theranos™
redefining healthcare

Syphilis Non-Treponemal Assay Development Report

Theranos, Inc.

October 10, 2011

Prepared by: Tam Dang

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. ASSAY INFORMATION

1.1 Assay Specifications

An enzyme linked immunosorbent assay (ELISA) was developed for the detection of human IgG and IgM specific to cardiolipin due to syphilis infections in human serum and plasma. The assay utilizes a cardiolipin capture surface and detection with alkaline phosphatase labeled mouse secondary antibodies. The assay is qualitative and uses commercial liquid quality control reagents. This report describes the assay feasibility and performance of the Syphilis Non-Treponemal Assay. Clinical data is required to further evaluate the assay and will be generated once a biosafety level 3 laboratory is available.

1.1.1 Reference Assays

The following commercial kit has been used in house as predicate methods:

- ASI VDRL Kit (Cat# 950010)

1.1.2 Materials and Methods

The capture reagent is a mixture of 0.03% cardiolipin, 0.9% cholesterol, and 0.2% lecithin in absolute ethanol and serves as the capture surface for the cardiolipin specific IgG and IgM. Coating of the surface requires no incubation time and detergent. The assay was performed without detergents and with a 1:25 sample dilution. The sample (plasma or serum) was diluted and then incubated on the capture surface for 10 minutes, the surface was washed with detergent free buffer, and then an alkaline phosphatase-labeled anti-human IgG and anti-human IgM antibody was incubated on the surface for 10 minutes. After the detection antibody incubation, another washing cycle was performed and the alkaline phosphatase substrate was incubated on the surface for 10 minutes, and the resulting chemiluminescence was read in Relative Light Units (RLU) on the Theranos System.

Table 1: Materials

Name	Supplier	Catalog #
Cardiolipin	Sigma	C0563-25MG
Lecithin	Sigma	P3556-100MG
Cholesterol	Sigma	C8667-1G
Mouse Anti-Human IgG-UNLB	Southern Biotech	9040-01
Mouse Anti-Human IgM	Abd Serotec	5278-5159
ASI RPR/VDRL Control Serum Set	ASI	905005
VIROTROL RPR Panel	Bio-Rad	01400
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Phospho Glo Substrate	KPL	55-60-04
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
10X PBS	Sigma	D1408-500ML
Ethanol	EMD	EX0285-3
Chloroform	Sigma	650498
Tris Buffered Saline	Sigma	T6664-10PAK
Theranos Cartridge	Theranos	
Theranos System	Theranos	

1.1.3 Preparation of Capture Reagent

The cardiolipin, lecithin, and cholesterol were prepared in chloroform at 10%, 40%, and 40% stocks. The stocks were further diluted to 0.03% cardiolipin, 0.9% cholesterol, and 0.2% lecithin using absolute ethanol. All preparations were carried out in a chemical fume hood.

1.1.4 Labeling of Detector Antibody

The mouse anti-human IgG (Southern Biotech, 9040-01) and mouse anti-human IgM (Abd Serotec, 5278-5159) were labeled with alkaline phosphatase according to kit instructions (Dojindo, LK13-10).

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.

2. ASSAY DEVELOPMENT

2.1 Capture Surface Saturation

The capture surface was optimized for saturation by comparing single aspirate coating to multiple aspirate coating. The capture reagent was aspirated and immediately dispensed with no incubation times. The data suggests that 1 aspirate was sufficient for tip saturation.

Table 2: Capture surface saturation

Coat	Sample	AVG	CV	Modulation
1 aspirate	Positive	34049	14%	84
	Weak	16377	15%	40
	Negative	407	5%	1
2 aspirate	Positive	26725	9%	58
	Weak	19508	2%	43
	Negative	458	5%	1
3 aspirate	Positive	26034	13%	41
	Weak	19169	14%	30
	Negative	635	38%	1
4 aspirate	Positive	24950	3%	71
	Weak	14066	11%	40
	Negative	352	35%	1

2.2 Capture Surface Titration

The capture surface was further optimized by titrating the capture reagent. The data show s that 0.03% cardiolipin, 0.9% cholesterol, and 0.2% lecithin had the highest signal and best modulations.

Table 3: Capture surface titration

Dilution	Sample	AVG	CV	Modulation
Neat	Positive	195814	10%	167.1
	Weak	34770	17%	29.7
	Negative	1172	6%	1.0
1:10	Positive	82650	11%	44.1
	Weak	16856	19%	9.0
	Negative	1873	20%	1.0
1:50	Positive	14405	10%	10.5
	Weak	4066	13%	3.0
	Negative	1370	3%	1.0
No Cardiolipin	Positive	1277	29%	3.0
	Weak	593	9%	1.4
	Negative	428	20%	1.0

2.3 Detergents

The effect of detergents added to the coating and assay wash buffers was tested. Phosphate buffered saline was used in the no detergent condition and 1X Enzo wash buffer was used in the detergent condition. Better signal and modulations were observed in the no detergent condition.

Table 4: Detergent in the assay wash buffer

Assay Wash Buffer	Sample	AVG	CV	Modulation
No Detergent	Positive	27406	21%	18.4
	Weak	7794	15%	5.2
	Negative	1488	11%	1.00
Detergent	Positive	2736	21%	7.7
	Weak	800	13%	2.2
	Negative	357	9%	1.0

Table 5: Detergent in the coating wash buffer

Coating Wash buffer	Sample	AVG	CV	Modulation
No Detergent	Positive	29772	17%	78.1
	Weak	14005	13%	36.7
	Negative	381	12%	1.0
Detergent	Positive	12965	16%	21.1
	Weak	6421	23%	10.4
	Negative	615	25%	1.0

2.4 Detection Antibody Titration

The optimum concentration of AP labeled anti-hIgG and anti-hIgM was determined by titrating both the anti-hIgG and anti-hIgM. A concentration of 100 ng/mL of AP labeled anti-hIgG and 25 ng/mL of AP labeled anti-hIgM gave the best assay modulation.

Table 4: Detection antibody titration

anti-hIgG-AP [ng/mL]	anti-hIgM-AP [ng/mL]	Sample	AVG	CV	Modulation
100	100	Positive	205183	4%	176.6
100	100	Weak	31648	5%	27.2
100	100	Negative	1162	12%	1.0
100	50	Positive	174184	15%	220.5
100	50	Weak	25119	11%	31.8
100	50	Negative	790	9%	1.0
100	25	Positive	158155	11%	216.6
100	25	Weak	31694	11%	43.4
100	25	Negative	730	7%	1.0
50	100	Positive	154041	15%	121.6
50	100	Weak	27172	6%	21.5
50	100	Negative	1267	2%	1.0
50	50	Positive	117531	6%	155.4
50	50	Weak	21309	7%	28.2
50	50	Negative	757	12%	1.0
50	25	Positive	100411	1%	171.9
50	25	Weak	14474	18%	24.8
50	25	Negative	584	6%	1.0
25	100	Positive	120954	2%	130.0
25	100	Weak	18903	8%	20.3
25	100	Negative	930	5%	1.0
25	50	Positive	89923	4%	140.9
25	50	Weak	10832	13%	17.0
25	50	Negative	638	6%	1.00
25	25	Positive	49380	5%	105.4
25	25	Weak	10598	15%	22.6
25	25	Negative	468.652	4%	1.0

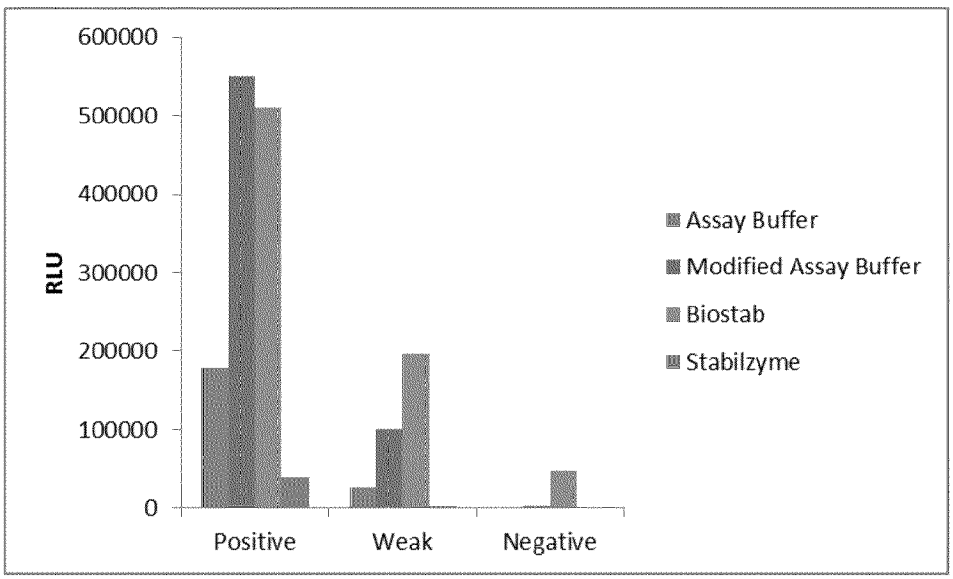
2.5 Detection Antibody Diluent

The optimum detection antibody diluent was determined by comparing standard assay buffer to Theranos AP Conjugate Stabilizer, Biostab, and Stabilzyme. The Theranos AP Conjugate Stabilizer contains 3% BSA, 0.05% NaN_3, 0.1mM Zn^{2+}, 5mM Mg^{2+} in TBS buffer. Based on comparable signal and modulations, the in-house stabilizer was selected.

Table 7: Detection antibody diluents

Dab Diluent	Sample	AVG	CV	Modulation
Assay Buffer	Positive	178273	6%	169
	Weak	26285	7%	25
	Negative	1058	10%	1
Theranos AP Conjugate Stabilizer	Positive	550611	8%	172
	Weak	100228	9%	31
	Negative	3194	8%	1
Biostab	Positive	510718	1%	11
	Weak	196350	8%	4
	Negative	47471	9%	1
Stabilzyme	Positive	39647	12%	69
	Weak	3669	12%	6
	Negative	576	7%	1

Figure 1: Detection antibody diluents



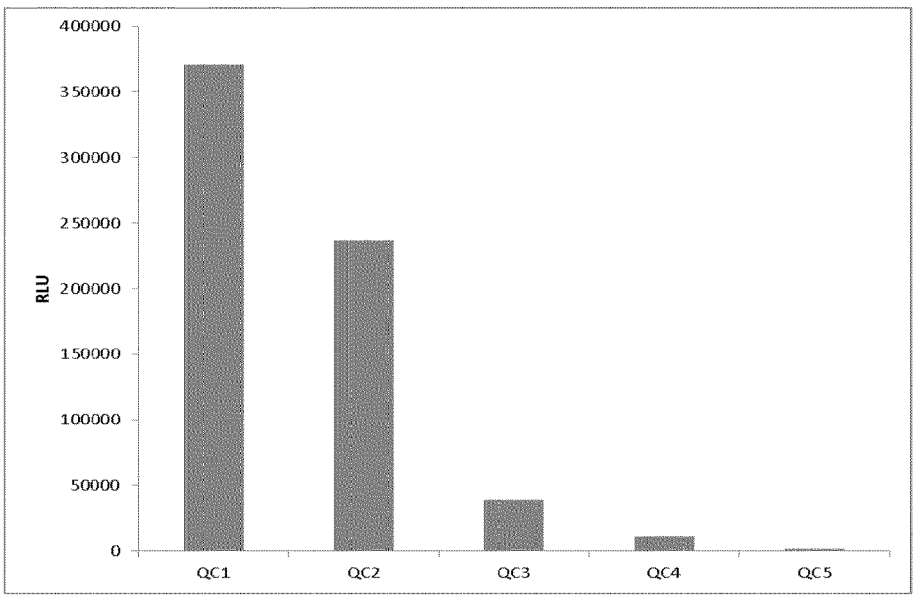
2.6 Quality Controls

Positive, weak, and negative controls were used for assay development and performance evaluation. QC1 and QC2 were positive controls. QC3 and QC 4 were weak controls, and QC5 was a negative control. These same liquid quality controls were used in commercial kits.

Table 8: Quality Controls

Description	Sample	AVG	CV	Modulation
Biorad Level 3	QC1	371289	5%	199.6
ASI Positive	QC2	236997	7%	127.4
ASI Weak	QC3	38628	12%	20.8
Biorad Level 2	QC4	11115	14%	6.0
Biorad Level 1	QC5	1860	3.6%	1.0

Figure 2: Performance of quality controls



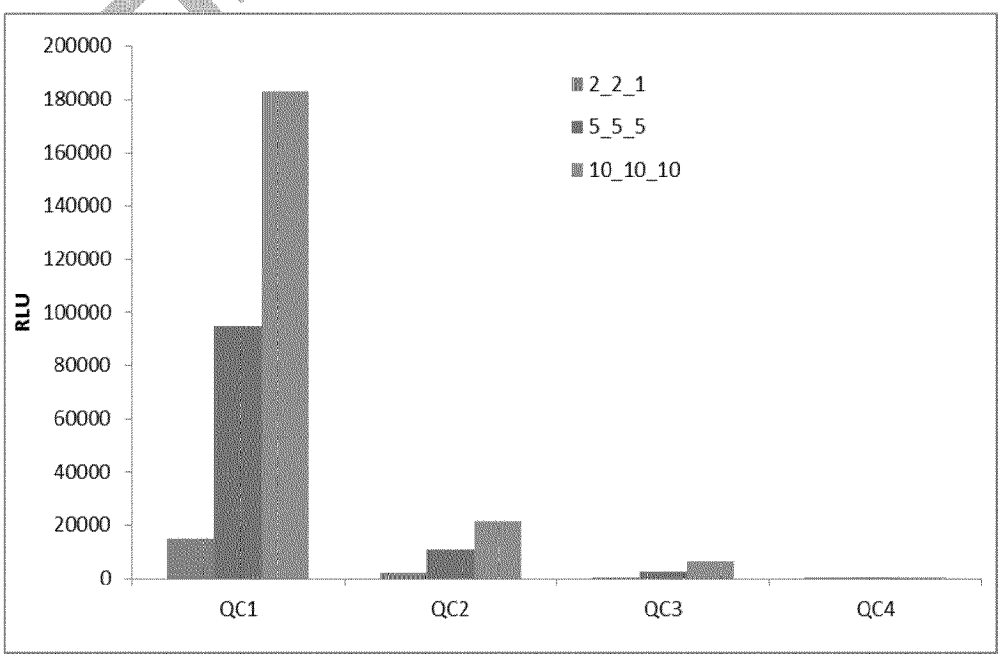
2.7 Edision Protocol Optimization

The assay was tested using the 2-2-1, 5-5-5, and 10-10-10 protocols at a 1:25 sample dilution. The 2-2-1 protocol gave the lowest signal and modulations compared to the 10-10-10 protocol. The 5-5-5 gave equivalent modulations as the 10-10-10 but lower signals. Either the 5-5-5 or 10-10-10 will work for the assay.

Table 9: Effect of assay protocols

Protocol	Description	Sample	AVG	CV	Modulation
2_2_1	Positive	QC1	15233	12%	106
2_2_1	Weak	QC2	2030	14%	14
2_2_1	Weak	QC3	516	12%	4
2_2_1	Negative	QC4	144	9%	1
5_5_5	Positive	QC1	94935	19%	272
5_5_5	Weak	QC2	10766	13%	31
5_5_5	Weak	QC3	2804	18%	8
5_5_5	Negative	QC4	349	8%	1
10_10_10	Positive	QC1	183327	14%	300
10_10_10	Weak	QC2	21638	9%	35
10_10_10	Weak	QC3	6483	21%	11
10_10_10	Negative	QC4	612	13%	1

Figure 3: Effect of assay protocols



2.8 Specificity

Assay specificity was determined by testing normal human serum and plasma, rheumatoid factor, lyme disease, and pregnancy samples. Only 1 out 36 (2.8%) total normal human serum and plasma were positive. Out of 11 pregnancy samples 1 was positive. There were no positives in the 7 rheumatoid factor samples. There were 4 positives in the 10 lyme disease samples. The assay is expected to have false positives due to other disease states. Confirmation of positives with a second clinical test is usually performed in the diagnosis of syphilis. More clinical data is required to evaluate false positives.

Table 10: Normal human serum (n = 16)

Description	Sample	AVG	CV
Control	QC1	25260	11%
Control	QC2	13582	6%
Control	QC3	463	16%
Male	BRH353850	1029	21%
Male	BRH353851	22112	7%
Male	BRH353854	1459	6%
Male	BRH353856	976	10%
Male	BRH353860	1062	7%
Male	BRH353865	124	22%
Male	BRH353866	897	8%
Male	BRH353871	689	15%
Female	BRH353874	2201	8%
Female	BRH353875	2245	7%
Female	BRH353876	1304	12%
Female	BRH353877	2110	2%
Female	BRH353879	1546	9%
Female	BRH353883	1615	17%
Female	BRH353890	3472	1%
Female	BRH353892	3344	5%

Figure 4: Normal human serum (n = 16)

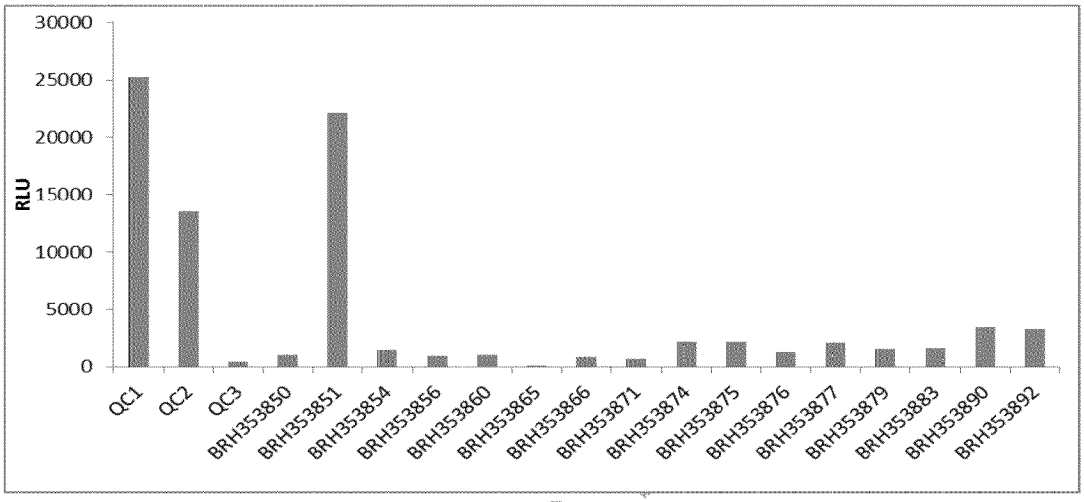


Table 11: Normal human plasma (n = 20)

Description	Sample	AVG	CV
Control	QC1	32419	20%
Control	QC2	19298	12%
Control	QC3	466	12%
S. Plasma	1010181500	891	22%
S. Plasma	1111212100	2251	9%
S. Plasma	1111216100	1365	6%
S. Plasma	1111206200	1715	8%
S. Plasma	1111121600	1256	5%
S. Plasma	1111228100	431	11%
S. Plasma	1111106100	1686	10%
S. Plasma	1111212000	1051	7%
S. Plasma	1111121400	1165	12%
S. Plasma	1111225000	1226	21%
S. Plasma	1011836600	3311	8%
S. Plasma	1011802400	1587	5%
S. Plasma	1011833800	1047	14%
S. Plasma	1111172200	2051	4%
S. Plasma	1011800800	559	14%
S. Plasma	1111102200	731	24%
S. Plasma	1021492900	712	5%
S. Plasma	1111228300	1563	4%
S. Plasma	1011848800	600	8%
S. Plasma	1111224300	946	11%

Figure 5: Normal human plasma (n=20)

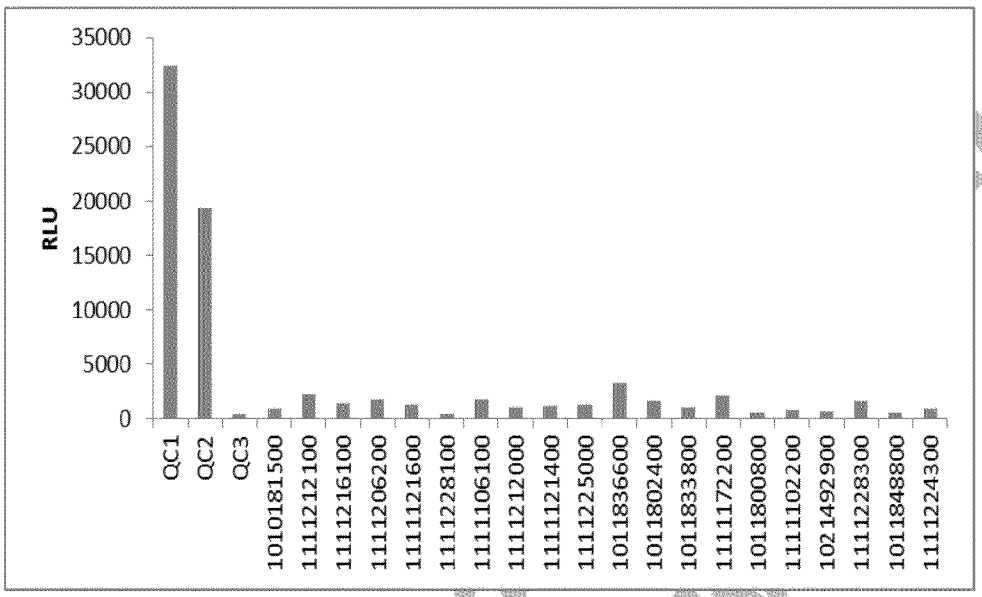


Table 12: Pregnancy samples (n=11)

Sample	AVG	CV
QC1	160529	18%
QC2	145482	7%
QC3	26736	22%
QC4	7888	11%
QC5	1632	19%
P11	902	10%
P12	1142	20%
P14	4652	9%
P15	16650	6%
P16	1077	22%
P18	2149	26%
P19	1071	28%
P20	1084	28%
S11	1100	6%
S16	1000	9%
S17	1621	4%

Figure 6: Pregnancy samples (n=11)

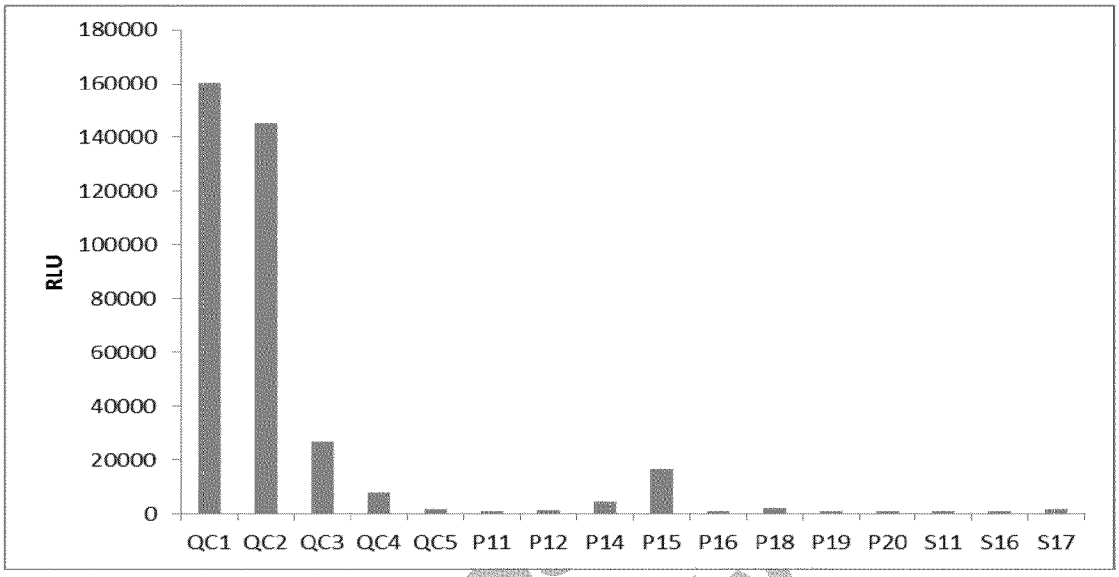


Table 13: Rheumatoid factor samples (n=7)

Sample	AVG	CV
QC1	160529	18%
QC2	145482	7%
QC3	26736	22%
QC4	7888	11%
QC5	1632	19%
SFB7886	5063	16%
SFB7885	5161	10%
SFB7887	2786	14%
SFB7891	3567	22%
SFB7888	4306	16%
SFB7892	5420	22%
SFB7893	4576	6%

Figure 7: Rheumatoid factor samples (n=7)

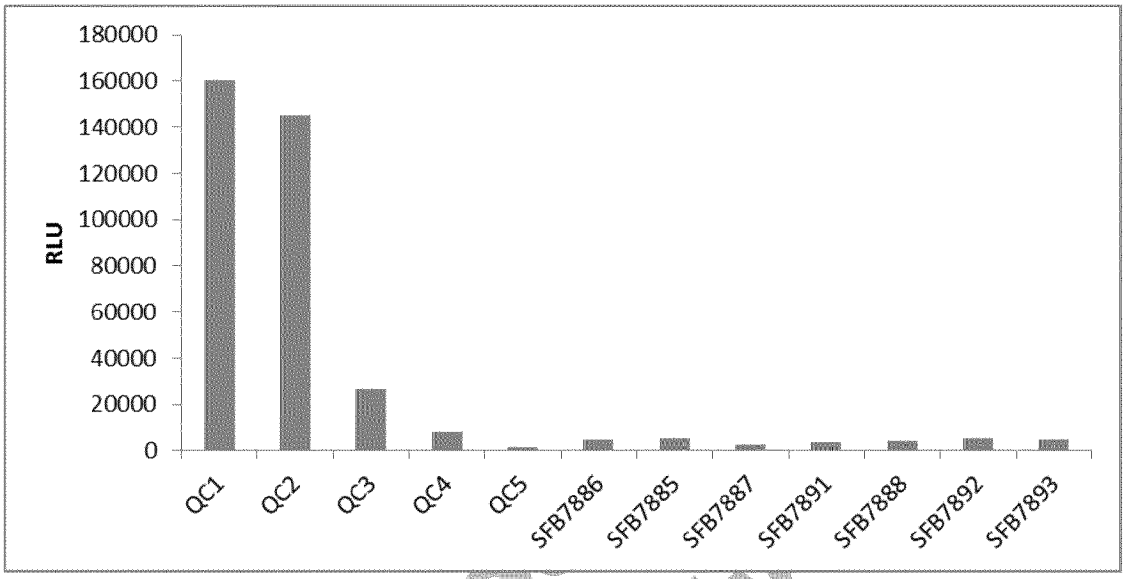
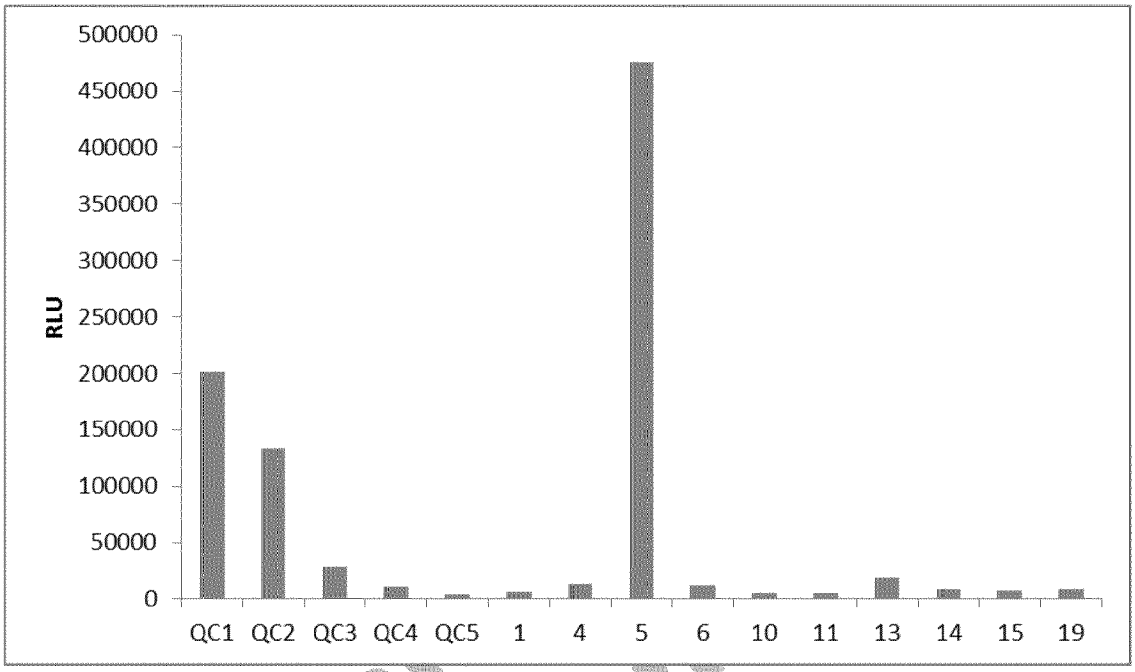


Table 14: Lyme disease samples (n=10)

Sample	AVG	CV
QC1	201571	7%
QC2	133148	29%
QC3	29027	13%
QC4	10721	17%
QC5	4510	7%
1	7119	5%
4	13280	11%
5	475265	3%
6	11790	10%
10	5741	17%
11	5465	11%
13	18504	22%
14	8835	33%
15	8054	19%
19	8914	18%

Figure 8: Lyme disease samples (n=10)



2.8 False Positives

Positive samples were confirmed visually on the VDRL reference test.

Table 15: Theranos and VDRL test

Description	Sample	Theranos	VDRL
Normal Serum	BRH353851	+	+
Pregnancy	P15	+	+
Lyme Disease	4	+	+
Lyme Disease	5	+	+
Lyme Disease	6	+	+
Lyme Disease	13	+	+

2.9 Effect of PSW and Coincubation Protocols

The assay was further tested using a post sample wash and coincubation protocols. The optimum protocol was still the Generic2_25X (10-10-10).

Table 16: Post sample wash and coincubation protocols

Protocol	Sample	AVG	CV	Modulation
Generic2_25X	QC1	968575	13%	169.6
Generic2_25X	QC2	593394	9%	103.9
Generic2_25X	QC3	109381	20%	19.1
Generic2_25X	QC4	33715	26%	5.9
Generic2_25X	QC5	5712	21%	1.0
Generic2_25X_PSW	QC1	2162439	6%	55.7
Generic2_25X_PSW	QC2	1814538	7%	46.7
Generic2_25X_PSW	QC3	546805	16%	14.1
Generic2_25X_PSW	QC4	252720	7%	6.5
Generic2_25X_PSW	QC5	38857	6%	1.0
Generic2_25x_Coincubation_10-10	QC1	53719	11%	32.0
Generic2_25x_Coincubation_10-10	QC2	26181	6%	15.6
Generic2_25x_Coincubation_10-10	QC3	4777	13%	2.8
Generic2_25x_Coincubation_10-10	QC4	3553	8%	2.1
Generic2_25x_Coincubation_10-10	QC5	1680	8%	1.0
Generic2_25x_Coincubation_5-5	QC1	20910	4%	21.6
Generic2_25x_Coincubation_5-5	QC2	13692	14%	14.1
Generic2_25x_Coincubation_5-5	QC3	2599	11%	2.7
Generic2_25x_Coincubation_5-5	QC4	1939	30%	2.0
Generic2_25x_Coincubation_5-5	QC5	968	12%	1.0

2.10 Effect of Sample Dilutions

The effect of sample dilutions was tested at 5x, 25x, and 100x dilutions. The assay worked equally well at 25x and 100x dilutions. Clinical data is required to further evaluate the effect of sample dilutions.

Table 17: The effect of sample dilutions

Sample Dilution	Sample	AVG	CV	Modulation
5x	QC1	1118202	10%	74.7
5x	QC2	913496	6%	61.0
5x	QC3	228411	12%	15.3
5x	QC4	83145	10%	5.6
5x	QC5	14965	8%	1.0
25x	QC1	1080011	13%	138.7
25x	QC2	784966	2%	100.8
25x	QC3	170759	5%	21.9
25x	QC4	55245	17%	7.1
25x	QC5	7786	15%	1.0
100x	QC1	803954	12%	228.0
100x	QC2	540459	6%	153.3
100x	QC3	81233	22%	23.0
100x	QC4	22312	13%	6.3
100x	QC5	3526	15%	1.0

2.11 Stability

Stability was tested at 4C and room temperature. At 4C, the assay was still stable after 7 months. At room temperature, the assay signal started decreasing after the 2nd week. The assay should be stored at 4C for long term stability.

Figure 9: Room Temperature

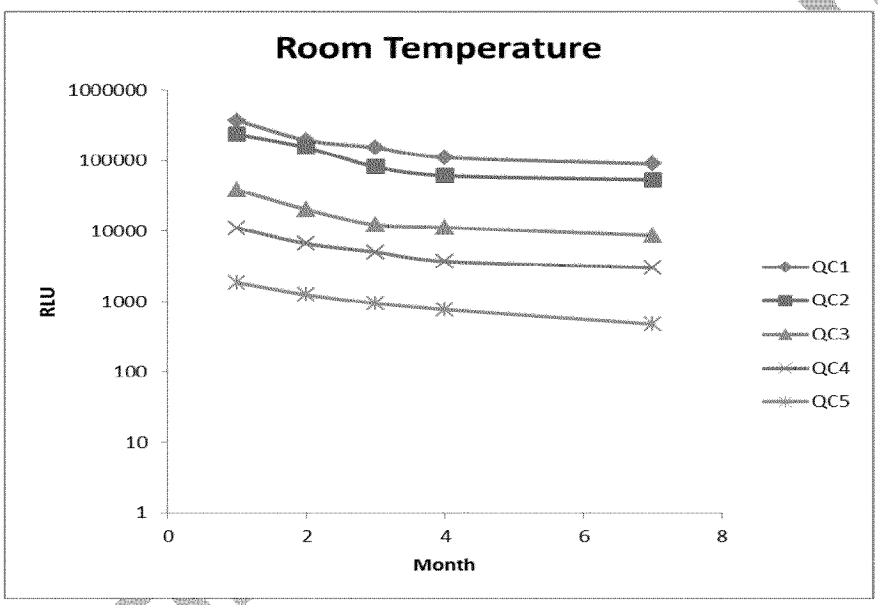
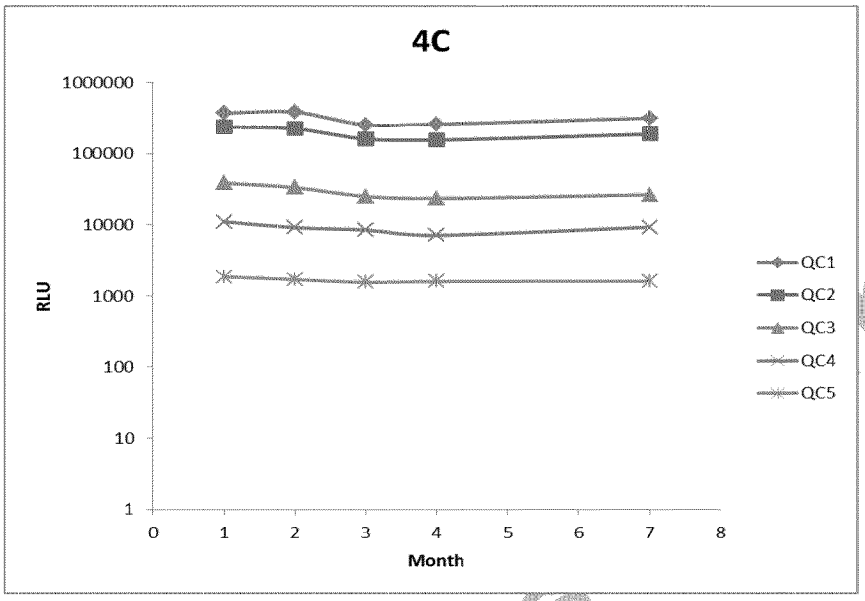


Figure 10: 4C



3. ASSAY SUMMARY

Table 18: Development Summary

Capture Reagent	0.03% cardiolipin, 0.9% cholesterol, 0.2% lecithin
Coating	1 aspirate, no incubation time, PBS wash, fix
Wash Buffer	1X PBS
Assay Buffer	3% BSA in TBS
Edison Protocol	Generic2_25X (10-10-10)
Anti-human IgG-AP	100 ng/mL in Theranos AP Conjugate Stabilizer
Anti-human IgM-AP	25 ng/mL in Theranos AP Conjugate Stabilizer
QC1	Biorad Level 3
QC2	ASI Positive
QC3	ASI Weak
QC4	Biorad Level 2
QC5	Biorad Level 1

4. CLINICAL EVALUATION

Table 19: Clinical Plan

Sample	Test Site	Purpose	Number of Samples	Vendor	Theranos	RPR	VDRL	FTA_ABS
Patient Samples	A	Prospective Study	300	Commercial, Blood Bank	yes	yes	yes	yes
Patient Samples	B	Prospective Study	300	Commercial, Blood Bank	yes	yes	yes	yes
Patient Samples	C	Prospective Study	300	Commercial, Blood Bank	yes	yes	yes	yes
Positive Samples	Theranos	Retrospective Study	300	Commercial, Blood Bank	yes	yes	yes	yes
Diagnosed Samples	Theranos	Primary-Untreated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Diagnosed Samples	Theranos	Secondary-Untreated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Diagnosed Samples	Theranos	Latent-Untreated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Diagnosed Samples	Theranos	Primary-Treated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Diagnosed Samples	Theranos	Secondary-Treated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Diagnosed Samples	Theranos	Latent-Treated	25	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Positive Preganancy Samples	Theranos	Retrospective Study	150	Commercial, Blood Bank, CDC	yes	yes	yes	yes
Drug users	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
ANA Positive	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
RF Positive	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
Lyme Disease	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
HSV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
CMV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
EBV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
HAV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
HIV 1 & 2	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
HTLV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
Heterophile	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
HCV	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
Anti-HBs	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes
Other STDs (GC, Chlamydia, HPV, Trichomonas)	Theranos	Cross Reactivity	25	Commercial, Blood Bank	yes	yes	yes	yes

References:

1. Evaluation of INNO-LIA Syphilis assay as a confirmatory test for syphilis. H.J. Hagedorn et al. http://jcm.asm.org/cgi/reprint/40/3/973
   (289 seronegative sera, 219 seropostiive sera, 23 indeterminate sera)
2. Evaluation of three serological tests manufactured in Belarus for the diagnosis of syphilis. I. Shimanskaya et al.
   http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1056
   (number of samples tested, n = 392)
3. Characterization of Treponema pallidum particle agglutination assay-negative sera following screening by treponemal total antibody enzyme immunoassays. P.A. Maple et al. http://cvi.asm.org/cgi/reprint/17/11/1718
   (number of samples tested, n = 226)
4. Evaluation of the Bio-Rad BioPlex 2200 syphilis multiplex flow immunoassay for the detection treponemal antibodies. E. Gomez et al. http://cvi.asm.org/cgi/reprint/17/6/966
   (number of samples tested, n = 1008)
5. Treponema-specific tests for serodiagnosis of syphilis: comparative evaluation of seven assays. M.J. Binnicker et al. http://jcm.asm.org/cgi/content/abstract/49/4/1313
   (number of samples tested, n = 303)
6. Diagnostics Direct, Syphilis Health Check 510k:
   http://www.accessdata.fda.gov/cdrh_docs/pdf10/K102400.pdf
7. Trinity Biotech, CAPTIA Syphilis-G 510k,
   http://www.accessdata.fda.gov/cdrh_docs/pdf/K001525.pdf