

25(OH) Vitamin D ELISA Kit

Catalog #ADI-900-215

96-Well Enzyme Immunoassay Kit

For use with human serum and plasma

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Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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Please read
entire booklet
before
proceeding with
the assay.

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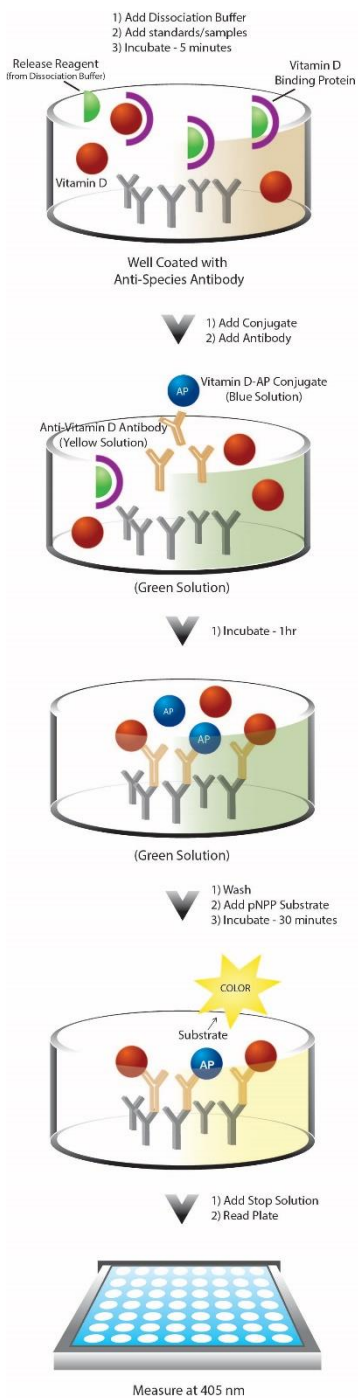
BACKGROUND

The Enzo Life Sciences 25(OH) Vitamin D Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of 25(OH) Vitamin D₃ and 25(OH) Vitamin D₂ 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 few¹. 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 D₃. Alternatively, Vitamin D can be ingested as parent Vitamin D₂ 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)₂Vitamin D, the biologically active form of Vitamin D². 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)³ are the reasons that the detection of the 25(OH) Vitamin D metabolite is used as the indicator for total Vitamin D concentration.

PRINCIPLE



1. Dissociation Buffer is added to wells coated with donkey anti-sheep IgG antibody. Standards and Samples are then added to these wells and dissociation of 25(OH) Vitamin D from Vitamin D Binding protein allowed to occur.
2. A solution of alkaline phosphatase conjugated 25(OH) Vitamin D₃ is then added, followed by a solution of sheep monoclonal antibody to 25(OH) Vitamin D.
3. During incubation at room temperature the antibody binds the 25(OH) Vitamin D from the sample/standard or conjugate in a competitive manner and is itself captured by the anti-sheep IgG antibody. The plate is then washed, leaving a complex with bound 25(OH) Vitamin D from samples or the Alkaline phosphatase conjugate.
4. A pNpp substrate solution is added initiating an alkaline phosphatase catalyzed reaction that generates a yellow color in the solution.
5. Stop solution is added to stop the substrate reaction and the resulting yellow color is read at 405nm. The amount of signal is inversely proportional to the level of 25(OH) Vitamin D in the sample.



Do not mix components from different kit lots or use beyond the expiration date of the kit.



Protect substrate from prolonged exposure to light.



Stop solution is caustic. Keep tightly capped.

MATERIALS SUPPLIED

1. **Dissociation Buffer:**
10 mL, Product No. 80-2454
MES buffer containing salts and releasing reagents
2. **25(OH) Vitamin D ELISA Components:**
Product No. 80-2483
 - ❖ **25(OH) Vitamin D₃ Standards – 40 µL each,**
Product No. 80-2462, 80-2463, 80-2464, 80-2465, 80-2466, 80-2467
One vial each, containing 1010, 279, 71.6, 24.4, 4.8 and 0.5 ng/mL of 25(OH) Vitamin D₃
 - ❖ **Sample Diluent:**
1.8 mL, Product No. 80-2457
Serum like diluent for NSB, Bo and samples
 - ❖ **25(OH) Vitamin D₃ Conjugate, 100x:**
50 µL, Product No. 80-2452
100x solution of Alkaline phosphatase conjugated 25(OH) Vitamin D₃
3. **Donkey anti-Sheep IgG Microtiter Plate:**
One plate of 96 wells, Product No. 80-0045
A clear plate of break-apart strips coated with a donkey anti-sheep polyclonal antibody
4. **25(OH) Vitamin D Antibody:**
5 mL, Product No. 80-2451
1X monoclonal antibody solution to 25(OH) Vitamin D
5. **25(OH) Vitamin D₃ Conjugate Diluent:**
6 mL, Product No. 80-2453
Tris buffer containing salts and protein
6. **Wash Buffer 4 Concentrate, 20x:**
20 mL, Product No. 80-2455
20X MES buffer containing salts and detergents
7. **pNpp Substrate:**
20 mL, Product No. 80-0075
A solution of p-nitrophenyl phosphate
8. **Stop Solution:**
5 mL, Product No. 80-0247
A solution of trisodium phosphate in water
9. **25(OH) Vitamin D Assay Layout Sheet:**
1 each, Product No. 30-0310
10. **Plate Sealer:**
3 each, Product No. 30-0012

STORAGE

Upon receipt, store entire kit at -20°C until needed. If the entire kit is not consumed within one experiment, please use storage temperature indicated on individual kit component labels as follows: Dissociation and Wash Buffers can be stored at room temperature and the Antibody solution, Conjugate Diluent and remaining microtiter plate wells at 4°C. All other kit components should be stored at -20°C. Shipping conditions may not reflect storage conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater or multichannel pipet for dispensing 50 µL and 200 µL
4. Graduated cylinders
5. A microplate shaker
6. Absorbent paper for blotting
7. Microplate reader capable of reading a 405 nm
8. Software (such as AssayBlaster™ catalog #ADI-28-0002) for calculating sample values from optical density readings utilizing a four parameter logistic curve fit.

SAMPLE HANDLING

The assay 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.



If buffers other than those provided are used, the end-user must determine the appropriate dilution and assay compatibility.



Sample handling procedures should be completed prior to reagent preparation.

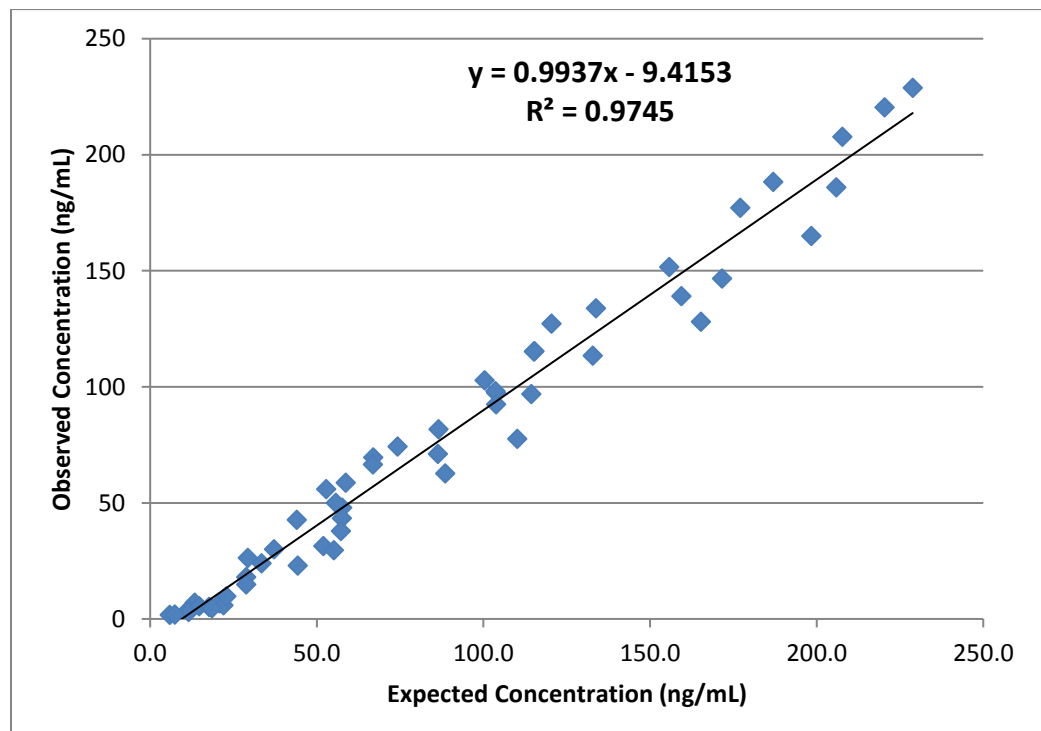
***Note:** Standards and Sample Diluent are prepared from human-derived serum and should be handled accordingly. Materials have been tested and found negative for anti-Human Immunodeficiency Virus (HIV 1 and 2), anti-Hepatitis C virus and anti-Hepatitis B surface antigen. Since no test offers complete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

Serum versus Plasma samples

Paired (n=10) human serum and EDTA plasma samples were evaluated for equivalence of 25(OH) Vitamin D concentrations in this assay. A linear regression of the matched samples data resulted in an equation of Plasma = (0.98)Serum + 4.4, $r^2=0.922$.

Linearity

Linearity was determined by dilution of eight high concentration samples. Expected versus observed results were plotted and analyzed by linear regression.



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.

Low Neat:	5.91			
High Neat:	175.98			
Dil Factor	Expected	Observed	% 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	
Avg. % Recovery				93.7

Low Neat:	12.7			
High Neat:	111.18			
Dil Factor	Expected	Observed	% 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	
Avg. % Recovery				84.0

Low Neat:	20.62			
High Neat:	60.56			
Dil Factor	Expected	Observed	% 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	
Avg. % Recovery				97.9

REAGENT PREPARATION

1. Wash Buffer 4, 1x

Prepare Wash Buffer 4 by diluting 20 mL of the supplied Wash Buffer 4 concentrate with 380 mL of deionized water. Store the diluted wash buffer at room temperature.

*Note: 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.

2. 25(OH) Vitamin D₃ Conjugate, 1x

Prepare conjugate by diluting 50 µL of the 100X conjugate stock with 5 mL of 25(OH) Vitamin D₃ Conjugate Diluent, mix thoroughly. For less than a full plate experiment, adjust volumes accordingly.

***Note:** the 1x Conjugate cannot be stored for later use and should be used in the assay within 1 hour of dilution/preparation.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4 °C.

1. Pipet 90 μ L of the Dissociation Buffer into all wells that will be used.
2. Pipet 10 μ L of Sample Diluent into the Bo and NSB wells.
3. Pipet 10 μ L of the standards and samples into the appropriate wells with Dissociation Buffer in them.
4. Incubate for 5 minutes with mixing on a plate shaker at room temperature.
5. Pipet 50 μ L of the prepared 1X Conjugate into each well.
6. Pipet 50 μ L of the Conjugate Diluent into NSB wells.
7. Pipet 50 μ L of the supplied Antibody into each well, except the NSB wells.
8. Seal the plate. Incubate for 1 hour with mixing on a plate shaker at room temperature.
9. Empty the contents of the wells and wash with an automated washer by adding 400 μ L of 1X Wash Buffer 4 to every well (for hand washing use 325 μ L 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.
10. Pipet 200 μ L of pNpp solution into each well.
11. Seal the plate. Incubate for 30 minutes with mixing on a plate shaker at room temperature.
12. Pipet 50 μ L of Stop Solution into each well.
13. Read optical density at 405 nm, blanking the plate reader against the NSB. If the plate reader is not capable of adjusting for an assigned blank, manually subtract the mean OD of the NSB from all readings.

Note: 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.



Bring all reagents to room temperature for at least 30 minutes prior to opening.



All standards should be run in duplicate.



Pipet the reagents to the sides of the wells to avoid possible contamination.



Prior to the addition of the substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variations in results.



Be sure to multiply sample concentrations by the dilution factor used during sample preparation.

CALCULATION OF RESULTS

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. Assay Blaster! Data analysis software (catalog # ADI-28-0002) is an easy-to-use and cost effective program that provides the options of point-to-point, 4PL and 5PL curve fitting options.

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

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.

$$\text{Average Net OD} = \text{Average OD} - \text{Average NSB OD}$$

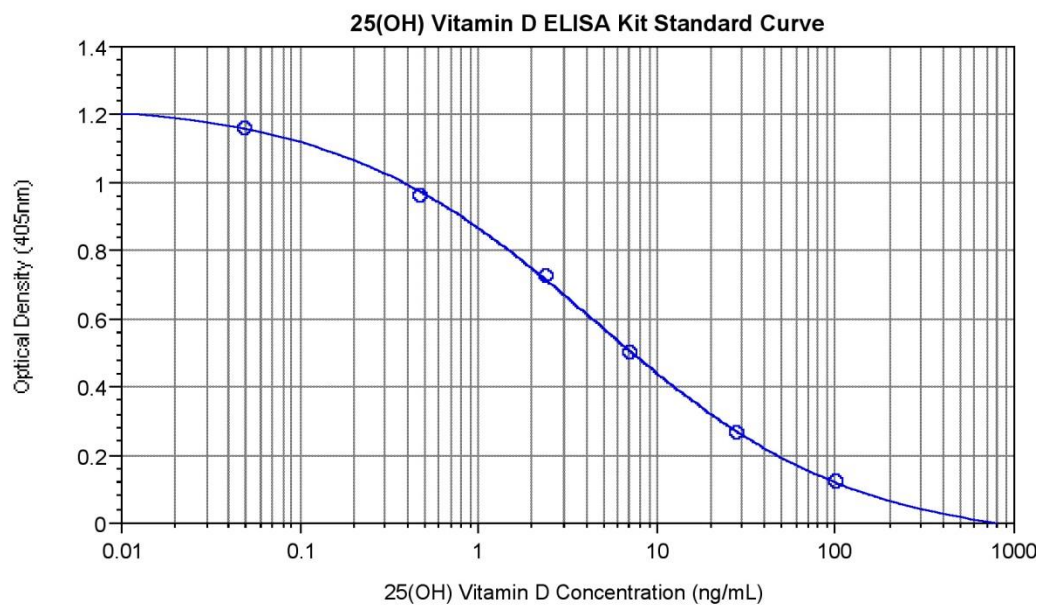
2. Plot the Net OD versus concentration of 25(OH) Vitamin D for the standards. Fit a curve through the data points (4PL curve fit is suggested). The concentration of 25(OH) Vitamin D in the unknown samples is then determined by interpolation from the standard curve. Be sure to account for the dilution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

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

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average Net OD	Percent Bound	25(OH) Vitamin D (ng/mL)
NSB	0.081	---	---
Bo	1.232	100%	0
S1	0.122	9.9	101
S2	0.264	21.4	27.9
S6	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



PERFORMANCE CHARACTERISTICS

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 D₃ 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 D₃.

Analyte	Cross Reactivity
25(OH) Vitamin D ₃	100%
25(OH) Vitamin D ₂	81.5%
Vitamin D ₃	0.30%
Vitamin D ₂	0.53%
1, 25(OH) ₂ Vitamin D ₃	467%
1, 25(OH) ₂ Vitamin D ₂	913%
24, 25(OH) ₂ Vitamin D ₃	5.9%
1 α (OH) Vitamin D ₃	0.52%
1 α (OH) Vitamin D ₂	0.58%
3-epi-25(OH) Vitamin D ₃	10.4%

The observed cross reactivity to the 1,25(OH)₂ Vitamin D₃ 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 D⁴, therefore contributing less than 2% of total concentrations reported.

Similarly, the small cross reactivity to the 3-epi-25(OH) Vitamin D₃ 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.

Biological Interferants

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 mg/mL did not interfere with assay results.

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.

Precision

Intra-assay precision

Intra-assay precision was determined by assaying 20 replicates of 3 Vitamin D controls within a single assay.

ng/mL	%CV
225.8	1.6
37.3	2.1
5.3	3.4

Inter-assay precision

Inter-assay precision was determined by measuring 2 Vitamin D controls in multiple assays (n=30) over several days.

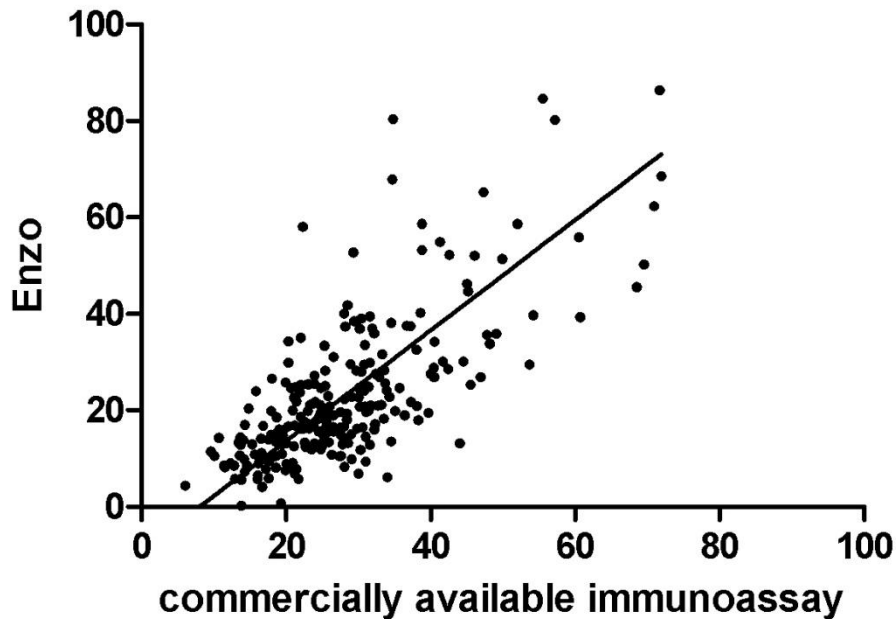
ng/mL	%CV
297.2	11.5
38.4	15.8

Single versus Duplicate measurements

Analysis of human serum samples (n=193) were carried out in duplicate with the mean values compared back to the individual values by paired Student's t test. The resulting analysis indicated that a single measurement was representative of a sample with a P value of 0.82, indicating no significant difference between singlicate or duplicate data sets.

Experimental Evaluation

A set of 248 human serum samples were analyzed by the Enzo 25(OH) Vitamin D ELISA and compared to analysis with a commercially available immunoassay. The resulting regression equation by Deming Analysis is $y=1.144x - 9.2$.



Data analysis was completed using Deming linear regression analysis. Sample values assigned by Enzo were plotted against those obtained via commercial immunoassay. The standard deviation (SD) of X-axis data was 4.68 ng/mL and SD of Y data was 8.66 ng/mL (95% CI). SD of X data was determined using data provided in the commercial product manual. SD of Y errors was determined by calculating the mean of the standard deviations determined from 2 independent runs of the 248-sample set.

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Product Manual

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