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Alpha Fetoprotein (AFP)Assay Development Report

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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"]

An enzyme linked immunosorbent assay (ELISA) was developed for the quantitative detection of alpha fetoprotein (AFP) in serum and plasma matrix. AFP, a member of the albuminoid superfamily, is a 70kD protein that is an important tumor maker in adult males and non-pregnant females. High levels of AFP are indicative of certain cancers and congenital defects. This report describes the assay development and performance of the Theranos AFP ELISA as aid to diagnosing certain tumors.

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

The following assay was used as predicament methods:

AFP, Siemens, Immulite 2000, Cat. L2KAP2

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

The Theranos AFP ELISA is a quantitative sandwich ELISA. The assay was developed using biotinylated anti-AFP antibodies coated on an ultravidin (UA) surface as the capture surface. AFP in serum or plasma binds specifically to the capture antibodies for 10 minutes followed by a wash cycle. After washing, AFP was detected using an AP labeled mouse monoclonal antibody to human AFP. After incubation with the detector antibody for10 minutes, another wash cycle was performed, and the alkaline phosphatase substrate added. The resulting chemiluminescence was read in relative light units (RLU) on the Theranos system.

Table [SEQ Table * ARABIC]: Materials

Item	Supplier	Catalog #
AFP Calibrator	NIBSC	AFP
Capture Antibody, mouse anti-AFP, clone AFP-Y1	MyBiosource	MBS592005
Detection Antibody, mouse anti-AFP, clone B492M	MyBiosource	MBS312605
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Theranos AP Stabilizer	Theranos	n/a
Tris Buffered Saline	Sigma	T6664-10PAK
Theranos Cartridge	Theranos	n/a
Theranos System	Theranos	n/a

1.1.3 Labeling of Detector Antibody

The mouse anti-human AFP clone B492M was labeled with alkaline phosphatase according to kit instructions (Dojindo, LK13-10).

1.1.4 Labeling of Capture Antibody

The biotinylation of anti-human AFP clone AFP-Y1 was performed according to kit instructions (Dojindo, LK10-10).

1.1.5 Preparation of Assay Buffer

The assay buffer was prepared by dissolving 1 packet of TBS into water and adding 10 mL of 10% azide, and 30 g of BSA to a final volume of 1000mL. The final composition of the assay buffer is 3% BSA, 50mM Tris, 138mM NaCl, 2.7mM KCl pH 8.0 in water. The assay buffer was filtered before use.

1.1.6 Preparation of Theranos AP Stabilizer

The conjugate diluent was prepared by adding $Zn^{2+}Cl$ and $Mg^{2+}Cl$ to the assay buffer to final concentrations of 0.1mM Zn^{2+} and 5mM Mg^{2+}.

1.1.7 Preparation of Calibrators

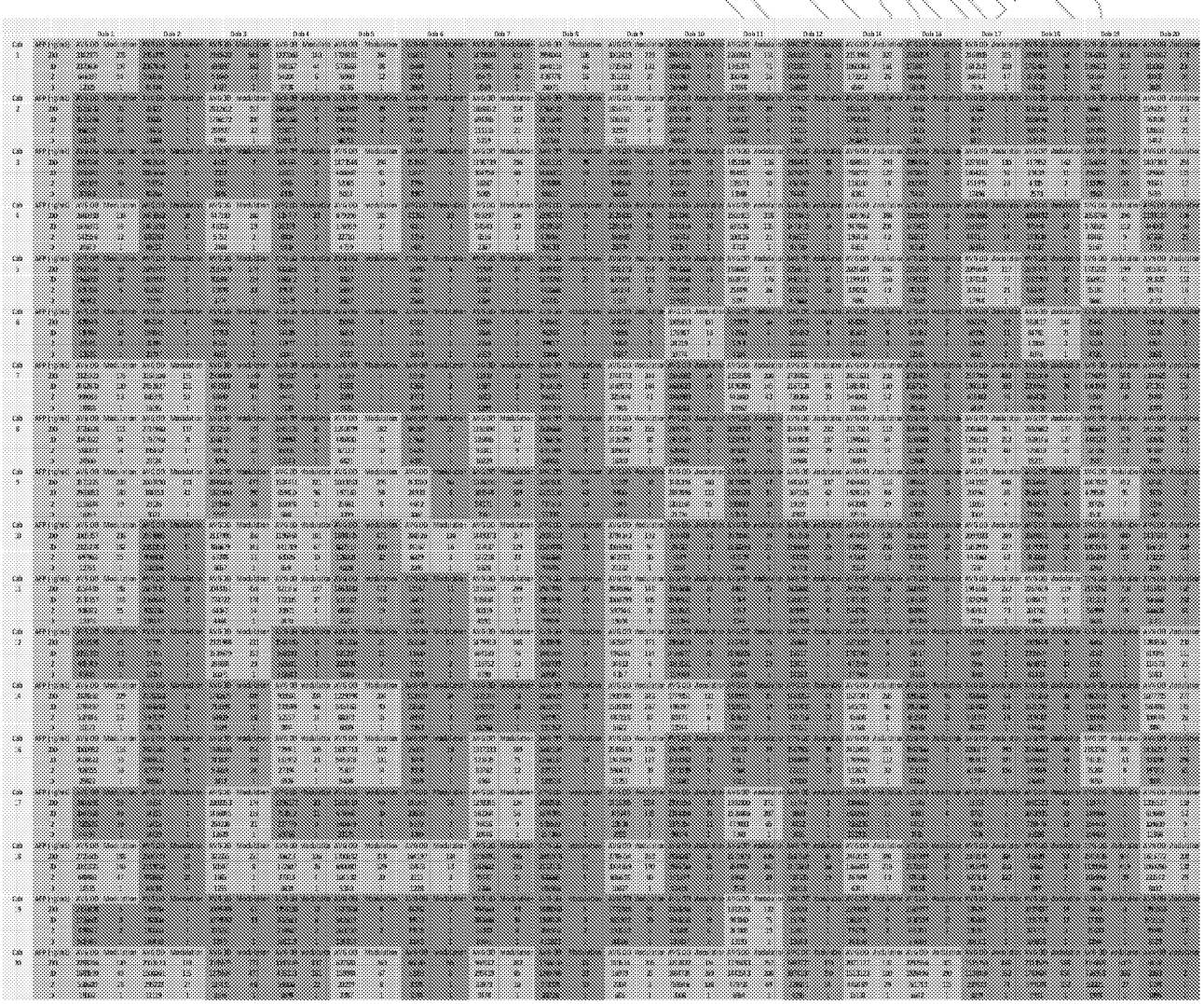
The AFP calibrators were prepared in assay buffer and stored at -80C.

2. ASSAY DEVELOPMENT

2.1 Microtiter Plate Screen (MTP)

Initial assay development was started by screening 18 antibodies to AFP. The results were summarized below in the table. The best antibodies which gave high modulations were highlighted in blue.

Table 2: MTP Screen



2.2 Cross Reactivity and Interference

The three best antibody pairs from MTP screening were further evaluated for cross reactivity. The pairs were capture antibody 7 with detection antibody 3, capture antibody 16 with detection antibody 3, and capture antibody 20 with detection antibody 19. There was no cross reactivity with the tested materials.

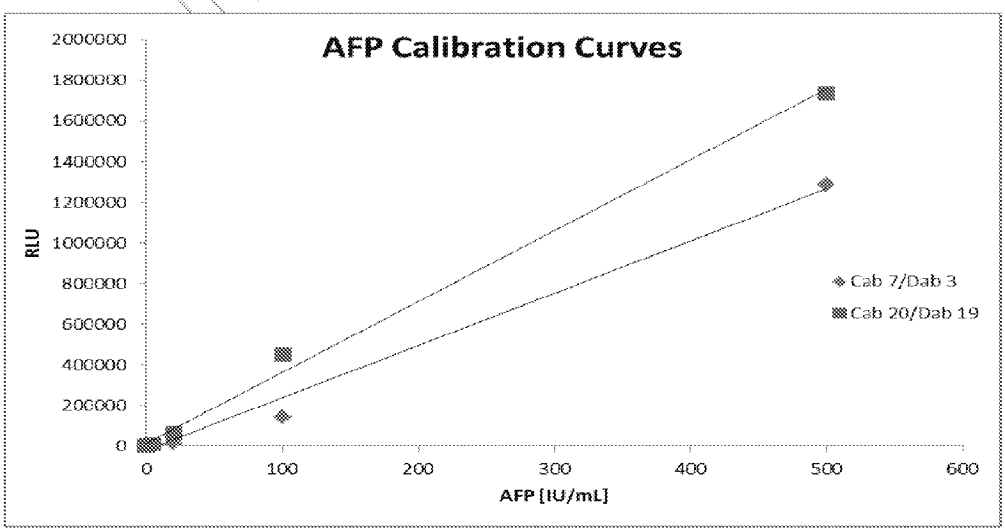
Table 3: Cross Reactivity

Test Material	Cross Reactivity
Alpha-I-Acid Glycoprotein	No
Human serum Albumin	No
Alpha-I-Antitrypsin	No
Chorionic Gonadotropin	No
Placental Lactogen	No
Prolactin	No
Hemoglobin	No
Transferrin	No
Ceruloplasmin	No

2.3 AFP Calibrators

The best antibody pair was narrowed down to capture antibody 20 with detection antibody 19. This antibody pair gave the most sensitivity using the WHO AFP calibrators. The Generic2_5X_PSW protocol was used.

Figure 1: AFP Calibrators



2.4 Assay Protocols

The assay was further optimized by comparing different 5x sample dilution protocols. The Generic2_5X_PSW protocol was the most sensitive.

Table 4: Assay Protocols

	Generic2_5X_PSW			Generic2_5X			Generic2_5X_Coincubation		
AFP [IU/mL]	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation
500.00	1668148	5%	687.25	1989602	17%	554.38	553837	4%	722.31
100.00	505393	14%	208.21	783326	11%	218.26	147001	5%	191.72
20.00	77478	2%	31.92	118334	5%	32.97	18211	9%	23.75
4.00	13235	7%	5.45	20791	11%	5.79	3527	9%	4.60
0.80	4433	13%	1.83	7639	11%	2.13	1081	12%	1.41
0.16	3748	17%	1.54	5013	10%	1.40	1011	17%	1.32
0.03	2937	11%	1.21	4080	13%	1.14	804	10%	1.05
0.00	2427	10%	1.00	3589	22%	1.00	767	4%	1.00

2.5 Assay Incubation Times

The assay incubation time was optimized by testing the 10-10-10, 5-5-5, and 2-2-1 assay protocols with the 5x sample dilution. The 10-10-10 protocol was selected for maximum sensitivity.

Table 5: Assay Incubation Times

	10_10_10			5_5_5			2_2_1		
AFP [IU/mL]	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation
500.00	2118022	7%	1242.11	977411	10%	1719.75	185259	20%	778.61
100.00	863981	24%	506.68	200049	18%	351.98	23344	15%	98.11
20.00	144831	10%	84.94	33351	18%	58.68	4388	25%	18.44
4.00	26498	15%	15.54	6534	20%	11.50	1114	22%	4.68
0.80	5666	27%	3.32	1954	24%	3.44	388	34%	1.63
0.16	2592	19%	1.52	1173	17%	2.06	287	15%	1.21
0.03	1835	23%	1.08	854	7%	1.50	279	9%	1.17
0.00	1705	18%	1.00	568	9%	1.00	238	10%	1.00

2.6 Capture Surface Titration

The optimum capture antibody concentration was determined by titrating the capture antibody at 1 ug/mL, 5 ug/mL, and 10 ug/mL (MyBiosource, MBS592005, clone AFP-Y1). The results with 10 ug/mL capture antibody showed higher modulation and sensitivity with this concentration.

Table 6: Capture Antibody Titration

	10 ug/mL Cab 20			5 ug/mL Cab 20			1 ug/mL Cab 20		
AFP [IU/mL]	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation	AVG RLU	CV	Modulation
500.00	1668148	5%	687.25	1452082	4%	504.25	577917	16%	141.26
100.00	505393	14%	208.21	416827	10%	144.75	102410	23%	25.03
20.00	77478	2%	31.92	60307	12%	20.94	16722	26%	4.09
4.00	13235	7%	5.45	11635	7%	4.04	6292	17%	1.54
0.80	4433	13%	1.83	4926	21%	1.71	4607	20%	1.13
0.16	3748	17%	1.54	3361	15%	1.17	4673	15%	1.14
0.03	2937	11%	1.21	3501	13%	1.22	4132	10%	1.01
0.00	2427	10%	1.00	2880	12%	1.00	4091	17%	1.00

2.7 Detector Antibody Titration

The optimum detection antibody concentration was determined by titrating the detector antibody (MyBiosource, MBS312605, clone B492M) at 25 ng/mL, 50 ng/mL, and 100 ng/mL. A 100 ng/mL concentration gave the best modulation and sensitivity.

Table 7: Detector Antibody Titration

Dab 19 [ng/mL]	AFP [IU/mL]	AVG RLU	CV	Modulation
100	500.00	1668148	5%	687.25
100	100.00	505393	14%	208.21
100	20.00	77478	2%	31.92
100	4.00	13235	7%	5.45
100	0.80	4433	13%	1.83
100	0.16	3748	17%	1.54
100	0.03	2937	11%	1.21
100	0.00	2427	10%	1.00
50	500.00	1468821	4%	615.51
50	100.00	344629	9%	144.42
50	20.00	46162	24%	19.34
50	4.00	9800	19%	4.11
50	0.80	4014	12%	1.68
50	0.16	2614	6%	1.10
50	0.03	2507	13%	1.05
50	0.00	2386	9%	1.00
25	500.00	1004642	3%	611.80
25	100.00	205188	18%	124.95
25	20.00	31013	12%	18.89
25	4.00	5583	26%	3.40
25	0.80	2855	19%	1.74
25	0.16	1833	5%	1.12
25	0.03	1731	23%	1.05
25	0.00	1642	15%	1.00

2.8 Effect of AP Conjugate Stabilizers

The optimum detection antibody stabilizer was tested by comparing the modulation using 3% BSA, Biostab, Stabilzyme, and Theranos AP. Theranos AP stabilizer gave the best modulations.

Table 8: Effect of AP Conjugate Stabilizers

Stabilizer	AFP [IU/mL]	AVG RLU	CV	Modulation
3% BSA	500.00	2372361	5%	605.18
3% BSA	100.00	1245006	9%	317.60
3% BSA	20.00	248654	24%	63.43
3% BSA	4.00	39826	9%	10.16
3% BSA	0.80	10839	20%	2.77
3% BSA	0.16	5614	6%	1.43
3% BSA	0.03	4409	27%	1.12
3% BSA	0.00	3920	18%	1.00
Biostab	500.00	1871306	5%	88.76
Biostab	100.00	526086	15%	24.95
Biostab	20.00	85395	20%	4.05
Biostab	4.00	15365	17%	0.73
Biostab	0.80	5713	14%	0.27
Biostab	0.16	15059	5%	0.71
Biostab	0.03	26110	58%	1.24
Biostab	0.00	21082	51%	1.00
Stabilzyme	500.00	1458426	11%	728.68
Stabilzyme	100.00	337412	1%	168.58
Stabilzyme	20.00	49466	12%	24.71
Stabilzyme	4.00	9352	23%	4.67
Stabilzyme	0.80	4196	16%	2.10
Stabilzyme	0.16	2801	14%	1.40
Stabilzyme	0.03	2317	15%	1.16
Stabilzyme	0.00	2001	16%	1.00
Theranos AP	500.00	2139131	10%	883.97
Theranos AP	100.00	670265	6%	276.98
Theranos AP	20.00	117689	7%	48.63
Theranos AP	4.00	18466	20%	7.63
Theranos AP	0.80	6897	16%	2.85
Theranos AP	0.16	3356	3%	1.39
Theranos AP	0.03	2685	14%	1.11
Theranos AP	0.00	2420	17%	1.00

2.9 Effect of Coating Buffers

The optimum coating buffer was tested by comparing Starting Block, Super Block, and Assay Buffer. Assay Buffer was comparable to the commercial blockers.

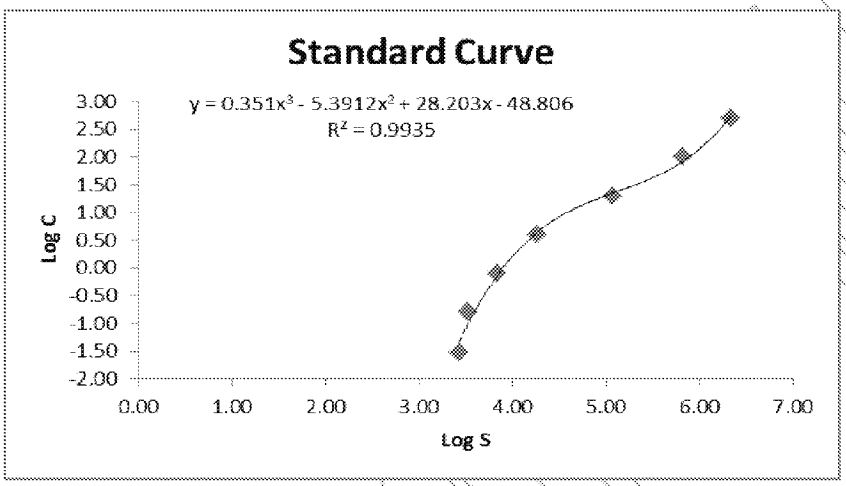
Table 9: Coating Buffers.

Coating	AFP [IU/mL]	AVG RLU	CV	Modulation
Starting Block	500.00	2141391	14%	1729.90
Starting Block	100.00	931494	17%	752.50
Starting Block	20.00	160457	6%	129.62
Starting Block	4.00	25420	17%	20.54
Starting Block	0.80	5687	17%	4.59
Starting Block	0.16	2552	17%	2.06
Starting Block	0.03	1487	8%	1.20
Starting Block	0.00	1238	13%	1.00
SuperBlock	500.00	2212637	9%	1417.79
SuperBlock	100.00	964141	9%	617.79
SuperBlock	20.00	151288	6%	96.94
SuperBlock	4.00	28001	16%	17.94
SuperBlock	0.80	5896	23%	3.78
SuperBlock	0.16	2472	16%	1.58
SuperBlock	0.03	1791	30%	1.15
SuperBlock	0.00	1561	22%	1.00
Assay Buffer	500.00	2118022	7%	1242.11
Assay Buffer	100.00	863981	24%	506.68
Assay Buffer	20.00	144831	10%	84.94
Assay Buffer	4.00	26498	15%	15.54
Assay Buffer	0.80	5666	27%	3.32
Assay Buffer	0.16	2592	19%	1.52
Assay Buffer	0.03	1835	23%	1.08
Assay Buffer	0.00	1705	18%	1.00

2.10 Effect of RF and HAMA Samples

The effect of RF and HAMA samples was tested and the results showed no interference to RF and HAMA samples. Sample R1 was both positive for AFP on the Theranos system and the Siemens.

Table 10: RF and HAMA Samples

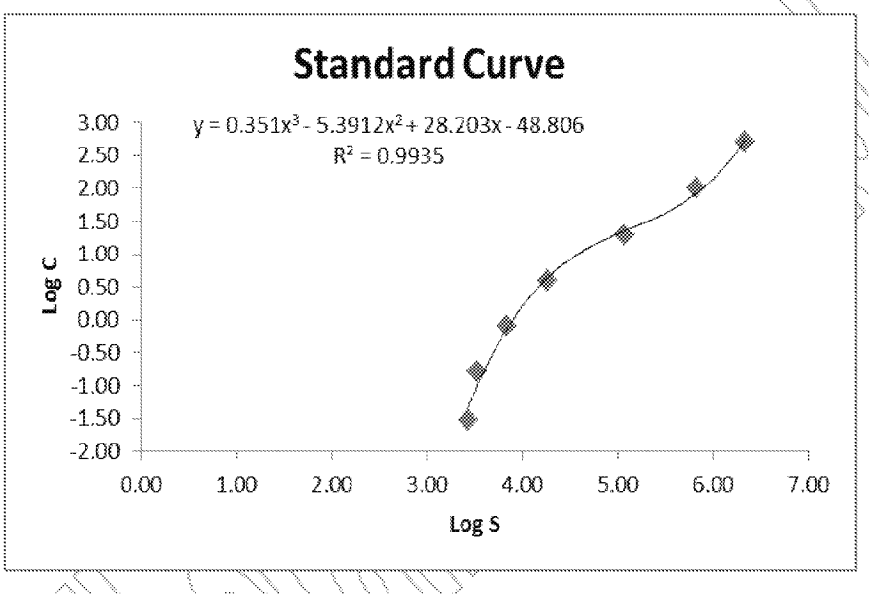


Description	Sample	AVG RLU	CV	Log S	Back-Cal	Siemens
Hama	G	4057	18%	3.61	0.18	0.80
Hama	H	5550	19%	3.74	0.43	2.96
Hama	I	2820	10%	3.45	0.05	1.05
Hama	J	3820	15%	3.58	0.15	1.04
Hama	K	4761	20%	3.68	0.29	2.12
Hama	L	2777	24%	3.44	0.05	0.73
Hama	M	2244	4%	3.35	0.02	0.64
RF	R1	351638	15%	5.55	45.70	64.30
RF	RA	14719	25%	4.17	3.18	n/a
RF	RB	2935	28%	3.47	0.06	n/a
RF	RC	3911	35%	3.59	0.16	n/a
RF	RD	4676	39%	3.67	0.27	n/a

2.11 Whole Blood and Plasma Screen

Whole blood and plasma was screened and the results showed no interference with WB and plasma. The back calculated AFP levels were all low.

Table 11: Whole Blood and Plasma Screen

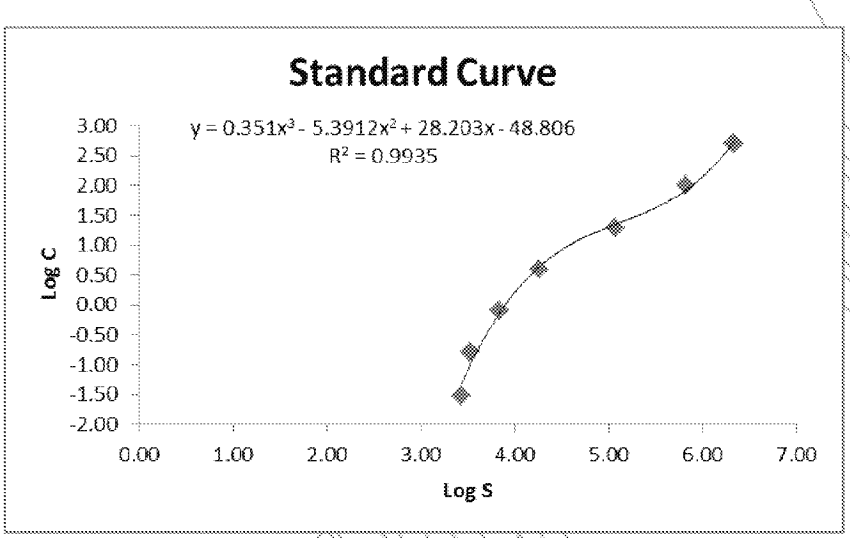


Description	Sample	AVG RLU	CV	Log S	Back-Cal
WB	1	2727	11%	3.44	0.05
WB	2	2843	2%	3.45	0.06
WB	3	2552	20%	3.41	0.04
WB	4	2877	21%	3.46	0.06
WB	5	2560	23%	3.41	0.04
EDTA Plasma	6	3973	1%	3.60	0.17
EDTA Plasma	7	4290	32%	3.63	0.21
EDTA Plasma	8	3378	8%	3.53	0.10
EDTA Plasma	9	3800	19%	3.58	0.15
EDTA Plasma	10	3147	25%	3.50	0.08

2.12 Hematocrit Effect

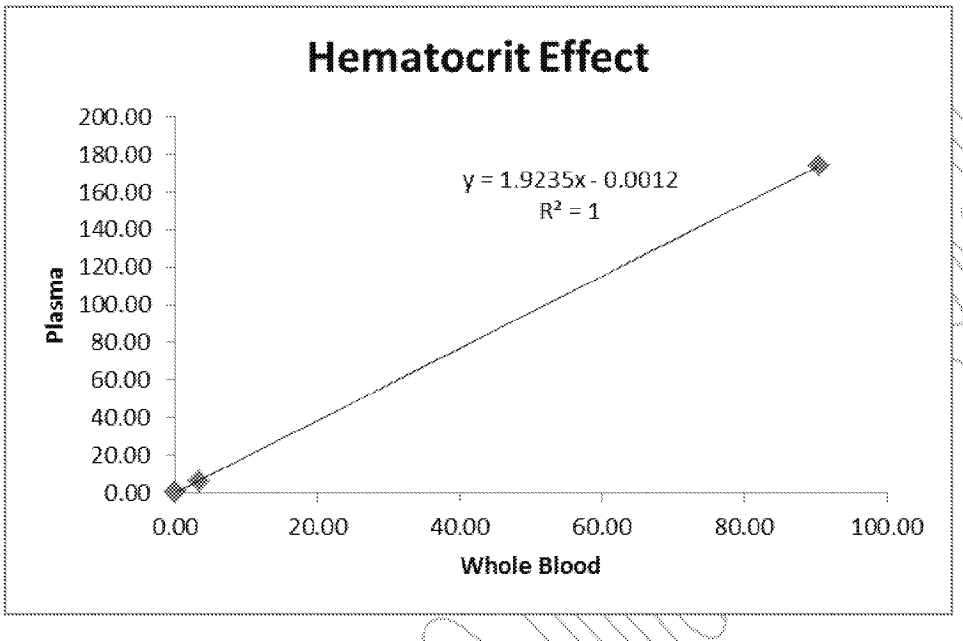
The hematocrit effect was tested and the results showed a hematocrit factor of 1.9x.

Table 12: Hematocrit Effect



Sample	Spike	AVG RLU	CV	Log S	Back-Cal	Recovery
Whole Blood	100	714894	11%	5.85	90.56	91%
Whole Blood	4	15307	8%	4.18	3.37	84%
Whole Blood	0.8	3666	17%	3.56	0.13	16%
Whole Blood	0	2445	14%	3.39	0.03	
Plasma	100	1161756	24%	6.07	174.21	174%
Plasma	4	23194	36%	4.37	5.92	148%
Plasma	0.8	6840	25%	3.84	0.72	91%
Plasma	0	3667	13%	3.56	0.13	

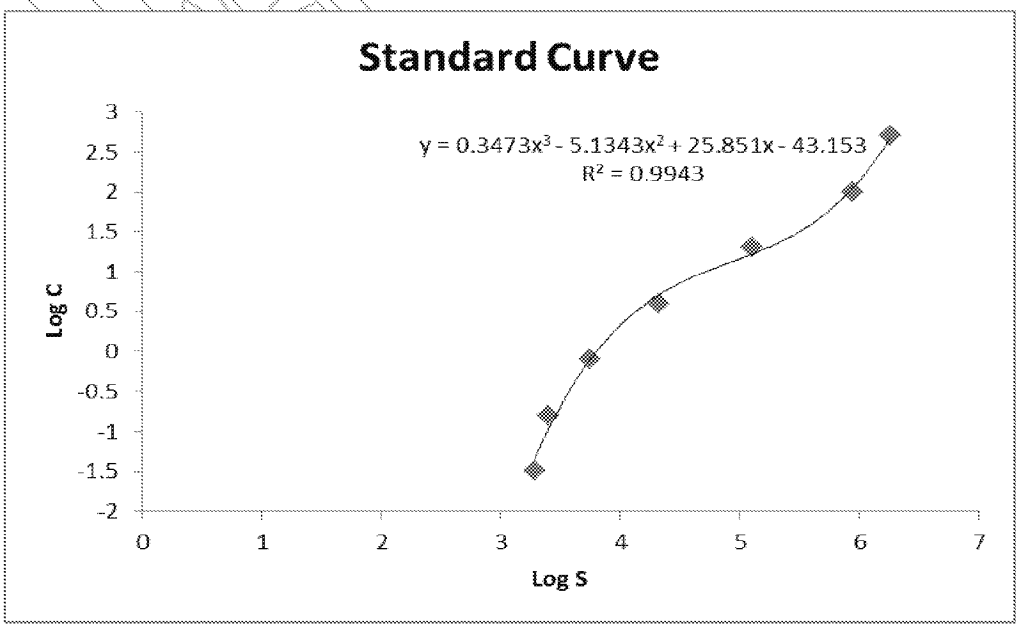
Figure 2: Hematocrit Effect



2.13 Clinical Correlation

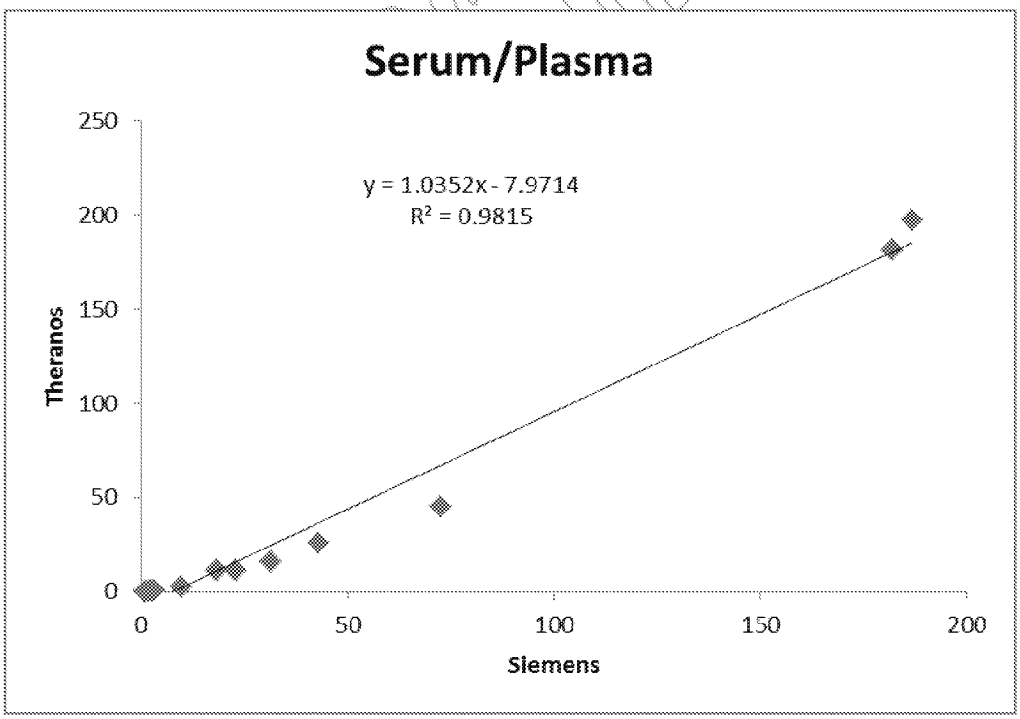
The clinical correlation was good with an R^2 value of 0.98. The Theranos test results were comparable to the Siemens test results.

Table 13: Clinical Correlation



Sample	AVG RLU	CV	Log S	Back-Cal	Matrix	Description	Siemens
1	453876	17%	5.66	44.96	EDTA Plasma	Pregnancy	72.60
7	1221727	12%	6.09	197.56	EDTA Plasma	Pregnancy	187.00
8	1167957	11%	6.07	181.15	EDTA Plasma	Pregnancy	182.00
14	64584	9%	4.81	11.27	Serum	Pregnancy	22.80
15	62409	7%	4.80	11.05	Serum	Pregnancy	18.10
27	251997	7%	5.40	25.97	Serum	Pregnancy	42.90
28	120257	18%	5.08	15.93	Serum	Pregnancy	31.30
46	3734	15%	3.57	0.32	EDTA Plasma	Pregnancy	1.65
48	11976	9%	4.08	2.73	EDTA Plasma	Pregnancy	9.84
55	2127	3%	3.33	0.07	Serum	Non-Viral Liver Disease	0.85
56	2726	29%	3.44	0.14	Serum	Non-Viral Liver Disease	0.74
57	3229	26%	3.51	0.22	Serum	Non-Viral Liver Disease	2.88
58	2656	9%	3.42	0.13	Serum	Non-Viral Liver Disease	2.26

Figure 3: Serum/Plasma Clinical Correlation



3. ASSAY SUMMARY

Table 14: Development Summary

AFP Calibrator	NIBSC, Cat. AFP
Capture Antibody	MyBiosurce, Cat. MBS592005, clone AFP-Y1
Wash Buffer	1X Enzo from 20X
Assay Buffer	3% BSA in TBS
Edison Protocol	Generic2_5X_PSW
Detector Antibody	MyBiosource, MBS312605, clone B492M
Detector Stabilizer	Theranos AP Conjugate Stabilizer
Sample Dilution	5X

4. CLINICAL EVALUATION

More clinical samples need to be tested to further validate the assay. Furthermore, the assay needs to be validated at more than 1 test center. At least 100 or more patients are needed for clinical evaluation.