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Human Total 25-OH Vitamin D IVD ELISA

GENERAL INFORMATION

A. Proprietary name: Human Total 25-OH Vitamin D IVD ELISA Kit

B. Catalog Number: RDKAP1971, 96 tests

C. Manufactured for: R&D Systems, a Bio-Techne brand

This package insert must be read in its entirety before using this product.

IVD For *In Vitro* Diagnostic use.

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I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of 25-hydroxyvitamin D_2 and D_3 (25-OH-D_2 and 25-OH-D_3) in serum. The test is a diagnostic tool and is to be used in conjunction with clinical findings.

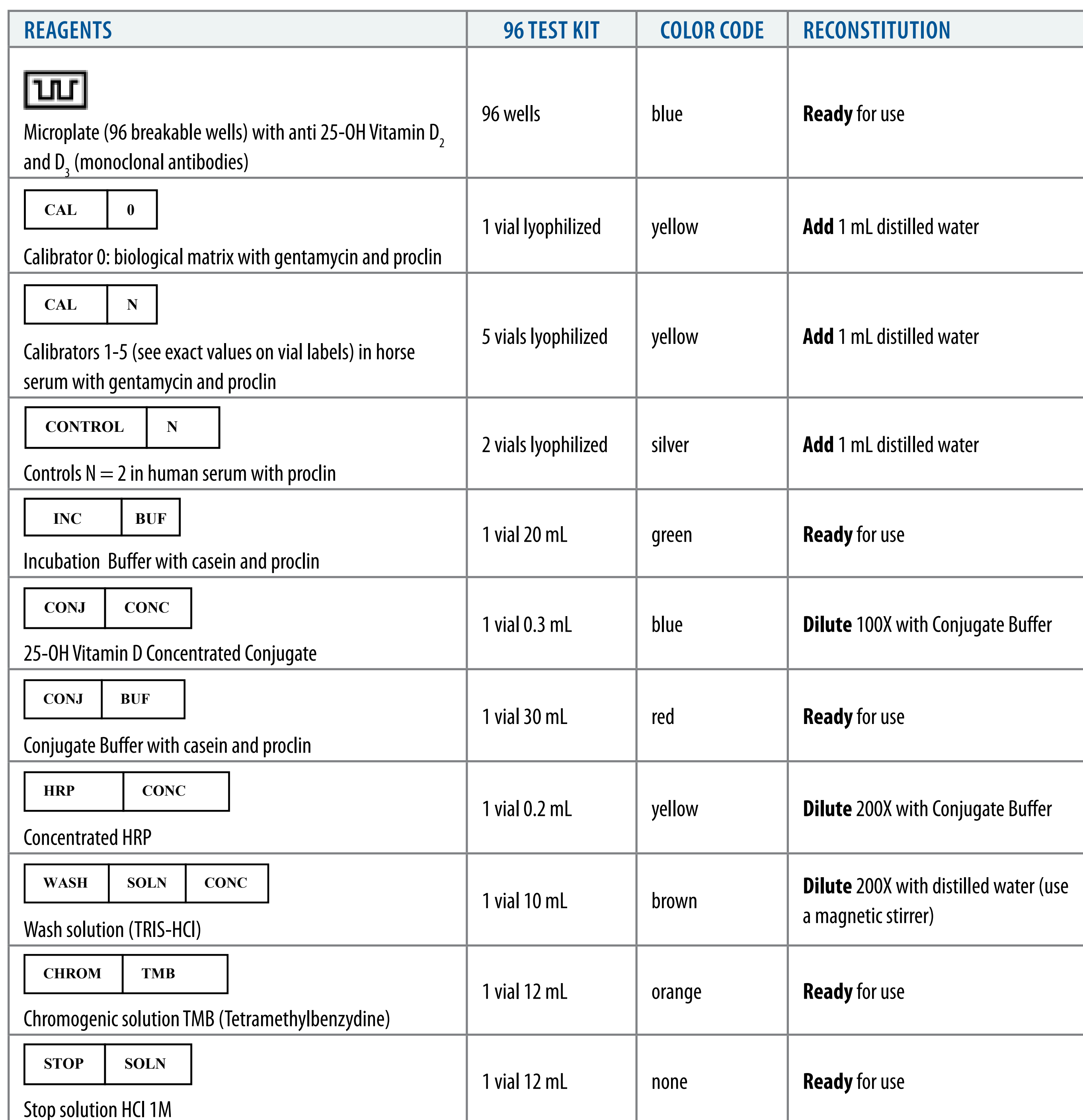
II. CLINICAL BACKGROUND

Vitamin D is the generic term used to designate Vitamin D_2 or ergocalciferol and Vitamin D_3 or cholecalciferol. Humans naturally produce Vitamin D_3 when the skin is exposed to ultraviolet sun rays. In the liver mainly, Vitamin D_3 is metabolized into 25-hydroxyvitamin D_3 (25-OH-D_3) which is the main form of Vitamin D circulating in the body. 25-OH-D_3 is a precursor for other Vitamin D metabolites and has also a limited activity by itself. The most active derivative is 1, 25-hydroxyvitamin D_3, produced in the kidney (or placenta) by 1-hydroxylation of 25-OH-D_3. 25-OH Vitamin D stimulates the intestinal absorption of both calcium and phosphorus and also bone resorption and mineralization. 25-OH Vitamin D might also be active in other tissues responsible for calcium transport for example, placenta, kidney, mammary gland and endocrine gland, parathyroid glands, and beta cells. Vitamin D_3 and Vitamin D_2 are also available by ingestion through food or dietary supplementation. As Vitamin D_2 is metabolized in a similar way to Vitamin D_3, both contribute to the overall Vitamin D status of an individual. It is the reason why it is very important to measure both forms of 25-OH Vitamin D equally for a correct diagnosis of Vitamin D deficiency, insufficiency or intoxication. Vitamin D deficiency is an important risk factor for rickets, osteomalacia, senile osteoporosis, and cancer and pregnancy outcomes. The measurement of both 25-OH Vitamin D forms is also required to determine the cause of abnormal serum calcium concentrations in patients. Vitamin D intoxication has been shown to cause kidney and tissue damage.

III. PRINCIPLES OF THE METHOD

The Human Total 25-OH Vitamin D IVD ELISA is a solid phase Enzyme Linked Immunosorbent Assay performed on microplates. During the first two hour incubation step, at room temperature, total 25-OH Vitamin D (D_2 and D_3) present in calibrators, controls and samples is dissociated from binding serum proteins to fix on binding sites of a specific monoclonal antibody. After one washing step, a fixed amount of 25-OH Vitamin D-labeled with biotin in presence of horseradish peroxidase (HRP), competes with labeled 25-OH Vitamin D_2 and 25-OH Vitamin D_3 present on the binding sites of the specific monoclonal antibody. After a 30 minute incubation at room temperature, the microplate is washed to stop the competition reaction. The Chromogenic solution (TMB) is added and incubated for 15 minutes. The reaction is stopped with the addition of Stop solution and the microplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is inversely proportional to the total 25-OH Vitamin D (D_2 and D_3) concentration. A calibration curve is plotted and the total 25-OH Vitamin D (D_2 and D_3) concentrations of the samples are determined by dose interpolation from the calibration curve.

IV. REAGENTS PROVIDED



REAGENTS	96 TEST KIT	COLOR CODE	RECONSTITUTION
Microplate (96 breakable wells) with anti 25-OH Vitamin D_2 and D_3 (monoclonal antibodies)	96 wells	blue	**Ready** for use
CAL 0 Calibrator 0: biological matrix with gentamycin and proclin	1 vial lyophilized	yellow	**Add** 1 mL distilled water
CAL N Calibrators 1-5 (see exact values on vial labels) in horse serum with gentamycin and proclin	5 vials lyophilized	yellow	**Add** 1 mL distilled water
CONTROL N Controls N = 2 in human serum with proclin	2 vials lyophilized	silver	**Add** 1 mL distilled water
INC BUF Incubation Buffer with casein and proclin	1 vial 20 mL	green	**Ready** for use
CONJ CONC 25-OH Vitamin D Concentrated Conjugate	1 vial 0.3 mL	blue	**Dilute** 100X with Conjugate Buffer
CONJ BUF Conjugate Buffer with casein and proclin	1 vial 30 mL	red	**Ready** for use
HRP CONC Concentrated HRP	1 vial 0.2 mL	yellow	**Dilute** 200X with Conjugate Buffer
WASH SOLN CONC Wash solution (TRIS-HCl)	1 vial 10 mL	brown	**Dilute** 200X with distilled water (use a magnetic stirrer)
CHROM TMB Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 12 mL	orange	**Ready** for use
STOP SOLN Stop solution HCl 1M	1 vial 12 mL	none	**Ready** for use

V. PRECAUTIONS AND WARNINGS

Safety

For *in vitro* diagnostic use only by trained personnel.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents and serum specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop solution contains HCl. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

VI. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kit components are stable until the expiry date, indicated on the label, if kept at 2-8 °C.
- After reconstitution, calibrators and controls are stable for eight weeks at 2-8 °C. For longer storage periods, aliquots should be made and kept at ≤ -20 °C for a maximum of 4 months. Avoid subsequent freeze-thaw cycles.
- Freshly prepared Working Wash solution should be used on the same day.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

VII. SUPPLIES NOT PROVIDED

The following materials are required but not provided in the kit:

1. Distilled water
2. Pipettes for delivery of: 50 µL,150 µL, 200 µL and 1.0 mL (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Plate shaker (300 to 700 rpm)
6. Washer for microplates
7. Microplate reader capable of reading at 450 nm and 630 or 650 nm (bichromatic reading)

VIII. REAGENT PREPARATION

A. Calibrator 0: Reconstitute Calibrator 0 with 1.0 mL distilled water.

B. Calibrators 1-5: Reconstitute Calibrators 1-5 with 1.0 mL distilled water.

C. Controls: Reconstitute the Controls with 1.0 mL distilled water.

D. Samples requiring dilution: For dilution of samples having concentrations of 25-OH Vitamin D above the highest calibrator concentration, use Control 1 or a serum sample with a concentration of 25-OH below 25 ng/mL and above 4.4 ng/mL (limit of quantification of the assay), as measured in this assay. Use Ctrl 1 or this sample to dilute 2X the out of curve samples. Take the concentration of the Ctrl 1 or the low sample into account when calculating the dilution result.
Note: *Use the concentration of Ctrl 1 measured in the same run as the dilution run, not the mean concentration on the Ctrl 1 label.*

Calculations: Sample value = (Measured value – F1 x Measured Ctrl 1) / F2 with the following values for F1 and F2:

Sample Dilution	F1	F2
1/2	0.5	0.5
1/4	0.75	0.25
1/8	0.875	0.125

Example: A sample out of the calibration curve is diluted 4 times with Ctrl 1, and is measured at 70 ng/mL. Ctrl 1 is measured in the same run at 20 ng/mL.

Dilution 4 times, F1 = 0.75; F2 = 0.25
Sample calculated value = (70 – 0.75 x 20)/0.25 = 220 ng/mL

E. Working HRP conjugate solution:

The Working HRP conjugate solution should be prepared during the first incubation and a minimum of 1 hour 45 minutes before its use (X.B.5).

Prepare an adequate volume of Working HRP conjugate solution by mixing the three reagents in the following sequence, vortexing where indicated: 1) Conjugate Buffer, 2) Concentrated Conjugate, 3) vortex, 4) Concentrated HRP, 5) vortex. ***The order of addition of these three reagents is critical to obtain reproducible optical densities.***

Determine the volume needed according to the number of strips used, as indicated in the table below. For example, for 6 strips (48 wells), add 100 µL of Concentrated Conjugate to 10 mL of Conjugate Buffer. Vortex to homogenize the solution. Add 50 µL of Concentrated HRP to the solution and vortex.

Until its use, keep the Working HRP conjugate solution at room temperature and avoid direct sunlight or use a brown glass vial for its preparation.

The preparation of Working HRP conjugate solution is not stable and must be discarded if not used.

Number of strips	Volume of Conjugate Buffer (mL)	Volume of Concentrated Conjugate (uL)	Volume of Concentrated HRP (uL)
1	3	30	15
2	5	50	25
3	6	60	30
4	8	80	40
5	9	90	45
6	10	100	50
7	12	120	60
8	14	140	70
9	16	160	80
10	18	180	90
11	20	200	100
12	22	220	110

F. Working Wash solution: Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash solution (200X). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.

IX. SPECIMEN COLLECTION AND PREPARATION

- This kit is suitable for serum samples.
- Serum samples must be kept at 2-8 °C.
- If the test is not run within 24 hours, **sample storage at ≤ -20 °C is recommended.**
- Avoid subsequent freeze-thaw cycles.

X. PROCEDURE

A. Handling notes

- Do not use the kit or components beyond expiry date.
- Do not mix materials from different kit lots.
- Bring all the reagents to room temperature prior to use.
- Thoroughly mix all reagents and samples by gentle agitation or swirling.
- Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
- Use a clean plastic container to prepare the Working Wash solution.
- In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
- For the dispensing of Chromogenic solution and Stop solution avoid pipettes with metal parts.
- High precision pipettes or automated pipetting equipment will improve the precision.
- Respect the incubation times.
- To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section XII paragraph G (Time delay).
- Prepare a calibration curve for each run, do not use data from previous runs.
- Dispense the Chromogenic solution within 15 minutes following the washing of the microplate.
- During incubation with Chromogenic solution, avoid direct sunlight on the microplate.

B. Procedure

1. Select the required number of strips for the run. It is recommended that all samples, controls, and calibrators be assayed in duplicate. The unused strips should be resealed in the bag with a desiccant and stored at 2-8 °C.
2. Secure the strips into the holding frame.
3. Pipette 50 µL of each calibrator, control and sample into the appropriate wells. A plate layout is provided to record standards and samples assayed.
4. Pipette 150 µL of Incubation Buffer into all the wells.
5. Incubate for 2 hours at room temperature, on a plate shaker (400 rpm). Prepare the Working HRP conjugate solution during the incubation and a minimum of 1 hour 45 minutes before its use.
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   a. dispensing 0.35 mL of Working Wash solution into each well
   b. aspirating the content of each well
8. Pipette 200 µL of the Working HRP conjugate solution into each well. Incubate the microplate for 30 minutes at room temperature, on a plate shaker (400 rpm).
9. Aspirate the liquid from each well.
10. Wash the plate 3 times by:
    a. dispensing 0.35 mL of Working Wash solution into each well
    b. aspirating the content of each well
11. Pipette 100 µL of the Chromogenic solution into each well within 15 minutes following the washing step.
12. Incubate the microplate for 15 minutes at room temperature, on a plate shaker (400 rpm), avoid direct sunlight.
13. Pipette 100 µL of Stop solution into each well.
14. Read the absorbance at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XII.

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. We recommend the use of computer assisted methods to construct the calibration curve. 4-parameter logistic function curve fitting is the preferred method. Reject obvious outliers.
4. By interpolation of the sample OD values, determine the 25-OH Vitamin D concentrations of the samples from the calibration curve.
5. If samples have been diluted refer to Section VIII for dilution and final concentration calculations.

XII. PERFORMANCE AND LIMITATIONS

A. Typical Data

The following data are for illustration only and should never be used instead of the real-time calibration curve.

25-OH ELISA		OD Units
Calibrator	0 ng/mL	2.66
	5.3 ng/mL	2.39
	15.0 ng/mL	1.83
	25.7 ng/mL	1.46
	54.3 ng/mL	0.81
	133 ng/mL	0.21

Note: 1 ng/mL=2.5 pmol/mL

B. Limits of Detection

The Limit of Blank (LoB), Limit of Detection (LoD), and the Limit of Quantitation (LoQ), were determined in accordance with the CLSI guideline EP17-A. The LoB was calculated by measuring the blank several times and calculating the 95th percentile of the distribution of the test values. The LoB was calculated to be 1.69 ng/mL. The LoD was calculated as described in the guideline. The LoD was calculated to be 2.81ng/mL. The LoQ was calculated by testing 5 samples of low value 14 times in different tests. The LoQ was calculated to be 4.39 ng/mL with a CV of 20 %.

C. Performance Characteristics

Cross reactivity of the Human Total 25-OH Vitamin D IVD ELISA Kit was determined by testing sera with spiked and unspiked cross reactants. The results are summarized in the following table:

Compound and Concentration	% Cross reaction
25-OH-Vitamin D_3 at 10 ng/mL	100
25-OH-Vitamin D_2 at 10 ng/mL	86
1, $25(OH)_2$-Vitamin D_3 at 200 ng/mL	20
1, $25(OH)_2$-Vitamin D_2 at 690 ng/mL	1.9
Vitamin D_3 at 200 ng/mL	2.9
Vitamin D_2 at 200 ng/mL	1.3
24, $25(OH)_2$-Vitamin D_3 at 20 ng/mL	>100
25, $26(OH)_2$-Vitamin D_3 at 4 ng/mL	>100
3-epi-25-OH-Vitamin D_3 at 20 µg/mL	0.1

The effect of potential interfering substances on samples using the Human Total 25-OH Vitamin D IVD ELISA Kit was evaluated. Different levels of Hemoglobin, Bilirubin Conjugated and Unconjugated, Triglyceride, Vitamin C, Biotin, and Zemplar in serum samples were tested on samples with different 25-OH Vitamin D concentrations. Our acceptance criterion was to have interference of less than 10 %. The tested substances did not affect the performance of the Human Total 25-OH Vitamin D IVD ELISA Kit.

Substance	25-OH Vitamin D (ng/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Hemoglobin	7.6	250	-0.6 %
		500	
	29.3	250	
		500	
	42.5	250	
		500	
Bilirubin Conjugated	6.0	50	-3.4 %
		100	
	21.6	50	
		100	
	38.6	50	
		100	
Bilirubin Unconjugated	7.6	50	2.5 %
		100	
	29.3	50	
		100	
	42.5	50	
		100	
Triglyceride	7.6	7.5	-4.3 %
		125	
		250	
		500	
	29.3	7.5	
		125	
		250	
		500	
	42.5	7.5	
		125	
		250	
		500	

Substance	25-OH Vitamin D (ng/mL)	Concentration of Interferent (mg/dL)	Mean % Variation
Vitamin C	6.0	1	2.5 %
		10	
		100	
	21.6	1	
		10	
		100	
	38.6	1	
		10	
		100	
Biotin	8.7	0.2	4.7 %
		2	
		4	
	19.8	0.2	
		2	
		4	
	36.1	0.2	
		2	
		4	
Zemplar	17.6	0.0013	-4.4 %
		0.0025	
		0.0050	
	33.5	0.0013	
		0.0025	
		0.0050	

D. Precision

The assay precision was calculated by running samples for a span of at least 20 days on 3 different lots. The results are summarized in the table below:

Intra-Assay Precision				Inter-Assay Precision			
Sample	n	<X> ± SD (ng/mL)	C.V. (%)	Sample	n	<X> ± SD (ng/mL)	C.V. (%)
A	24	5.5 ± 0.4	7.8	A	39	17.7 ± 1.3	7.4
B	35	27.4 ± 1.6	5.7	B	10	26.3 ± 1.2	4.7
C	35	43.0 ± 1.2	2.7	C	10	42.1 ± 1.8	4.3
D	24	81.2 ± 2.0	2.5	D	21	85.4 ± 7.8	9.2

SD : Standard Deviation, CV: Coefficient of Variation

E. Reproducibility

The reproducibility of the assay was done by testing three samples in duplicate for five days, twice a day, at three sites with two technicians per site. The mean results are summarized in the table below:

Sample	n	ng/mL		Within Run	Between Run	Between Day	Between Tech	Between Site	Total
1	57	25.5	SD	0.22	0.61	0.98	1.54	2.21	2.59
			CV	0.3 %	0.9 %	3.8 %	6.0 %	8.7 %	10.2 %
2	57	52.9	SD	0.64	1.57	1.11	2.28	4.29	5.19
			CV	0.9 %	2.3 %	2.1 %	4.3 %	8.1 %	9.8 %
3	59	124.9	SD	1.00	1.74	1.84	3.39	4.98	6.25
			CV	1.4 %	2.5 %	1.5 %	2.7 %	4.0 %	5.0 %

F. Accuracy

Recovery was assessed by adding different levels of 25-OH Vitamin D to samples. The results are summarized in the table below:

Recovery Test			
Added 25-OH-Vit.D_3 (ng/mL)	**Recovery (%)**	**Added 25-OH-Vit.D_2 (ng/mL)**	**Recovery (%)**
0	100	0	100
25	96	25	105
50	92	50	95

A sample with the concentration known throughout the measurable range was tested at equidistant dilutions, according to the dilution protocol in Section VIII, to determine the linear range of the assay. A linear regression analysis was performed. The results are summarized in the following table:

Sample Dilution		Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	Slope	Y-Intercept	R^2	Recovery (%)
1/1		101.8	101.8	1.02	-1.91	>0.98	100
1/2	with a Ctrl 1 measured at 27.1 ng/mL	64.4	62.9				98
1/4		45.7	52.0				114
1/8		36.4	34.8				96
1/16		31.7	33.6				106

The linear range of the assay was found to be 33.6 - 101.8 ng/mL.

G. Time delay

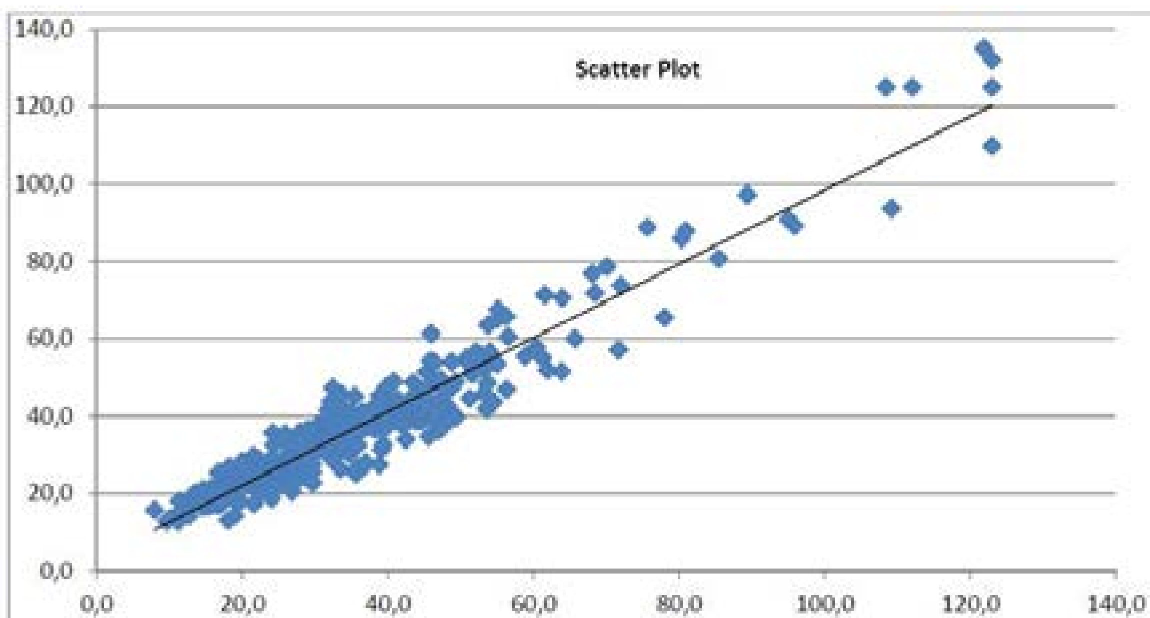
The results of the time delay test between the last calibrator and sample dispensing are shown in the following table:

	Time Delay		
	0 min (ng/mL)	10 min (ng/mL)	20 min (ng/mL)
Sample 1	27.9	30.5	30.2
Sample 2	49.5	47.5	49.0

Assay results remain accurate even when Incubation Buffer is dispensed 10 and 20 minutes after the Calibrator has been added in the coated wells.

H. Method comparison

The performance of the Human Total 25-OH Vitamin D IVD ELISA Kit was determined by conducting a correlation study tested at three different sites using a total of 356 samples. The samples were tested on both the Human Total 25-OH Vitamin D IVD ELISA Kit and a commercially available 25-OH Vitamin D ELISA test. The results ranged from 8.0 ng/mL to 123.0 ng/mL, the correlation coefficient between the two methods was 0.917, with the 95 % confidence interval of 87.6 % to 93.6 %, a slope of 0.954 and the y-intercept of 3.05. The following graph summarizes the results:



XIII. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practices.
- It is recommended that controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practice to check visually the curve fit selected by the computer.

XIV. EXPECTED VALUES

Dietary intake, race, season and age are known to affect the normal levels of 25-OH Vitamin D_3 The performance of this assay has not been established in a pediatric population.

Each laboratory should establish its own range based on their local population.

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

Level	ng/mL
Deficient	<10
Insufficient	10-29
Sufficient	30-100
Potential Toxicity	>100

Reference ranges have been established based on 150 apparently healthy individuals. The individual patient serum samples used were obtained from a certified commercial source and were collected from an FDA Licensed Donor Center with informed consent. 50 samples were from Northern US (Pennsylvania), 50 samples were from Central US (Tennessee), and 50 samples were from Southern US (Florida). Samples were collected in the winter months (January-March) from individuals between the ages of 21-92 years old and included both light skin and dark skin population. The donors from which samples were collected were not taking Vitamin D supplements, had no family history of parathyroid or calcium regulatory disease, had no history of kidney, liver, parathyroid, calcium related disease or bariatric surgery, and were not taking any medications known to affect absorption or catabolism of Vitamin D. The following table is the summary or results:

	Florida	Tennessee	Pennsylvania	Overall
Highest Conc. (ng/mL)	88.6	71.4	54.6	88.6
Lowest Conc. (ng/mL)	6.1	4.9	5.9	4.9
Median Conc. (ng/mL)	20.8	17.2	14.3	17.3

Only the central 95 % (2.5 % - 97.5 %) of the results observed were used.

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XVI. SUMMARY OF THE PROTOCOL

	Calibrators (uL)	Sample(s) Controls (uL)
Calibrators (0-5)	50	——
Controls, Samples	——	50
Incubation Buffer	150	150
Incubate for 2 hours at room temperature with continuous shaking at 400 rpm. Prepare the Working HRP conjugate solution during the incubation and a minimum of 1 hour 45 minutes before its use. **The sequence of preparation is critical, see VIII. E. Reagent Preparation** Aspirate the contents of each well. Wash 3 times with 350 µL of Working Wash solution and aspirate.		
Working HRP conjugate	200	200
Incubate for 30 minutes at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 350 µL of Working Wash solution and aspirate.		
Chromogenic solution	100	100
Incubate for 15 min at room temperature with continuous shaking at 400 rpm.		
Stop solution	100	100
Read on a microplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

NOTES

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