Rubella IgM Assay Development Report

Theranos, Inc.

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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"]

Rubella also known as German measles is a disease caused by the Rubella virus. This disease is often mild and the attacks are often unnoticed. The disease can last one to three days. Children recover more quickly than adults. Infection of the mother by Rubella virus during pregnancy can be serious because if the mother is infected within the first 20 weeks of pregnancy, the child may be born with congenital rubella syndrome (CRS), which entails a range of serious incurable illnesses. Spontaneous abortion occurs in up to 20% of cases.

IgM antibodies specific for Rubella are present in people recently infected by Rubella virus but these antibodies can persist for over a year and a positive test result needs to be interpreted with caution. The presence of these antibodies along with the characteristic rash confirms the diagnosis.

This assay is designed to qualitatively determine Rubella IgM in human plasma and serum.

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

The following assay has been used in house as the predicate method:

- Siemens Immulite 2000 Rubella IgM

(Qualitative Detection of Rubella IgM in Serum or Plasma)

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

Rubella antigen coated surface serves as the capture surface for the Rubella IgM antibody assay. The sample (plasma or serum) is diluted and then incubated on the capture surface for 10 minutes, the surface is washed, and then an alkaline phosphatase(AP)-labeled anti-human IgM antibody is incubated on the surface for 10 minutes. After the detection antibody incubation, another washing cycle is performed and the alkaline phosphatase substrate is incubated on the surface for 10 minutes, and the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Carbonate-Bicarbonate buffer	Sigma	C3041
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Theranos Alk Phos Antibody Conjugate Stabilizing Buffer (0.1 mM Zn^{2+}, 5 mM Mg^{2+} in 3% BSA with 0.05 % Sodium Azide in 50mM TBS pH 8.0)	Theranos	
StartingBlock™ (TBS) Blocking Buffer	Thermo Scientific	37542
Theranos Substrate (in house)	Theranos	
AXSYM RUBELLA M CTL	Abbott Laboratories, Inc	04B4610

Table [SEQ Table * ARABIC]: Antigens

Antigen #	Vendor	Product Code	Description
1	Genway	11-511-248285	Rubella Antigen (HPV-77) Native Protein
2	Mybiosource	MBS318660	Rubella RSVP (Rubella Spike Viral Protein) Antigen Antigen

Table [SEQ Table * ARABIC]: Detection Antibodies

DAb #	Supplier	Catalog #	Description
1	AbD Serotec	5278-5159	Mouse Anti Human IgM
2	Genway	25-787-278105	Goat Anti-Human IgM (u chain specific)
3	Novus	NB500-468	Human IgM, Fc Fragment Antibody (CH2)

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

2.1 Capture Surface: Antigen Screen (MTP)

Clinical samples were screened on Siemens Immulite 2000 Rubella IgM kit to determine positive and negative Rubella IgM samples. Commercially available antigens were then screened with these positive and negative samples on a microtiter plate (MTP).

Different assay formats were tested on the microtiter plate (MTP) screen. The first format that was tested was the sandwich ELISA; ultraavidin coating of the biotinylated Anti-human IgM (with SH group) as a capture and using the antigen conjugated with alkaline phosphatase as detection. Another format that was tested was the direct coat method where each antigen was prepared in Carbonate-Bicarbonate buffer for direct coating on the Nunc-384 well plates. All antigens were coated at 10 ug/ml. A sample diluted 1:50 was added to the surface followed by wash steps. The detection antibody (D#1,2,3) concentration was 100 ng/ml in 3% BSA in TBS blocking buffer. According to the literature, capture # 2 is supposed to be more specific for Rubella IgM since it is a recombinant antigen where the nucleocapsid is removed and hence reduces the non-specificity issue. The modulation for sandwich elisa format was good and the modulation for direct coat was better. Both Antigens #1 and #2 showed modulation between positive and negative samples but antigen # 2 had a much better modulation because the antigen is specific to IgM and hence Antigen #2 was chosen for further development. The data is summarized in table 4

Table [SEQ Table * ARABIC]: Capture Surface Screen (MTP)

Antigen #	**1**		**2**	
[Antigen], ug/ml	**10ug/ml**		**10ug/ml**	
Sample#	**Mean Value**	**CV%**	**Mean Value**	**CV%**
Negative: #1	12054	7	1525	28
#2	13503	22	1546	26
Mean Negative	**12778**		**1535**	
Positive: #3	159369	15	86932	7
Mean Positive	**159369**		**86932**	
Negative Control	6959	25	755	20
Positive Control	48597	15	24838	16
Positive control/negative control	7		33	
Positive control/ Mean normal	4		16	
Mean positive /Mean normal	12		57	

2.2 Effect of different Detection Antibodies on Theranos System

Antigen #1 and Antigen 2 was tested with the different detection antibodies. These antigens were screened on the Theranos system at 10 µg/mL direct coat. Three different detection antibodies were tested and the concentration was 100 ng/ml in StabilZyme AP buffer. Clinical samples were tested on the above mentioned predicate method, and then used as the test set on the Theranos system. A sample dilution of 1:50 was manually done and the protocol was run at the 10, 10,10 minutes incubation time.

There was a significant improvement in terms of modulation between the three detection antibodies. Dab # 3 gave the best modulation with much less background and was selected for further optimization. Dab # 2 is a possible back-up detection antibody; the data is summarized in Tables 5 and 6.

Table [SEQ Table * ARABIC]: Detection Antibodies on Theranos System with Antigen 1

Dab	**1 at 100ng/ml**		**2 at 100ng/ml**		**3 at 100ng/ml**	
[Antigen # 1], 10 ug/ml	**10ug/ml**		**10ug/ml**		**10ug/ml**	
Sample#	**Mean Value**	**CV%**	**Mean Value**	**CV%**	**Mean Value**	**CV%**
Negative: #1	63019	18	311909	6	12054	7
#2	80257	13	405213	3	13503	22
Mean Negative	**71638**		**358561**		**12778**	
Positive: #3	536963	16	2551660	6	159369	15
Mean Positive	**536963**		**2551660**		**159369**	
Negative Control	13307	17	46829	8	6959	25
Positive Control	239335	12	1599134	15	48597	15
Positive control/negative control	18		34		7	
Positive control/ Mean normal	3		4		4	
Mean positive /Mean normal	7		7		12	

Table [SEQ Table * ARABIC]: Detection Antibodies on Theranos System with Antigen 2

Dab	**1 at 100ng/ml**		**2 at 100ng/ml**		**3 at 100ng/ml**	
[Antigen # 2], 10ug/ml	**10ug/ml**		**10ug/ml**		**10ug/ml**	
Sample#	**Mean Value**	**CV%**	**Mean Value**	**CV%**	**Mean Value**	**CV%**
Negative: #1	57021	13	48028	14	1525	28
#2	49754	14	76181	19	1546	26
Mean Negative	**53388**		**62105**		**1535**	
Positive: #3	443127	30	2514412	6	86932	7
Mean Positive	**443127**		**2514412**		**86932**	
Negative Control	2335	21	9059	14	755	20
Positive Control	159804	16	1215569	18	24838	16
Positive control/negative control	68		134		33	
Positive control/ Mean normal	3		20		16	
Mean positive /Mean normal	8		40		57	

2.3 Capture Antigen Surface Titration on Theranos System[TC " Capture Antigen Surface Antigen " \f C \l "1"]

The direct coat antigen surface was titrated at levels: 10, 5 and 2.5 µg/mL. Table 5 summarizes the results of Antigen #2 and Detection Antibody # 3 at 100ng/mL. 5 µg/mL provides the best modulation between the pooled positive and pooled normal clinical samples as well as between positive and negative control and thus was finalized as the capture antigen surface concentration. The pooled rubella IgM positive clinical sample had a high rubella IgM concentration and hence a 5 point curve was made with serial dilutions between pooled positive and pooled negative.

Table [SEQ Table * ARABIC]: Capture Antigen Surface Titration

	10 µg/mL		5 µg/mL		2.5 µg/mL	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	1005	27	1030	12	885	27
Positive Control	24112	29	52153	10	33856	17
Point 1 (Pooled Negative)	2431	16	2145	14	2326	19
Point 2 (1:8)	16836	11	28886	13	17839	9
Point 3 (1:4)	27467	25	46844	8	30367	21
Point 4 (1:2)	53738	17	92274	18	60469	6
Point 5 (Pooled positive)	132100	9	202516	12	151514	8
Positive control/negative control	24		51		38	
Positive control/ Pooled Negative	10		24		15	
Pooled positive /Pooled Negative	54		94		65	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative
*Point 3 : 1 part of pooled positive and 3 parts of pooled negative
*Point 4 : 1 part of pooled positive and 1 part of pooled negative

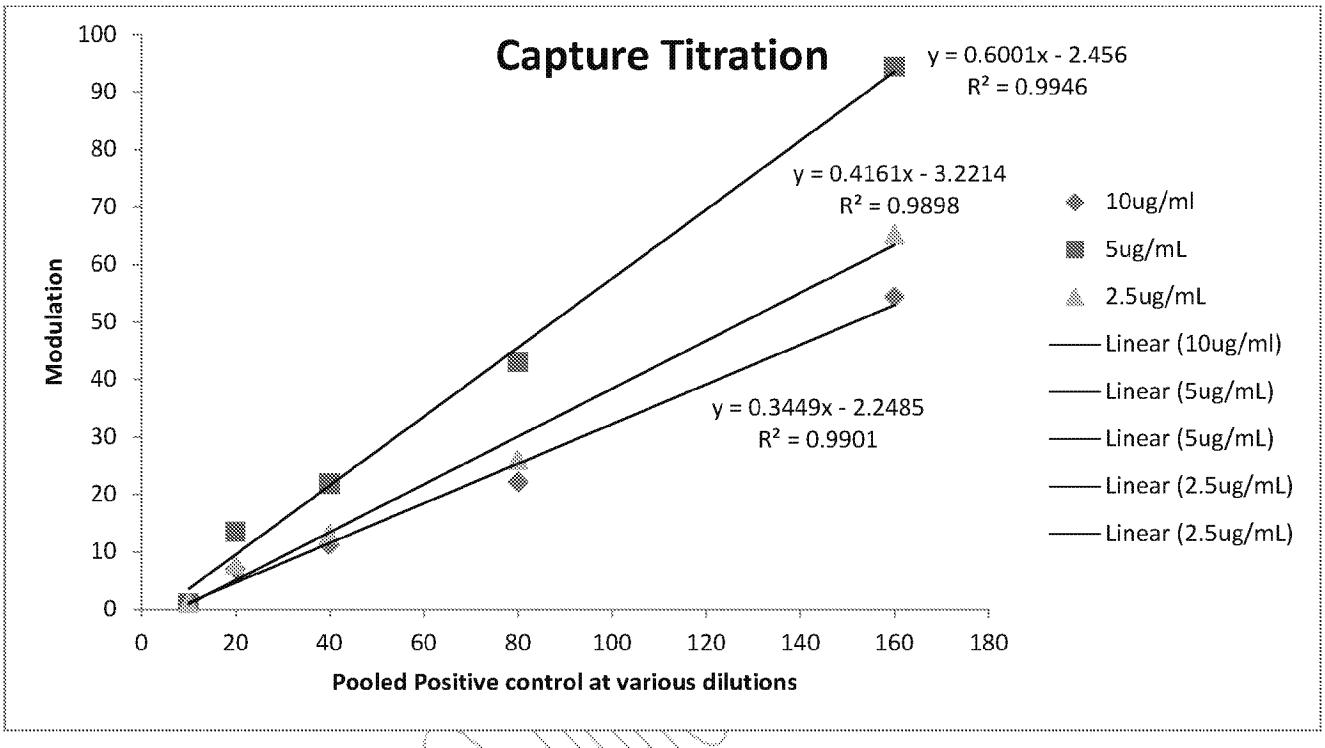

Figure [SEQ Figure * ARABIC] : Capture Antigen Surface Titration (10, 5 and 2.5 µg/mL)

2.4 Detection Titration on Theranos System

[TC " Capture Antigen Surface Antigen " \f C \l "1"]

The AP conjugated detection antibody was titrated at levels 100, 50 and 25ng/mL. All the three levels gave good modulation. The best modulation was achieved with 100 ng/mL but 25ng/mL gave the second best modulation with lower RLU signal and hence 25ng/mL was chosen for further optimization. Data is summarized in Table 8

Table [SEQ Table * ARABIC]: Detection Conjugate Titration

	100ng/ml		50ng/ml		25ng/ml	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	1107	16	693	13	476	33
Positive Control	48715	17	26979	12	14248	21
Point 1 (Pooled Negative)	2172	12	1445	15	729	13
Point 2 (1:8)	20325	8	12177	20	6866	14
Point 3 (1:4)	42863	13	24870	13	12493	21
Point 4 (1:2)	89448	24	52356	15	27112	15
Point 5 (Pooled positive)	181043	8	94198	15	57390	11
Positive control/negative control	44		39		30	
Positive control/ Pooled Negative	22		19		20	
Pooled positive /Pooled Negative	83		65		79	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative
*Point 3 : 1 part of pooled positive and 3 parts of pooled negative
*Point 4 : 1 part of pooled positive and 1 part of pooled negative

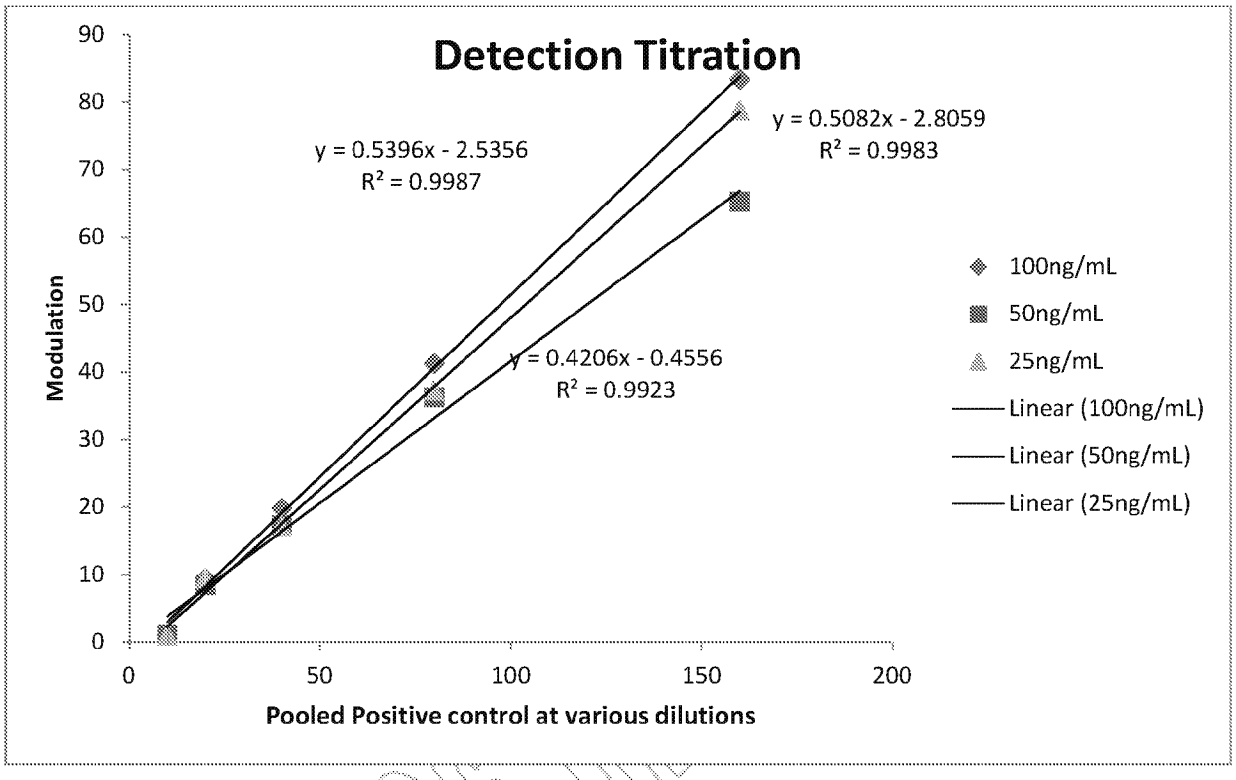

Figure [SEQ Figure * ARABIC] : Detection Conjugate Titration (100, 50, 25ng/ml)

2.5 Effect of Detection Conjugate Stabilizer

Two commercial and one in house formulated alkaline phosphatase stabilizers were tested as detection antibody diluents, with the anti-human IgM Dab at 25 ng/mL. The samples were diluted 1:50 in Surmodics (Assay Diluent). Signal modulation was best with Theranos AP conjugate stabilizer (In house) and it was thus used for further optimization. Table 9 and figure 3 summarizes the results.

Table [SEQ Table * ARABIC]: Effect of Detection Conjugate Stabilizer

	In house AP stabilizer		Bio Stab		Stabilzyme	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	830	17	1907	22	1556	36
Positive Control	66237	11	63083	11	13375	15
Point 1 (Pooled Negative)	2531	14	3703	8	1367	21
Point 2 (1:8)	29557	11	25012	10	6910	11
Point 3 (1:4)	61696	13	55986	4	14244	10
Point 4 (1:2)	111156	15	87900	12	22879	11
Point 5 (Pooled positive)	197029	16	175903	15	47693	12
Positive control/negative control	80		33		9	
Positive control/ Pooled Negative	26		17		10	
Pooled positive /Pooled Negative	78		48		35	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative
*Point 3 : 1 part of pooled positive and 3 parts of pooled negative
*Point 4 : 1 part of pooled positive and 1 part of pooled negative

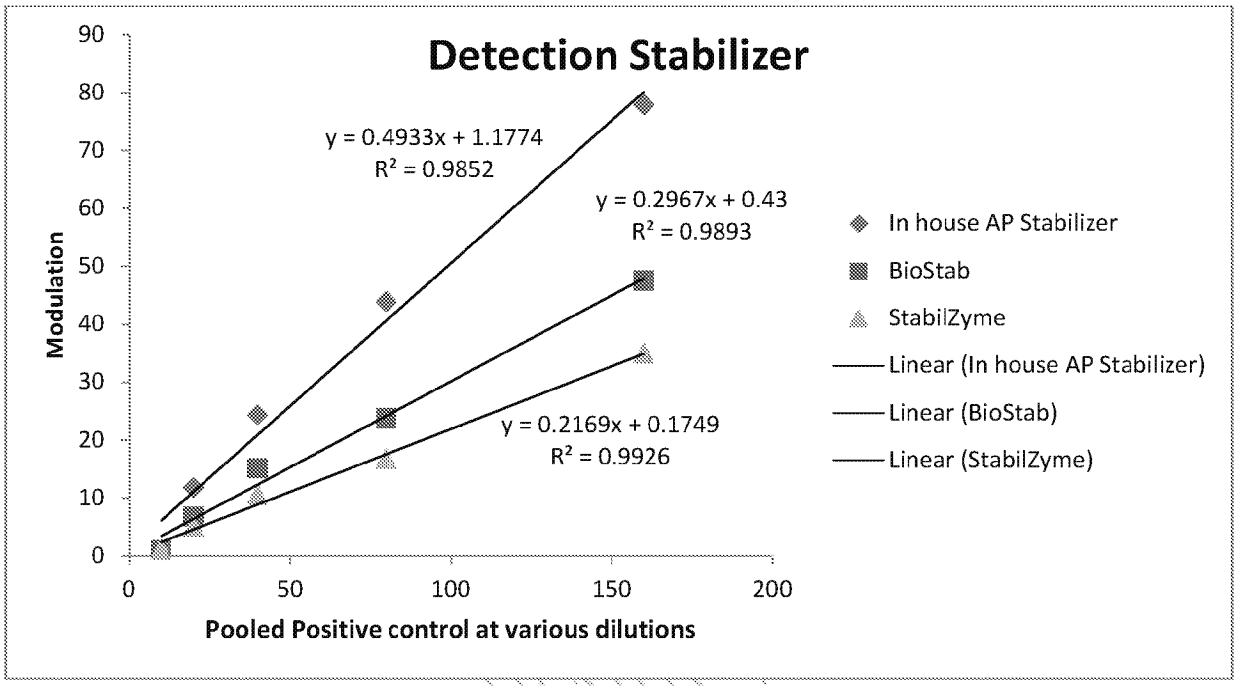

Figure [SEQ Figure * ARABIC] : Effect of detection conjugate stabilizer

2.6 Effect of Assay Diluent

Two commercially available blockers (StartingBlock™ and Surmodics) and one in-house blocking buffer were tested as diluents for the assay. Data was compared to the control diluent which was the blocking buffer consisted of 3% BSA and 0.05% sodium azide in TBS. There was not a lot of difference in modulation between each diluent and hence Starting Block was chosen for further optimization because it had low background. The data is summarized in Table 10.

Table [SEQ Table * ARABIC]: Effect of Assay Diluent

	In house Blocking Buffer		Starting Block		Surmodics	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	522	24	433	17	830	17
Positive Control	78987	14	49073	9	66237	11
Point 1 (Pooled Negative)	3369	8	2556	12	2531	14
Point 2 (1:8)	33728	23	20445	34	29557	11
Point 3 (1:4)	51224	3	45366	13	61696	13
Point 4 (1:2)	118188	12	84681	10	111156	15
Point 5 (Pooled positive)	235608	23	168641	10	197029	16
Positive control/negative control	151		113		80	
Positive control/ Pooled Negative	23		19		26	
Pooled positive /Pooled Negative	70		66		78	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative
*Point 3 : 1 part of pooled positive and 3 parts of pooled negative
*Point 4 : 1 part of pooled positive and 1 part of pooled negative

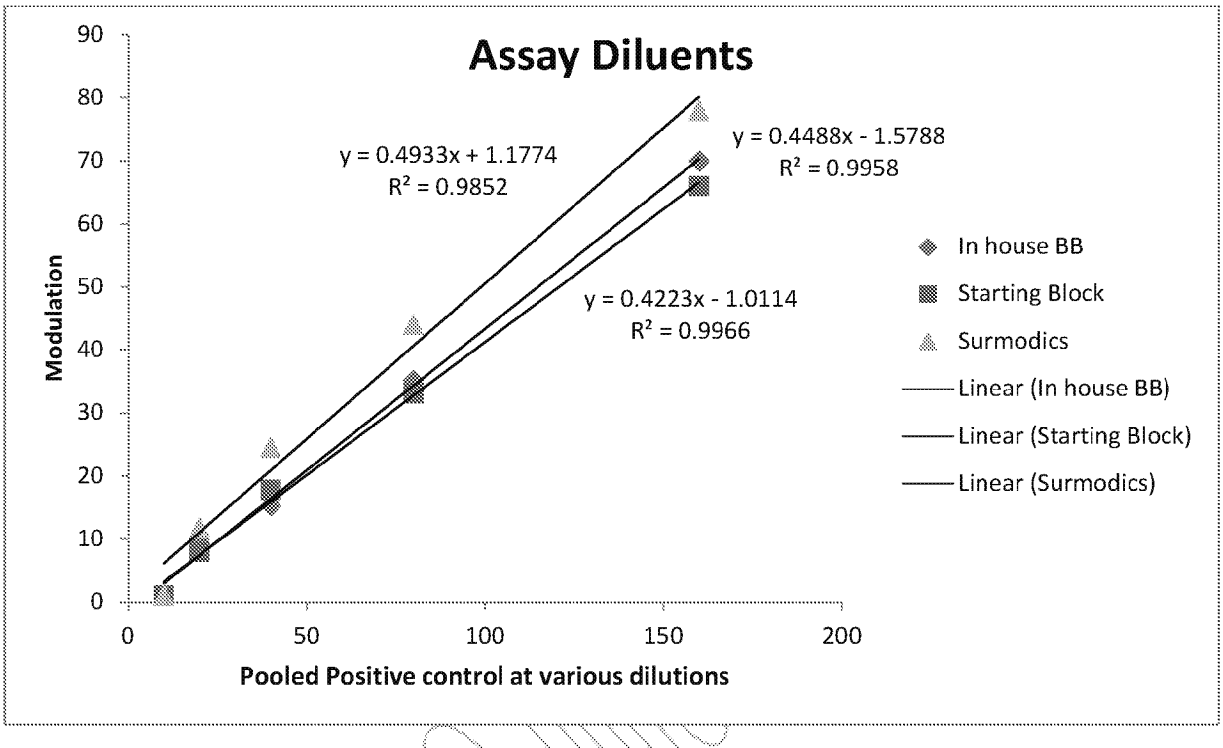

Figure [SEQ Figure * ARABIC] : Effect of different assay diluents

2.7 Effect of Sample Dilution[TC “Effect of Sample dilution" \f C \l "1"]

The effect of sample dilution was tested with final sample dilution factors of 1:50, 1:100 and 1:500 PSW into 3% BSA in TBS blocking buffer. Modulation between pooled positive and negative sera was best at 100 fold sample dilution. However, 50 fold sample dilution is also reasonably good. We can observe of a greater reduction in the signal from negative samples compared to the reduction in signal from the positive samples. Results are summarized in Table 13.

Table [SEQ Table * ARABIC]: Effect of sample dilution

	50x_PSW		100x_PSW		25x_PSW	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	554	14	464	14	259	26
Positive Control	81086	9	40373	10	10903	12
Point 1 (Pooled Negative)	3268	7	1792	14	830	10
Point 2 (1:8)	33731	14	18560	11	5136	10
Point 3 (1:4)	59615	5	34254	15	7861	31
Point 4 (1:2)	99861	27	73486	6	16701	17
Point 5 (Pooled positive)	196119	50	137336	4	34865	8
Positive control/negative control	146		87		42	
Positive control/ Pooled Negative	25		23		13	
Pooled positive /Pooled Negative	60		77		42	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative
*Point 3 : 1 part of pooled positive and 3 parts of pooled negative
*Point 4 : 1 part of pooled positive and 1 part of pooled negative

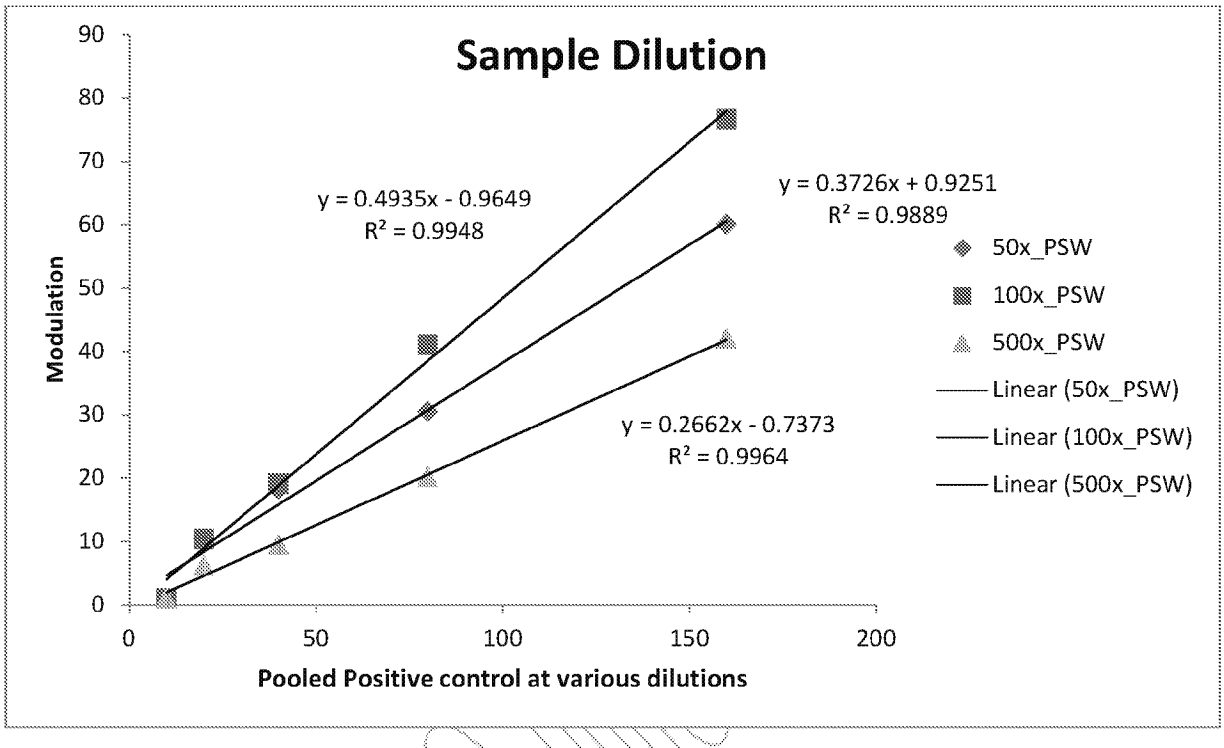

Figure [SEQ Figure * ARABIC] : Effect of sample dilution

2.8 Effect of changing reagent incubation time [TC "Effect of changing reagent incubation time" \f C \l "1"]

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times respectively of 10, 10, 10; 5, 5, 5 and 2, 2, 1 minutes. 10, 10, 10 minutes incubation time gave the best modulation and was thus chosen for further optimization.

Table [SEQ Table * ARABIC]: Effect of reagent incubation time

	10, 10, 10		5, 5, 5		2, 2, 1	
Sample ID	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Negative Control	464	14	237	17	175	13
Positive Control	40373	10	10981	18	936	14
Point 1 (Pooled Negative)	1792	14	851	22	201	14
Point 2 (1:8)	18560	11	4492	5	502	8
Point 3 (1:4)	34254	15	8586	11	861	21
Point 4 (1:2)	73486	6	15885	18	1689	17
Point 5 (Pooled positive)	137336	4	28981	17	2775	23
Positive control/negative control	87		46		5	
Positive control/ Pooled Negative	23		13		5	
Pooled positive /Pooled Negative	77		34		14	

*Point 2 : 1 part of pooled positive and 7 parts for pooled negative

*Point 3 : 1 part of pooled positive and 3 parts of pooled negative

*Point 4 : 1 part of pooled positive and 1 part of pooled negative

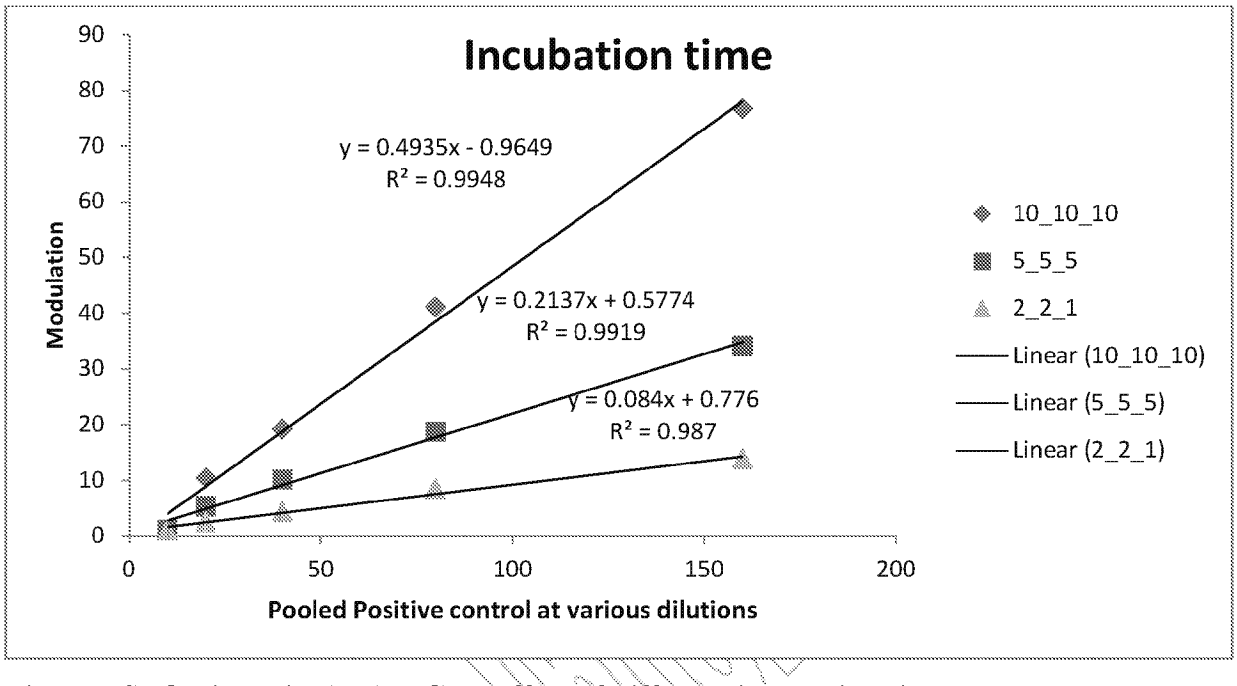

Figure [SEQ Figure * ARABIC] : Effect of different incubation times

2.9 HAMA and Rf Positive Sample Testing

5 HAMA positive and 9Rf positive sera obtained from a commercial source were tested on the Theranos Rubella IgM Assay and on the predicate method. All the HAMA samples were negative but 2 out of the 9 RF sera gave high RLUs (RLU values were close to low positives) but they gave out as negative on the predicate method. The two false positive RF sera (RF3, RF9) were tested with various assay diluents and the RLU values were still high. The next step was to test the effect of HBR in assay diluent to reduce the false positives. The data is shown in table 13 and 14.

Table [SEQ Table * ARABIC]: HAMA and Rf positive sample screen

Samples	**Inter-Cartridge**		**Siemens**
	Mean RLU	**CV%**	**Immulite**
HAMA positive			
H1	678	23	NEG
H2	2692	12	NEG
H3	993	44	NEG
H4	780	13	NEG
H5	1722	23	NEG
RF Positive			
RF 1	6533	4	NEG
RF 2	580	11	NEG
RF 3	33766	54	NEG
RF 4	960	9	NEG
RF 5	7489	29	NEG
RF 6	16167	15	NEG
RF 7	5877	15	NEG
RF 8	488	29	NEG
RF 9	44954	16	NEG
Mean Negative	**2964**		

Table [SEQ Table * ARABIC] : Effect of different Assay diluents on RF serum

Assay Diluent	Inter-Cartridge Mean RLU RF 3	CV%	Inter-Cartridge Mean RLU RF 9	CV%
Low cross buffer	43308	12	31597	34
Sea Block	115857	16	129359	6
In house BB	130898	18	126211	3
Surmodics	70160	11	59014	18
Starting Block	33766	22	44954	16
Pierce Protein Free	30809	29	34587	17
Super Block	73574	5	49130	20

2.10 Effect of HBR in assay diluent (RF troubleshooting)

The effect of adding Heterophilic blocking reagent (HBR) to the assay diluent was tested to reduce the false positives of RF samples. Adding HBR to the assay diluent reduced the RLU values of Rf positive by many folds as seen in table 15. It was decided to include HBR in the diluent since it does help in mitigating any non-specific binding and HBR titration was done as a following step.

Table [SEQ Table * ARABIC]: Effect of HBR in assay diluent

Sample	Control diluent (Starting Block)		Starting block plus 400 µg/mL HBR	
	Inter-Cartridge RLU			
	Mean	CV%	Mean	CV%
RF 3	33766	22	1121	12
RF 9	44954	16	1675	14
Pooled Negative	729	13	550	16
Pooled positive	57390	11	17487	16
RF Sera/ Pooled Negative	54		3	
Pooled positive /pooled normal	79		32	

2.11 HBR titration in diluent

Clinical samples as well as the RF samples were assayed at 400, 100, 50, 25, 15 and 10 µg/mL of HBR spiked into the assay diluent (starting block), the control data had the assay diluent without HBR. Data is summarized in Table 9 and 10. 100 µg/mL of HBR was finalized as the final concentration of HBR in the assay diluent because the modulation was approximately the same throughout the different concentration of HBR and the value of RF started increasing again with lower HBR levels.

Table [SEQ Table * ARABIC]: HBR titration in assay diluent (part 1)

	Control diluent (Starting Block)		Starting block plus 400 µg/mL HBR		Starting block plus 100 µg/mL HBR	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
RF 3	33766	22	1121	12	1270	12
RF 9	44954	16	1675	14	1689	1
Pooled Negative	729	13	550	16	634	25
Pooled positive	57390	11	17487	16	20059	21
RF Sera/ Pooed Negative	54		3		2	
Pooled positive /pooled normal	79		32		32	

Table [SEQ Table * ARABIC] : HBR titration in assay diluent (part 2)

	Starting block plus 50 µg/mL HBR		Starting block plus 25 µg/mL HBR		Starting block plus 15 µg/mL HBR		Starting block plus 10 µg/mL HBR	
Control	**Inter-Cartridge RLU**							
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
RF 3	1266	12	1376	13	1580	14	4163	5
RF 9	1738	8	1736	19	2074	21	3372	36
Pooled Negative	627	18	505	28	510	16	883	18
Pooled positive	22823	22	23462	20	21717	15	22276	15
RF Sera/ Pooed Negative	2		3		4		4	
Pooled positive /pooled normal	36		36		43		25	

2.12 Detection Titration after adding HBR in assay Diluent

The effect of adding Heterophilic blocking reagent (HBR) to the assay diluent was tested to reduce the false positives of RF but it also reduced the modulation with pooled positive and pooled negative. Hence a detection titration was performed to see if it will be possible to increase the modulation. 5 clinical negative, 5 clinical positives and 5 RF sera were run on the following conditions (5ug/ml Direct coat of antigen # 2, 100x PSW, Starting Block +100ug/mL HBR), The AP conjugated detection antibody was titrated at levels 50 and 25ng/mL. 50ng/mL gave a better modulation and was thus chosen as the final condition.

Table [SEQ Table * ARABIC] : Detection Titration

	25ng/ml		50ng/ml	
Sample ID	**Inter-Cartridge RLU**			
	Mean	**CV%**	**Mean**	**CV%**
Pooled RF	1147	16	2713	18
Pooled Negative	782	15	1415	17
Pooled Positive	10125	20	26552	18
Pooled Rf/Pooled Negative	1		2	
Pooled Positive/Pooled Negative	13		19	

2.13 Clinical Sample Correlation and Cut off Determination

Normal donor plasma (N=30) were obtained and tested on Rubella IgM Siemens Immulite 2000 (Predicate method) and in the Theranos System. The Theranos cutoff value was determined by taking the mean RLU of the normal samples plus 5 times the standard deviation of the 40 normal samples (Table 19). The sample RLU divided by the cutoff value yields the Antibody Index. The following criteria was applied to categorize the result as positive (red), negative (green) or borderline (yellow).

Ab Index > 1.1
Ab Index > 0.9, < 1.1
Ab Index < 0.9

Table [SEQ Table * ARABIC]: Rubella IgM assay : Cut off Determination

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	Theranos Result
1	678	19	0.12	NEG
2	784	18	0.13	NEG
3	599	23	0.10	NEG
4	1659	14	0.29	NEG
5	3119	13	0.54	NEG
6	1512	13	0.26	NEG
7	1077	15	0.19	NEG
8	1296	14	0.22	NEG
9	674	12	0.12	NEG
10	753	14	0.13	NEG
11	398	21	0.07	NEG
12	354	16	0.06	NEG
13	374	16	0.06	NEG
14	866	19	0.15	NEG
15	430	15	0.07	NEG
16	1703	21	0.29	NEG
17	545	16	0.09	NEG
18	1049	19	0.18	NEG
19	563	10	0.10	NEG
20	520	28	0.09	NEG
21	5051	16	0.87	NEG
22	460	23	0.08	NEG
23	1355	19	0.23	NEG
24	510	17	0.09	NEG
25	556	27	0.10	NEG
26	628	19	0.11	NEG

27	748	15	0.13	NEG
28	433	18	0.07	NEG
29	1470	17	0.25	NEG
30	550	18	0.09	NEG
MEAN	**1024**			
CUT OFF (mean+5SD)	**5812**			

Out of the 30 normals tested all were negative on the Theranos assay based on the aforementioned cutoff computation. These same samples were all negative on the predicate method data showed excellent correlation with the Theranos result (Table 20).

For Clinical sample correlation, a total of 40 clinical samples were tested on Theranos assay and on 1 FDA approved commercial kit and the results are summarized in table 20.

All except 1 clinical sample (sample # 31) showed excellent correlation. One sample which came out as positive on the predicate method showed negative on Theranos System, but from the RLU value, it is noticed that sample # 31 could have been a false positive as after adding the HBR the value of RLU dropped drastically (from ~ 20,000 to ~1860) but none of the other positives had the same issue; hence it can be concluded that sample # 31 was a false positive and also showing that the assay has excelled specificity to Rubella IgM .

Table [SEQ Table * ARABIC]: Clinical Samples on Theranos vs. Commercial kits

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	Theranos Result	Siemens Immulite 2000
1	678	19	0.12	NEG	NEG
2	784	18	0.13	NEG	NEG
3	599	23	0.10	NEG	NEG
4	1659	14	0.29	NEG	NEG
5	3119	13	0.54	NEG	NEG
6	1512	13	0.26	NEG	NEG
7	1077	15	0.19	NEG	NEG
8	1296	14	0.22	NEG	NEG
9	674	12	0.12	NEG	NEG
10	753	14	0.13	NEG	NEG
11	398	21	0.07	NEG	NEG
12	354	16	0.06	NEG	NEG
13	374	16	0.06	NEG	NEG
14	866	19	0.15	NEG	NEG

15	430	15	0.07	NEG	NEG
16	1703	21	0.29	NEG	NEG
17	545	16	0.09	NEG	NEG
18	1049	19	0.18	NEG	NEG
19	563	10	0.10	NEG	NEG
20	520	28	0.09	NEG	NEG
21	5051	16	0.87	NEG	NEG
22	460	23	0.08	NEG	NEG
23	1355	19	0.23	NEG	NEG
24	510	17	0.09	NEG	NEG
25	556	27	0.10	NEG	NEG
26	628	19	0.11	NEG	NEG
27	748	15	0.13	NEG	NEG
28	433	18	0.07	NEG	NEG
29	1470	17	0.25	NEG	NEG
30	550	18	0.09	NEG	NEG
31	1860	18	0.32	NEG	POS
32	17170	24	2.95	POS	POS
33	19527	12	3.36	POS	POS
34	12273	12	2.11	POS	POS
35	21516	15	3.70	POS	POS
36	22716	17	3.91	POS	POS
37	35221	10	6.06	POS	POS
38	9261	18	1.59	POS	POS
39	20684	22	3.56	POS	POS
40	43491	15	7.48	POS	POS
MEAN	1024				
CUT OFF (mean+5SD)	5812				

2.14 HAMA and Rf Positive Sample Testing

10 HAMA positive and 9 Rf positive sera obtained from a commercial source were tested on the Theranos Rubella IgM assay. Out of the 19 samples tested, all were negative on Theranos System showing excellent correlation.

Table [SEQ Table * ARABIC] : HAMA and RF testing on Theranos System

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	Theranos Results	Siemens Immulite 2000
HAMA positive					
H1	838	12	0.14	NEG	NEG
H2	1181	12	0.20	NEG	NEG
H3	686	15	0.12	NEG	NEG
H4	1093	14	0.19	NEG	NEG
H5	2142	11	0.37	NEG	NEG
H6	1855	11	0.32	NEG	NEG
H7	1249	22	0.21	NEG	NEG
H8	423	5	0.07	NEG	NEG
H9	455	35	0.08	NEG	NEG
H10	648	10	0.11	NEG	NEG
Rf Positive					
RF1	2120	11	0.36	NEG	NEG
RF2	994	42	0.17	NEG	NEG
RF3	3186	18	0.55	NEG	NEG
RF4	758	12	0.13	NEG	NEG
RF5	2097	4	0.36	NEG	NEG
RF6	3094	15	0.53	NEG	NEG
RF7	2689	12	0.46	NEG	NEG
RF8	1137	17	0.20	NEG	NEG
RF9	4026	21	0.69	NEG	NEG
MEAN		**1024**			
CUT OFF (mean+5SD)		**5812**			

2.15 Specificity (Cross Reactivity Sample Testing)

Literature mentioned that Rubella IgM assay could have cross reactivity with other infectious diseases like Infectious mononucleosis, ANA, Parvovirus and CMV. Positive sera or QC controls of various infectious diseases were tested on the Theranos Rubella IgM Assay and on the predicate kit. All Samples came out negative showing that the Rubella IgM assay has high specificity.

Table [SEQ Table * ARABIC]: Cross reactivity testing with various infectious disease positive samples

Samples	Inter-Cartridge Mean	Inter-Cartridge CV%	Theranos Ab Index	Theranos Results Test	Siemens Immulite 2000
ParvoVirus 7 IgM	602	11	0.10	NEG	NEG
HAV IgM	2118	17	0.36	NEG	NEG
Virotrol II Hbs	699	25	0.12	NEG	NEG
ANA26	603	20	0.10	NEG	NEG
ANA27	1053	19	0.18	NEG	NEG
ANA28	1493	20	0.26	NEG	NEG
ANA29	555	31	0.10	NEG	NEG
ANA30	1142	19	0.20	NEG	NEG
CMV QC	783	18	0.13	NEG	NEG
Mono 3	848	23	0.15	NEG	NEG
Mean Negative+5SD	**5812**				

2.16 Whole Blood, Plasma Screen and Spiked Recoveries

To verify the response of the assay in whole blood and to verify any matrix effects one normal/negative sample was spiked with Rubella IgM Positive sample and screened. A total of 5 point dilution was made and the dilution linearity was tested. The same whole blood was then centrifuged and the rubella IgM sample was spiked into the plasma to test for the results. There was excelled correlation between whole blood and plasma and the linearity for dilution factor was also very good. Data is summarized is the table below.

Table [SEQ Table * ARABIC] : Whole Blood vs Plasma screen (spike recovery)

	Whole Blood		Theranos	Theranos	Plasma		Theranos	Theranos
Sample ID	Inter-Cartridge RLU Mean	CV%	Ab Index	Results	Inter-Cartridge RLU Mean	CV%	Ab Index	Results
Point 1 (Normal Clinical)	326	23	0.07	NEG	290	21	0.06	NEG
Point 2 (1:8)	3722	17	0.77	NEG	4293	20	0.88	NEG
Point 3 (1:4)	8128	22	1.67	POS	8351	6	1.72	POS
Point 4 (1:2)	18504	11	3.81	POS	15815	7	3.26	POS
Point 5 (Pooled positive)	29472	2	6.07	POS	32877	10	6.77	POS

*Point 2 : 1 part of pooled positive and 7 parts for normal
*Point 3 : 1 part of pooled positive and 3 parts of normal
*Point 4 : 1 part of pooled positive and 1 part of normal

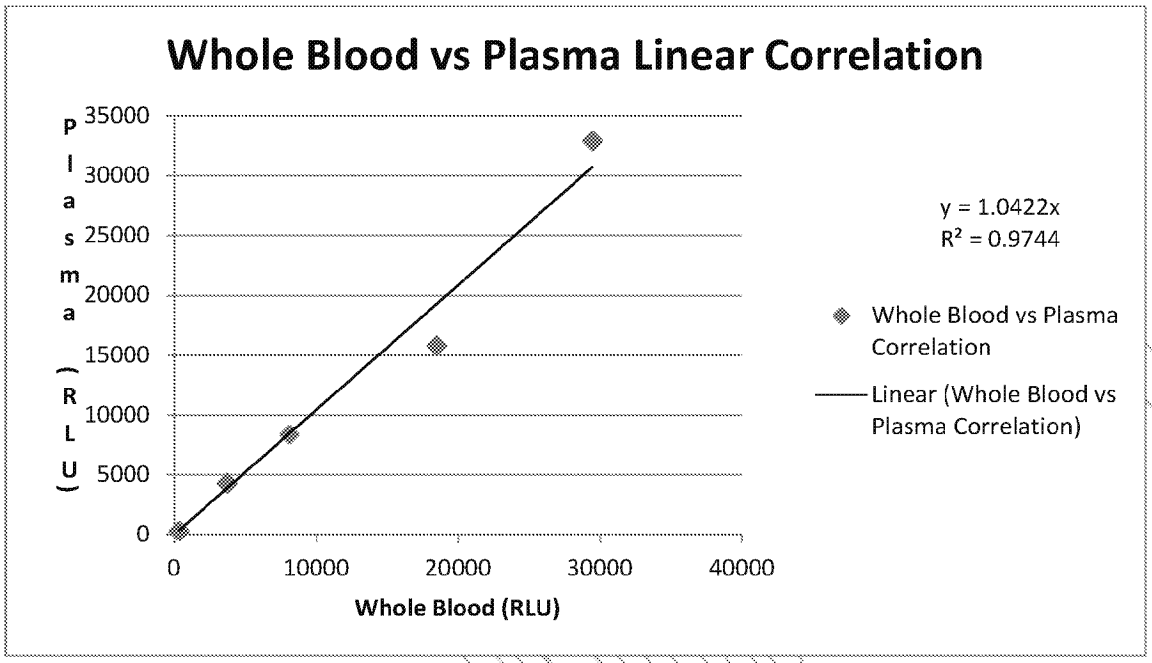

Figure [SEQ Figure * ARABIC] : Whole Blood versus Plasma Spike recovery

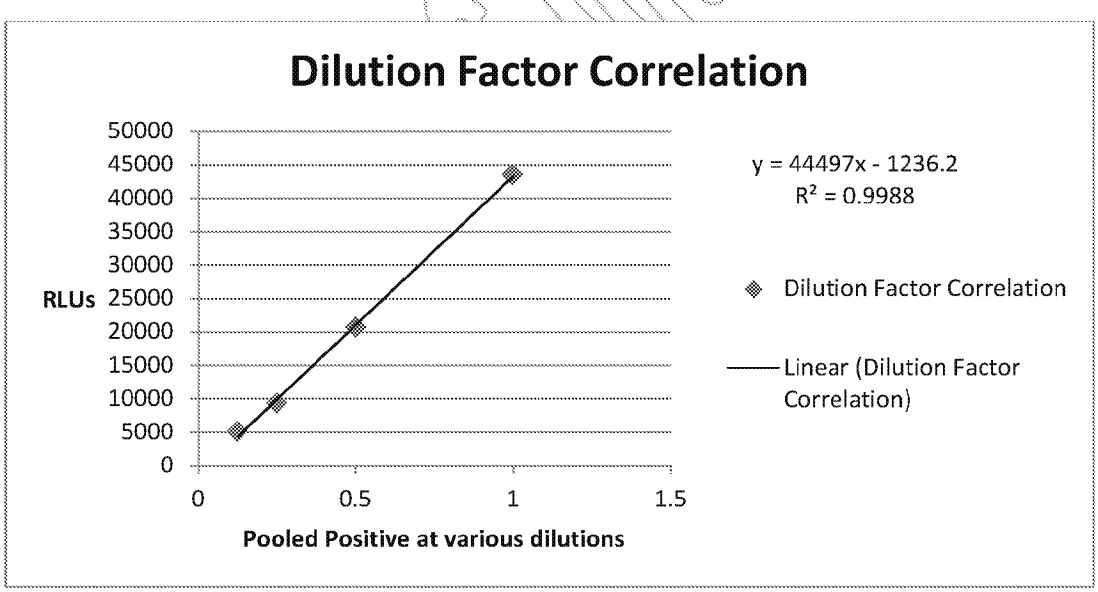

Figure [SEQ Figure * ARABIC] : Dilution Factor linear correlation (Point 1 to Point 5)

2.17 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light for 12 weeks