Mouse/Rat 25-OH Vitamin D ELISA

Catalog Number: VID21-K01

For Research Use Only. Not for use in diagnostic procedures.

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INTENDED USE

The Eagle Biosciences Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is intended for use in the quantitative determination of total 25-OH Vitamin D (Vitamin D2 and Vitamin D3) in serum and EDTA plasma. This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is for Research Use Only.

ASSAY BACKGROUND

The group of compounds referred to as Vitamin D, are actually fat soluble steroidal prehormones. The main forms which occur in the body are Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol). The active form of these molecules is Dihydroxyvitamin D3 (1, 25$(OH)_2$ D_3). Vitamin D3 is formed in the skin by photolysis of 7-dehydrocholesterol by ultraviolet radiation from sunlight. It is transported in blood circulation bound to proteins to the liver where it is hydroxylated. Further hydroxylation occurs in the kidneys to produce the most active form. Vitamin D levels are highest in newborns and decrease exponentially throughout life. Sufficient circulating levels of vitamin D are necessary for healthy bone maintenance and cell metabolism. Recent studies have shown that it may also lower incidents of certain cancers. Insufficient levels of Vitamin D can result in osteoporosis and bone fracture in the elderly, secondary hyperparathyroidism, abnormal cell metabolism and even increased incidents of cancer. Severe deficiency may lead to rickets in children and osteomalacia in adults. Disease associated with Vitamin D deficiency may also include: impaired immunity, increased autoimmunity, myopathy, diabetes mellitus, and an increased risk of colon, breast, and prostate cancers. Abnormally high levels (> 200 ng/ml) of Vitamin D leads to Vitamin D toxicity and may cause hypercalcaemia.

ASSAY PRINCIPLE

This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is designed developed and produced for the quantitative measurement of total 25-OH Vitamin $D_{2/3}$ in serum utilizing the competitive immunoassay technique. This assay utilizes a monoclonal antibody that binds to both 25-OH Vitamin D2 and 25-OH Vitamin D3 equally. Assay calibrators, controls and test samples are added directly to wells of a microtiter plate that is coated with specific anti-25-OH Vitamin D2, D3 antibody. A buffer designed to release Vitamin D from binding proteins is then added to the wells. After the first incubation period, unbound material is washed away and biotinylated Vitamin D analogue is added to the wells and binds to remaining antibody sites. After the second incubation period, unbound biotin-D is washed away and horseradish peroxidase (HRP) conjugated streptavidin is added to each well. During the third incubation step, an immune complex of well coated "vitamin D antibody – vitamin D, biotin D and HRP conjugated streptavidin" is formed. The unbound matrix is removed in the subsequent washing steps. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction, which is terminated with an acidic reagent (ELISA stop solution). The absorbance is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is inversely proportional to the amount of total 25-OH Vitamin $D_{2/3}$ in the test sample. A calibration curve is generated by plotting the absorbance versus the respective Vitamin D concentration for each calibrator on a 4-parameter or point to point curve fitting. The concentration of total 25-OH Vitamin $D_{2/3}$ in test samples is determined directly from this calibration curve.

REAGENTS: Preparation and Storage
This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit must be stored at 2 – 8 °C upon receipt. Vitamin D is sensitive to heat and light. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.

1. **Vitamin D Antibody Coated Microplate**
   One microplate with twelve by eight strips (96 wells total) coated with anti-Vitamin D2/D3antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.
2. **HRP - Streptavidin**
   One bottle containing **11.5 mL** ready to use HRP labeled streptavidin in a stabilized protein matrix. This reagent should be stored in 2-8ºC and is stable until the expiration date on the kit box.
3. **Biotinylated Vitamin D Analogue**
   One bottle containing **11.5 mL** of ready to use biotin-Vitamin D analogue in a stabilized buffer matrix with preservative. This reagent should be stored in 2-8ºC and is stable until the expiration date on the kit box.
4. **Vitamin D Assay Buffer**
   One bottle containing **15 ml** of ready-to-use buffered matrix. This buffer releases Vitamin D from its binding proteins. This reagent may be stored in room temperature and/or 2-8ºC and is stable until the expiration date on the kit box.
5. **ELISA Wash Concentrate**
   One bottle containing **30 mL** of 30-fold concentrate. Before use, the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.
6. **ELISA HRP Substrate**
   One bottle containing **12 mL** of ready to use tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.
7. **ELISA Stop Solution**
   One bottle containing **12 mL** of ready to use stop solution. This reagent may be stored at 2-8ºC or room temperature and is stable until the expiration date on the kit box.
8. **Vitamin D Calibrators 0 to 5**
   Six vials contain 0.5 mL each of ready to use liquid 25-OH Vitamin D3 in a bovine serum albumin-based matrix with a non-azide preservative. Refer to the vial for exact concentration. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.

9. **Vitamin D Controls**
   Two vials each contain **0.5 mL** of ready to use liquid Vitamin D3 in a human serum-based matrix with a non-azide preservative. Refer to vials for concentration range for each control. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.

SAFETY PRECAUTIONS

The reagents of the Mouse/Rat 25-OH Vitamin D ELISA Assay Kit must be used in professional laboratory. Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 25 µl, 100 µl, 500 µL, etc.
2. Disposable pipette tips suitable for above volume dispensing.
3. Aluminum foil.
4. Plastic microtiter well cover or polyethylene film.
5. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
6. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
7. ELISA plate shaker.

SPECIMEN COLLECTION

Serum, EDTA-plasma and citrate plasma samples were validated with this Mouse/Rat 25-OH Vitamin D ELISA Assay Kit. Only 50 µL total (25 µL each) of human EDTA-plasma or serum is required for the 25-OH Vitamin D measurement in duplicate. No special preparation of individual is necessary prior to specimen collection. Collect whole blood with Vacutainer and separate the serum or plasma from cells according to manufacturer's instruction. Serum and plasma samples can be stored at room temperature for 3 days. For longer term storage, sample can be kept at – 15ºC. Avoid more than three freeze-thaw cycles of specimen.

Animal serum Total 25-OH Vitamin D from bovine/calf, goat, horse, chicken, mouse, and equine can be detected using this kit.

ASSAY PREPARATION

1. Reagent Preparation(1)Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.

ASSAY PROCEDURE

Test Configuration

Row	Strip 1	Strip 2	Strip 3
A	Cal 0	Cal 4	SAMPLE 1
B	Cal 0	Cal 4	SAMPLE 1
C	Cal 1	Cal 5	SAMPLE 2
D	Cal 1	Cal 5	SAMPLE 2
E	Cal 2	Control 1	SAMPLE 3
F	Cal 2	Control 1	SAMPLE 3
G	Cal 3	Control 2	SAMPLE 4
H	Cal 3	Control 2	SAMPLE 4

1. Add **25 µl** of calibrators, controls and test samples into the designated microwells.
2. Add **100 µl** Vitamin D Assay Buffer to each well.
3. **Seal** the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate at room temperature (20ºC – 25ºC) for 60 minutes.
4. **Wash** each well 5 times by dispensing **350 µL** of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
5. Add **100 µl** of Biotinylated Vitamin D Analogue to each well.
6. **Seal** the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate at room temperature (20 ºC - 25 ºC) for 30 minutes.
7. **Wash** each well 5 times by dispensing **350 µL** of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
8. Add **100 µL** of Streptavidin-HRP into each of the wells.
9. **Seal** the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate at room temperature (20 ºC - 25 ºC) for 20 minutes.
10. **Wash** each well 5 times by dispensing **350 µL** of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
11. Add **100 µl** TMB reagent to each of the wells.
12. **Cover** plate with aluminum foil, and incubate at room temperature (20 ºC - 25 ºC) for 20 minutes, static.

13. Immediately add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.
14. **Read** the absorbance at 450 nm. 4-parameter curve is recommended.

PROCEDURAL NOTES

1. Vitamin D is sensitive to heat and light. It is important to avoid direct exposure to these conditions.
2. It is recommended that all calibrators and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results. It is recommended to add external controls to each assay.
3. Keep light sensitive reagents in the original amber bottles.
4. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
5. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the Mouse/Rat 25-OH Vitamin D ELISA Assay test.
6. Incubation times or temperatures other than those stated in this insert may affect the results.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. It is important to seal the plate properly to avoid evaporation.

INTERPRETATION OF RESULTS

1. It is recommended to use a 4-parameter calibrator curve fitting
2. Calculate the average absorbance for each pair of duplicate test results.
3. The calibration curve is generated by the corrected absorbance of all calibration levels on the ordinate against the calibrator concentration. Appropriate computer assisted data reduction programs should be used for the calculation of results.
4. The total 25 OH Vitamin D concentrations for the test samples are read directly from the calibration curve using their respective average absorbance

LIMITATION OF THE PROCEDURE

1. This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit requires serum or plasma sample for testing.
2. Serum or plasma samples from different species may show different matrix background.
3. For sample values greater than 150 ng/mL, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100). The best dilution matrix is vitamin D free human serum.
4. Cell culture or tissue culture samples should be validated with total binding and other performance specifications before being used.

5. Severely hemolyzed samples, icteric or lipaemic sample should not be used
6. If Spike Recovery is desired, use controls to spike into the samples.

QUALITY CONTROL

The performance of the Mouse/Rat 25-OH Vitamin D ELISA Assay Kit was determined a correlation study test using an FDA approved kit 25-OH Vitamin D ELISA test. To assure the validity of the results each assay should include adequate controls with known Vitamin D levels. We recommend that all assays include the laboratory's own Vitamin D controls in addition to those provided with this kit.

EXPECTED VALUES

Dietary intake, race, season and age are known to affect the normal levels of Total 25-OH Vitamin D. The following data is provided for guidance only.

It is important for each laboratory to establish its own reference ranges, which may better represent its typical population and region.

Recent literature has suggested the following ranges for the classification of Total 25-OH Vitamin D status:

Level	Concentration (ng/mL)
Severe Deficiency	<10
Insufficient	10 - 24
Optimal	25 - 100
Potential toxicity	>100

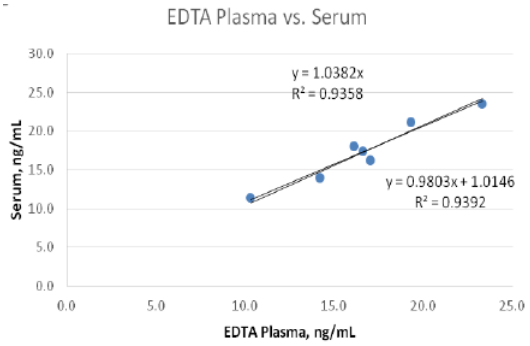
The Endocrine Society Clinical Practice Guideline (2011) has suggested a higher target level of at least 30 ng/ml:

Level	Concentration (ng/mL)
Deficiency	<20
Insufficient	20 - 29
Sufficiency	30 - 100

We have validated the above reference range with 56 apparently healthy individuals. Donors that were not taking Vitamin D supplements from which samples were collected were tested. Patient EDTA plasma and serum were used to obtain the summarized data below.

	Concentration (ng/mL)
Mean	32.4`
Highest	74.6
Lowest	12.6

Donor serum and EDTA plasma paired samples were correlated using this kit. The result yielded an excellent slope and correlation.

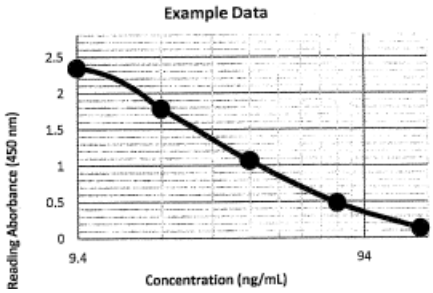


EXAMPLE DATA

A typical absorbance data and the resulting standard curve from are represented.

This curve should not be used in lieu of a calibrator curve run with each assay.

Well ID	Reading Absorbance (450nm)		
	Readings	Average	B/B0
Calibrator Level 0: 0 ng/mL	3.159	3.159	100%
	3.195		
Calibrator Level 1: 9.4 ng/mL	2.729	2.726	86%
	2.717		
Calibrator Level 2: 18.8 ng/mL	2.181	2.152	68%
	2.123		
Calibrator Level 3: 37.5 ng/mL	1.219	1.258	40%
	1.297		
Calibrator Level 4: 75 ng/mL	0.468	0.454	14%
	0.440		
Calibrator level 5: 150 ng/mL	0.159	0.158	5%
	0.156		



PERFORMANCE CHARACTERISTICS

Sensitivity

The Limit of Blank (LoB) was calculated by measuring the Calibrator zero in 16 replicates and calculating the 95th percentile of the distribution of the test values. The LoB was calculated to be
1.000ng/mL

The Limit of Detection (LoD) was calculated by measuring the Calibrator 0, 1, and a low sample and calculating the 95th percentile of the distribution of the test values. The LoD was calculated to be 4.781ng/mL

The Limit of Quantitation (LoQ) was calculated to be 8.558ng/mL.

Specificity

Cross reactivity of this Total 25-OH Vitamin D ELISA kit was determined by testing sera with spiked and unspiked cross reactants. The results are as follows:

Compound and Concentration	Cross Reaction %
25 OH Vitamin D3 at 10 ng/mL	100
25 OH Vitamin D2 at 10 ng/mL	100
1,25(OH)2 Vitamin D3 at 200 ng/mL	20
1,25(OH)2 Vitamin D2 at 690 ng/mL	1.9
Vitamin D3 at 200 ng/mL	2.9
Vitamin D2 at 200 ng/mL	1.3
24,25(OHJ2 Vitamin D3 at 20 ng/mL	>100
24,26(OHJ2 Vitamin D3 at 4 ng/mL	>100
3-epi 25OH Vitamin D3 at 20 ng/mL	0.1

Reproducibility and Precision

The intra-assay precision was validated by measuring three samples in sixteen (16) replicate determinations. The inter-assay precision was validated by measuring three samples in twelve (12) different assays in duplicate. The results are as follows:

	Intra Assay			Inter Assay		
Sample	1	2	3	1	2	3
Mean (ng/mL)	94.6	50.2	26.2	22.2	60.5	48.6
Standard Deviation	1.4	2.8	2.4	1.7	4.6	2.5
CV (%)	1.4	5.5	9.0	7.5	7.6	5.2

Linearity

Three (3) calibrators were diluted with standard matrix and tested. The results are as follows:

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator A	**-**	**135.1**	**-**
80%	117.9	108.1	109%
60%	94.2	81.1	116%
40%	58.0	54.0	107%
20%	28.5	27.0	105%
Calibrator B	**-**	**120.6**	**-**
80%	102.4	96.5	106%
60%	73.9	72.4	102%
40%	46.3	48.3	96%
20%	24.9	24.1	104%
Calibrator C	**-**	**87.2**	**-**
80%	68.7	69.8	98%
60%	49.0	52.3	94%
40%	34.1	34.9	98%
20%	17.9	17.4	102%

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator A	**-**	**146.8**	**-**
80%	124.7	117.4	106%
60%	98.4	88.1	112%
40%	60.7	58.7	103%
20%	27.4	29.3	93%
Calibrator B	**-**	**129.6**	**-**
80%	109.6	103.7	106%
60%	77.1	77.8	99%
40%	47.9	51.9	92%
20%	22.0	25.9	85%
Calibrator C	**-**	**91.5**	**-**
80%	74.4	73.2	102%
60%	50.3	54.9	92%
40%	30.8	36.6	84%
20%	16.5	18.3	90%

Spike Recovery

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Calibrator level 1	**9.4**	**-**	**-**
+ Calibrator Level 3: 37.5 ng/mL	26.4	23.45	113%
+ Calibrator Level 4: 75.0 ng/mL	41.7	42.2	99%
+ Calibrator Level 5: 150.0 ng/mL	85.3	79.7	107%
Calibrator Level 2	**18.8**	**-**	**-**
+Calibrator Level 3: 37.5 ng/mL	29.836	28.15	106%
+ Calibrator Level 4: 75.0 ng/mL	48.15	46.9	103%
+ Calibrator Level 5: 150.0 ng/mL	93.95	84.4	111%
Calibrator Level 3	**37.5**	**-**	
+ Calibrator Level 4: 75 ng/mL	54.931	56.25	98%
+ Calibrator Level 5: 150.0 ng/mL	106.491	93.75	114%

Calibrator Level 4	**75**	**-**	**-**
+ Calibrator Level 5: 150.0 ng/mL	128.5	112.5	114

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	**65.5**	**-**	**-**
+ Sample 1 67.8	66.7	66.6	100%
+ Sample B 68.0	76.1	66.6	114%
+ Sample C 20.0	44.5	42.7	104%
Sample 2	**43.7**	**-**	**-**
+ Sample A 67.8	58.9	55.8	106%
+ Sample B 68.8	54.9	55.8	98%
+ Sample C 20.0	30.1	31.9	94%
Sample 3	**19.1**	**-**	**-**
+ Sample A 67.8	39.6	43.5	91%
+ Sample B 68.8	39.4	43.4	91%
+ Sample C 20.0	19.6	19.6	100%

Interference

	Results (ng/mL)	Bias (%)	Amount (mg/mL
Test Control	**13.3**	**-**	**-**
Bilirubin	13.0	-2%	10
	12.9	-3%	2
	13.4	1%	0.4
Test Control	**18.7**	**-**	**-**
Hemoglobin	20.4	9%	10
	18.4	-2%	2
	17.7	-5%	0.4
Lipids	18.5	-1%	200
	18.2	-3%	40
	15.8	-16%	8
Test Control	**38.6**	**-**	**-**
Bilirubin	41.1	6%	10
	39.8	3%	2
	37.6	-3%	0.4
Test Control	**63.1**	**-**	**-**
Hemoglobin	64.5	2%	10
	67.1	6%	2
	69.3	10%	0.4
Lipids	64.2	2%	200
	59.4	-6%	40
	52.9	-16%	8

Assay Delay

The assay delay was tested using real human samples. The samples were added after the calibrators in different times. The results are as follows.

Samples	Concentration (ng/mL)	Bias (%)
Sample 1	**18.516**	**-**
After 5 min	20.414	10%
After 15 min	18.094	-2%
After 25 min	19.481	5%
Sample 2	**65.839**	**-**
After 5 min	68.271	4%
After 15 min	57.089	-13%
After 25 min	62.023	-6%

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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.