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Parathyroid Hormone (PTH) Assay Development Report

Theranos, Inc.

Sept 18, 2012

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1. ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

1.1 Assay Specifications[TC "Assay Specifications" \f C \l "3"]

This assay is designed to detect intact human Parathyroid Hormone (PTH) in human whole blood, plasma and serum. The assay has a reportable range of 2 to 800 pg/mL, and is calibrated using WHO First International Standard for PTH (NIBSC 79/500) and the Siemens Immulite 2000XP.

1.1.1 Reference Assays [TC "Reference Assays and Standards" \f C \l "3"]

The following predicate method was used:

- Siemens Immulite 2000XP

1.1.2 Analyte Specifications

Intact PTH is a labile peptide and can be broken down into inactive fragments by endogenous blood proteases. Collected whole blood samples should be immediately placed on ice and should be processed within 4 hours of collection. If samples are not to be tested immediately, the whole blood should be separated into plasma or serum and stored at 4°C for up to 8 hours or frozen for future testing.

1.1.3 Materials and Methods[TC "Materials and Methods" \f C \l "1"]

A biotin-labeled mouse monoclonal anti-C-terminal PTH antibody coated on an avidin surface serves as the capture surface for the sandwich ELISA. The sample (whole blood, plasma or serum) is diluted and mixed with an alkaline-phosphatase-labeled mouse monoclonal anti-N-Terminal PTH antibody, and the mixture incubated on the capture surface for 10 minutes. After the incubation, the surface is washed and the substrate is incubated on the surface for 10 minutes, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Native Human PTH	NIBSC	79/500
Mouse Anti-C-Terminal PTH Antibody (CAb)	Immunodiagnostik	AK 1403.2
Mouse Anti-N-Terminal PTH Antibody (DAb)	Genway	GWB-E2AA46
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Biotin Labeling Kit (SH)	Dojindo	LK10
Alkaline Phosphatase Substrate	Theranos	T-ALKP-SB01
Carbonate-bicarbonate buffer	Sigma	C3041
Starting Block (TBS)	Pierce	37542

2. ASSAY DEVELOPMENT[TC "ASSAY OPTIMIZATION" \F C \L "2"]

1.2 Antibody Screening [TC "Detection Antibody Conjugate Verification" \f C \l "1"]

To determine the optimal pair for the PTH ELISA, combinations of C-Terminal and N-Terminal antibodies were tested. The screening was performed with calibrators diluted 1:10 into assay buffer in a co-incubation format with 10ng/mL final DAb concentration with sample mixture, 10 ug/mL CAb coated on the tips and a 10-10 minute incubation. Four rounds of antibody screening were completed, with candidate pairs from each round tested against clinical samples.

Table [SEQ Table * ARABIC]: Antibody Information

Antibody #	Vendor	Catalog #	Immunogen	Clone	Host
1	AbD Serotec	7070-6206	1-34	3H9	Mouse
2	Genway	GWB-E2AA46	1-34	BGN/1F8	Mouse
3	SIGMA	WH0005741M5	32-115	4A2	Mouse
4	SIGMA	SAB1401320	1-115		Rabbit
5	US Biological	P3109-39	1-34		Rabbit
6	US Biological	P3109-14B	1-115	11H97	Mouse
7	US Biological	P3109-14C	1-115	11H98	Mouse
8	US Biological	P3109-07	1-34	8.BG.10	Mouse
9	QED Biosciences	58004	53-83		Mouse
10	QED Biosciences	58005	1-34		Mouse
11	syd labs	PA002898-C0293	not specified		Rabbit
12	Genway	20-322-392050-E16	1-34		Mouse
13	Genway	20-322-392050-1B7	1-34		Mouse
14	Genway	20-322-392050-E5	44-68		Mouse
15	Genway	20-322-392050-G7	44-68		Mouse
16	Immundiagnostik	AK 1104.2	1-38	A1/70	Mouse
17	Immundiagnostik	AK 1103.2	1-37		Mouse
18	DiaSource	51.149.16	44-68	14H5 1C7	Mouse
19	US Biological	P3109	70-84		Goat
20	Fitzgerald	70-XG68	53-84		Goat
21	Fitzgerald	70-XG67	1-34		Goat
25	DiaSource	53.149.06	1-34		Sheep
26	abcam	ab14498	53-85		Mouse
26	Immundiagnostik	AK1403.2	53-84	D1.1	Mouse
27	Immuquest	IQ399	1-34	BAM87	Mouse
28	Immundiagnostik	A 1111.2	1-34		Rabbit
29	Immundiagnostik	A 1112.2	1-34		Rat
30	Immundiagnostik	A 1114.3	1-34		Goat

Antibody #	Vendor	Catalog #	Immunogen	Clone	Host
31	Immundiagnostik	A 1115.1	1-10		Rabbit
32	Immundiagnostik	A 1117.2	1-34		Rabbit
33	Immundiagnostik	AK1102.2	1-34	77/78	Mouse
34	Immundiagnostik	AK1105.2	1-38	B1/70	Mouse
35	Immundiagnostik	AK1106.2	1-10	L7-13A-B7	Mouse
36	Immundiagnostik	AK1107.2	1-38	A1/64	Mouse
37	Immundiagnostik	AK1108.2	1-38	B2-82	Mouse
38	Immundiagnostik	AK1109.2	1-10	L7-12A-F12	Mouse
39	Immundiagnostik	AK1110.2	1-10	L7-9-F10	Mouse
40	Immundiagnostik	A 1018.2	44-68		Rabbit
41	Immundiagnostik	A 1113.2	44-68		Goat
42	Immundiagnostik	A 1411.2	53-84		Rabbit
44	Immundiagnostik	AK1404.2	53-84	D1.5	Mouse
45	IBT	PA-593-9	1-34		Rabbit
46	IBT	MABP3-OP-4	1-34	OP-4	Mouse

Table [SEQ Table * ARABIC]: Summary of Best-Modulating Antibody Pairs Round 4

CAb	DAb	[PTH] pg/mL	Mean RLU	CV %	Mod
19	2	400	6966	20.7	18
		40	1187	21.5	3
		0	386	5.1	
26	2	400	5558	10.5	12
		40	823	13.9	2
		0	474	17.2	
44	2	400	2271	14.4	4
		40	843	17.2	2
		0	548	22.1	
19	29	400	48737	23.0	40
		40	5754	23.3	5
		0	1223	41.9	
26	29	400	35600	9.6	97
		40	3785	18.7	10
		0	368	6.3	
43	29	400	10183	20.5	4
		40	1173	5.5	2
		0	483	43.2	
44	29	400	3686	26.6	4
		40	602	18.9	2
		0	388	37.1	

1.3 Antibody Screen for Response to Clinical Samples

In order to determine that a correct dose response to clinical samples was shown, all finalist pairs were screened with clinical samples. Most candidate pairs showed high cross reactivity to PTH fragments, resulting in no correlation to reported concentrations for clinical samples, especially sample 685. Only 2 final pairs were discovered to show a correct response to clinical samples; CAb 26 with DAb 2 and CAb 26 with DAb 29. The final pair chosen was CAb 26 with DAb 29 due to significantly higher modulation, and DAb 2 as a backup. There was no backup CAb identified.

Table [SEQ Table * ARABIC]: Clinical Sample Screen Candidates from Rounds 1-3 (1:5 sample dilution)

CAb	DAb	Sample Id	[Reported] pg/mL	[CLIA/Alpco Result] pg/mL	Mean RLU	CV %
18	26	BRH545685	7.0	18.7	6962	9.2
		BRH545695	48.9	19.5	394	19.7
		BRH545710	601.9	172.0	520	9.7
18	3	BRH545685	7.0	18.7	137653	7.3
		BRH545695	48.9	19.5	380	14.6
		BRH545710	601.9	172.0	530	19.3
18	6	BRH545685	7.0	18.7	33068	2.4
		BRH545695	48.9	19.5	512	11.6
		BRH545710	601.9	172.0	491	7.4
26	18	BRH545685	7.0	18.7	15594	26.8
		BRH545695	48.9	19.5	390	16.8
		BRH545710	601.9	172.0	326	17.6
18	12	BRH545685	7.0	18.7	59765	69.6
		BRH545695	48.9	19.5	566	6.2
		BRH545710	601.9	172.0	597	6.5
18	13	BRH545685	7.0	18.7	11487	2.4
		BRH545695	48.9	19.5	610	16.7
		BRH545710	601.9	172.0	632	7.9
18	7	BRH545685	7.0	18.7	30374	13.9
		BRH545695	48.9	19.5	641	0.7
		BRH545710	601.9	172.0	753	15.2
18	9	BRH545685	7.0	18.7	85582	31.0
		BRH545695	48.9	19.5	621	11.9
		BRH545710	601.9	172.0	779	8.2
26	2	BRH545684	0	18.7	524	22.2
		BRH545695	48.9	19.5	1052	9.5

	BRH545710	601.9	172.0	2049	25.3

Table [SEQ Table * ARABIC]: Clinical Sample Screen Candidates from Round 4 (1:10 sample dilution)

CAb	DAb	Sample Id	[Reported], pg/mL	[CLIA Result], pg/mL	Mean RLU	CV %
26	29	999-146-2	115	29.9	3692	10.1
		999-146-10	156	13.2	1125	9.0
		999-146-11	308	122.0	10538	45.8
		999-146-9	353	114.0	12986	36.6
19	29	999-146-2	115	29.9	161474	58.9
		999-146-10	156	13.2	332878	35.5
		999-146-11	308	122.0	34378	26.2
		999-146-9	353	114.0	20509	17.2

1.4 Training Set (C26, D29)

To confirm the response to clinical samples, 4 serum and 4 Lithium Heparin plasma samples were tested. The response to the clinical samples matched the reported results with the exception of one outlier sample. When compared to the CLIA lab results, the serum samples and the plasma samples each correlated well but on 2 very different slopes. However the reported values via Bayer Centaur and the CLIA lab results were also tracking by matrix. It was decided to proceed with this antibody pair and perform further matrix tests.

Table [SEQ Table * ARABIC]: Training Set Results (C26, D29)

Type	Sample Id	[Reported], pg/mL	[CLIA Result], pg/mL	[Theranos Result], pg/mL
Serum	999-146-2	115.0	29.9	31.4
	999-146-9	353.0	114.0	111.4
	999-146-10	156.0	13.2	0.1
	999-146-11	308.0	122.0	97.9
Li-Hep Plasma	BRH545687	36.9	34.5	4.6
	BRH545689	31.3	64.1	5.5
	BRH545702	62.6	172.0	18.2
	BRH545703	76.3	395.0	62.8

Reference methods:

Serum samples reported pre-aliquot values via Bayer Centaur (model not specified)
LiHep samples reported pre-aliquot values via Siemens Immulite (model not specified)
CLIA lab post-aliquot results via Siemens Immulite 2000XPi

Figure [SEQ Figure * ARABIC]: Training Set Theranos vs. Reported Results (C26, D29)

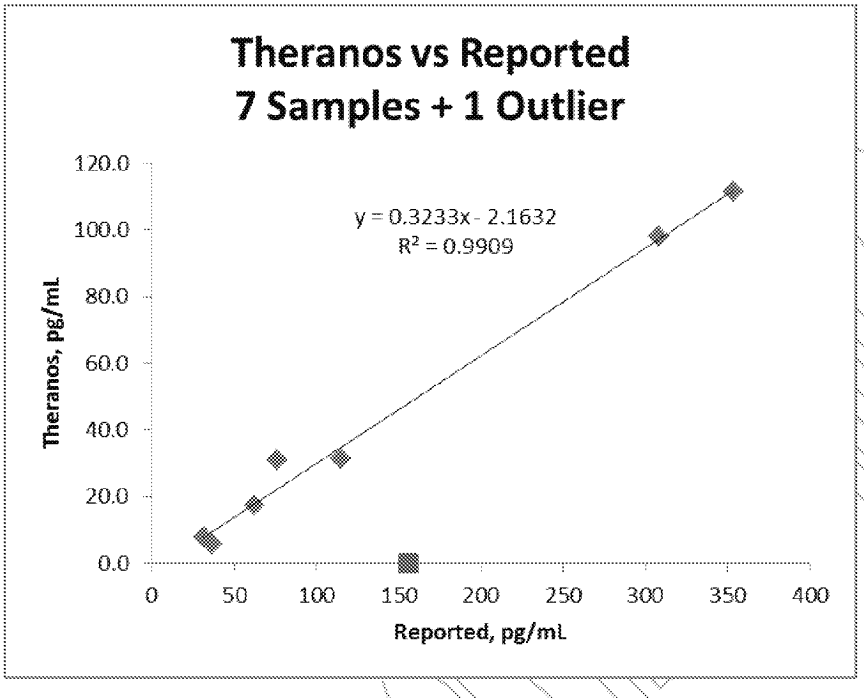


Figure [SEQ Figure * ARABIC]: Training Set Theranos (C26, D29) vs. Siemens Immulite 2000XP Results

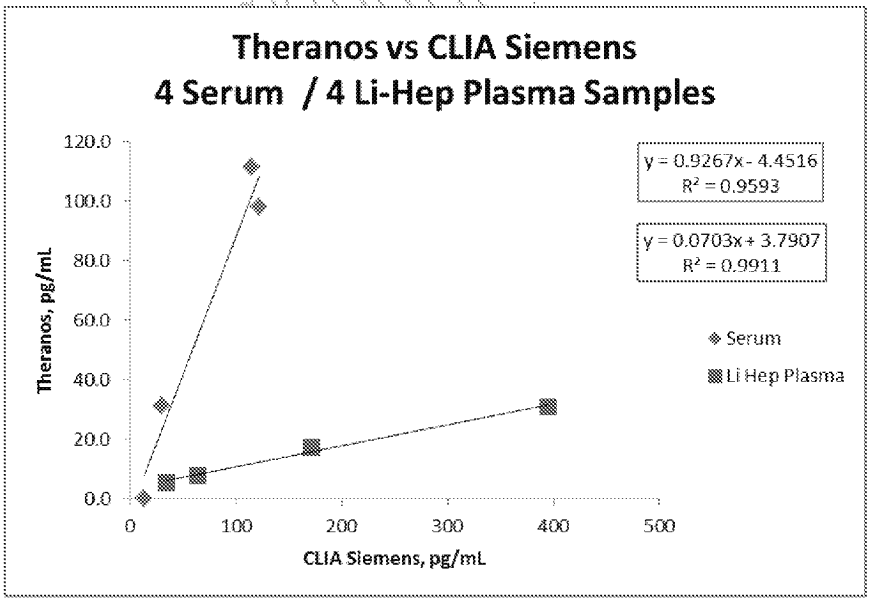
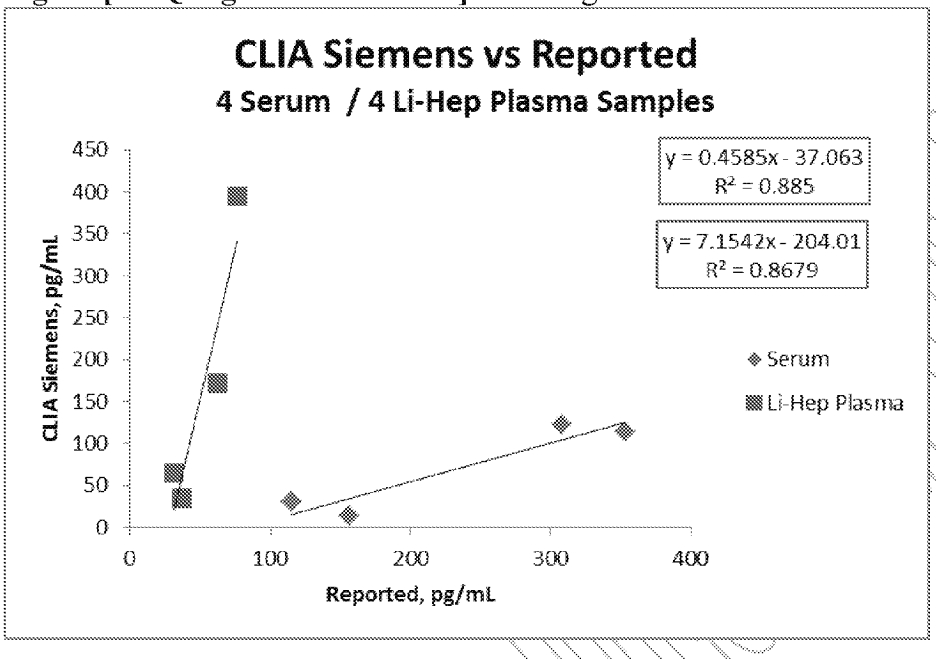


Figure [SEQ Figure * ARABIC]: Training Set CLIA Lab Result vs. Reported Centaur Result



1.5 Effect of Diluent

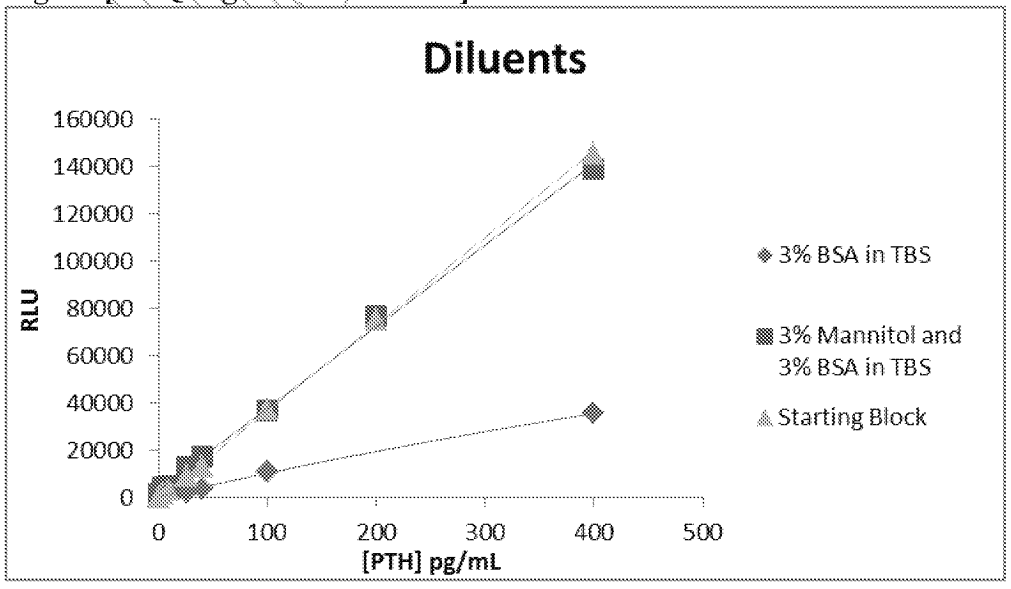
Some literature reports that the addition of mannitol to assay diluent may help to stabilize intact PTH. Three diluents were tested for the Theranos PTH assay. The addition of mannitol to the 3% BSA in TBS assay buffer resulted in higher signal but also higher background, decreasing the signal to background ratio compared to the control assay diluent (3% BSA in TBS).

Pierce Starting Block (TBS) showed a significantly better signal to background. Starting Block was chosen as the assay diluent.

Table [SEQ Table * ARABIC]: Effect of Diluent

	3% BSA in TBS		3% Mannitol and 3% BSA in TBS		Starting Block (TBS)	
[PTH] pg/mL	Mean RLU	CV %	Mean RLU	CV %	Mean RLU	CV %
400	35600	9.6	138976	6.3	145495	5.9
200	-	-	76694	8.1	75252	6.8
100	10852	16.5	36665	1.4	37335	8.9
40	3785	18.7	17737	7.0	13314	9.3
26	2505	18.4	13177	4.2	9190	8.6
10	2615	3.8	5080	30.3	3649	11.7
4	-	-	4705	5.4	1588	14.9
0	368	6.3	2030	26.6	265	6.0
S/B @ 1000 pg/mL	97		68		550	
S/B @ 25 pg/mL	7		3		14	
S/B @ 10 pg/mL	-		2		6	

Figure [SEQ Figure * ARABIC]: Effect of Diluent



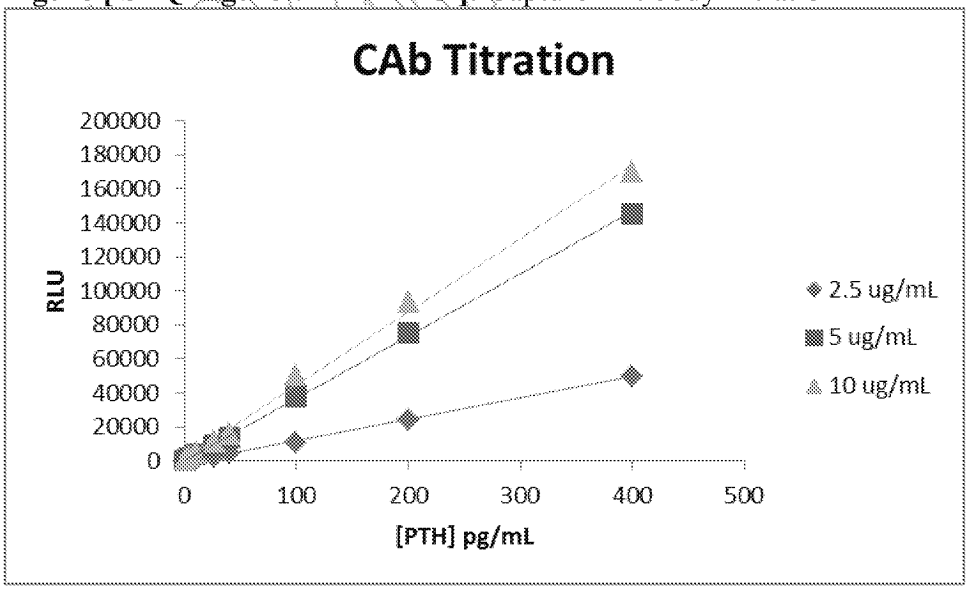
1.6 Capture Antibody Titration

The capture antibody was titrated at 10, 5 and 2.5 ug/mL on the 20 ug/mL UltraAvidin surface. The response was very similar at 5 and 10 ug, mL. At 2.5 ug/mL the modulation was significantly lower and CVs were significantly higher. A coating concentration of 10 ug/mL was chosen as the final condition based on the signal to background observed.

Table [SEQ Table * ARABIC]: Capture Antibody Titration

[PTH] pg/mL	2.5 ug/mL		5 ug/mL		10 ug/mL	
	Mean	CV %	Mean	CV %	Mean	CV %
400	50060	21.2	145495	5.9	169704	3.4
200	24302	15.2	75252	6.8	93190	2.6
100	10716	25.8	37335	8.9	50309	13.0
40	4649	12.3	13314	9.3	15985	8.0
26	2622	10.5	9190	8.6	11419	18.0
10	1278	19.6	3649	11.7	4492	2.3
4	591	7.4	1588	14.9	2090	2.0
0	145	31.6	265	6.0	278	17.1
Avg CV %		18.0		9.0		8.3
S/B @ 1000	346		550		610	
S/B @ 10	9		14		16	

Figure [SEQ Figure * ARABIC]: Capture Antibody Titration



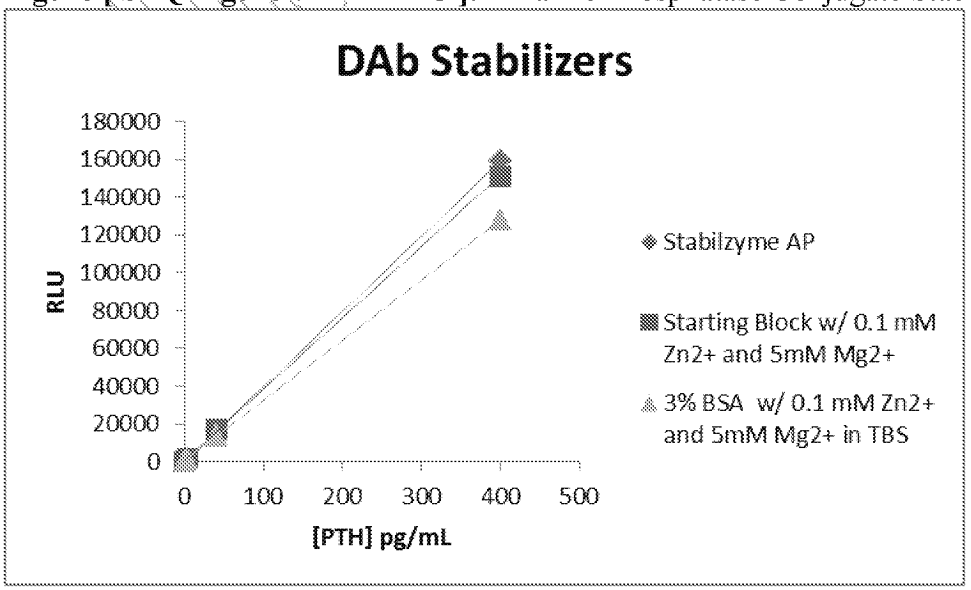
1.7 Alkaline Phosphatase Conjugate Stabilizer (C26, D29)

Various alkaline phosphatase stabilizing diluents were tested; Stabilzyme AP, a commercial stabilizer by Surmmodics, and in-house formulations made with Zn^{2+} and Mg^{2+} spiked into either 3% BSA in TBS or Pierce Starting Block. The Starting Block formulation showed the best modulation, however Stabilzyme AP produced a similar response and is widely used for long term stability of alkaline phosphatase conjugates. It was noted that the introduction of BSA in the detection antibody stabilizer as well as in assay diluent caused loss of modulation in this assay. Stabilzyme AP was chosen as the DAb diluent.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Conjugate Stabilizers (C26, D29)

Condition	[PTH] pg/mL	Mean RLU	CV %	Modulation
Stabilzyme AP	400	159332	9.7	585
	40	16183	7.0	59
	4	1666	17.3	6
	0	272	14.0	
Starting Block w/ 0.1 mM Zn^{2+} and 5mM Mg^{2+}	400	151285	6.6	629
	40	16972	7.9	71
	4	1544	13.6	6
	0	240	19.2	
3% BSA w/ 0.1 mM Zn^{2+} and 5mM Mg^{2+} in TBS	400	127846	5.2	380
	40	13621	14.4	41
	4	1448	10.4	4
	0	336	45.8	

Figure [SEQ Figure * ARABIC]: Alkaline Phosphatase Conjugate Stabilizers (C26, D29)



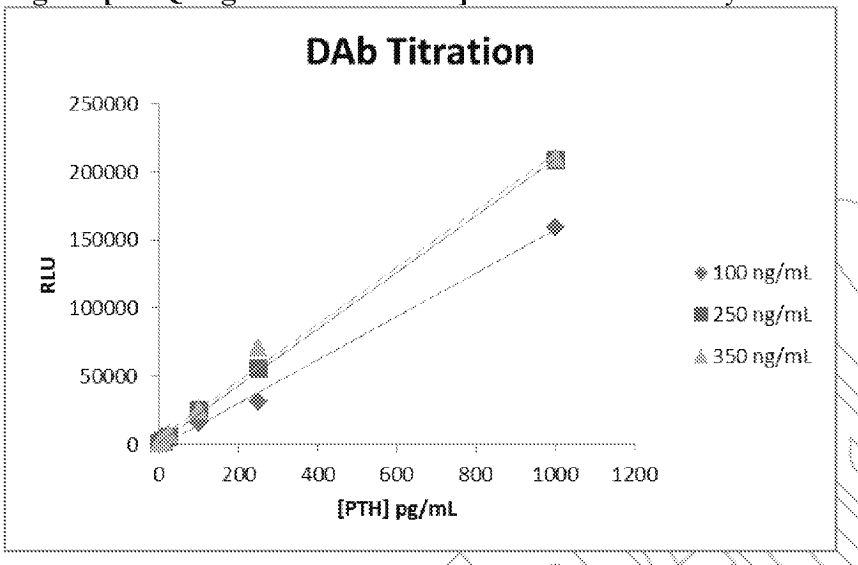
1.8 Detection Antibody Titration (C26, D29)

The detection antibody was titrated at a loading concentration of 350, 250 and 100 ng/mL. The final concentration in the sample mixture is diluted 10-fold during sample preparation. A loading concentration of 100 ng/mL produced the best signal to background across the range and at the low end, and was chosen as the final assay condition.

Table [SEQ Table * ARABIC]: Detection Antibody Titration (C26, D29)

[DAb], ng/mL	[PTH] pg/mL	Mean RLU	CV %	Modulation
100	400	159332	9.7	585
	100	31402	19.7	115
	40	16183	7.0	59
	10	3730	15.3	14
	4	1666	17.3	6
	0	272	14.0	
250	400	208792	14.2	360
	100	55712	10.1	96
	40	25063	11.2	43
	10	5840	20.3	10
	4	2181	7.0	4
	0	580	14.7	
350	400	210163	6.4	380
	100	71188	22.8	129
	40	25212	19.6	46
	10	7839	17.4	14
	4	3212	23.0	6
	0	553	29.0	

Figure [SEQ Figure * ARABIC]: Detection Antibody Titration (C26, D29)



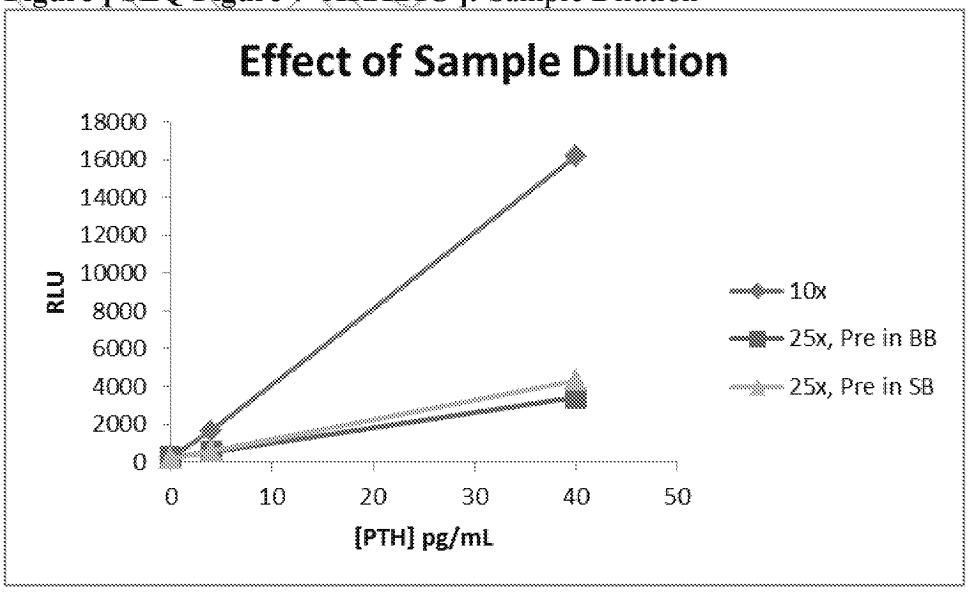
1.9 Sample Dilution

To test the effect of sample dilution on the assay and to determine if a predilution into standard 3% BSA assay buffer could be performed for ease of future multiplexing, 3 sample dilutions were tested; 1:10 sample dilution into Starting Block, a 1:5 dilution into 3% BSA/TBS followed by another 1:5 dilution into Starting Block, and a 1:25 final dilution into only Starting Block. Since the target sensitivity for the assay is less than 5 pg/mL, the 1:10 sample dilution directly into Starting Block was preferred. If a higher sample dilution is required, 1:25 would result in loss of sensitivity and making a predilution into 3% BSA buffer would further impact the sensitivity of the assay. Ideally the sample diluent should be only Starting Block, and no greater than 1:10.

Table [SEQ Table * ARABIC]: Sample Dilution

Final Dilution	Diluent	[PTH] pg/mL	Mean RLU	CV %	Modulation
10x	Starting Block	40	16183	7.0	59
		4	1666	17.3	6
		0	272	14.0	
25x	1:5 in 3% BSA Blocking Buffer, then 1:5 in Starting Block	40	3415	6.9	13
		4	539	11.4	2
		0	254	20.6	
25x	Starting Block	40	4332	20.9	23
		4	561	8.7	3
		0	190	16.0	

Figure [SEQ Figure * ARABIC]: Sample Dilution



1.1 Standard Curve (C26, D29)

To determine optimized assay sensitivity and calibrate the clinical sample training set and spike recovery, a standard curve was generated. The assay calibrators were adjusted to the Siemens Immulite. The LLOQ was 2 pg/mL.

Table [SEQ Table * ARABIC]: Standard Curve (C26, D29)

[PTH] pg/mL	Signal, RLU		Conc, pg/mL		
	Mean	CV %	Mean	CV %	% Recovery
400	111757	13.8	382.5	14.4	96
200	63139	11.5	214.9	12.0	107
100	29114	20.4	97.1	20.6	97
40	11406	11.8	37.6	12.0	94
26	8445	20.2	27.7	20.6	107
10	3195	13.1	10.0	14.2	100
4	1453	8.4	4.0	10.6	100
2	858	4.8	1.9	7.7	96
1	612	14.3	OORL	-	-
0	233	22.3	OORL	-	-

Conc = 2789.535 * (((943035.398 - 336.016) / (RLU - 336.016)) - 1) ^ (1 / -1.030)
Signal Min = 769, Signal Max = 127792

1.2 Plasma and Serum Spike Recovery (C26, D29)

Spike Recovery was tested in serum, EDTA plasma and Lithium Heparin plasma collected from the same donor. The 10x calibrators were made in assay buffer containing Roche Complete Protease Inhibitor Cocktail. These calibrators were spiked into each matrix 1:10 including a buffer control to determine any effect of the protease inhibitor on the assay response. The spiked samples were also submitted to the CLIA lab for measurement on the Siemens Immulite 2000XPi PTH assay. The CLIA lab assay was not validated for lithium heparin plasma.

Recovery in all 3 test matrixes was excellent in the Theranos assay. Recovery was slightly lower than expected in the Siemens assay especially in the lithium heparin plasma, but results were comparable to the Theranos assay.

Matrix effects did not explain the observed difference in results between Theranos and Siemens Immulite for Lithium Heparin plasma clinical samples compared to serum clinical samples.

Table [SEQ Table * ARABIC]: Plasma and Serum Spike Recovery (C26, D29)

Matrix	Nominal [PTH] pg/mL	CLIA Result (Siemens Immulite 2000XP)			Theranos Result				
					Signal, RLU		Conc, pg/mL		
		Result, pg/mL	Minus Endogenous	% Recovery	Mean	CV %	Mean	CV %	% Recovery
Buffer Control	350				46142	7.8	377.5	8.1	108
	175				20013	13.0	165.0	12.5	94
	65				6319	22.4	54.3	22.3	83
	30				4444	15.7	40.3	12.6	134
	10				2299	17.6	18.3	21.2	183
	0				295	20.2	OORL		
EDTA Plasma	350	239.0	223.0	64	38983	10.9	317.7	11.1	91
	65	63.3	47.3	73	7237	17.5	62.1	17.2	95
	10	23.5	7.5	75	1521	7.9	10.8	10.9	108
	0	16.0	0.0		256	0.3	OORL		
LiHep Plasma	350	184.0	169.1	48	40653	9.7	331.5	9.8	95
	65	55.2	40.3	62	7420	11.0	63.6	10.8	98
	10	21.8	6.9	69	1603	32.3	9.1	17.1	91
	0	14.9	0.0		236	21.5	OORL		
Serum	350	239.0	225.8	65	46480	8.8	380.5	9.1	109
	65	59.1	45.9	71	9534	9.1	81.2	8.8	125
	10	23.0	9.8	98	2216	22.3	17.5	27.1	175
	0	13.2	0.0		318	12.9	OORL		

Figure [SEQ Figure * ARABIC]: Dose Response in Buffer, Serum and Plasma Matrix (C26, D29)

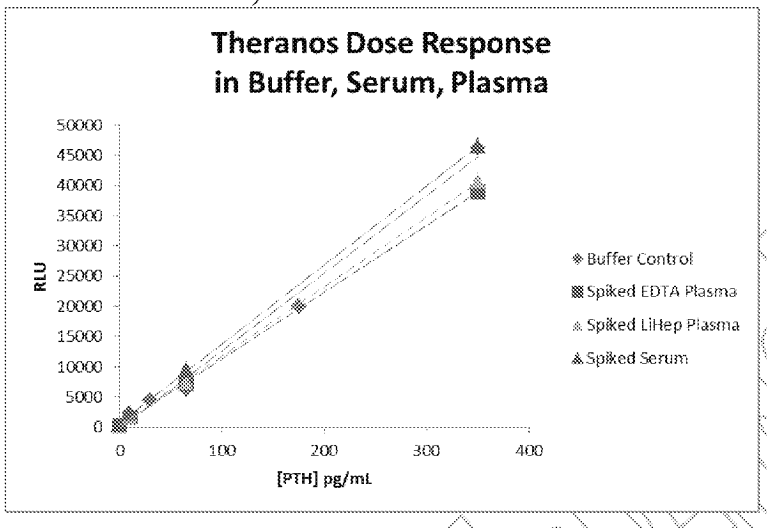


Figure [SEQ Figure * ARABIC]: Plasma and Serum Spike Recovery (C26, D29)

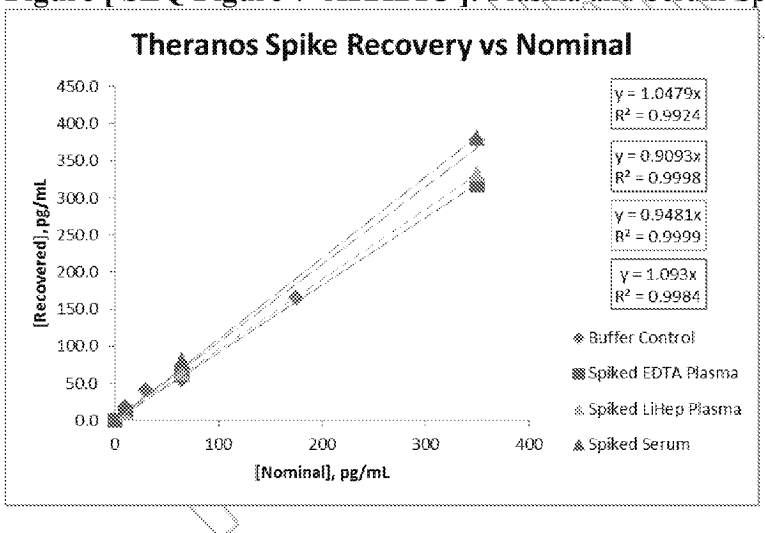
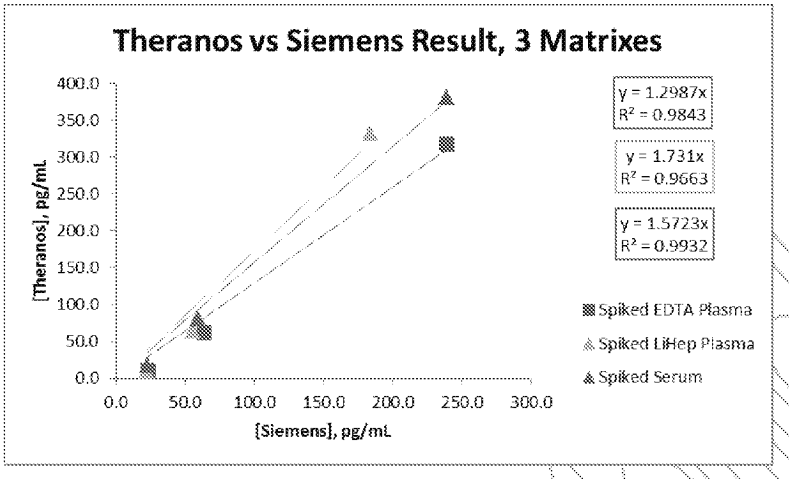


Figure [SEQ Figure * ARABIC]: Theranos (C26, D29) vs. Siemens Immulite Result, Spiked Serum and Plasma



1.3 Clinical Samples (C26, D29)

A set of clinical samples consisting of 9 serum samples (Sunny Lab) and 30 lithium heparin plasma samples (Bioreclamation) were tested on the Theranos system and in the CLIA lab on the Siemens Immulite 2000XP.

Correlation between the CLIA Siemens Immulite result and the reported results were poor, however the samples had been shipped and frozen/thawed for aliquoting at least once since the reported value measurement. The reported results for the serum set and the plasma set were also based on different instruments, therefore comparing the Siemens result to the reported results for serum versus plasma was not viable.

Correlation of the Theranos result to the Siemens Immulite result was consistently different for serum samples compared to plasma samples. For serum, the correlation and slope was acceptable. However for plasma samples the recovery in the Theranos assay was only 10% compared to the Siemens, as was seen in the initial test set of 4 serum and 4 plasma samples.

Previous spike recovery of native PTH in EDTA plasma, lithium heparin plasma and serum showed there was no baseline matrix effect in the Theranos assay, yet the low recovery of endogenous PTH in clinical plasma samples compared to serum was consistent.

Figure [SEQ Figure * ARABIC]: Clinical Samples (C26, D29)

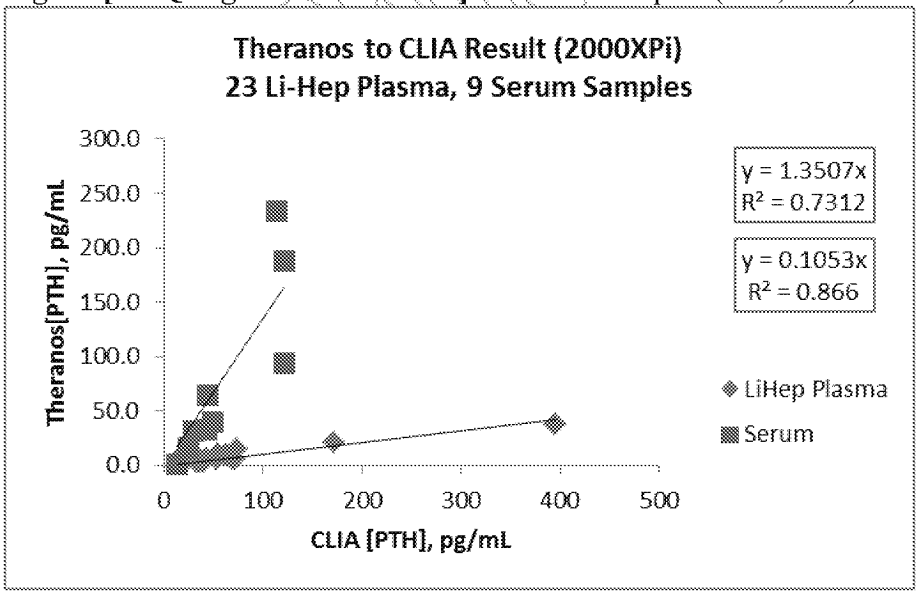


Table [SEQ Table * ARABIC]: Clinical Samples (C26, D29)

Matrix	Sample ID	Reported, pg/mL	CLIA, pg/mL	Theranos, pg/mL
Serum	01	115	49.3	39.2
	02	115	29.9	31.9
	05	273	44.4	64.1
	06	123	24.4	16.2
	07	196	122	93.2
	09	353	114	233.7
	10	156	13.2	0.4
	11	308	122	187.3
	12	118	42.4	32.7
LiHep Plasma	317	0	53.1	5.6
	318	0	0	OORL
	686	17.7	17.7	4.1
	687	36.9	34.5	6.2
	688	14.6	3.45	OORL
	689	31.3	64.1	9.0
	690	13.1	15.5	3.8
	691	22.5	30.8	8.8
	692	24.2	15.3	3.7
	693	53.7	32	3.8
	694	39.3	14.5	5.2
	695	48.9	NES	6.7
	696	35.2	NES	11.6
	697	37.7	42.9	5.3
	698	39.4	73.6	14.5
	699	51.1	54.3	10.8
	700	16.2	20	1.8
	701	87.2	NES	2.8
	702	62.6	172	21.0
	703	76.3	395	37.4
	704	58.4	26.7	OORL
	705	57.1	39.5	3.0
	706	91.3	70.9	5.9
	707	74.1	35.9	3.5
	708	57.6	8.37	OORL
	709	92.3	16.6	1.8
	711	80.9	33.5	3.4
	712	99.2	70.3	5.6
	713	61.7	62.1	8.7
	715	80.6	40	3.0

1.4 Protease Inhibitor Test

To determine if a protease inhibitor in the diluent is necessary, lithium heparin plasma samples that showed low results in the Theranos assay compared to the Siemens were tested with and without Roche Complete Protease Inhibitor cocktail in the assay diluent. Although previously tested in the matrix spike recovery experiment, buffer control samples were run at 4 levels to ensure that there was no effect of the protease cocktail on the assay dose response. When the protease inhibitor was added to the diluent, recovery of the controls was excellent, there was no effect on the dose response of the assay.

The lithium heparin plasma sample results were not significantly different with or without the protease inhibitor. Protease inhibitor does not appear to be necessary for the assay and does not improve response to clinical plasma samples.

However the addition of the Roche Complete Protease Inhibitor cocktail to samples or diluent will not affect the assay and may be desireable for certain multiplex formats.

Table [SEQ Table * ARABIC]: Control Recovery with Protease Inhibitor in Diluent

[PTH] pg/mL	Conc, pg/mL		
	Mean	CV %	% Recovery
100	35779	11.0	119.6
26	7996	7.5	25.6
4	1541	7.9	4.4
0	224	36.7	OORL

Table [SEQ Table * ARABIC]: LiHep Plasma Sample Results with Protease Inhibitor in Diluent

Sample ID	[Reported], pg/mL	[CLIA], pg/mL	Without Protease Inhibitor				With Protease Inhibitor			
			Signal, RLU		Conc, pg/mL		Signal, RLU		Conc, pg/mL	
			Mean	CV %	Mean	CV %	Mean	CV %	Mean	CV %
693	53.7	32.0	1388	18.0	3.8	23.1	1229	15.4	3.5	12.7
697	37.7	42.9	1814	12.6	5.3	15.0	2327	6.7	7.0	7.6
704	58.4	26.7	565	10.5	OORL	-	603	8.2	OORL	-
707	74.1	35.9	1296	3.9	3.5	5.2	1140	13.1	2.9	18.1

1.5 Normal Plasma Screen (C26, D29)

To further assess the problem with recovery of endogenous PTH in plasma samples, a set of 12 plasma samples from normal donors were obtained and tested in the CLIA lab on the Siemens Immulite 2000XP and on the Theranos assay. The whole blood was collected in ETDA tubes and immediately placed on ice, and plasma was prepared and frozen within 4 hours of collection. The CLIA results were within the normal range of 15 – 65 pg/mL for 10 of 12 samples, with 2 samples showing slightly higher PTH levels.

In the Theranos assay, signal for all 12 samples was not distinguishable from background. Sample #12 – which showed a high PTH level in the Siemens assay, was also tested with Roche Complete Protease Inhibitor in the assay diluent, but addition of the protease inhibitor did not improve recovery of the sample.

Table [SEQ Table * ARABIC]: Normal Plasma Screen (C26, D29)

Sample ID	CLIA Result, pg/mL	Theranos Result		
		Signal, RLU		Conc, pg/mL
		Mean RLU	CV %	Mean Conc
1	43.2	328	17.6	OORL
2	29.0	199	30.0	OORL
3	42.2	332	32.9	OORL
4	36.5	219	11.9	OORL
5	15.9	307	29.9	OORL
6	76.3	236	12.3	OORL
7	23.3	213	39.5	OORL
8	11.9	380	52.7	OORL
9	61.6	202	19.6	OORL
10	47.0	198	34.1	OORL
11	36.4	239	30.0	OORL
12	70.5	320	12.3	OORL
12 w/PI	70.5	331	13.1	OORL

1.6 Amelioration of Poor Recovery of Endogenous PTH in Plasma

The difference in plasma versus serum clinical samples was not explained by spike recovery of native PTH into EDTA plasma, lithium heparin plasma, and serum, since spike recovery was excellent in all matrixes. It is also not explained by the reported difference in PTH measurements take from serum and plasma from the same sample [Twomey et al, 2005], since plasma samples measured by the Siemens instrument showed expected results.

The following experiments were performed to improve recovery of endogenous PTH in plasma samples. With all of the following conditions, the normal plasma samples continued to show signal indistinguishable from background:

- Addition of 2 brands of protease inhibitor cocktails (serine protease inhibitors) into the assay diluent with and without EDTA.
- Spiking of the plasma samples directly with protease inhibitor cocktail.
- Adding 5% mannitol in assay buffer.
- Increasing DAb concentration by 10-fold to saturate binding in case of interference by complement.
- Pre-incubating the DAb with the sample an additional 10 minutes.
- A 2 step assay format in which the sample is incubated first on the capture surface and then exposed to the DAb instead of co-incubating the DAb and sample in the presence of CAb coated on the surface.
- Testing of Theranos assay and Siemens Immulite for cross reactivity against PTH fragments – no cross reactivity was measured in either assay.

After completing the tests above, it was decided to test the normal plasma samples with the backup pair C26, D2 which was originally not preferred due to significantly lower modulation and lack of sensitivity.

Results (shown in the next section) were promising and showed that despite the low modulation under the un-optimized conditions, the combination of C26, D2 was able to detect endogenous intact PTH in the normal plasma samples.

Therefore, the assay was re-optimized with the backup pair C26, D2.

1.7 Normal Plasma Screen (C26, D2)

As previously noted, the original antibody pair chosen (C26, D29) showed very low recovery of endogenous PTH in plasma samples and was unable to detect PTH in a set of normal plasma samples. Ten of the previous-collected and frozen normal plasma samples were tested with the backup pair C26, D2 and calibrated on the standard curve shown below, using conditions optimized for the original pair.

With the new detection antibody, a range of values for PTH was detected in the normal plasma samples.

Table [SEQ Table * ARABIC]: Standard Curve (C26, D2)

[PTH] pg/mL	Signal, RLU		Conc, pg/mL		
	Mean RLU	CV %	Mean Conc	CV %	% Recovery
400	6592	11.6	383.6	15.6	96
200	4031	11.2	213.1	12.3	107
40	1002	7.7	38.0	12.3	95
26	769	10.3	24.0	19.3	92
10	450	3.3	7.4	8.7	74
4	429	7.5	6.5	19.3	163
2	281	16.2	1.9	48.5	96
0	311	15.0	-	-	-

Table [SEQ Table * ARABIC]: Normal Plasma Screen (C26, D2)

Sample ID	Signal, RLU		Conc, pg/mL	
	Mean RLU	CV %	Mean Conc	CV %
1	779	7.6	24.6	14.3
2	560	6.3	12.5	14.2
3	1002	13.6	38.0	21.8
4	540	10.9	11.6	25.2
5	490	0.5	9.1	1.3
6	1006	13.0	31.8	0.4
7	485	16.8	9.1	39.4
8	429	3.3	6.5	8.7
9	880	9.2	30.6	15.9
10	637	13.7	16.6	28.9

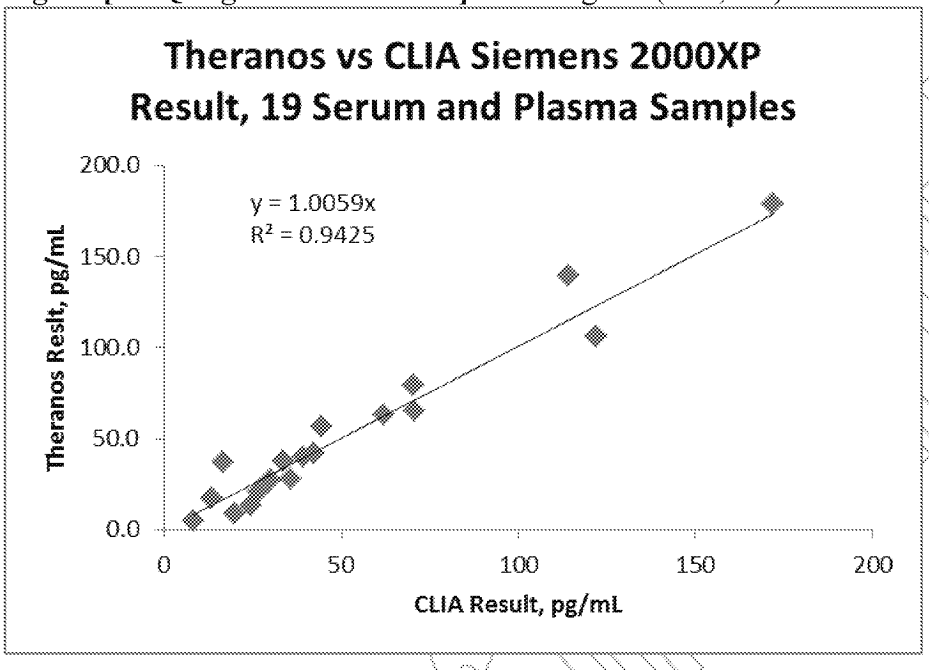
1.8 Training Set

To verify that changing the detection antibody eliminated the problem with measuring endogenous PTH in plasma compared to serum, a training set of 8 serum samples and 11 lithium heparin plasma samples was tested on the Theranos System with C26, D2 and compared to the CLIA Siemens Immulite 2000XP result. The correlation for the combined set was excellent, with slope of 1.0 and r^2 of 0.9425. When plotted separately, there was no difference in the correlation between Theranos and Siemens for serum or plasma samples. Therefore, the assay was re-optimized to improve sensitivity with the new detection antibody.

Table [SEQ Table * ARABIC]: Training Set (C26, D2)

Sample Type	Sample ID	CLIA, pg/mL	Theranos, pg/mL
Serum	02	29.9	27.3
	05	44.4	57.0
	06	24.4	13.1
	07	122	106.3
	09	114	139.4
	10	13.2	17.6
	11	122	106.1
	12	42.4	41.8
LiHep plasma	700	20	8.7
	702	172	179.1
	704	26.7	20.9
	705	39.5	39.8
	706	70.9	65.0
	707	35.9	28.3
	708	8.37	4.9
	709	16.6	37.3
	711	33.5	37.5
	712	70.3	79.4
	713	62.1	62.9

Figure [SEQ Figure * ARABIC]: Training Set (C26, D2)



1.9 Detection Antibody Titration (C26, D2)

The optimal concentration of detection antibody in Stabilzyme AP was determined by titrating loading concentrations of 100, 250 and 500 ng/mL. Signal to background was not improved by increasing DAb concentration.

[DAb] ng/mL	[PTH] pg/mL	Mean RLU	CV %	Modulation
100	400	10921	3.9	33.0
	40	1043	11.8	3.2
	4	501	10.5	1.5
	0	331	8.7	
250	400	24142	17.2	27.4
	40	3800	12.4	4.3
	4	1088	18.5	1.2
	0	881	21.9	
500	400	57400	9.1	33.6
	40	6078	9.3	3.6
	4	2366	9.9	1.4
	0	1709	10.1	

1.10 Assay Format Optimization

Previous testing showed that co-incubating the sample and detection antibody on the capture surface improved sensitivity significantly compared to a 2 step format. To further increase sensitivity, 2 additional assay formats were tested – all with a 1:10 sample dilution and 10 minute co-incubation and substrate incubation times. In one format, the sample diluted and incubated in solution with the DAb for 10 minutes before being incubated on the CAb-coated surface. In the second test, biotinylated CAb was mixed with the sample and the AP-labeled DAb and then introduced to an UltraAvidin capture surface. Neither format showed improvement in modulation.

Table [SEQ Table * ARABIC]: Assay Formats (C26, D2)

Format	[PTH] pg/mL	Mean RLU	CV %	Mod
Control (10, 10 minute co-incubation)	400	6592	11.6	21
	40	1002	7.7	3
	4	429	7.5	1
	0	311	15.0	
10 Minute pre-incubation of DAb and Sample	400	4213	5.9	19
	40	1692	7.2	8
	4	249	14.9	1
	0	218	17.3	
CAb and DAb both in solution	400	2634	40.7	8.
	40	2091	82.3	6
	4	376	0.2	1
	0	326	15.2	

1.11 Blocking Buffers for Capture Surface Coating

Since Starting Block showed improved assay response compared to 3% BSA in TBS, it was tested as a CAb surface-coating blocker and fixative as well. Starting Block showed a slight improvement in signal to background compared to BSA blocking buffer. Therefore, Starting Block was chosen as the final coating condition.

Table [SEQ Table * ARABIC]: Blocking Buffers for Capture Surface Coating

Coating Buffers	[PTH] pg/mL	Mean RLU	CV %	Modulation
3% BSA	250	6652	15.6	17
	25	1063	22.9	3
	0	395	18.9	
Starting Block	250	6107	9.3	23
	25	784	6.2	3
	0	264	26.3	

1.12 Sample Dilution (C26, D2)

To determine if a lower sample dilution would have a significant impact on assay sensitivity, the assay was tested with the original 1:10 sample dilution and a 1:5 sample dilution. Using a 1:5 sample dilution would as expected increase the modulation and sensitivity of the assay however the difference was not as significant as expected, and due to multiplexing constraints it was preferred to continue with a 1:10 sample dilution.

Table [SEQ Table * ARABIC]: Sample Dilution (C26, D2)

		Signal, RLU			Conc, pg/mL		
Sample Dilution	**[PTH] pg/mL**	**Mean RLU**	**CV %**	**Mod**	**Mean**	**CV %**	**% Recovery**
10x	800	12053	7.2	33.9	839.1	18.6	105
	200	5621	17.3	15.8	196.7	23.1	98
	40	1466	12.5	4.1	42.8	18.4	107
	10	743	5.7	2.1	10.8	15.7	108
	4	484	10.4	1.4	2.6	42.4	66
	2	472	9.6	1.3	2.4	37.9	119
	1	412	21.2	1.2	1.5	92.0	155
	0	356	11.7		0.7	53.9	
5x	800	19990	8.9	59.8	806.0	19.5	101
	200	9573	7.9	28.6	209.4	11.3	105
	40	2273	9.9	6.8	38.0	12.5	95
	10	1019	8.8	3.0	10.5	17.8	105
	4	651	5.4	1.9	3.6	14.7	91
	2	559	5.5	1.7	2.3	16.4	117
	1	425	19.1	1.3	1.0	50.8	104
	0	334	29.2		0.5	83.1	

1.13 Alkaline Phosphatase Conjugate Stabilizer (C26, D2)

Detection antibody conjugate stabilizers were re-tested for the new detection antibody. With the original pair a stabilizer made with Starting Block showed slight improvement over Stabilzyme AP but the advantage was not necessary for that pair since the original pair already showed strong modulation.

For the new pair both Starting Block-based stabilizer and BioStab showed a significant increase in modulation compared to Stabilzyme AP, with Starting Block-based stabilizer showing increased modulation at 10 pg/mL compared to Stabilzyme AP and Biostab.

Starting Block with 0.1 mM Zn^{2+} and 5mM Mg^{2+} was chosen as the detection antibody conjugate stabilizer, with BioStab as the backup condition.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Conjugate Stabilizer (C26, D2)

DAb Diluent/Stabilizer	[PTH] pg/mL	Mean RLU	CV %	Modulation
Stabilzyme	200	5621	17.3	16
	10	743	5.7	2
	0	356	11.7	
Biostab	200	6054	5.6	32
	10	410	13.5	2
	0	190	17.5	
Starting Block w/ 0.1 mM Zn^{2+} and 5mM Mg^{2+}	200	4790	12.8	29
	10	442	7.9	3
	0	163	6.0	

1.14 Detection Antibody Titration in Starting Block and Biostab (C26, D2)

Since Starting Block with 0.1 mM Zn^{2+} and 5mM Mg^{2+} and Biostab both decreased the assay background and increased modulation, the DAb concentration was re-titrated in both of these diluents to determine the optimal condition.

The best condition was 500 ng/mL loading concentration of DAb in Starting Block-based stabilizer, the backup condition is 100 ng/mL DAb in Biostab.

Table [SEQ Table * ARABIC]: Detection Antibody Titration in Starting Block and Biostab

Stabilizer	[DAB] ng/mL	[PTH] pg/mL	Mean RLU	CV %	Mod
Starting Block w/ 0.1 mM Zn2+ and 5mM Mg2+	100	200	4790	12.8	29
		10	479	20.0	3
		0	163	6.0	
	500	200	23334	9.4	37
		10	2066	2.9	3
		0	632	27.2	
	1000	200	39742	9.3	32
		10	3793	12.9	3
		0	1246	44.0	
BioStab	100	200	6054	5.6	32
		10	410	13.5	2
		0	190	17.5	
	500	200	26774	11.0	17
		10	2650	36.9	2
		0	1548	19.1	
	1000	200	44669	6.4	18
		10	5511	8.7	2
		0	2443	10.0	

1.15 Determination of LLOQ and ULOQ

With the final optimized conditions, a 10 point calibration curve was run and the LLOQ and ULOQ were determined along with other assay parameters using the Theranos proprietary calibration software. The assay LLOQ was 2 pg/mL and the ULOQ was 800 pg/mL, exceeding the target sensitivity for the assay of at least 4 pg/mL.

Table [SEQ Table * ARABIC]: Standard Curve with Final Assay Conditions

[PTH] pg/mL	Signal, RLU		Back-Calculated Conc, pg/mL		
	Mean RLU	CV %	Mean	CV %	% Recovery
800	75308	5.8	852.08	5.5	107
400	32197	12.3	379.48	11.8	95
200	17229	24.9	205.32	25.0	103
100	7913	12.5	91.55	13.6	92
40	4100	6.5	43.14	7.8	108
26	3050	17.3	29.82	22.3	115
10	1232	14.6	7.91	24.8	79
4	893	7.1	4.40	13.6	110
2	610	6.8	2.06	3.9	103
0	330	27.1	OORL		

Conc = 814.291 * (((7.510 - 2.202) / (log10(S) - 2.202)) - 1) ^ (1 / -0.346)
Signal Min = 566, Signal Max = 81659
[LINK Excel.Sheet.12 "\\\\theranos.local\\folders\\Projects\\Experiment Log\\E0700 - E0799\\E0747\\Edison 3.0\\09-13-12_PTH_Final_LLOQ.xlsx" "All Data!R66C1:R75C3" \a \f 5 \h * MERGEFORMAT][SHAPE * MERGEFORMAT]

1.16 Cross Reactivity

Fragments of PTH were obtained from ProSpec Bio and NIBSC for cross reactivity testing. The Theranos iPTH shows no cross reactivity for any of the PTH fragments and is specific for intact PTH.

Table [SEQ Table * ARABIC]: Cross Reactivity

Test Substance	[Test Substance], pg/mL	Signal, RLU		Conc, pg/mL	
		Mean RLU	CV %	Mean Conc	CV %
PTH 1-34 fragment	1000	419	0.7	OORL	-
PTH 39-84 fragment	1000	353	2.9	OORL	-
PTH 44-68 fragment	1000	379	11.8	OORL	-
PTH 53-84 fragment	1000	402	9.1	OORL	-
PTH 7-34 fragment	1000	337	23.9	OORL	-

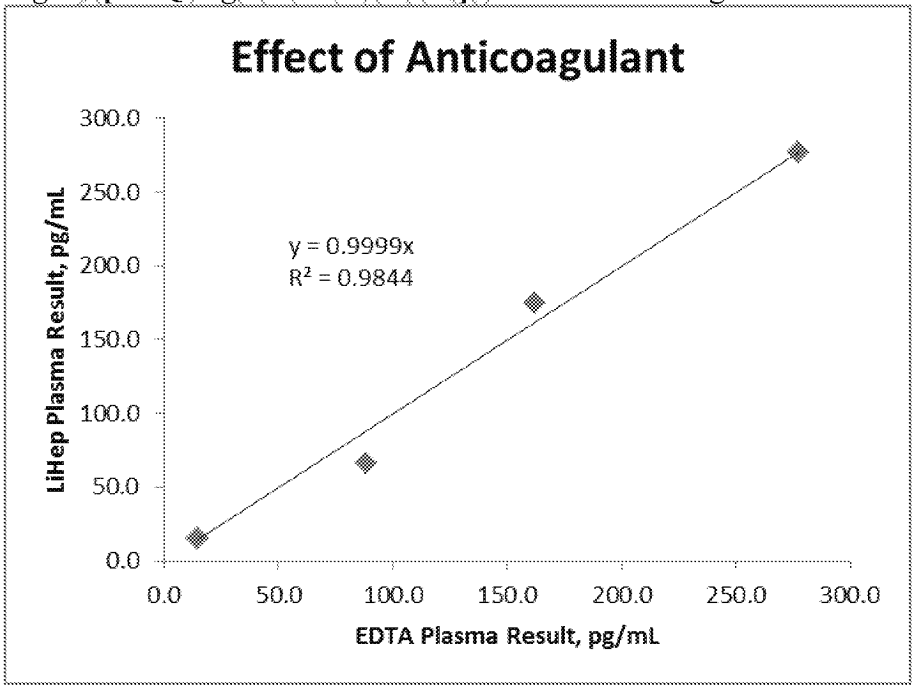
1.17 Effect of Anticoagulant

To determine if the choice of EDTA or lithium heparin anticoagulant for blood collection influenced the assay result, spike recovery was tested in EDTA and lithium heparin plasma. Whole blood was stored at 4°C for 36 hours before plasma preparation, resulting in depletion of endogenous PTH levels. The remaining endogenous levels were unaffected by the choice of anticoagulant. Spike recovery was excellent and equivalent in both matrixes. Either ETDA or lithium heparin anticoagulant may be used for this assay.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant

Anticoagulant	Spiked [PTH] pg/mL	Conc, pg/mL			
		Mean Conc	CV %	Minus Endogenous	% Recovery
EDTA	250	277.1	12.9	262.7	105
	150	162.2	14.5	147.9	99
	75	88.3	28.4	74.0	99
	0	14.4	22.9	0.0	
Li-Hep	250	277.1	5.8	262.1	105
	150	174.3	5.4	159.3	106
	75	66.2	17.5	51.2	68
	0	14.9	11.6	0.0	

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant



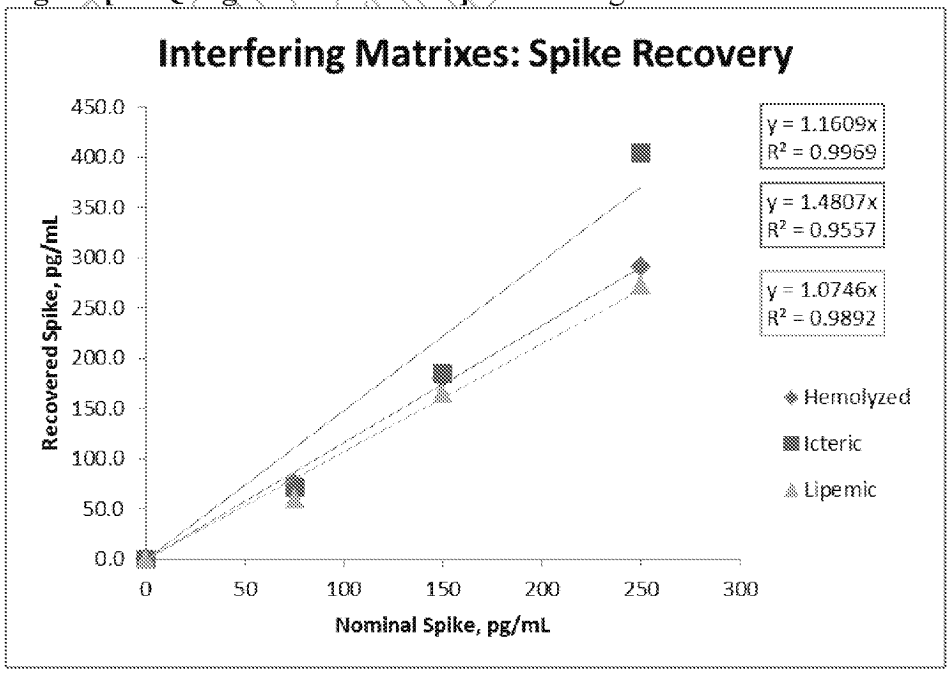
1.18 Interfering Matrixes

To determine if grossly lipemic, icteric or hemolyzed samples influence the result of this assay, PTH was spiked into each of these matrixes and spike recovery was calculated. Recovery was good in lipemic and hemolyzed serum, however icteric serum showed over-recovery. Grossly icteric samples may produce erroneous results in this assay.

Table [SEQ Table * ARABIC]: Interfering Matrixes

Matrix	Spiked [PTH] pg/mL	Conc, pg/mL Mean Conc	CV %	Minus Endogenous	% Recovery
Hemolyzed	250	290.1	9.6	290.1	116
	150	179.7	8.3	179.7	120
	75	76.3	17.1	76.3	102
	0	OORL	-	0.0	
Icteric	250	416.5	8.9	404.5	162
	150	196.8	14.2	184.8	123
	75	83.3	26.8	71.3	95
	0	12.0	16.2	0.0	
Lipemic	250	304.3	24.3	273.1	109
	150	195.4	13.2	164.2	109
	75	90.8	5.6	59.6	79
	0	31.2	18.4	0.0	

Figure [SEQ Figure * ARABIC]: Interfering Matrixes



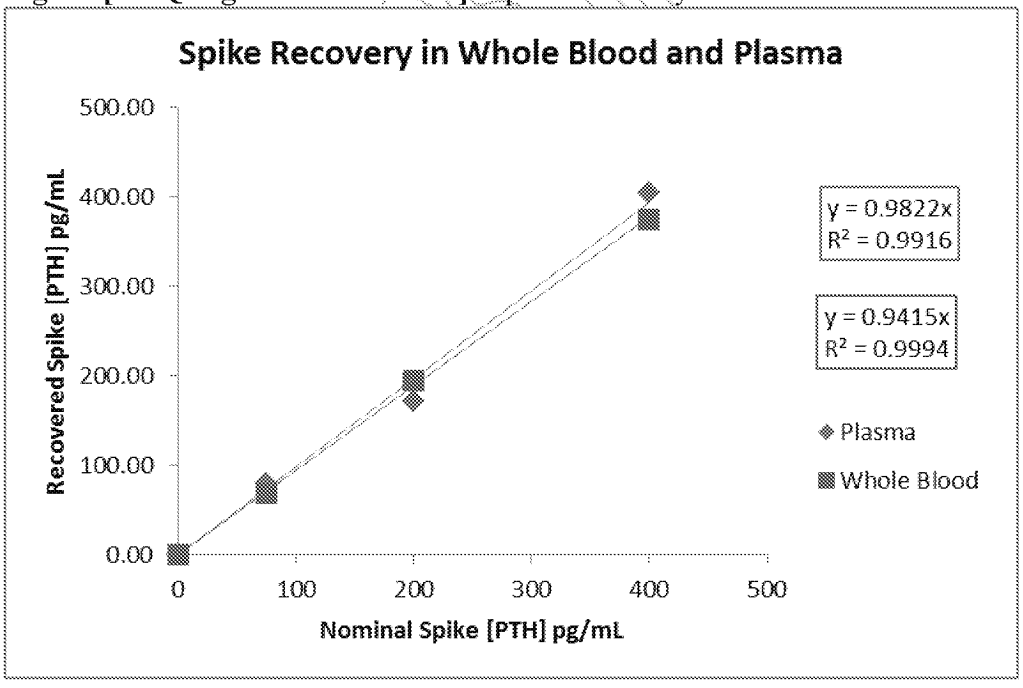
1.19 Spike Recovery in Whole Blood and Plasma

Spike recovery in EDTA whole blood and plasma was tested in the Theranos System. Spike recovery was excellent in both whole blood and in plasma.

Table [SEQ Table * ARABIC]: Spike Recovery in Whole Blood and Plasma

Matrix	Spiked [PTH] pg/mL	Conc, pg/mL Mean Conc	CV %	Minus Endogenous	% Recovery
Plasma	400	421.3	10.1	403.7	101
	200	189.6	8.5	172.0	86
	75	98.6	12.0	81.0	108
	0	17.6	32.0	0.0	
Whole Blood	400	388.0	5.3	373.7	93
	200	208.8	4.7	194.6	97
	75	83.5	8.2	69.3	92
	0	14.3	9.1	0.0	

Figure [SEQ Figure * ARABIC]: Spike Recovery in Whole Blood and Plasma



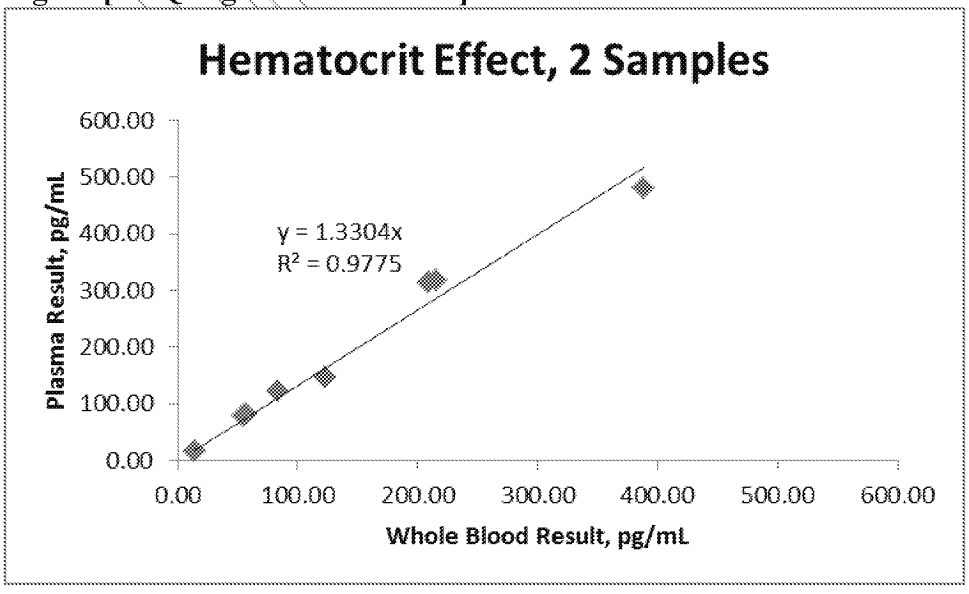
1.20 Hematocrit Effect

The Theranos System is designed to automatically prepare plasma from whole blood, however in some cases it may be desireable to measure analytes directly in whole blood. In order to determine the hematocrit effect, spiked whole blood was measured on the Theranos System, then plasma prepared from the spiked whole blood was measured and the results were compared. The results indicate that PTH does concentrate into plasma, however the result measured in the plasma is slightly lower than the expeceted 1.6-fold increase based on average hematocrit, indicating there could be some loss of PTH during the plasma prepartion process. Loss of expected PTH in plasma compared to whole blood could possibly be through association with blood cells and subsequent removal by centrifugation – for example neutrophils, B and T cells have PTH receptors [Geara et al, 2010]. With the 2 samples tested the recovery slope was consistent, but if whole blood measurement is desired and the results are to be compared to plasma or serum results, further testing should be done.

Table [SEQ Table * ARABIC]: Hematocrit Effect

	Whole Blood, pg/mL		Plasma from Whole Blood, pg/mL	
Sample ID	**Mean Conc**	**CV %**	**Mean Conc**	**CV %**
W070512002328	388.0	5.3	481.1	11.7
	208.8	4.7	314.6	12.3
	83.5	8.2	122.6	13.1
	14.3	9.1	16.5	26.6
W070512002121	216.1	6.6	317.5	4.2
	123.4	6.6	146.9	15.8
	56.7	24.0	80.8	7.8
	55.1	18.3	79.6	30.3

Figure [SEQ Figure * ARABIC]: Hematocrit Effect



1.21 Clinical Correlation

To validate the measurement of clinical samples, a total of 8 serum samples and 25 lithium heparin plasma samples were tested in the Theranos System and on the Siemens Immulite 2000XP. The correation between the Theranos System result and the Siemens result was excellent.

Figure [SEQ Figure * ARABIC]: Clinical Correlation Theranos to Siemens Immulite 2000XP

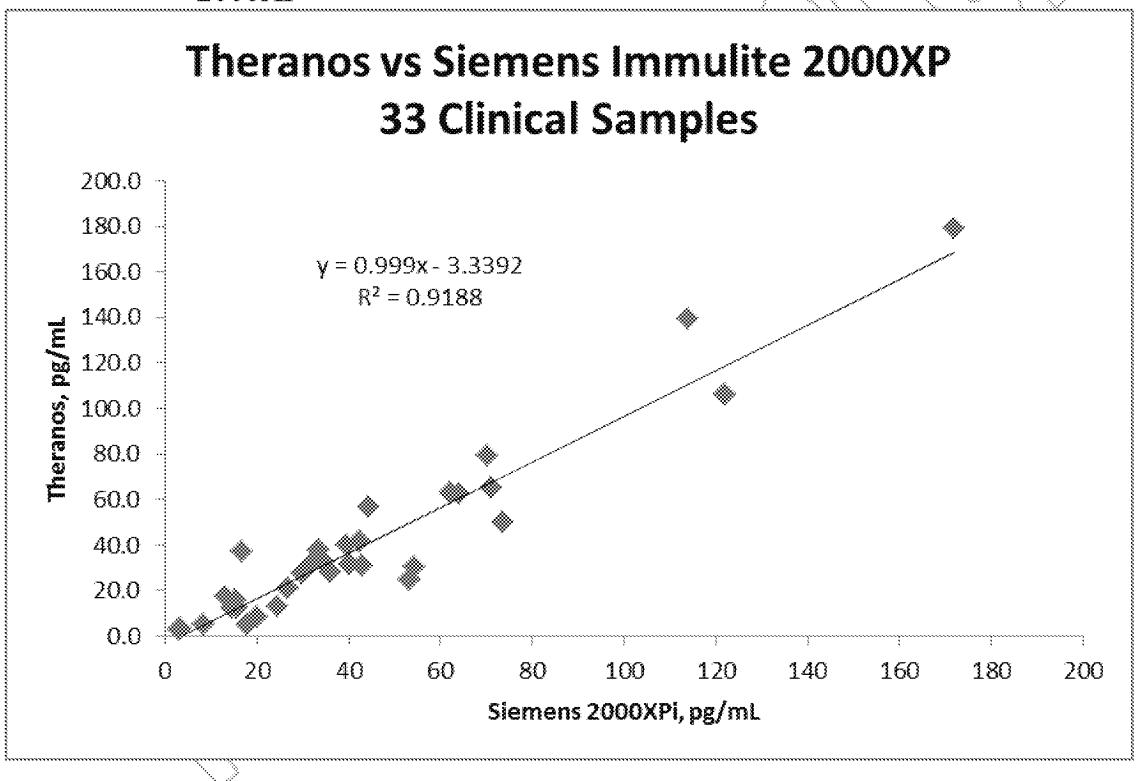


Table [SEQ Table * ARABIC]: Clinical Correlation Theranos to Siemens Immulite 2000XP

Sample Matrix	Sample ID	Siemens Immulite, pg/mL	Theranos, pg/mL
Serum	2	29.9	27.3
	5	44.4	57.0
	6	24.4	13.1
	7	122.0	106.3
	9	114.0	139.4
	10	13.2	17.6
	11	122.0	106.1
	12	42.4	41.8
LiHep Plasma	317	53.1	24.9
	318	3.0	2.8
	686	17.7	5.4
	687	34.5	32.8
	688	3.5	3.7
	689	64.1	62.4
	690	15.5	12.6
	692	15.3	15.8
	693	32.0	32.2
	694	14.5	12.4
	697	42.9	30.9
	698	73.6	50.3
	699	54.3	30.4
	700	20.0	8.7
	702	172.0	179.1
	704	26.7	20.9
	705	39.5	39.8
	706	70.9	65.0
	707	35.9	28.3
	708	8.4	4.9
	709	16.6	37.3
	711	33.5	37.5
	712	70.3	79.4
	713	62.1	62.9
	715	40.0	31.4

1.22 Stability

Stability studies are ongoing.

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