), anti-Hepatitis C virus and anti-Hepatitis B surface antigen. Since no test offers complete assurance that infectious agents are absent, the reagents should be handled in accordance at Biosafety Level 2.

11. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls (NSB and Bo) and samples in duplicate.

12.1 Pipette 90 µL of the Dissociation Buffer into all wells that will be used.

12.2 Pipette 10 µL of Sample Diluent into the Bo (Maximum binding) and NSB (Non- Specific Binding) wells.

12.3 Pipette 10 µL of the standards and samples into the appropriate wells with Dissociation Buffer in them.

12.4 Incubate for 5 minutes with mixing on a plate shaker at room temperature.

12.5 Pipette 50 µL of the prepared 1X Conjugate into each well.

12.6 Pipette 50 µL of the Conjugate Diluent into NSB wells.

12.7 Pipette 50 µL of the supplied Antibody into each well, except the NSB wells.

12.8 Seal the plate. Incubate for 1 hour with mixing on a plate shaker at room temperature.

12.9 Empty the contents of the wells and wash with an automated washer by adding 400 µL of 1X Wash Buffer 4 to every well (for hand washing use 325 µL per well). Aspirate wells and repeat 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

12.10 Pipette 200 µL of pNpp solution into each well.

12.11 Seal the plate. Incubate for 30 minutes with mixing on a plate shaker at room temperature.

12.12 Pipette 50 µL of Stop Solution into each well.

12.13 After blanking the plate reader against the substrate, read optical density at 405nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

ΔNote: The optimal speed for each shaker will vary and may range from 120-700 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.

13. Calculations

Several options are available for the calculation of the concentration of 25(OH) Vitamin D in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

The concentration of 25(OH) Vitamin D can be calculated as follows:

13.1 Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

Average Net OD = Average OD - Average NSB OD

13.2 Plot the Net OD versus concentration of 25(OH) Vitamin D for the standards. Fit a curve through the data points (4PL curve fit is suggested). The concentration of 25(OH) Vitamin D in the unknown samples is then determined by interpolation from the standard curve. Be sure to account for the dilution factor during the sample dissociation step.

13.3 Samples with concentrations outside of the standard curve range will need to be re-analyzed using an additional dilution step.

14. Typical data

Typical standard curve – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Sample	Average Net OD	Percent Bound	25(OH) Vitamin D (ng/mL)
NSB	0.081	N/A	N/A
Bo	1.232	100	0
S1	0.122	9.9	101
S2	0.264	21.4	27.9
S3	0.502	40.7	7.16
S4	0.722	58.6	2.44
S5	0.963	78.4	0.48
S6	1.161	94.4	0.05

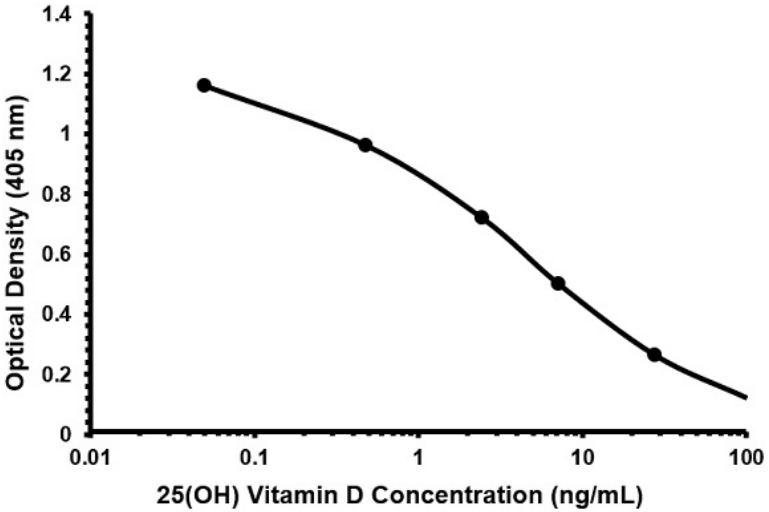


Figure 1. 25(OH) Vitamin D ELISA Kit (ab213966) Standard Curve

15. Typical sample values

SENSITIVITY –

The biological sensitivity of the assay is 1.98 ng/mL. The sensitivity was determined by interpolation at 2 standard deviations below the mean signal at a concentration of 0 ng/mL analyte (n=20) using data from 25 standard curves.

RECOVERY –

Recovery was determined by mixing paired serum samples that have low and high endogenous 25(OH) Vitamin D concentrations. These were mixed at specific ratios and the measured Vitamin D concentrations compared to the expected values based on the neat sample concentrations.

Neat Low Sample (L) = 5.91			
Neat High Sample (H) = 175.98			
Dilution Factor	Expected value	Observed value	% Recovery
3L:1H	48.43	42.22	87.2
2L:1H	62.59	58.98	94.2
1L:1H	90.95	86.48	95.1
1L:2H	119.30	112.74	94.5
1L:3H	133.47	130.04	97.4
		Average Percentage Recovery	93.7

Neat Low Sample (L) = 12.77			
Neat High Sample (H) = 111.18			
Dilution Factor	Expected value	Observed value	% Recovery
3L:1H	37.32	31.92	85.5
2L:1H	45.52	36.19	79.5
1L:1H	61.94	51.41	83.0
1L:2H	78.35	67.39	86.0
1L:3H	86.56	74.27	85.8
		Average Percentage Recovery	84.0

Neat Low Sample (L) = 20.62			
Neat High Sample (H) = 60.56			
Dilution Factor	Expected value	Observed value	% Recovery
3L:1H	30.60	28.70	93.8
2L:1H	33.93	32.58	96.0
1L:1H	40.59	39.61	97.6
1L:2H	47.25	45.79	96.9
1L:3H	50.57	53.23	105.3
		Average Percentage Recovery	97.9

PRECISION –

Intra-assay precision:

Sample	Number of measures	Mean (ng/mL)	CV%
1	20	225.8	1.6
2	20	37.3	2.1
3	20	5.3	3.4

Inter-assay precision:

Sample	Number of assays	Mean (ng/mL)	CV%
1	30	297.2	11.5
2	30	38.4	15.8

Specificity

The cross reactivity for related compounds was determined by serially diluting cross reactants in assay buffer beginning at a concentration of fifty times the 25(OH) Vitamin D_3 high standard. These samples were then measured in the assay and percent cross reactivity determined by normalizing ED50 values to that of 25(OH) Vitamin D_3.

Analyte	Cross Reactivity
25(OH) Vitamin D3	100%
25(OH) Vitamin D2	81.5%
Vitamin D3	0.30%
Vitamin D2	0.53%
1, 25(OH)2 Vitamin D3	467%
1, 25(OH)2 Vitamin D2	91.3%
24, 25(OH)2 Vitamin D3	5.9%
1a(OH) Vitamin D3	0.52%
1a(OH) Vitamin D2	0.58%
3-epi-25(OH) Vitamin D3	10.4%

The observed cross reactivity to the $1,25(OH)_2$ Vitamin D_3 metabolite is not of major concern to the overall reported values of the assay since the circulating levels of this metabolite are approximately 1000 fold lower than that of 25(OH) Vitamin D, therefore contributing less than 2% of total concentrations reported.

Similarly, the small cross reactivity to the 3-epi-25(OH) Vitamin D_3 metabolite will only be of concern to investigators performing research involving infants 1yr and younger as this population is where the epi-metabolite can be found in significant concentrations5. However, at this level of cross reactivity the epi-metabolite if present would contribute no more than 4% of the total reported Vitamin D concentration.

Interference

Potential biological interferants (hemoglobin, bilirubin, triglycerides and cholesterol) were tested beginning at 5 mg/mL. Hemoglobin up to 0.3 mg/mL and bilirubin, triglycerides and cholesterol up to 5 g/mL did not interfere with assay results.

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check Pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check Pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	All components 4°C. Keep TMB substrate solution protected from light.

16. Troubleshooting

17. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | +86-21-5110-5938 | 400-628-6880
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abcam.com | +64-(0)9-909-7829