Vitamin D Assay Development Report

Theranos, Inc.

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1 Purpose

This document describes the studies conducted during the development of the Theranos Total 25-hydroxyvitamin D assay.

2 Scope

This report includes relevant experiments in the development of the Theranos Total 25-hydroxyvitamin D assay.

3 Definitions

Term	Definition
25(OH)Vitamin D	25-hydroxyvitamin D, (interchangeably used with 25-OH VitD, Vitamin D)
25(OH)VitaminD2	25-hydroxyvitamin D2
25(OH)ViamintD3	25-hydroxyvitamin D3
1,25(OH)2VitD3	1,25-dihydroxyvitamin D3
1,25(OH)2VitD2	1,25-dihydroxyvitamin D2
EDTA	Ethylenediamine tetraacetic acid; an anticoagulant; EDTA is synonymous with K_2EDTA; the acronyms are used interchangeably
TSCD	Theranos Sample Collection Device
RLU	Relative Light Units
MDL	Medical Decision Level
RF	Rheumatoid factor
HAMA	Human anti-mouse antibodies

4 Assay Principle

The Theranos total 25-hydroxyvitamin D assay is a competitive in vitro diagnostic immunoassay for the quantitative determination of total 25-hydroxyvitamin D (25-hydroxyvitamin D2 and 25-hydroxyvitamin D3) in human serum, plasma and whole blood. In this assay, the sample is treated a sample treatment buffer that allows the release of the bound Vitamin D Binding protein (VDBP) and other interfering proteins. The mixture is neutralized with a pH specific reagent to inactivate the treatment, a capture solution is added to the mixture and then incubated. After this incubation, the alkaline phosphatase-labeled conjugate is added to the mixture and co-incubated with the solid surface. The solid surface is coated with secondary antibodies. After a number of washes to remove any unbound sample, the solid surface is incubated with a chemiluminescent substrate and RLUs are measured using the M100. The RLU data is used to calculate each sample's 25(OH)D concentration by comparison with RLUs from an on-board calibration curve.

A greater amount of total 25-Hydroxyvitamin D in the sample results in lower binding of the 25(OH)VitD-AP to the capture antibody. Thus the signal generated by the assay is inversely proportional to the concentration of 25-Hydroxyvitamin D in the sample.

5 Reference Assay

5.1 The following assay was used as reference method:
Vitamin D, Diazyme EIA Assay (assay range 8.3-143.6 ng/ml)

6 Method Comparison

6.1 Procedure: 94 sourced clinical samples were tested in triplicate, averaged and back calculated based on an on-board calibration curve.

6.2 Acceptance Criteria: Slope within 20% of 1.0, y-intercept within 20% of 0, bias confidence intervals < 10% at each MDL.

6.3 Results: The table and figure below summarizes the method comparison results for 94 sourced clinical samples tested on the Theranos benchtop 25(OH) Vitamin D assay. Medical decision levels were selected by using the endpoints of an assay reference range determined by the reference method, Diazyme EIA.

*Table [SEQ Table * ARABIC]: Method Comparison Data*

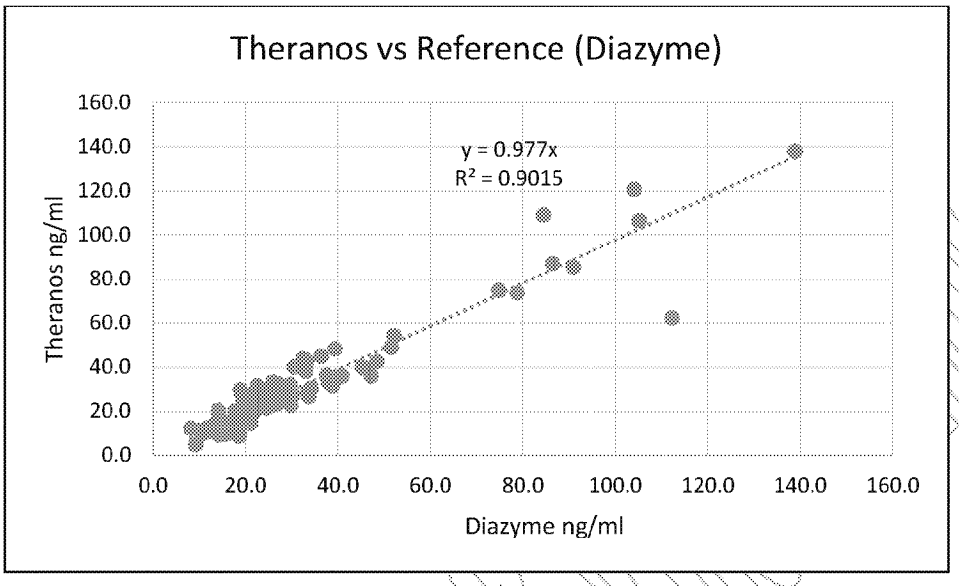
Sample No	Diazyme ng/ml	Theranos ng/ml
1	25.9	22.8
2	19.5	14.5
3	29.8	22.6
4	38.8	31.4
5	34.2	30.5
6	21.2	15.0
7	17.8	12.7
8	27.1	32.3
9	33.4	28.4
10	30.2	27.1
11	22.3	20.8
12	20.6	21.8
13	27.9	24.8
14	17.7	14.7
15	19.5	23.8
16	12.0	12.5
17	22.5	26.6
18	14.7	11.5
19	9.9	11.4
20	25.6	24.6
21	19.2	19.9
22	19.4	20.0
23	20.7	21.5
24	17.4	13.3
25	13.5	13.7
26	26.0	25.1
27	18.629	9
28	33.678	26
29	22.131	23
30	13.35	14
31	18.545	17
32	39.344	48
33	48.4	42.4
34	52.1	54.1
35	47.2	36.0
36	45.3	39.8
37	51.5	48.9
38	105.2	106.4
39	104.1	120.8
40	138.9	137.8
41	91.0	85.4
42	84.4	109.0
43	74.8	74.9

Sample No	Diazyme ng/ml	Theranos ng/ml
51	23.8	27.2
52	21.4	20.3
53	18.9	29.8
54	19.2	16.0
55	10.409	10
56	14.487	11
57	20.924	14
58	21.648	20
59	16.074	15
60	24.136	27
61	21.433	19
62	15.536	12
63	32.559	44
64	15.774	9
65	20.446	22
66	19.411	19
67	24.233	23
68	22.503	32
69	25.911	33
70	22.28	30
71	21.766	24
72	17.779	20
73	29.658	31
74	40.851	36
75	37.822	33
76	30.515	40
77	36.331	45
78	26.833	27
79	40.159	35
80	15.486	11
81	33.202	42
82	24.272	30
83	11.27	10
84	26.814	23
85	14.19	9
86	37.51	36
87	9.243	5
88	8.196	12
89	17.415	16
90	32.996	38
91	14.119	20
92	20.697	25
93	21.411	25

44	86.443	87
45	78.782	74
46	112.281	62
47	20.4	21.7
48	19.1	28.4
49	14.2	18.4
50	24.5	21.3

94	29.688	32

*Figure [SEQ Figure * ARABIC]: Method Comparison Plot*



6.4 Conclusion: From the fit data, it appears that slope, intercept and bias are within acceptance criteria.

7 *Analytical Specificity (Cross Reactivity)*

7.1 Procedure: Cross reactivity of the Theranos 25(OH) Vitamin D assay was characterized by spiking cross reactants at concentrations shown in Table 3 into the calibrator matrix and using the equation below to calculate percent cross reactivity. x_M is the back calculated measured value of the spiked calibrator in ng/mL, x_T is the true value of the calibrator in ng/mL and x_C is the concentration of spiked cross reactant in ng/mL.

$$\% \textbf{ Cross Reactivity} = 100 * {(x_M - x_T)}/{x_C}$$

7.2 Acceptance Criteria: The absolute value of the percent cross reactivity must be less than 30%

7.3 Results: Cross reactivity samples were tested in triplicate, averaged and back calculated using an on-board calibration curve. All samples with percent cross reactivity > 30% were called 'discrepant'. No drug interference samples tested had cross reactivity greater than 0%. There is reactivity between 80-120% for 25(OH)VitD2 and 25(OH)VitD2 which shows that the Theranos assay can detect 25(OH)VitD2 and 25(OH)VitD2 equally.

Additional drug interferents testing is on-going.

*Table [SEQ Table * ARABIC]: Cross Reactivity Data*

Cross Reactant	Conc (ng/ml)	% CR
Acetaminophen	400000	0%
Acetaminophen	120000	0%
Acetylcysteine		
Acetylsalicylic Acid	1000000	0%
Acetylsalicylic Acid	640000	0%
Ibuprofen	10000000000	0%
Ibuprofen	2000000000	0%
25-OHD3	50	89%
25-OHD2	50	114%
1,25(OH)2VitD3	30	83%
1,25(OH)2VitD2	30	293%
VitaminD3	1000	0%
VitaminD2	1000	0%
3-epi-25-OH VitD3	100	55%
3-epi-25-OH VitD2	100	125%
(24S)24,25 (OH)2 VitD3	100	0%
Paricalcitol	24	165%

7.1 Conclusion: Although there is cross reactivity with 1,25(OH)2VitD3, 1,25(OH)2VitD2, 3-epi-25-OHVitD3, 3-epi-25-OHVitD2 and Paricalcitol, there is no clinical concern since physiologically, these metabolites are present in the body a 1000-fold lower than 25-OH Vitamin D and thus will not affect the assay performance.

8 Interference

8.1 Procedure: Interference testing was based on CLSI guideline EP07-A2. Effects of spiked interferents and native interferents were tested as described below.

Spiked Interferents. The effect of known interferents (endogenous or exogenous) was evaluated using three levels of 25-OH Vit D. The interferents were tested at one spiked level and up to three times the physiologic or therapeutic range. Percent interference was calculated with the equation below. x_M is the back calculated measured value of the spiked calibrator in ng/mL and x_T is the value of the control calibrator determined by the reference method in ng/ml.

$$\%\ \boldsymbol{Interference} = \mathbf{100} * {}^{(x_M - x_T)}/_{x_T}$$

Native Interferents. Samples with known amounts of interferents were tested on our reference method, Diazyme EIA, so 25-OH Vitamin D values could be determined. Interference samples were then run on the Theranos assay in triplicate, averaged and back calculated based on a simultaneously-run calibrator curve. Percent interference was calculated with the equation below. x_M is the back calculated measured value of the sample in ng/ml on the Theranos assay and x_T is the value of the sample in ng/ml on the Diazyme EIA reference assay.

$$\%\ \boldsymbol{Interference} = \mathbf{100} * {}^{(x_M - x_T)}/_{x_T}$$

8.2 Acceptance Criteria: The absolute value of the percent interference must be less than 30%

8.3 Results: There was no interference with RF, HAMA and total protein samples. Hemoglobin, bilirubin, triglyceride and biotin samples did show some interference more than 30%. However, this interference is inconsistent which makes this results particularly disconcerting.

*Table [SEQ Table * ARABIC]: Interference Data*

Interferent	Samples	Diazyme ng/ml	Spike Interferent Conc (ng/ml)	% Interference
Hemoglobin	Hb1	86.357	200 mg/dL	-34%
	Hb2	22.289	200 mg/dL	12%
	Hb3	8	200 mg/dL	45%
Bilirubin	B1	81.116	20 mg/dL	6%
	B2	21.177	20 mg/dL	18%
	B3	6.669	20 mg/dL	56%
Triglycerides	Tr1	64.368	500 mg/dL	-31%
	Tr2	24.663	500 mg/dL	-4%
	Tr3	8.097	500 mg/dL	24%
Total Protein	TP1	88.75	0.5 g/dL	-17%
	TP2	22.285	0.5 g/dL	11%
	TP3	16.405	0.5 g/dL	-9%
Biotin	Bt1	104.484	30 ng/ml	-22%
	Bt2	33.477	30 ng/ml	-19%
	Bt3	18.177	30 ng/ml	-33%
RF_Contrived Samples	M1-R1	24.093	8725.5 IU/ml	-4%
	M1-R2	22.857	7756 IU/ml	4%
	F16-R1	23.516	8725.5 IU/ml	-3%
	F16-R2	28.886	7756 IU/ml	-25%
RF_Native Samples	R34	34.2	149.2 IU/ml	-11%
	R35	21.2	144.6 IU/ml	-29%
	R78	17.8	35.1 IU/ml	-29%
	R80	27.1	32.4 IU/ml	19%
HAMA_Contrived Samples	M4-H1	9.153	900000 ng/ml	11%
	M4-H2	8.652	800000 ng/ml	20%
	F12-H1	12.585	900000 ng/ml	3%
	F12-H2	13.789	800000 ng/ml	-11%
HAMA_Native Samples	H47	33.4	12.4 IU/ml	-15%
	H48	30.2	39.5 IU/ml	-10%
	H49	22.3	119.1 IU/ml	-7%
	H51	20.6	17.5 IU/ml	6%

8.4 Conclusion: There is some interference with bilirubin, triglyceride and biotin samples but it the interference does not seem to correlate with sample concentration or affect assay performance. There is some interference with Hemoglobin at 200mg/dL and studies to titrate the hemoglobin is on-going.

9 *Matrix Comparison*

9.1 Procedure: In order to demonstrate matrix equivalence for the Theranos total 25-hydroxyvitamin D assay, matched venous serum, venous K_2-EDTA and TSCD K_2-EDTA samples were collected from 20 subjects in-house. Matched samples were tested on the same plate to minimize plate to plate variability. Triplicate measurements for each serum sample were averaged and values were back calculated from a calibration curve run with the samples. For the measurement of the other two matrices, a difference measure was calculated as follows:

Difference measure is the percent difference between sample back calculated value and the reference back calculated value

$$Difference\ measure = {(x_M - x_{ref})}/{x_{ref}}, \%$$

where x_M is the back calculated value of the matrix being tested and x_{ref} is the serum reference value.

9.2 Acceptance Criteria: The absolute value of the difference measure must be less than 20%

9.3 Results: Table 1 summarizes the matrix comparison results for 20 in-house matched samples tested on the Theranos benchtop total 25(OH)D assay.

*Table [SEQ Table * ARABIC]: Matrix Comparison Data*

	Reference ng/ml	Theranos ng/ml		
Sample	**Venous Serum**	**Venous Serum**	**Venous EDTA**	**TSCD EDTA**
1	27.948	24.8	26.3	28
2	17.698	14.7	15.0	22.0
3	19.471	23.8	29.2	29.5
4	11.966	12.5	14.8	14.5
5	22.462	26.6	30.4	32
6	14.738	11.5	14.9	12.0
7	14.823	11.4	15.6	13.3
8	25.566	24.6	32	27.7

9	19.198	19.9	23.4	21.3
10	19.377	20.0	23.0	22.2
11	20.658	21.5	22.1	19.8
12	17.393	13.3	15.6	14.5
13	13.528	13.7	14.4	13.5
14	26.006	25.1	25.7	27.9
15	14.044	16.0	14.3	16.6
16	33.678	26.4	25.8	28.7
17	22.131	23.3	28.1	26.0
18	13.35	14.3	16.3	17.7
19	18.545	16.6	23.6	18.6
20	39.59	56	53	62

*Figure [SEQ Figure * ARABIC]: Theranos Comparison of EDTA vs. Serum (Venous)*

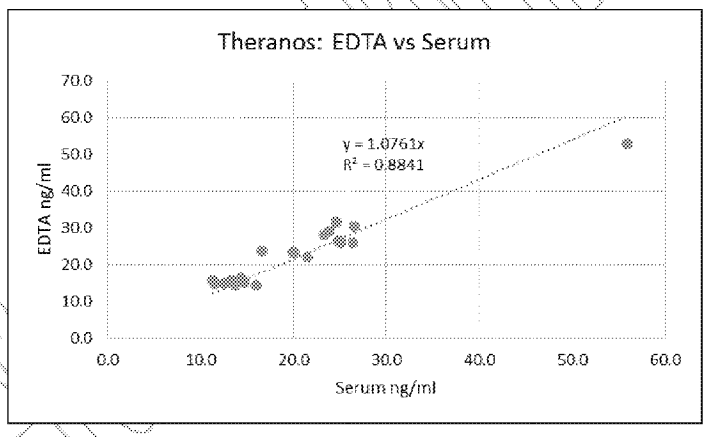


*Figure [SEQ Figure * ARABIC]: Theranos Comparison of EDTA TSCD vs. Serum (Venous)*

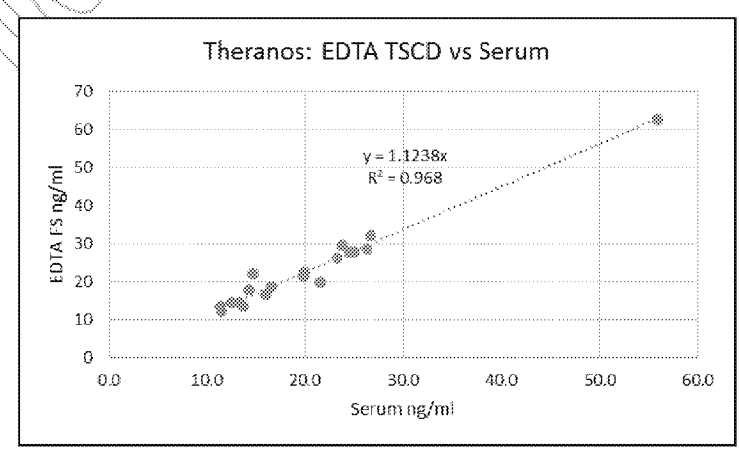
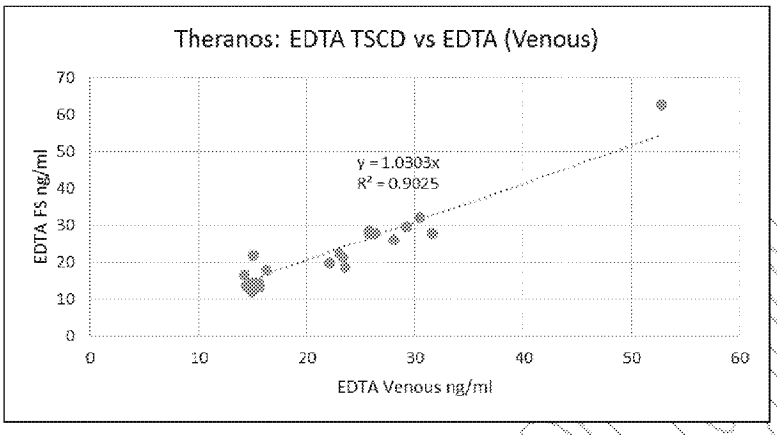


*Figure [SEQ Figure * ARABIC]: Theranos Comparison of EDTA TSCD vs. EDTA (Venous)*



9.4 Conclusion: There is minimal bias among the different matrices tested.

10 Hook Effect

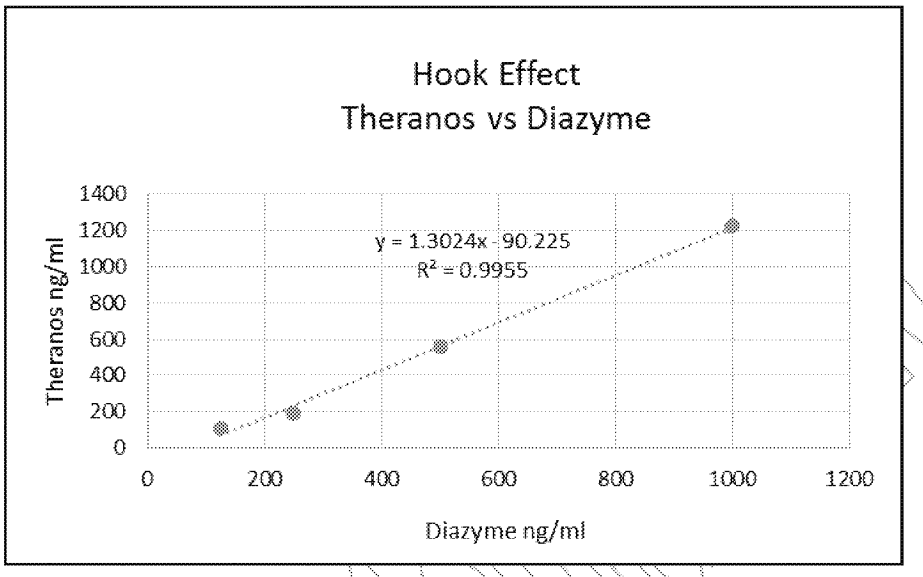
10.1 The goal of this experiment was to test for hook effect by analyzing samples with increasing analyte concentration on the Theranos assay

10.2 Procedure: 5 samples with increasing 25-OHVitD concentration were analyzed with Theranos system.

10.3 Acceptance criteria: increasing 25-OHVitD concentration should lead to increasing RLUs. There should be a linear response when RLUs are plotted against Theranos antibody index

10.4 Results: the following plot shows a linear trend when RLUs are plotted against Theranos antibody index, with an $R^2 = 0.9955$

*Figure [SEQ Figure * ARABIC]: Hook Effect*



10.5 Conclusion: Acceptance criteria is met. No hook effect is observed.

11 Dilution Linearity

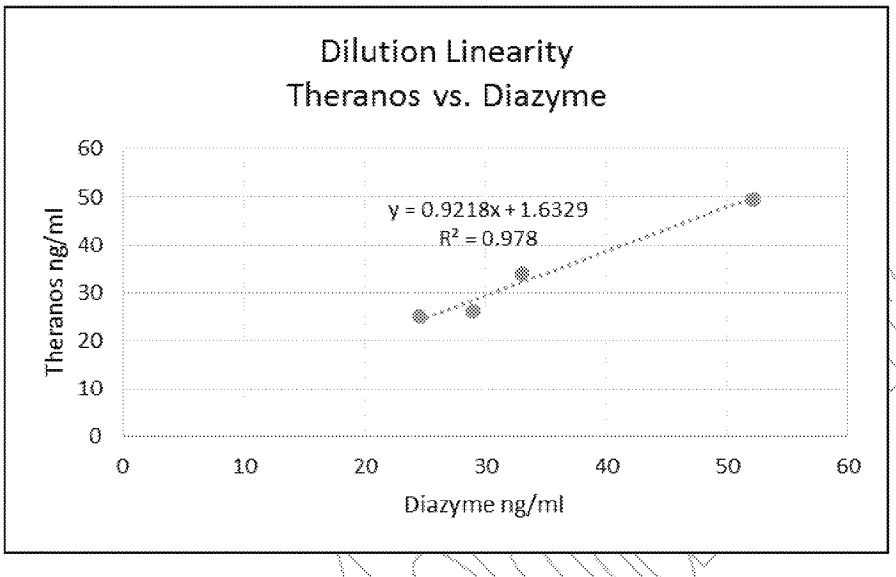
11.1 The goal of this experiment was to test for linearity by diluting a high contrived sample across a range with on the Theranos assay

11.2 Procedure: 9 samples with increasing 25-OHVitD concentration were analyzed with Theranos system.

11.3 Acceptance criteria: There should be a linear response when RLUs are plotted against Theranos antibody index

11.4 Results: The following plot shows a linear trend when RLUs are plotted against Theranos antibody index, with an $R^2 = 0.978$

*Figure [SEQ Figure * ARABIC]: Dilution Linearity*



11.5 Conclusions: Acceptance criteria is met.

12 Stability

Theranos 25-OH VitD assay stability studies are ongoing.

13 Conclusions

The benchtop Theranos 25-OHVit D assay has been tested with 94 clinical samples, 16 cross reactivity samples and 31 interference samples. Some interference with bilirubin, triglyceride and biotin was detected, but this interference was only detected less than 50% of the time and did not correlate with sample concentration. Interference at 200mg/dl of Hemoglobin was detected an on-going tests are being done to calculate the threshold. Slope, intercept and bias met acceptance criteria for clinical studies. The assay shows no cross reactivity that would affect assay performance. The matrix comparison demonstrates equivalence across matrices.

14 References

14.1 EP07-A2 Interference Testing in Clinical Chemistry