Human IgM Assay Development Report

Theranos, Inc.

February 2013

Prepared by: Tiffany Zhou/Mi Yan

TABLE OF CONTETNS

LIST OF TABLES

LIST OF FIGURES

1. ASSAY INFORMATION

1.1 Analyte information

Human Immunoglobulin M (IgM) is one of the five antibody isotypes that produced by B cells. IgM is the third most common serum immunoglobulin; it composes about 10% of total immunoglobulin. IgM exists in monomeric form on B cell surface and in secreted forms as pentamer. The molecular weight of pentameric form IgM is about 970 KDa that each heavy (mu) chain is 65KDa. Because of the large molecular weight of IgM, it cannot diffuse well, and it's mainly found intravascular.

IgM is an important component in immune system. It's the first immunoglobulin to be made by B cells when stimulated by antigen. IgM possesses high avidity and it's a good complement fixing immunoglobulin due to its pentameric structure, thus IgM is very efficient in leading to the microorganism cell lysis. IgM can also cause cell agglutination. The antibody-antigen immune complex is then destroyed by complement fixation or receptor mediated endocytosis by macrophages. IgM is the first immunoglobulin express in the fetus (around 20 weeks).

Normal adult serum concentration of IgM is from 0.5-3mg/ml. Depressed level of IgM is normally associated with lymphoid aplasia, agammaglobulinemia, heavy chain disease, and chronic lymphocytic leukemia. Whereas elevated level of IgM can be observed in chronic infections, Waldenstroms macroglobulinemia, hepatitis, rheumatoid arthritis.

1.2 Assay specifications

This assay determines the concentration of IgM in human serum, plasma (EDTA and lithium-heparin), and whole blood. The assay has a quantification range of 0.038mg/mL to 5.11mg/mL (3.8mg/dL to 511mg/dL).

1.3 Reference assay

The following assay was used as reference method:

SIEMENS ADVIA 1800

1.4 Materials and methods

A sandwich immunoassay using anti-human IgM antibodies was developed for the quantitative determination of human IgM in serum, plasma, and whole blood.

In this assay, a mouse anti-human monoclonal antibody was used as capture antibody of IgM determination. Reaction tips were first coated with Ultra-avidin, followed by a layer of biotinylated capture antibody. Serum, plasma, or whole blood samples were diluted 25,000 folds with sample diluent and incubated with capture antibody coated tips. A mouse anti-human IgM monoclonal antibody was conjugated with alkaline phosphatase and used as detection antibody. Detection antibody conjugate was incubated with reaction tips after sample incubation and sample wash. After the second incubation, the tips were washed with wash buffer and incubated with AP substrate. The chemiluminescence results were measured and reported as Relative Light Units (RLU). A calibration curve was generated by plotting the measured response (RLU) vs. concentration of each calibrator. IgM concentration of unknown sample was calculated from calibration curve.

Table [SEQ Table * ARABIC]: Human IgM assay materials in final assay procedure

Name	Supplier	Catalog number
Human IgM whole molecule (myeloma)	Jackson Immuno Lab	009-000-012
Mouse anti-human IgM monoclonal antibody	Life Technologies (Invitrogen)	54900
Mouse anti-human IgG	Novus	NB110-7092

monoclonal antibody		
Tris buffer (powder)	Sigma	T6664
Bovine serum albumin	Sigma	A3059
Sucrose	Sigma	S5016
5% Sodium Azide solution	VWR	101320-516
Carbonate-bicarbonate buffer	Sigma	C3041
1M Magnesium chloride solution	Sigma	M1028
0.1M Zinc Chloride solution	Sigma	39059
TBST (powder)	Sigma	T9039
UltraAvidin	Leinco	A110
AP substrate	In house	Current Lot 11102012-A
In house biotin labeling kit	In house	
AP conjugation kit	Dojindo	LK13
Superblock (TBS) blocking buffer	Thermo Scientific	37542
Protein free assay diluent	SurModics	Sm01-1000

1.5 Raw data storage

Raw data of assay development were stored in Elog #844 and Theranos notebook #404 (partial of data).

2 ASSAY DEVELOPMENT

2.1 Initial antibody screening on MTP

2.1.1 Initial antibody screening on MTP with calibrators

During initial assay development stage, 11 commercial anti-IgM antibodies and 2 Theranos binder anti-IgM biotinylated antibodies were screened for binding of human IgM on micro titer plate (MTP).

All the commercial antibodies were labeled with Theranos in house biotin-SH kits. All these antibodies were also conjugated with alkaline phosphatase using Dojindo AP labeling kit-SH (cat LK13). Total 13 biotinylated conjugated and 11 AP conjugates were paired with each other for initial MTP screening.

Method:

The MTP was first coated with UltraAvidin (UA) at 20ug/ml in coating buffer and then coated with Biotin labeled antibody at 5ug/ml in blocking buffer. Human IgM calibrators at 4mg/ml, 1mg/ml, 0.05mg/ml and 0mg/ml were hand diluted in blocking buffer 25,000 folds and incubated with coated antibodies. Then detection antibody-AP conjugated were diluted in blocking buffer at 50ng/ml and incubated after sample incubation. Finally AP substrate was added to each well and Relative Luminescence Unit (RLU) was measured by a plate reader. Modulations for each antibody pair were calculated using RLU of each sample concentration level divided by the RLU of background (buffer blank, no IgM).

Results:

Many antibody pairs showed good modulations. Base on the overall response 11 pairs of antibodies were move on the cross reactivity and interference tests on the MTP plate.

Table 2: Antibodies screened on MTP

Ab. Number	Vendor	Cat #	Product
1	My BioSource	MBS560678	Mouse Anti-Human IgM Monoclonal Antibody
2	My BioSource	MBS560396	Goat Anti-IgM Polyclonal Antibody
3	Southern Biotech	9022-01	Mouse anti Human IgM
4	Southern Biotech	9020-01	Mouse anti Human IgM-UNLB
5	Bachem Americas Inc.	T-1327.0200	Mouse Anti-IgM (human) Monoclonal Antibody (IgG1)
7	NOVUS Bio	NBP1-78603	Mouse anti-Human IgM Antibody (P5E2)
8	NOVUS Bio	NBP1-48764	Rat Anti-IgM Monoclonal Antibody (IgM kappa)
9	NOVUS Bio	NB110-7089	Mouse Anti-IgM Monoclonal Antibody (IgG1)
10	NOVUS Bio	NB110-7092	Mouse Anti-IgM Monoclonal Antibody (IgG2b)
16	Life Technologies (Invitrogen)	54900	Mouse anti-Human IgM
17	Antibodies-online	ABIN343875	Mouse anti-Human IgM
A9	Theranos	N/A	Biotinylated anti-human IgM Fab raised in E. coli
D1	Theranos	N/A	Biotinylated anti-human IgM Fab raised in E. coli

Table 3: Initial antibody screening on MTP

antibody #	D1	D2	D3	D4	D5	D7	D8	D9	D10	D16	D17
C1											
C2											
C3											
C4											
C5											
C7											
C8											
C9											
C10											
C16											
C17											
Inhouse C-A9											
Inhouse C-D1											

	Excellent modulation >600
	Good modulation >500
	Fair modulation >300

	Little modulation >50
	No or poor modulation

Table 4: 11 pairs of antibodies selected for cross reactivity and interference

C4/D10	C7/D10	C16/D10	
C4/D1	C2/D3		
C-A9/D3	C-A9/D10		
C-A9/D9	C-D1/D3	C-D1/D9	C-D1/D10

2.1.2 Cross reactivity and interference tests

Human IgA, IgD, IgE, and IgG were tested for cross reactivity and interference on MTP for all eleven pairs of antibodies selected from the initial MTP screening round.

Table 5: Human immunoglobulin used for cross reactivity and interference tests

Name	**Supplier**	**Cat#**	**Lot#**	**Conc. in tests (mg/ml)**	**Conc. in normal serum (mg/ml)**
Human IgA, serum	Jackson Lab	009-000-01	103094	4	0.68-3.78
Human IgD	Abcam	Ab91022	GR84036-1	0.2	< or = 0.153
Human IgE	Abbiotec	250202		0.005	< or = 0.0007
IgG whole molecule	Jackson Lab	009-000-003	104362	20	6.5-17

Methods:

Previous methods used in initial antibody screening were used here. However in cross reactivity test instead of using IgM calibrator, the above cross reactants which hand diluted 25,000 folds in blocking buffer were added as samples. For interference test, the above reactants that spiked into IgG calibrators at each concentration were used as samples.

Results:

All eleven pairs of IgM antibodies didn't show any cross reactivity against other immunoglobulin. Out of these 11 pairs, 9 pairs were tested for interference, and 6 pairs showed no interference with other immunoglobulin. Comparing the modulation, cross reactivity, and interference of all 6 pairs of IgM antibody, three pairs of antibodies C4/D1 C16/D10 C-A9/D9 were selected for further evaluation on Theranos system.

Table 6: Results of cross reactivity

		C4 / D10		**C7/D10**		**C16/D10**		**C4/D1**	
Samples	Concentration	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulatio
IgM	0mg/ml	132.7	1	134.1	1	173.6	1	211	1
IgA	4mg/ml	82.4	1	103.3	1	96.6	1	191.4	1
IgD	200ug/ml	79.6	1	113.1	1	128.8	1	148.1	1

IgE	5ug/ml	124.3	1	132.7	1	133	1	190	1
IgG	20mg/ml	97.8	1	110.3	1	138.6	1	149.5	1

		C2/D3		**C-A9/D3**		**C-D1/D3**		**C-A9/D9**	
Samples	Concentration	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulatio
IgM	0mg/ml	244.5	1	237.5	1	216.4	1	265.1	1
IgA	4mg/ml	338.1	1	379.4	1	161.6	1	356.8	1
IgD	200ug/ml	128.5	1	262.7	1	150.3	1	393.5	1
IgE	5ug/ml	121.6	0	309.1	1	210.8	1	397.7	2
IgG	20mg/ml	149.5	1	320.4	1	127.9	1	348.3	1

		C-D1/D9		**C-A9/D10**		**C-D1/D10**	
Samples	Concentration	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
IgM	0mg/ml	258.1	1	254	1	285	1
IgA	4mg/ml	165	1	218.7	1	381	1
IgD	200ug/ml	183.3	1	213.1	1	214.5	1
IgE	5ug/ml	169.2	1	290.7	1	246.9	1
IgG	20mg/ml	191.8	1	217.3	1	299.1	1

Table 7: Results of interference

C4 / D1	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	86730	938	96774.2	112	93759.1	97	98715.9	105	97876.5	99
1	26381	285	28402.9	108	29275.0	103	30275.8	103	31088.0	103
0.1	2678	29	3087.6	115	3147.4	102	3598.9	114	3566.2	99
0.05	1324	14	1620.9	122	1704.2	105	1564.6	92	1805.8	115
0	92	1	121.5	100	194.0	100	166.8	100	155.9	100
C2 / D3	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	56880	682	58695.1	108	56153.2	97	59888.0	114	66367.8	147
1	29844	358	31004.6	107	31405.3	110	30181.5	102	31873.1	113
0.1	4970	60	5170.8	105	5531.6	113	5167.1	105	6865.9	142
0.05	2406	29	3015.1	121	3018.7	121	2677.8	107	3040.5	122
0	83	1	165.0	100	317.3	100	239.3	100	121.5	100
C-A9 / D3	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery

4	55072	551	66626.3	141	58786.6	112	69240.7	151	67948.1	146
1	24443	244	27162.5	119	27646.1	123	28647.9	130	27242.5	120
0.1	3636	36	3981.7	93	4096.2	96	3934.4	92	4310.7	101
0.05	1540	15	2158.1	108	2101.7	106	1699.9	91	2207.2	110
0	100	1	85.5	100	201.8	100	83.6	100	165.4	100
C-D1 / D3	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	42505	349	41463.7	96	33389.5	69	39471.1	89	39627.4	90
1	16392	135	20139.2	132	18666.5	119	20044.6	131	18786.5	120
0.1	3005	25	3508.9	143	3121.7	124	3708.9	153	2638.1	101
0.05	1496	12	1754.5	118	1576.3	102	1478.1	93	1419.9	88
0	122	1	89.1	0	192.7	100	149.1	100	123.6	100
C-A9 / D9	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	23203	182	24286.3	105	22613.2	97	24149.5	104	23713.4	102
1	5983	47	6166.9	103	6161.4	103	6402.3	107	6272.7	105
0.1	777	6	698.8	91	704.3	91	753.5	100	970.7	137
0.05	487	4	434.2	90	458.0	98	372.2	69	560.1	134
0	128	1	125.9	100	125.9	100	142.3	100	178.8	100
C-D1 / D9	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	16268	106	13875.6	84	11770.1	70	12381.3	74	15059.7	92
1	4527	30	4340.6	96	4039.5	89	4351.5	96	4497.5	99
0.1	619	4	777.3	141	562.0	93	538.2	88	875.8	163
0.05	332	2	470.7	146	335.7	86	313.8	76	525.5	170
0	153	1	122.2	100	167.9	100	166.0	100	467.1	100
C-A9 / D10	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	50228	349	52575.1	105	50987.0	102	53619.8	108	53497.7	107
1	13719	95	14475.2	106	15891.9	117	16369.7	120	16971.3	125
0.1	1543	11	1318.3	85	1861.6	125	2277.3	155	2802.4	192
0.05	764	5	853.3	112	918.9	114	1021.1	129	1148.7	147
0	144	1	134.9	100	169.6	100	186.0	100	227.9	100
C-D1 / D10	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	

IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	27326	227	28615.0	105	29023.4	104	26459.8	98	30458.4	106
1	8362	69	9840.4	118	9271.5	115	8771.9	106	9218.6	114
0.1	1315	11	1500.6	114	1276.3	101	1693.9	139	1761.3	146
0.05	520	4	1055.7	203	733.0	110	888.0	136	840.5	128
0	120	1	242.5	100	220.6	100	224.3	100	289.9	100
C16 / D10	**IgM**		IgG 20mg/ml		IgA 4mg/ml		IgD 200ug/ml		IgE 5ug/ml	
IgM (mg/ml)	Mean RLU	Modulation	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery	Mean RLU	% Recovery
4	86349	986	77722.8	90	72650.9	81	75258.0	85	73796.6	83
1	25597	292	27565.4	108	24175.6	94	25852.3	101	23456.8	91
0.1	2868	33	2780.4	97	2785.9	97	2816.9	98	2592.5	90
0.05	1589	18	1507.0	95	1534.4	101	1342.8	87	1311.8	85
0	88	1	94.9	100	175.1	100	109.5	100	122.2	100

2.2 Antibody screening on Theranos system

2.2.1 Training sets of three pairs of antibodies

2.2.1.1 Training sets of three pairs of antibodies (Dab 10ng/ml)

From MTP screening, three pairs of antibodies were chosen to screen on readers with calibrators, clinical samples and control materials.

Methods:

All calibrators, clinical samples, and control materials were first hand diluted 25,000X in blocking buffer. Edison Protocol Generic2_ND_5-5-5 was used for the test. The reaction tips were first coated with UA at 20ug/ml in coating buffer and then with Biotin-labeled antibodies at 5ug/ml in blocking buffer. Hand diluted samples were loaded to cartridge to incubate with coated tips for 10mins. Detection antibody-AP conjugates were diluted at 10ng/ml in blocking buffer and incubated after sample incubation for another 10mins. Tips were then washed and incubated with AP substrate for 10mins. RLU was measured for each tip. IgM concentration of each sample was calculated from the calibration curve obtained from three pairs of antibody respectively. Percentage of recovery was calculated as concentration measured by Theranos method vs. by reference method (SIEMENS ADVIA).

Training set contained total 5 normal male clinical samples, 10 normal female clinical samples, 5 RF IgM samples, 3 HBs IgM samples, 4 HAV IgM samples, and 1 Rubella IgM pooled positive sample.

Control materials included:

- Bio-rad Lyphochek immunoassay plus control level 1, 2, 3. Ref # 370, exp: 1-31-2015, lot #: level 1: 40271; level 2: 40272; level 3: 40273.
- Bio-rad Liquichek ToRCH plus IgM positive sample. Ref # 229.
- Bio-rad Liquichek ToRCH plus IgM negative sample. Ref # 230.
- IRMM reference material. Cat # ERM-DA470K/IFCC, certified IgM value: 0.723mg/ml, uncertainty: 0.027mg/ml.

Results:

Total 28 clinical samples and 6 control materials were tested for the IgM level with the final three pairs of antibody. Between the two commercial pairs C4/D1 and C16/D10, C16/D10 gave a better correlation and recovery when comparing to reference method. The binder capture C-A9 with commercial detection D9 had a low recovery and poor correlation at 10ng/ml detection antibody concentration when comparing to reference method. However in house binder C-A9 was a preferred choice of capture antibody, another training set for C-A9/D9 with detection antibody increasing to 50ng/ml.

Table 8: Training set result of C4/D1 antibody pair

Samples	IgM conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	IgM conc. by Theranos (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	1426439	9%	4.99	100
Calibrator #2 2mg/ml	2.61	1078854	6%	2.66	102
Calibrator #3 1mg/ml	1.36	678778	7%	1.29	95
Calibrator #4 0.5mg/ml	0.72	386297	6%	0.74	103
Calibrator #5 0.1mg/ml	0.14	92195	5%	0.19	139
Calibrator #6 0.05mg/ml	0.07	40361	5%	0.07	107
Calibrator #7 0mg/ml	0.02	999	18%	-0.03	
Biorad Lyphochek level 1	0.80	275736	10%	0.55	69
Biorad Lyphochek level 2	0.79	287625	11%	0.57	73
Biorad Lyphochek level 3	0.83	267806	8%	0.54	64
Biorad ToRCH Positive Control	1.70	479441	10%	0.90	53
Biorad ToRCH Negative Control	0.48	159620	11%	0.33	69
IRMM Standard	0.77	300025	18%	0.59	77
Normal male clinical sample-1	0.84	292056	15%	0.58	69
Normal male clinical sample-2	1.13	276997	4%	0.55	49
Normal male clinical sample-3	1.34	378856	14%	0.73	54
Normal male clinical sample-4	1.24	486730	14%	0.91	74
Normal male clinical sample-5	0.52	153580	12%	0.32	62
Normal female clinical sample-1	1.15	350855	8%	0.68	59
Normal female clinical sample-2	1.01	287646	13%	0.57	57
Normal female clinical sample-3	0.67	168430	8%	0.35	52
Normal female clinical sample-4	1.49	382742	5%	0.73	49
Normal female clinical sample-5	1.81	487815	3%	0.91	51
Normal female clinical sample-6	1.31	382837	1%	0.73	56
Normal female clinical sample-7	0.78	259573	10%	0.52	67
Normal female clinical sample-8	1.45	454252	15%	0.86	59
RF IgM clinical sample-53	1.12	363846	15%	0.70	63
RF IgM clinical sample-54	0.67	184732	10%	0.38	57
RF IgM clinical sample-60	2.08	551459	6%	1.03	50

HBs IgM clinical sample-7	0.65	225446	5%	0.46	71
HBs IgM clinical sample-24	0.91	309688	7%	0.61	67
HBs IgM clinical sample-25	0.67	205442	7%	0.42	63
HAV IgM clinical sample-1	1.11	286307	8%	0.57	51
HAV IgM clinical sample-2	0.95	295486	5%	0.59	61
HAV IgM clinical sample-3	1.63	441036	8%	0.83	51
HAV IgM clinical sample-4	0.73	225584	24%	0.46	63
Rubella IgM pooled positive control	0.62	171530	24%	0.36	58

Table 9: Training set result of C16/D10 antibody pair

Samples	IgM conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	IgM conc. by Theranos (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	969303	8%	4.99	100
Calibrator #2 2mg/ml	2.61	661974	11%	2.66	102
Calibrator #3 1mg/ml	1.36	332722	13%	1.20	89
Calibrator #4 0.5mg/ml	0.72	231057	10%	0.84	117
Calibrator #5 0.1mg/ml	0.14	55460	11%	0.19	140
Calibrator #6 0.05mg/ml	0.07	23285	11%	0.06	94
Calibrator #7 0mg/ml	0.02	489	11%	-0.03	
Biorad Lyphochek level 1	0.80	207299	11%	0.76	94
Biorad Lyphochek level 2	0.79	203469	10%	0.74	95
Biorad Lyphochek level 3	0.83	236415	19%	0.86	103
Biorad ToRCH Positive Control	1.70	345897	26%	1.25	74
Biorad ToRCH Negative Control	0.48	120110	16%	0.44	92
IRMM Standard	0.77	187013	14%	0.69	89
Normal male clinical sample-1	0.84	184014	6%	0.68	81
Normal male clinical sample-2	1.13	202056	10%	0.74	66
Normal male clinical sample-3	1.34	248973	1%	0.91	68
Normal male clinical sample-4	1.24	271501	6%	0.99	80
Normal male clinical sample-5	0.52	98964	8%	0.36	70
Normal female clinical sample-1	1.15	233654	17%	0.85	74
Normal female clinical sample-2	1.01	218043	12%	0.80	79
Normal female clinical sample-3	0.67	123654	15%	0.46	68
Normal female clinical sample-4	1.49	280249	8%	1.02	68
Normal female clinical sample-5	1.81	392263	15%	1.42	79
Normal female clinical sample-6	1.31	286757	27%	1.04	80
Normal female clinical sample-7	0.78	174453	9%	0.64	82
Normal female clinical sample-8	1.45	315187	6%	1.14	79

RF IgM clinical sample-53	1.12	249258	5%	0.91	81
RF IgM clinical sample-54	0.67	137910	9%	0.51	76
RF IgM clinical sample-60	2.08	360346	18%	1.30	63
HBs IgM clinical sample-7	0.65	134728	17%	0.50	77
HBs IgM clinical sample-24	0.91	186626	28%	0.68	75
HBs IgM clinical sample-25	0.67	142395	12%	0.53	78
HAV IgM clinical sample-1	1.11	232249	10%	0.85	76
HAV IgM clinical sample-2	0.95	215456	4%	0.79	83
HAV IgM clinical sample-3	1.63	254604	15%	0.93	57
HAV IgM clinical sample-4	0.73	141424	14%	0.52	71
Rubella IgM pooled positive control	0.62	102073	20%	0.38	61

Table 10: Training set results of C-A9/D9 antibody pair (Dab 10ng/ml)

Samples	IgM conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	IgM conc. by Theranos (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	303516	15%	5.00	100
Calibrator #2 2mg/ml	2.61	167100	22%	2.60	100
Calibrator #3 1mg/ml	1.36	101165	28%	1.39	102
Calibrator #4 0.5mg/ml	0.72	57593	5%	0.69	97
Calibrator #5 0.1mg/ml	0.14	12292	13%	0.14	99
Calibrator #6 0.05mg/ml	0.07	4868	6%	0.07	99
Calibrator #7 0mg/ml	0.02	621	20%	0.03	129
Biorad Lyphochek level 1	0.80	81026	16%	1.05	131
Biorad Lyphochek level 2	0.79	78880	12%	1.02	129
Biorad Lyphochek level 3	0.83	84725	20%	1.11	133
Biorad ToRCH Positive Control	1.70	145041	17%	2.18	128
Biorad ToRCH Negative Control	0.48	39877	28%	0.45	94
IRMM Standard	0.77	61537	10%	0.75	97
Normal male clinical sample-1	0.84	52579	16%	0.62	75
Normal male clinical sample-2	1.13	55726	10%	0.67	59
Normal male clinical sample-3	1.34	73211	21%	0.93	69
Normal male clinical sample-4	1.24	58419	13%	0.71	57
Normal male clinical sample-5	0.52	23377	37%	0.26	49
Normal female clinical sample-1	1.15	58378	17%	0.71	61
Normal female clinical sample-2	1.01	58248	16%	0.70	70
Normal female clinical sample-3	0.67	28420	14%	0.31	47
Normal female clinical sample-4	1.49	43335	9%	0.50	34

Normal female clinical sample-5	1.81	121167	9%	1.74	96
Normal female clinical sample-6	1.31	67778	21%	0.84	65
Normal female clinical sample-7	0.78	43335	9%	0.50	64
Normal female clinical sample-8	1.45	69554	24%	0.87	60
Normal female clinical sample-9	1.10	53557	16%	0.64	58
Normal female clinical sample-10	0.20	8900	30%	0.11	52
RF IgM clinical sample-51	1.01	53014	25%	0.63	62
RF IgM clinical sample-52	1.24	70768	21%	0.89	72
RF IgM clinical sample-53	1.12	76929	24%	0.99	88
RF IgM clinical sample-54	0.67	43623	54%	0.50	75
RF IgM clinical sample-60	2.08	196228	31%	3.16	152
HBs IgM clinical sample-7	0.65	58399	34%	0.71	109
HBs IgM clinical sample-24	0.91	103714	8%	1.43	157
HBs IgM clinical sample-25	0.67	85798	23%	1.13	167
HAV IgM clinical sample-1	1.11	125847	16%	1.83	164
HAV IgM clinical sample-2	0.95	112177	22%	1.58	165
HAV IgM clinical sample-3	1.63	152054	24%	2.31	142
HAV IgM clinical sample-4	0.73	91605	17%	1.22	168
Rubella IgM pooled positive control	0.62	53694	13%	0.64	104

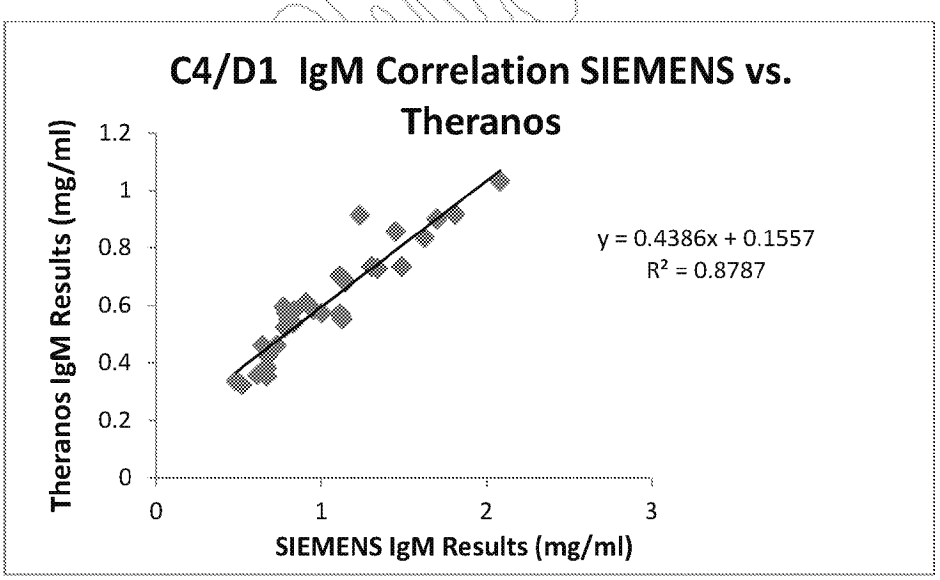

Figure 1: Training set clinical sample correlation of C4/D1

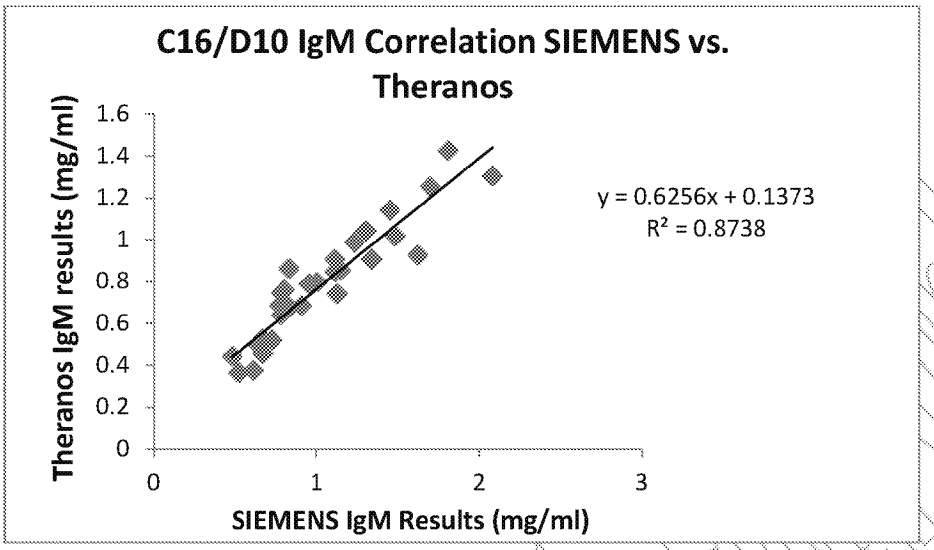

Figure 2: Training set clinical sample correlation of C16/D10

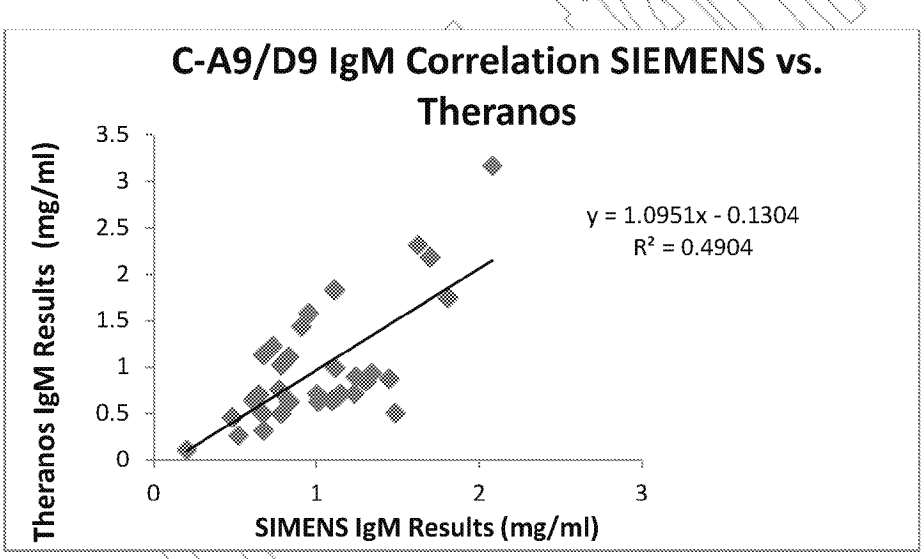

Figure 3: Training set clinical sample correlation of C-A9/D9 (10ng/ml)

2.2.1.2 Training sets of C-A9/D9 (Dab 50ng/ml)

Methods:

Since C-A9/D9 clinical correlation was poor at Dab 10ng/ml, Dab 50ng/ml was prepared and training set was reran with the same set of clinical samples and control samples. Method was the same as mentioned in 2.2.1.1 except detection antibody concentration increased to 50ng/ml.

Results:

C-A9/D9 clinical samples correlation comparing to SIEMENS result improved with good % recovery at 50ng/ml detection antibody concentration. Thus antibody pair C4/D1 (Dab 10ng/ml) and C-A9/D9 (Dab 50ng/ml) were selected as final pairs for further selection decision.

Table 11: Training set results of C-A9/D9 antibody pair (Dab 50ng/ml)

Samples	IgM conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	IgM conc. by Theranos (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	848666	10%	5.00	100
Calibrator #2 2mg/ml	2.61	684656	10%	2.61	100
Calibrator #3 1mg/ml	1.36	403134	40%	1.36	100
Calibrator #4 0.5mg/ml	0.72	184658	21%	0.71	98
Calibrator #5 0.1mg/ml	0.14	42042	20%	0.15	105
Calibrator #6 0.05mg/ml	0.07	27018	5%	0.09	131
Calibrator #7 0mg/ml	0.02	2694	13%	0.00	
Biorad Lyphochek level 1	0.80	271044	11%	1.00	125
Biorad Lyphochek level 2	0.79	270176	9%	1.00	127
Biorad Lyphochek level 3	0.83	431588	15%	1.44	172
Biorad ToRCH Positive Control	1.70	471186	3%	1.54	91
Biorad ToRCH Negative Control	0.48	235768	16%	0.89	183
IRMM Standard	0.77	300562	8%	1.09	141
Normal male clinical sample-1	0.84	334683	8%	1.19	142
Normal male clinical sample-2	1.13	339537	8%	1.20	107
Normal male clinical sample-3	1.34	320060	15%	1.15	86
Normal male clinical sample-4	1.24	402004	6%	1.36	110
Normal male clinical sample-5	0.52	148148	7%	0.57	109
Normal female clinical sample-1	1.15	285498	17%	1.05	91
Normal female clinical sample-2	1.01	375492	12%	1.29	129
Normal female clinical sample-3	0.67	220225	21%	0.83	124
Normal female clinical sample-4	1.49	472167	28%	1.54	104
Normal female clinical sample-5	1.81	567107	4%	1.87	103
Normal female clinical sample-6	1.31	480131	14%	1.57	120
Normal female clinical sample-7	0.78	295222	8%	1.08	137
Normal female clinical sample-8	1.45	563059	16%	1.85	128
Normal female clinical sample-9	1.10	470536	9%	1.54	140
Normal female clinical sample-10	0.20	96786	7%	0.36	178
RF IgM clinical sample-51	1.01	358842	28%	1.25	124
RF IgM clinical sample-52	1.24	468285	3%	1.53	123
RF IgM clinical sample-53	1.12	355792	6%	1.24	111
RF IgM clinical sample-54	0.67	214715	16%	0.82	122
RF IgM clinical sample-60	2.08	676336	9%	2.54	122
HBs IgM clinical sample-7	0.65	223151	8%	0.84	131
HBs IgM clinical sample-24	0.91	343483	24%	1.21	133
HBs IgM clinical sample-25	0.67	254458	27%	0.95	141
HAV IgM clinical sample-1	1.11	390139	13%	1.33	120

HAV IgM clinical sample-2	0.95	325242	17%	1.16	122
HAV IgM clinical sample-3	1.63	438858	13%	1.45	89
HAV IgM clinical sample-4	0.73	236713	11%	0.89	122
Rubella IgM pooled positive control	0.62	202860		0.77	125

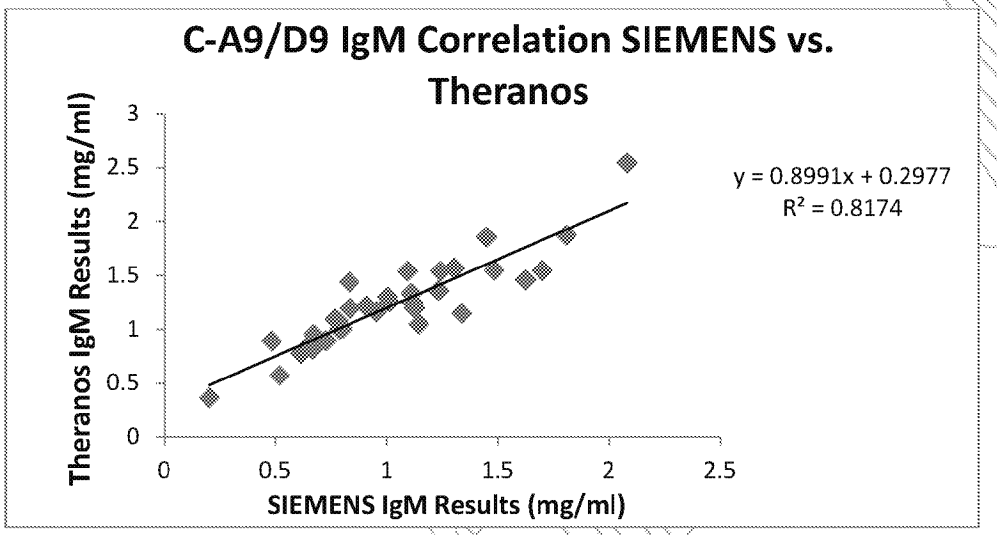

Figure 4: Training set clinical sample correlation of C-A9/D9 (Dab 50ng/ml)

2.2.2 Cross reactivity test on Theranos readers

Besides four other types of immunoglobulin that tested for cross reactivity on MTP previously, five more cross reactants were tested for cross reactivity on readers with the final two pairs of antibody C16/D10 and C-A9/D9.

Methods:

First calibration curve was run using the new Edison protocol Generic2_25,000X. Then cross reactants were loaded as sample. IgM % recovery from each cross reactants was back calculated based on the calibration curve.

Results:

Both final pairs of antibodies C16/D10 and C-A9/D9 didn't cross react with IgA, IgD, IgE, IgG, Prealbumin, transferrin, ferritin, CRP, or human serum albumin. The final decision was made that only the commercial antibody pair C16/D10 moved forward for reader optimization*.

(* First part of the assay was developed by another person, thus some information was unable to be found when this assay reported was prepared.)

Table 12: Cross reactivity results of antibody pair C-A9/D9 (Dab 50ng/ml)

Samples	SIEMEMS IgM conc. (mg/ml)	mean RLU	%CV	Modulation	Theranos IgM conc. (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	500512	42%	319	5.00	100

Calibrator #2 2mg/ml	2.61	371351	38%	237	2.64	101
Calibrator #3 1mg/ml	1.36	234405	16%	149	1.29	95
Calibrator #4 0.5mg/ml	0.72	144832	25%	92	0.76	106
Calibrator #5 0.1mg/ml	0.14	22829	33%	15	0.14	99
Calibrator #6 0.05mg/ml	0.07	11636	30%	7	0.07	107
Calibrator #7 0mg/ml		1568	33%	1		
IgG (20 mg/ml)		980	22%	1	0	
IgA (4 mg/ml)		1147	44%	1	0	
IgD (200 ug/ml)		687	22%	0	0	
IgE (5000ng/ml)		2493	27%	2	0	
Prealbumin (5 mg/ml)		1211	54%	1	0	
Transferrin (10 mg/ml)		957	65%	1	0	
Ferritin (1500ng/ml)		880	40%	1	0	
CRP (C-reactive protein) (10 mg/l)		2245	47%	1.4	0	
Human Serum Albumin (100 mg/ml)		783	42%	0	0	

Table 13: Cross reactivity results of antibody pair C16/D10 (Dab 10ng/ml)

Samples	SIEMEMS IgM conc. (mg/ml)	mean RLU	%CV	Modulation	Theranos IgM conc. (mg/ml)	% recovery
Calibrator #1 4mg/ml	5.00	504474	16%	3782	5.01	100
Calibrator #2 2mg/ml	2.61	260038	20%	1949	2.56	98
Calibrator #3 1mg/ml	1.36	170855	8%	1281	1.47	108
Calibrator #4 0.5mg/ml	0.72	88971	21%	667	0.62	87
Calibrator #5 0.1mg/ml	0.14	18909	6%	142	0.12	89
Calibrator #6 0.05mg/ml	0.07	13503	36%	101	0.10	141
Calibrator #7 0mg/ml		133	12%	1		
IgG (20 mg/ml)		157	53%	1	0	
IgA (4 mg/ml)		197	37%	1	0	
IgD (200 ug/ml)		102	18%	1	0	
IgE (5000ng/ml)		131	9%	1	0	
Prealbumin (5 mg/ml)		217	43%	2	0	
Transferrin (10 mg/ml)		142	39%	1	0	
Ferritin (1500ng/ml)		107	14%	1	0	
CRP (C-reactive protein) (10 mg/l)		110	17%	1	0	
Human Serum Albumin (100 mg/ml)		122	10%	1	0	

2.3 Assay optimization

2.3.1 Titration of capture antibody

Methods:

Capture concentration titration was done by coating with C16 in blocking buffer at 2.5ug/ml, 5ug/ml and 10ug/ml respectively. Detection antibody concentration was kept at 10ng/ml in blocking buffer. Edison protocol Generic2_25000X was used for capture titration.

Results:

With detection antibody at 10ng/ml, capture antibody at 5ug/ml seemed to saturate the coating surface. Increasing the coating concentration to 10ug/ml only lowered the signal/background. 5ug/ml coating concentration was selected as the final concentration for its high modulation, low background, the high sensitivity.

Table 14: Results of C16 capture titration

	10ug/ml			5ug/ml			2.5ug/ml		
calibrator	mean RLU	%CV	modulation	mean RLU	%CV	modulation	mean RLU	%CV	modulation
#1 4mg/ml	319032	15%	1696	310106	39%	2064	257819	32%	1092
#2 2mg/ml	237577	28%	1263	252506	40%	1681	214309	23%	908
#3 1mg/ml	172409	35%	916	110488	12%	735	155027	27%	657
#4 0.5mg/ml	56316	37%	299	60097	35%	400	48216	34%	204
#5 0.25mg/ml	47174	24%	251	41311	38%	275	34093	21%	144
#6 0.12mg/ml	13648	33%	73	12734	44%	85	22208	47%	94
#7 0.06mg/ml	6626	26%	35	7826	17%	52	6510	25%	28
#8 0.03mg/ml	3569	36%	19	2754	15%	18	3205	26%	14
#9 0mg/ml	188	40%	1	150	38%	1	236	39%	1

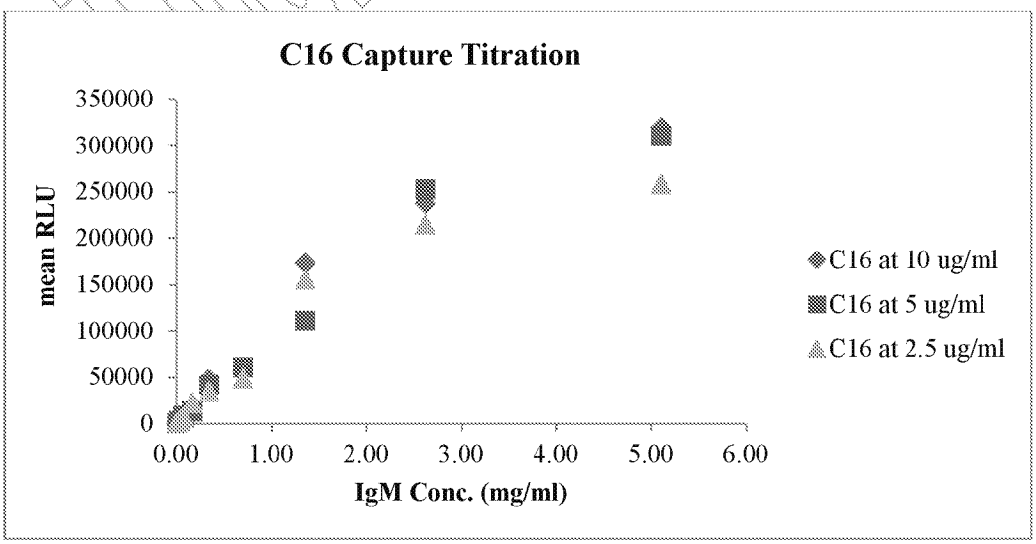

Figure 5: C16 capture titration

2.3.2 Comparison of capture coating buffer

Methods:

Capture antibody was prepared at 5ug/ml in different buffers for comparison of capture coating buffer. Beside in-house blocking buffer (3% BSA-TBS, pH 8.0), Starting block, SEA block, and Super block were also used to prepare the capture solution. Detection antibody was kept at 10ng/ml and the same Edison protocol Generic2_25000 was used. Samples included 9 IgM calibrators and Bio-rad lyphochek control at level 1, 2, and 3.

Results:

Starting block showed a highest background comparing the other three buffers. SEA block also had a higher background comparing to 3% BSA blocking buffer and Super block. Between 3% BSA blocking buffer and Super block, Bio-rad lyphochek controls showed better recovery at each level in Super block. Super block was selected as the final coating buffer.

Table 15: Results of coating buffer comparison

	3% BSA			**Starting Block**		
Calibrators	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	379728	16%	95	298209	17%	98
Calibrator #2	181328	29%	88	185613	29%	107
Calibrator #3	110522	34%	103	101671	32%	106
Calibrator #4	50506	48%	91	45171	29%	89
Calibrator #5	23506	32%	85	25012	31%	100
Calibrator #6	15611	23%	115	9427	29%	78
Calibrator #7	7425	29%	114	4753	11%	84
Calibrator #8	4459	33%	134	3188	41%	114
Calibrator #9	162	23%		2155	42%	
Biorad Control #1	50486	15%	82	40953	14%	73
Biorad Control #2	41566	12%	69	51517	25%	93
Biorad Control #3	52815	13%	80	49271	11%	82
	SEA Block			**Super Block**		
Calibrators	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	314580	15%	74	309131	21%	102
Calibrator #2	205227	32%	93	153797	28%	99
Calibrator #3	123426	52%	109	89100	41%	111
Calibrator #4	53357	42%	90	42675	34%	102
Calibrator #5	27837	28%	95	21317	33%	103
Calibrator #6	13370	55%	94	13530	53%	135
Calibrator #7	9004	52%	136	5837	46%	125
Calibrator #8	3466	32%	104	2938	40%	128
Calibrator #9	593	56%		149	34%	

Biorad Control #1	55004	34%	85	44657	15%	97
Biorad Control #2	46842	7%	73	47059	8%	104
Biorad Control #3	56454	21%	80	46243	11%	93

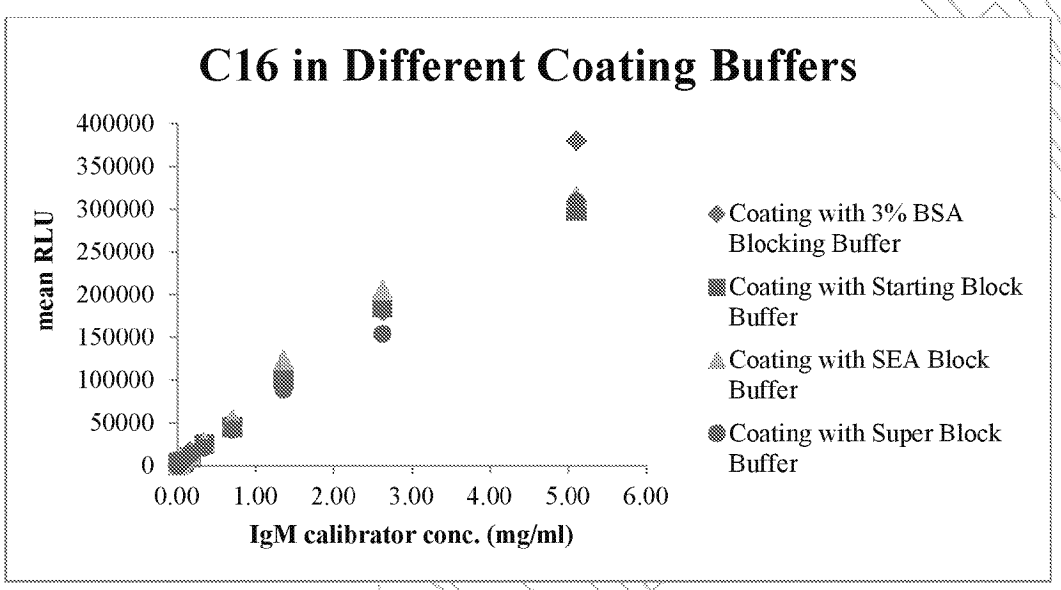

Figure 6: C16 coating buffer comparison

2.3.3 Comparison of sample diluent

Sample diluent comparison was done by using Thermo Scientific Super block, SurModics protein free assay diluent, Pierce protein free (TBS) buffer, Candor low cross buffer, and Starting block as sample diluent. Comparing the results including background, signal, modulation, sensitivity, %CV, and % recovery to original condition where 3% BSA blocking buffer was first used as sample diluent.

Mcthods:

Total six different diluents were tried as sample diluent: 3% BSA blocking buffer, Super block, SurModics protein free assay diluent, Pierce protein free buffer, Candor low cross buffer, and Starting block. All the other assay conditions were kept the same with C16 coated at 5ug/ml in Super block and detection antibody at 10ng/ml in 3% BSA blocking buffer. Edison protocol Generic2_25000X was used, and calibration curve was run for each sample diluent condition respectively along with Bio-rad lyphochek controls at three levels, WHO international standard NIBSC code: 67/086, and two RF positive samples.

Results:

The CV of most sample diluents was high except SurModics protein free assay diluent. With acceptable sample and control recovery SurModics protein free assay diluent was chosen as the final sample diluent.

Table 16: Results of sample diluent comparison

	3% BSA	Super Block

Calibrator	RLU	% CV	% Recovery	RLU	% CV	% Recovery
Calibrator #1	217954	10%	104	152019	44%	121
Calibrator #3	71414	25%	104	29265	24%	106
Calibrator #5	12934	38%	101	5560	46%	100
Calibrator #7	3603	34%	128	2037	31%	155
Calibrator #9	109	16%		92	9%	
Biorad Control #1	33608	25%	104	13610	21%	103
Biorad Control #2	33749	25%	106	22368	40%	156
Biorad Control #3	36305	21%	102	20857	20%	135
WHO Standard	28932	19%	89	12780	9%	94
RF sample #4	37748	22%	69	17955	35%	78
RF sample #7	85863	39%	114	34423	10%	115
	Protein Free Buffer (SurModics)			**Protein Free Buffer (Pierce)**		
Calibrator	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	101123	26%	118	138635	55%	107
Calibrator #3	21698	43%	109	25844	15%	102
Calibrator #5	5988	19%	108	6949	43%	102
Calibrator #7	1571	21%	121	1934	9%	120
Calibrator #9	95	25%		121	19%	
Biorad Control #1	8387	18%	69	20484	39%	140
Biorad Control #2	7649	16%	63	23231	40%	162
Biorad Control #3	7634	25%	58	28531	42%	184
WHO Standard	9983	21%	80	13161	12%	85
RF sample #4	14682	21%	76	15946	29%	65
RF sample #7	14321	25%	67	24926	19%	95
	LowCross Buffer			**Starting Block Buffer**		
Calibrator	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	24678	29%	53	139101	8%	99
Calibrator #3	5438	36%	78	75773	8%	82
Calibrator #5	1242	39%	83	17421	37%	82
Calibrator #7	449	17%	98	2792	27%	82
Calibrator #9	158	30%		150	15%	
Biorad Control #1	3129	14%	91	31746	23%	57
Biorad Control #2	3127	37%	92	28448	10%	53
Biorad Control #3	4110	19%	105	27542	20%	47
WHO Standard	2818	18%	82	30986	29%	54

RF sample #4	5808	23%	104			
RF sample #7	6147	23%	101			

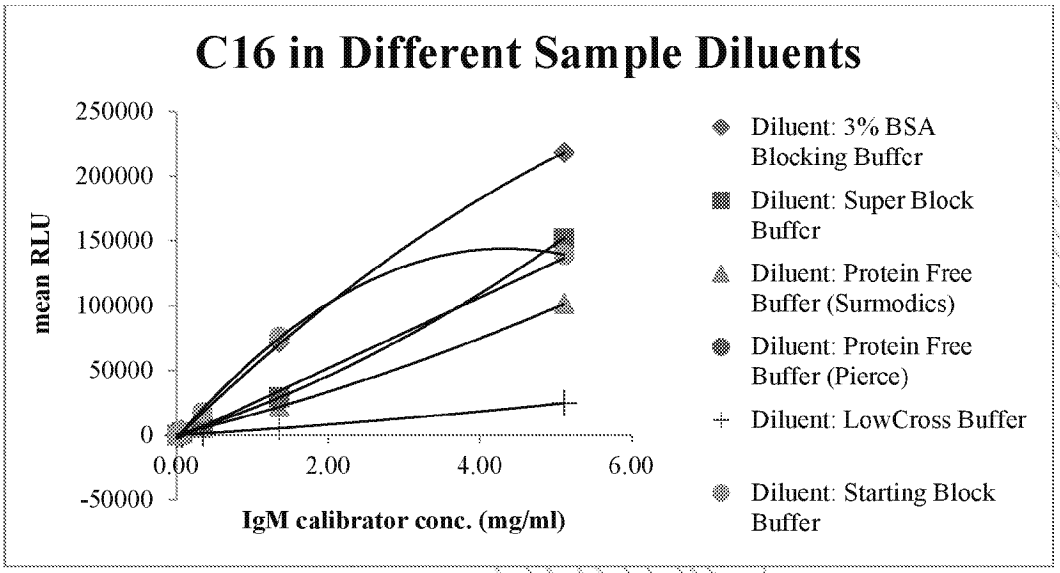

Figure 7: C16 sample diluents comparison

2.3.4 Comparison of detection conjugate stabilizers

Methods:

With capture antibody at 5ug/ml in super block, detection conjugate was prepared at 10ng/ml in SurModics StabilZyme-AP stabilizer, Biostab AP conjugate stabilizer, and Theranos in house AP stabilizer. All samples were diluted in SurModics protein free assay diluent, and Edison protocol Generic2_25000X was used to cmpare the effect of different AP stabilizer.

Results:

Among the three AP stabilizers, Theranos in house AP stabilizer showed the overall better modulation, lower overall CV, and acceptable control recovery. Thus Theranos in house AP stabilizer was chosen as the final detection conjugate AP stabilizer.

Table 17: Results of detection conjugate AP stabilizer comparison

	StabilZyme			**BioStab**			**Inhouse AP Stabilizer**		
Calibrators	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	196386	36%	112	869008	23%	180	381645	37%	133
Calibrator #2	64500	20%	87	505378	24%	62	161857	21%	108
Calibrator #3	38461	34%	111	455710	27%	101	82562	40%	108
Calibrator #4	15785	17%	102	274711	9%	102	41237	16%	106

Calibrator #5	8852	56%	126	163989	22%	122	18179	39%	98
Calibrator #6	2830	23%	90	62480	5%	92	7022	25%	79
Calibrator #7	1752	61%	118	45113	43%	128	5145	50%	124
Calibrator #8	928	38%	115	21425	53%	11	2061	9%	95
Calibrator #9	105	28%		466	25%		375	26%	
Biorad Control #1	16309	31%	96	300319	36%	103	41568	33%	98
Biorad Control #2	20939	42%	119	453351	13%	178	48514	26%	115
Biorad Control #3	25116	48%	127	395397	26%	134	48402	42%	105
WHO Standard	17385	23%	98	287390	62%	94	40621	18%	92

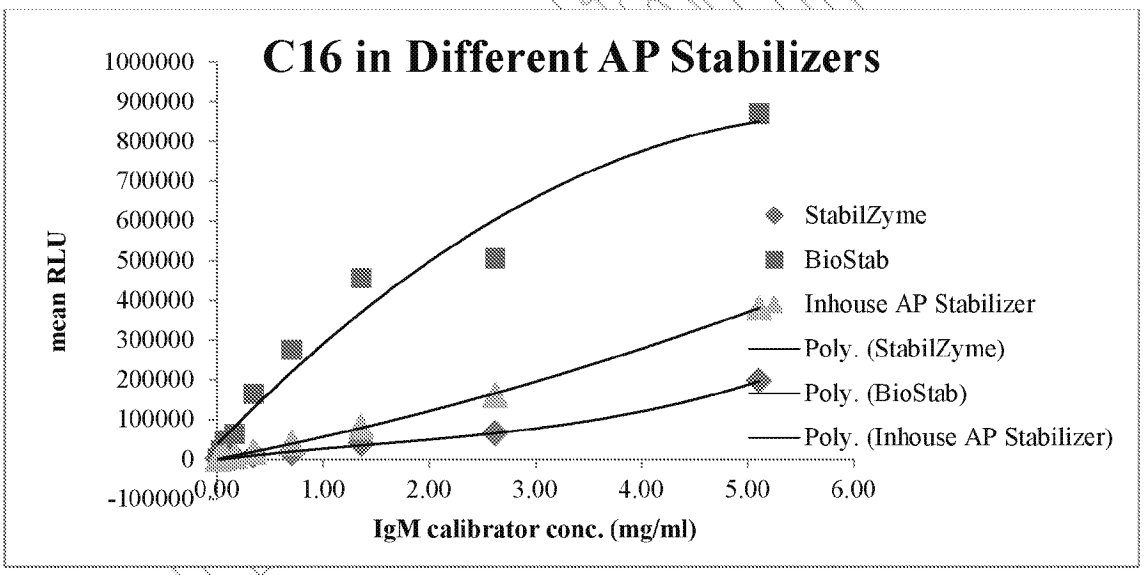

Figure 8: C16 detection conjugate AP stabilizer comparison

2.3.5 Titration of detection antibody

Methods:

Titration of detection antibody concentration was done by preparing detection conjugate in Theranos in house AP stabilizer at 5ng/ml, 10ng/ml, and 20ng/ml respectively. Capture antibody was kept at 5ug/ml in Super block, SurModics protein free assay diluent was used as sample diluent, and Edison protocol Generic2_25000X was used to run the calibration curve and control material at each detection antibody concentration respectively.

Results:

Detection antibody at 5ng/ml in Theranos in house AP stabilizer gave the best overall modulation and good control material recovery, thus 5ng/ml detection antibody was selected as the final choice.

Table 18: Results of detection conjugate titration

	D10 at 20ng/ml			D10 at 10ng/ml			D10 at 5ng/ml		
Calibrators	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**	**RLU**	**% CV**	**% Recovery**
Calibrator #1	466799	28%	102	381645	37%	133	159033	17%	100
Calibrator #2	239049	32%	101	161857	21%	108	73331	47%	90
Calibrator #3	103214	29%	84	82562	40%	108	31634	37%	76
Calibrator #4	62492	11%	96	41237	16%	106	14286	44%	66
Calibrator #5	38818	36%	119	18179	39%	98	13357	58%	127
Calibrator #6	26144	39%	162	7022	25%	79	5411	34%	110
Calibrator #7	8058	40%	90	5145	50%	124	2433	44%	113
Calibrator #8	3814	25%	61	2061	9%	95	1020	11%	114
Calibrator #9	615	14%		375	26%		161	18%	
Biorad Control #1	65880	33%	93	41568	33%	98	22488	16%	95
Biorad Control #2	91503	22%	131	48514	26%	115	27029	47%	115
Biorad Control #3	93256	24%	122	48402	42%	105	22859	16%	89
WHO Standard	70889	2%	97	40621	18%	92	18693	5%	76

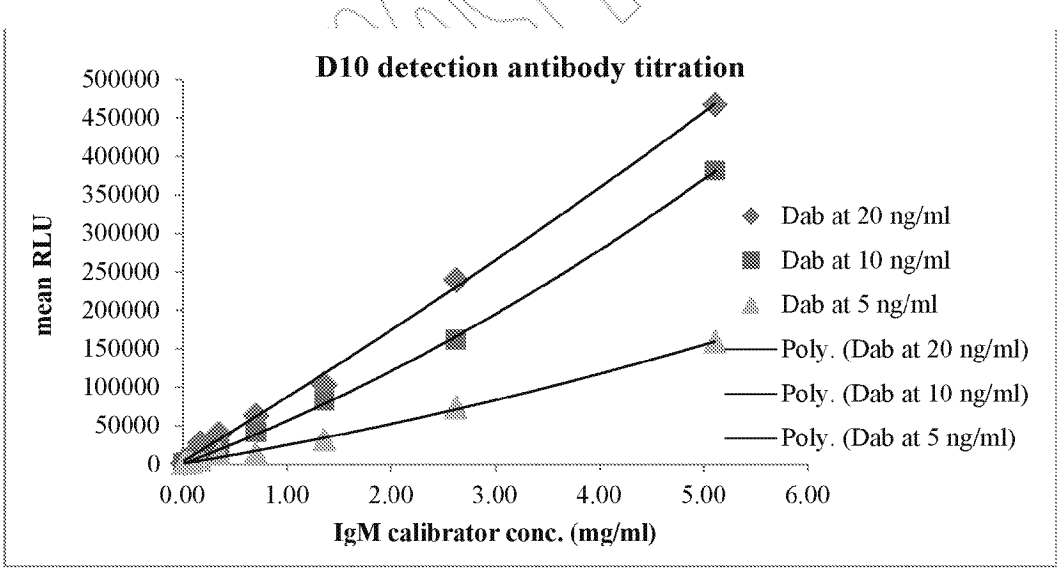

Figure 9: D10 detection antibody titration

2.3.6 Incubation time testing

Method:

With the final assay conditions of all reagents including C16 5ug/ml in Super block, D10 10ng/ml in Theranos in house AP stabilizer, and SurModics protein free assay diluent as sample diluent, new incubation time of 5-5-5 was

test to check the effect of incubation time at each incubation step. Protocol Generic2_25000X_5-5-5 was used for the test of incubation time.

Results:

Short incubation time resulted in lower background and lower signal with still good recovery of control material. To save assay time, Generic2_25000X_5-5-5 was chosen over the Generic2_25000X as the final assay protocol.

Table 19: Incubation time comparison

	Generic2_25000x			Generic2_25000X_5-5-5		
Calibrators	**RLU**	**%CV**	**% recovery**	**RLU**	**%CV**	**% recovery**
Calibrator #1	381645	37%	133	85498	21%	104
Calibrator #2	161857	21%	108	41355	38%	108
Calibrator #3	82562	40%	108	22383	19%	126
Calibrator #4	41237	16%	106	9013	41%	112
Calibrator #5	18179	39%	98	3896	22%	105
Calibrator #6	7022	25%	79	1877	36%	96
Calibrator #7	5145	50%	124	1371	22%	135
Calibrator #8	2061	9%	95	741	17%	91
Calibrator #9	375	26%		230	17%	
Biorad Control #1	41568	33%	98	9591	28%	108
Biorad Control #2	48514	26%	115	10117	40%	114
Biorad Control #3	48402	42%	105	8478	13%	90
WHO Standard	40621	18%	92	9534	30%	104

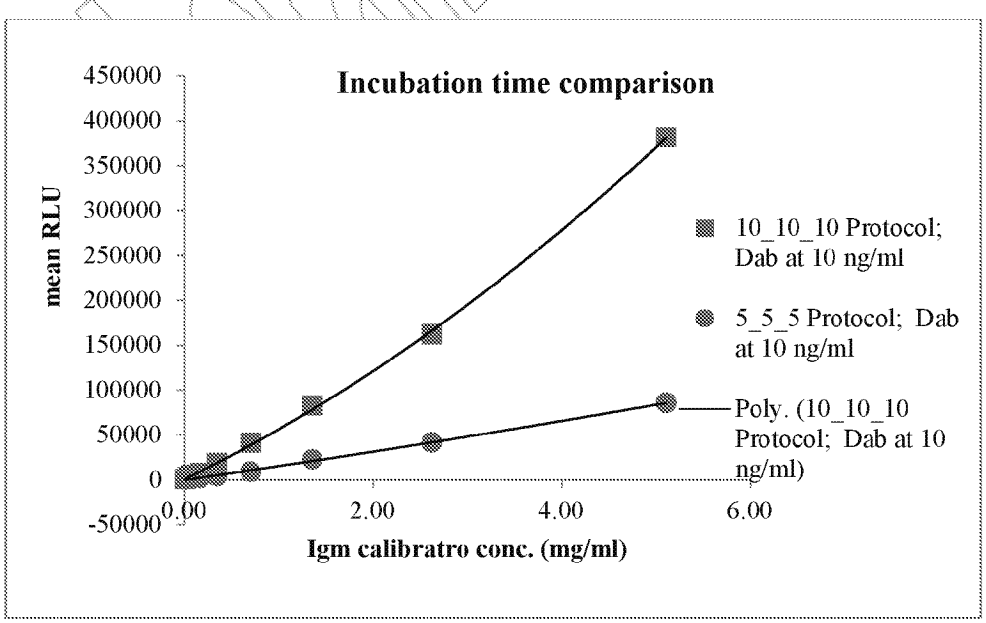

Figure 10: Incubation time comparison

2.3.7 Edison protocol optimization

Based on the existing Edison protocol Generic2_25000_5-5-5, a new protocol Generic2_25000X_5-5-5_PSW was generated. The purpose of adding post sample wash in the new protocol was in the hope of improving background, CV, modulation, and clinical sample recovery.

Methods:

IgM calibrators and control materials were run under these two protocols: Generic2_25000X_5-5-5 and Generic2_25000X_5-5-5_PSW. The tips were coated at 5ug/ml in superblock, and detection antibody was prepared at 10ng/ml in Theranos in house AP stabilizer, and the sample diluent was SurModics assay diluent (protein free). Percentage of recovery was calculated by comparing Theranos results vs. SIEMENS ADVIA results.

Results:

Addition of PSW dramatically decreased the %CV without affecting modulation or control material recovery. As a result, Edison protocol Generic2_25000X_5-5-5_PSW was chosen as the final protocol for this assay.

Table 20: Results of Edison protocol optimization

	Generic2_25000x_5-5-5_PSW			**Generic2_25000X_5-5-5**		
Calibrators	**RLU**	**%CV**	**% recovery**	**RLU**	**%CV**	**% recovery**
Calibrator #1	75792	19%	115	85498	21%	104
Calibrator #2	24987	24%	85	41355	38%	108
Calibrator #3	13767	29%	96	22383	19%	126
Calibrator #4	8206	34%	115	9013	41%	112
Calibrator #5	4199	17%	121	3896	22%	105
Calibrator #6	1786	42%	96	1877	36%	96
Calibrator #7	1035	18%	102	1371	22%	135
Calibrator #8	663	24%	105	741	17%	91
Calibrator #9	289	15%		230	17%	
Biorad Control #1	7192	14%	92	9591	28%	108
Biorad Control #2	9865	10%	126	10117	40%	114
Biorad Control #3	8605	23%	101	8478	13%	90
WHO Standard	7317	12%	91	9534	30%	104

2.4 Clinical samples testing

2.4.1 IgM calibrator validation

Methods:

Human IgM whole molecule (myeloma) was obtained from JacksonImmunoLab, catalog #: 009-000-012. The liquid came in with stated IgM concentration of 4.4mg/ml. calibrators were prepared by diluted the stock liquid in 3% BSA, then calibrators were aliquot, freed and stored at -80C. IgM concentration of one set of calibrator was sending to be tested on SIEMENS ADVIA for validation purpose.

Results:

Based on the SIEMENS IgM test results, all calibrators at each concentration came out higher than the nominal value. When using these calibrators for further recovery calculation, SIEMENS IgM results were signed to each calibrator.

Table 21: IgM calibrator validation on SIEMENS ADVIA

Sample #	Sample Information	SIEMENS IgM Results (mg/ml)	Nominal Conc. (mg/ml)
1	4 mg/ml IgM Standard	5.109	4
2	2 mg/ml IgM Standard	2.624	2
3	1 mg/ml IgM Standard	1.358	1
4	0.5 mg/ml IgM Standard	0.705	0.5
5	0.25 mg/ml IgM Standard	0.347	0.25
6	0.12 mg/ml IgM Standard	0.168	0.12
7	0.06 mg/ml IgM Standard	0.078	0.06
8	0.03 mg/ml IgM Standard	0.038	0.03
9	0 mg/ml IgM Standard	0	0

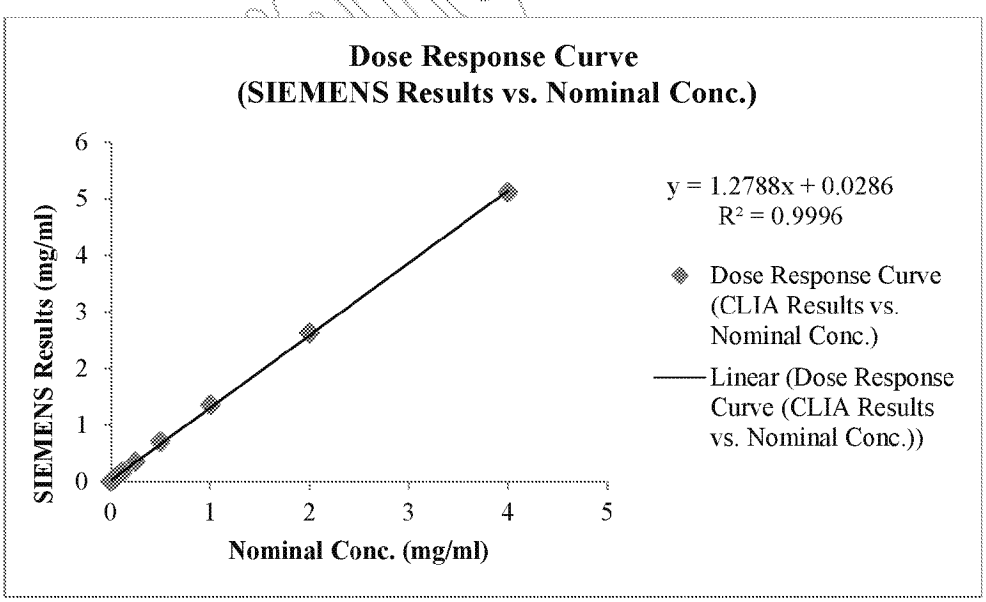

Figure 11: Calibrator validation

2.4.2 HAMA and RF positive samples

Methods:

Ten HAMA and ten RF positive samples were analyzed with final assay condition. All samples were also tested for IgM level on SIEMENS ADVIA. Back calculation was based on the calibration curve which was run under the same assay condition.

Results:

From the comparison of the difference between Theranos data and SIEMENS data, the results showed that HAMA and RF positive samples didn't affect the IgM analysis. IgM value in HAMA and RF positive samples correlated well between Theranos method and reference method.

Table 22: Results of HAMA and RF positive samples

Clinical samples	mean RLU	%CV	SIEMENS IgM conc. (mg/ml)	Theranos IgM conc. (mg/ml)	% recovery
RF Sample # 9247532	101771.7	7.8	4.86	4.82	99
RF Sample # 9247533	85124.2	17.0	4.47	4.03	90
RF Sample # 2096156	46594.1	23.3	2.31	2.21	96
RF Sample # 2096164	46010.8	22.3	2.12	2.18	103
RF Sample # 2096171	31133.1	16.8	1.57	1.47	94
RF Sample # 2096172	18269.3	18.4	0.85	0.86	101
RF Sample # 2096180	15649.6	11.3	0.87	0.74	85
RF Sample # 2108353	15134.5	17.2	0.62	0.72	115
RF Sample # 2108855	41230.4	9.0	1.88	1.95	104
RF Sample # 2110778	69838.0	19.8	2.97	3.31	111
HAMA Sample # 825860	40263.8	15.4	2.02	1.91	94
HAMA Sample # 942853	7038.3	22.8	0.31	0.33	106
HAMA Sample # 1291420	35716.3	13.5	1.81	1.69	94
HAMA Sample # 1291429	14415.0	14.9	0.69	0.68	99
HAMA Sample # 1291430	31193.1	20.1	1.57	1.48	94
HAMA Sample # 1291434	18403.4	21.0	1.05	0.87	83
HAMA Sample # 1291446	25128.0	24.4	1.43	1.19	83
HAMA Sample # 1291449	19659.9	13.7	1.12	0.93	83
HAMA Sample # 1291454	13286.1	15.4	0.73	0.63	86
HAMA Sample # 1291469	48628.8	12.6	2.47	2.30	93

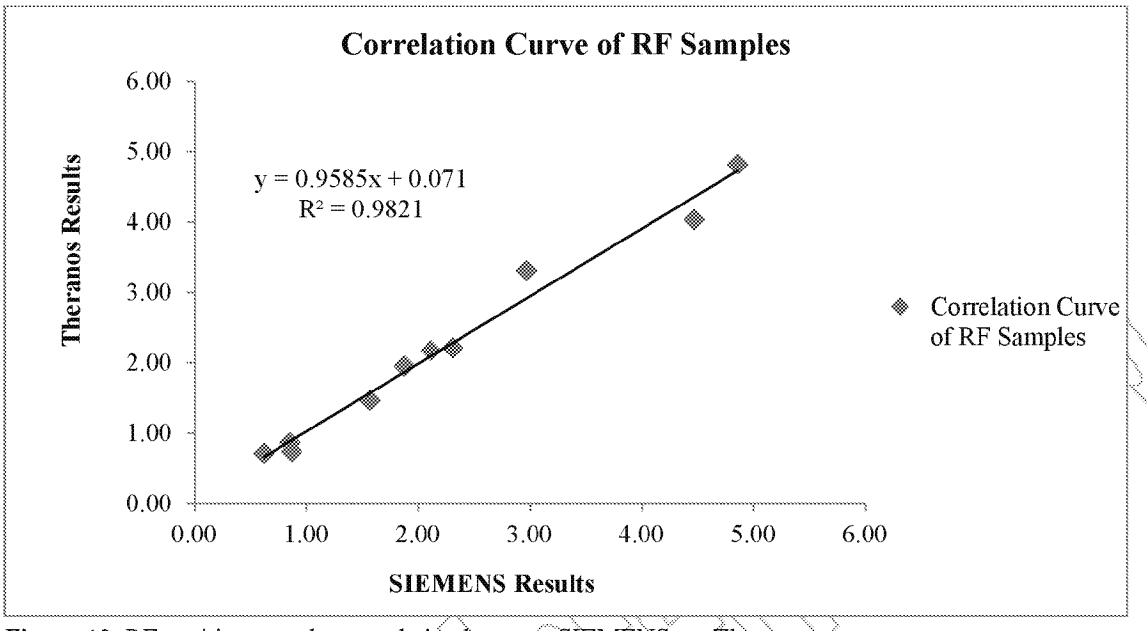

Figure 12: RF positive samples correlation between SIEMENS vs. Theranos

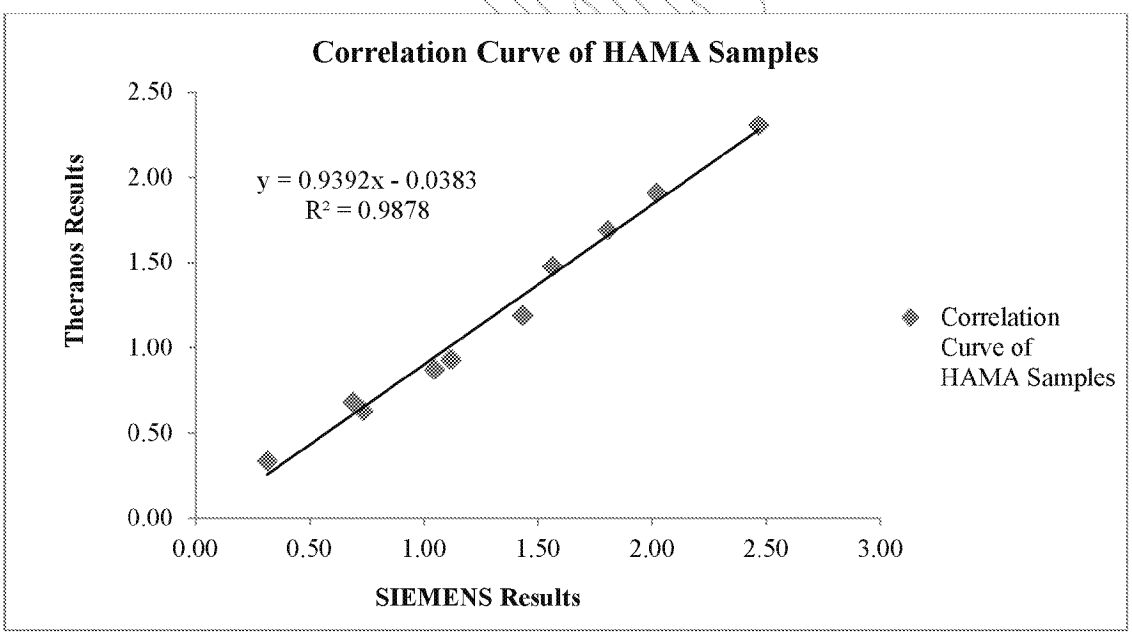

Figure 13: HAMA positive samples correlation between SIEMENS vs. Theranos

2.4.3 Matrix effect

Methods:

Three hemolyzed, three icteric, and three lipemic samples were tested for IgM recovery on the Theranos system with final assay conditions. The IgM concentration was back calculated based on the calibrator curve which was run under the same condition.

Results:

The above three different matrix didn't show any effect on IgM recovery in Theranos system when comparing the results to reference method. Thus this assay was valid to test hemolyzed, icteric, and lipemic samples.

Table 23: Results of matrix effect test

Sample Name:	lot #	mean RLU	%CV	SIEMENS IgM conc. (mg/ml)	Theranos IgM conc. (mg/ml)	% Recovery
Hemolyzed	0107-027-00707	18922	16%	1.09	0.90	82
	0107-027-00705	8483	20%	0.46	0.40	87
	0107-027-00704	11996	24%	0.69	0.57	82
Icteric Sample	2137057	13956	18%	0.60	0.66	110
	2137058	25040	13%	1.35	1.19	88
	2137059	8793	24%	0.50	0.42	83
Lipemic Sample	1511708	42270	19%	2.18	2.00	92
	1820265	12908	23%	0.67	0.61	92
	1820289	13001	12%	0.75	0.62	82

2.4.4 Hematocrit effect and anticoagulant effect

Methods:

Whole blood, EDTA plasma, and lithium-heparin plasma samples obtained from ten donors (5 male and 5 female). All samples were analyzed with final assay procedure. Hematocrit effect was evaluated by comparing IgM results in whole blood vs. EDTA plasma from the same donor. EDTA plasma and lithium-heparin plasma from the same donor were also analyzed to compare the effect of anticoagulant.

Results:

Samples from ten donors collected in pairs of whole blood, EDTA plasma, and lithium heparin plasma were analyzed. Hematocrit factor was calculated to be 1.7 from the slope of plotting IgM results from EDTA plasma vs. results from whole blood.

IgM results from serum, EDTA plasma and lithium heparin plasma correlated well each other without showing significant difference in between each anticoagulant. This assay could be used to analyzed whole blood (Hematocrit =1.70), EDTA plasma, and lithium heparin plasma.

Table 24: IgM results of whole blood and plasma matching pairs

Sample Name:		Whole Blood (EDTA)	Plasma (EDTA)	Plasma (Li-Heparin)	Serum
Normal Male # 1	RLU	8269	18252	17321	14893
	CV	15%	22%	21%	22%
	Theranos IgM conc. (mg/ml)	0.39	0.86	0.82	0.70
Normal Male # 2	RLU	6476	10138	10212	11080
	CV	13%	14%	18%	24%
	Theranos IgM conc. (mg/ml)	0.31	0.48	0.48	0.52

Normal Male # 3	**RLU**	9182	15729	19291	14840
	CV	9%	19%	19%	23%
	Theranos IgM conc. (mg/ml)	0.43	0.74	0.91	0.70
Normal Male # 4	**RLU**	13948	26787	27153	25337
	CV	17%	21%	9%	12%
	Theranos IgM conc. (mg/ml)	0.66	1.27	1.29	1.20
Normal Male # 5	**RLU**	20806	35426	37541	36309
	CV	20%	17%	21%	12%
	Theranos IgM conc. (mg/ml)	0.98	1.68	1.78	1.72
Normal Female # 1	**RLU**	8074	12437	13966	14334
	CV	20%	18%	18%	21%
	Theranos IgM conc. (mg/ml)	0.38	0.59	0.66	0.68
Normal Female # 2	**RLU**	10496	17286	19099	14238
	CV	22%	14%	19%	20%
	Theranos IgM conc. (mg/ml)	0.50	0.82	0.90	0.67
Normal Female # 3	**RLU**	10094	17247	18267	13318
	CV	13%	18%	21%	11%
	Theranos IgM conc. (mg/ml)	0.48	0.82	0.86	0.63
Normal Female # 4	**RLU**	20827	33164	30755	35923
	CV	17%	20%	17%	22%
	Theranos IgM conc. (mg/ml))	0.99	1.57	1.46	1.70
Normal Female # 5	**RLU**	19122	35690	35900	29915
	CV	22%	15%	24%	17%
	Theranos IgM conc. (mg/ml)	0.91	1.69	1.70	1.42

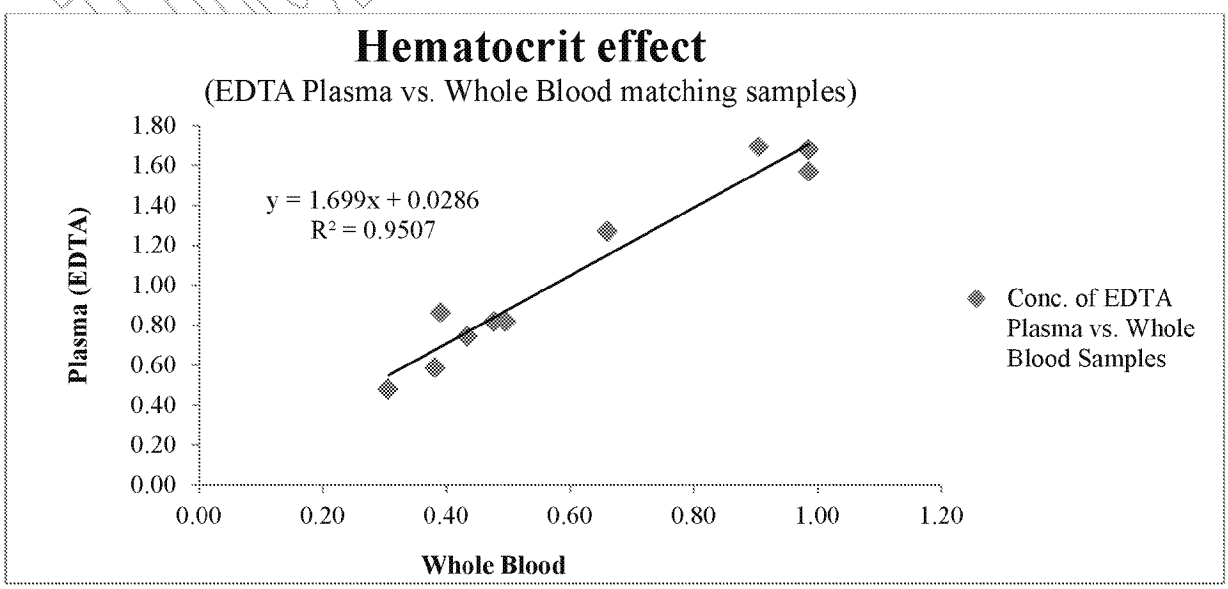

Figure 14: Hematocrit effect

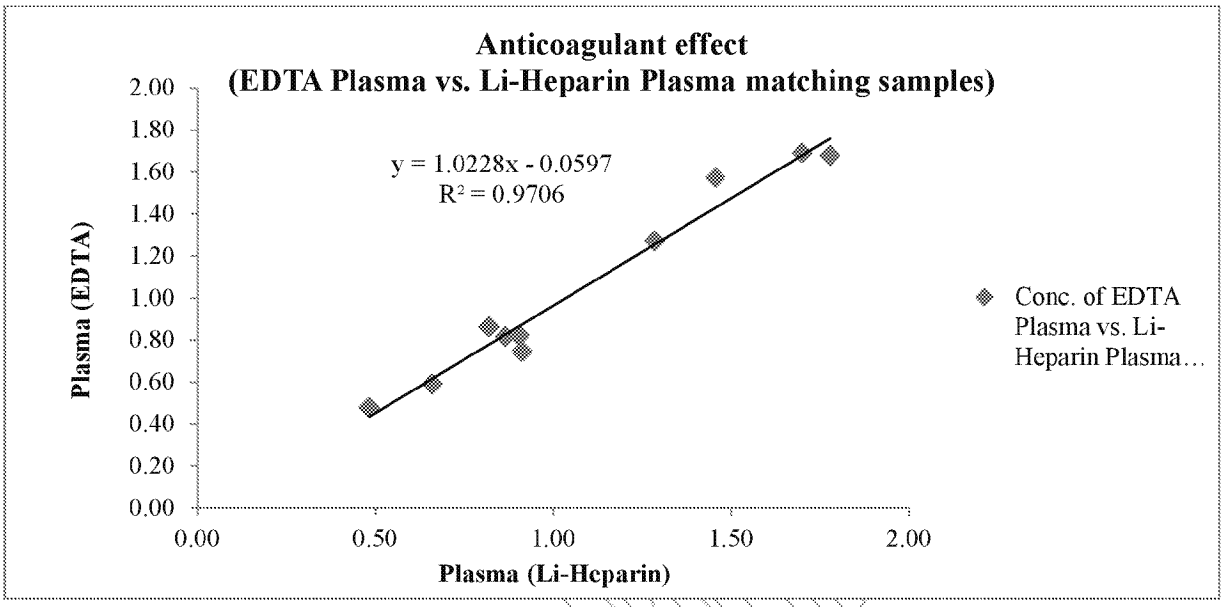

Figure 15: Anticoagulant effect (EDTA plasma vs. li-hep plasma)

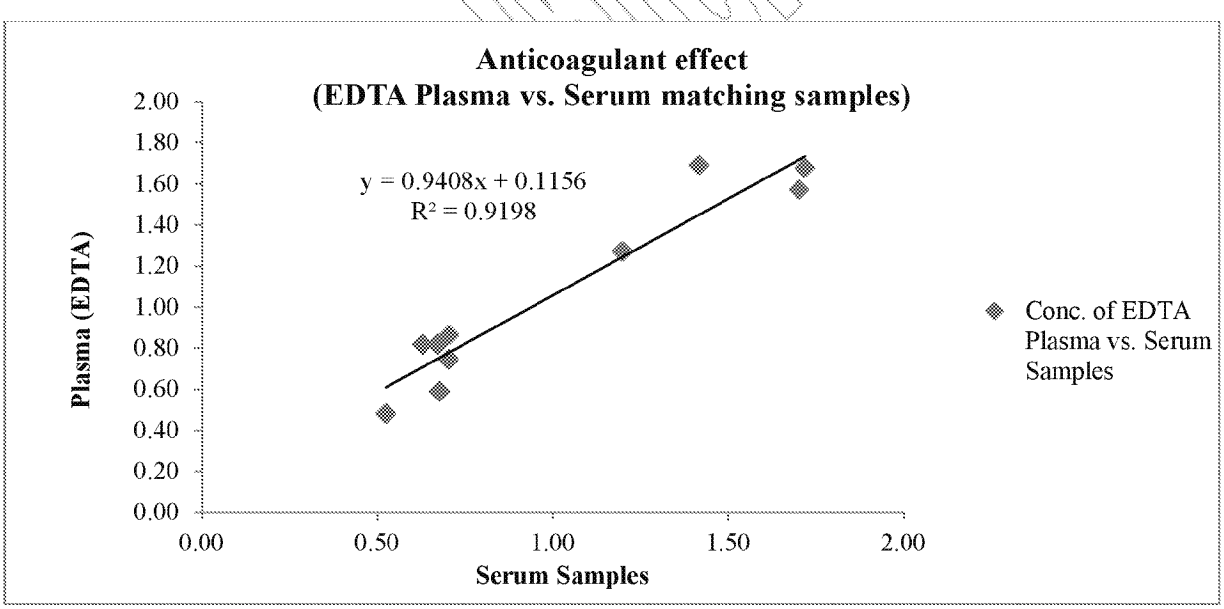

Figure 16: Anticoagulant effect (EDTA plasma vs. serum)

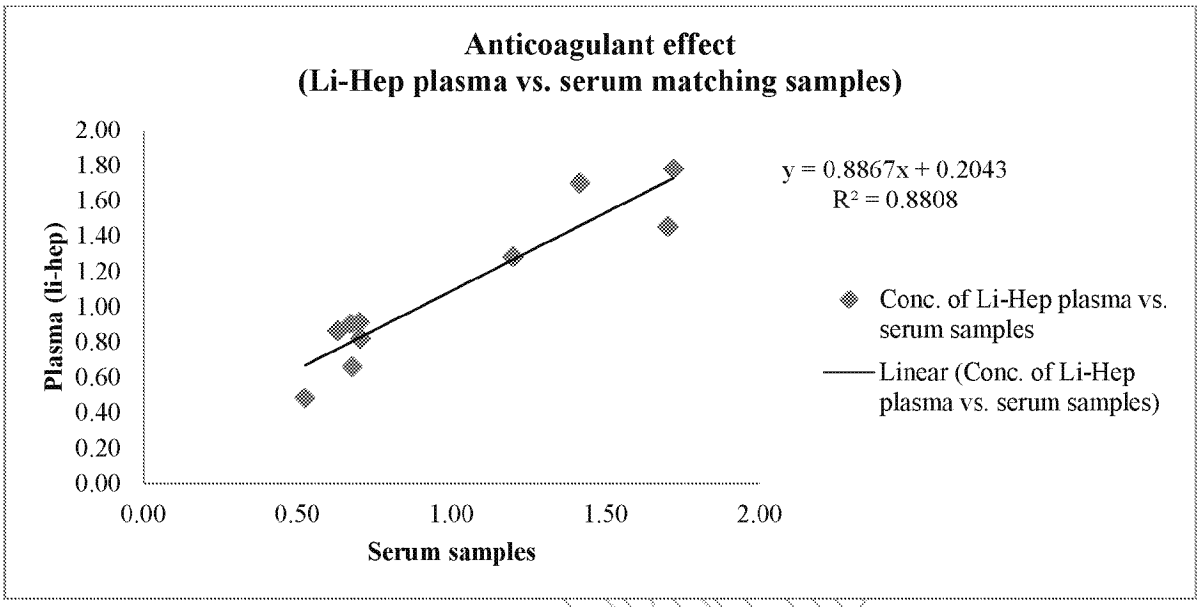

Figure 17: Anticoagulant effect (Li-hep plasma vs. serum)

2.4.5 Calibration curve run with final assay condition

Final assay condition C16 5ug/ml in Superblock, D10 detection antibody 10ng/ml in Theranos in house AP stabilizer, SurModics protein free assay diluent was sample diluent. Calibration curve was generated under this assay condition with final Edison protocol Generic2_25000X_5-5-5_PSW and data was analyzed by Dexter.

Table 25: IgM final calibration curve

Sample Id	SIEMENS IgM value (mg/ml)	mean RLU	%CV	Theranos IgM conc. (mg/ml)	% recovery
Calibrator 1	5.109	143850	3.0	4.907	96.0
Calibrator 2	2.624	87548	23.0	2.742	104.5
Calibrator 3	1.358	49888	2.1	1.527	112.4
Calibrator 4	0.705	20578	22.0	0.654	92.7
Calibrator 5	0.347	10422	15.6	0.344	99.3
Calibrator 6	0.168	6467	6.4	0.216	128.5
Calibrator 7	0.078	2380	9.9	0.071	90.9
Calibrator 8	0.038	1550	11.4	0.039	103.1
Calibrator 9	0	301	13.7		

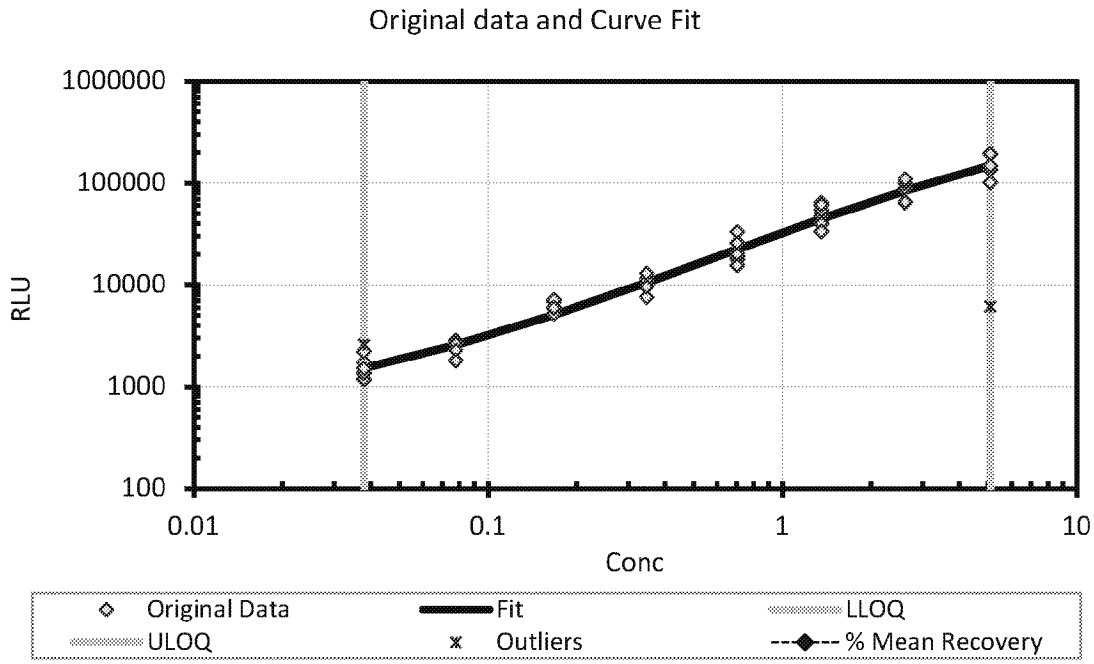

Figure 18: Calibration curve generated by Dexter

Table 26: Calibration curve parameters

Model Type	LogLin 4PL
Model Equation	Log10(RLU)=b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	conc=b3*(((b2-b1)/(log10(RLU)-b1))-1)^(1/b4)
b1	2.525
b2	6.019
b3	0.598
b4	-0.531
LLOQ	0.04 mg/ml
ULOQ	5.11 mg/ml
LLOQ accuracy	103%
LLOQ precision	17.30%
ULOQ accuracy	98%
ULOQ precision	6.40%

2.4.6 Control material assay validation

Total eight control materials were run with the final assay condition to validate the assay. All IgM concentration from each control was back calculated using the Dexter generated calibration curve data. The results were compared to the SIEMENS results and the two correlated well with each other.

Table 27: Eight control material information

Control sample	**Information**

Bio-rad liquichek immunoassay plus control level 1	Ref: 360 Lot: level 1 40801
Bio-rad liquichek immunoassay plus control level 2	Ref: 360 Lot: level 2 40802
Bio-rad liquichek immunoassay plus control level 3	Ref: 360 Lot: level 3 40803
WHO international standard IgG,A,M, human serum	NIBSC code: 67/086
IRMM Human serum	ERM-DA470K/IFCC
Bio-rad lyphochek immunoassay plus control level 1	Ref: 370 Lot: level 1 40271
Bio-rad lyphochek immunoassay plus control level 2	Ref: 370 Lot: level 2 40272
Bio-rad lyphochek immunoassay plus control level 3	Ref: 370 Lot: level 3 40273

Table 28: Results control materials IgM concentration Theranos vs. SIEMENS

Sample Id	SIEMENS IgM value (mg/ml)	mean RLU	%CV	Theranos IgM conc. (mg/ml)	% recovery
Biorad Lyphochek Level 1	0.774	11756	17.8	0.386	91.8
Biorad Lyphochek Level 2	0.765	33286	11.4	1.030	114.6
Biorad Lyphochek Level 3	0.837	24632	6.6	0.774	94.9
WHO international std.	0.847	27625	10.4	0.862	101.8
IRMM DA-470K	0.723	22660	12.4	0.716	99.0
Biorad Liqchek Level 1	0.421	23757	2.2	0.748	96.7
Biorad Liqchek Level 2	0.898	27173	19.1	0.849	111.0
Biorad Liqchek Level 3	0.816	25076	19.8	0.787	94.0

2.4.7 Serum samples of different disease from SeraCare

Forty serum samples from different patients were obtained from SeraCare. Samples were analyzed by Theranos method and SIEMENS method for IgM level. The results difference between the two methods was calculated. Data from Theranos method and SIEMENS method correlated well.

Table 29: Results of 40 diseased serum samples from SeraCare

Sample Id	Disease	SIEMENS IgM value (mg/ml)	mean RLU	%CV	Theranos IgM conc. (mg/ml)	% recovery	% difference
SeraCare 1	CMV IgM	0.606	18874	11.2	0.603	100	0
SeraCare 2	CMV IgM	0.597	16902	20.3	0.544	91	-9
SeraCare 3	CMV IgM	0.589	15789	20.2	0.510	87	-13
SeraCare 4	CMV IgM	0.645	19593	18.8	0.624	97	-3
SeraCare 5	CMV IgM	2.276	65154	13.4	2.002	88	-12
SeraCare 6	HAV IgM	1.364	38552	17.2	1.186	87	-13
SeraCare 7	HAV IgM	1.489	40178	18.3	1.234	83	-17
SeraCare 8	HAV IgM	4.159	141988	16.4	4.827	116	16
SeraCare 9	HAV IgM	1.227	32047	5.4	0.993	81	-19
SeraCare 10	HAV IgM	2.328	61714	6.5	1.893	81	-19
SeraCare 11	Hepatitis B core IgM	1.150	27504	6.2	0.859	75	-25
SeraCare 12	Hepatitis B core IgM	0.522	17152	15.3	0.551	106	6

SeraCare 13	Hepatitis B core IgM	0.572	16601	11.7	0.535	93	-7
SeraCare 14	Hepatitis B core IgM	2.195	87860	1.8	2.753	125	25
SeraCare 15	Hepatitis B core IgM	1.138	39840	8.7	1.224	108	8
SeraCare 26	Mumps IgM	0.898	22694	22.0	0.717	80	-20
SeraCare 27	Mumps IgM	1.967	73915	8.4	2.285	116	16
SeraCare 28	Mumps IgM	2.613	76949	15.2	2.385	91	-9
SeraCare 29	Mumps IgM	1.962	61733	21.2	1.893	97	-3
SeraCare 30	Rubella IgM	0.834	23248	13.0	0.733	88	-12
SeraCare 31	Rubella IgM	1.193	37805	11.7	1.163	98	-2
SeraCare 32	Rubella IgM	1.107	32588	10.5	1.009	91	-9
SeraCare 33	Rubella IgM	0.583	14710	15.1	0.477	82	-18
SeraCare 34	Rubella IgM	0.905	24503	9.0	0.770	85	-15
SeraCare 35	VZV IgM	0.566	19003	3.0	0.607	107	7
SeraCare 36	VZV IgM	0.382	11365	9.5	0.374	98	-2
SeraCare 37	VZV IgM	1.401	44533	11.9	1.365	97	-3
SeraCare 38	VZV IgM	0.882	25384	25.8	0.796	90	-10
SeraCare 39	VZV IgM	0.927	28894	19.8	0.900	97	-3
SeraCare 40	Cardiolipin IgM	2.759	107089	7.5	3.440	125	25
SeraCare 41	Cardiolipin IgM	2.398	84243	7.1	2.629	110	10
SeraCare 42	Cardiolipin IgM	2.297	64438	19.7	1.979	86	-14
SeraCare 43	Cardiolipin IgM	2.516	92681	18.7	2.920	116	16
SeraCare 44	Cardiolipin IgM	0.995	35103	15.1	1.083	109	9
SeraCare 45	JO-1 IgM	1.882	51831	10.3	1.586	84	-16
SeraCare 55	RF IgM	4.901	133182	16.5	4.458	91	-9
SeraCare 56	RF IgM	4.546	150382	12.7	5.192	114	14
SeraCare 57	RF IgM	5.307	173532	3.7	6.266	118	18
SeraCare 58	RF IgM	6.520	151119	5.1	5.224	80	-20
SeraCare 59	RF IgM	6.320	185064	14.8	6.843	108	8

2.4.8 Summary of all clinical samples analysis

Total 40 serum disease serum samples and 8 control materials were analyzed by Theranos method final assay condition. Overall, the results from Theranos method correlated well with SIEMENS method.

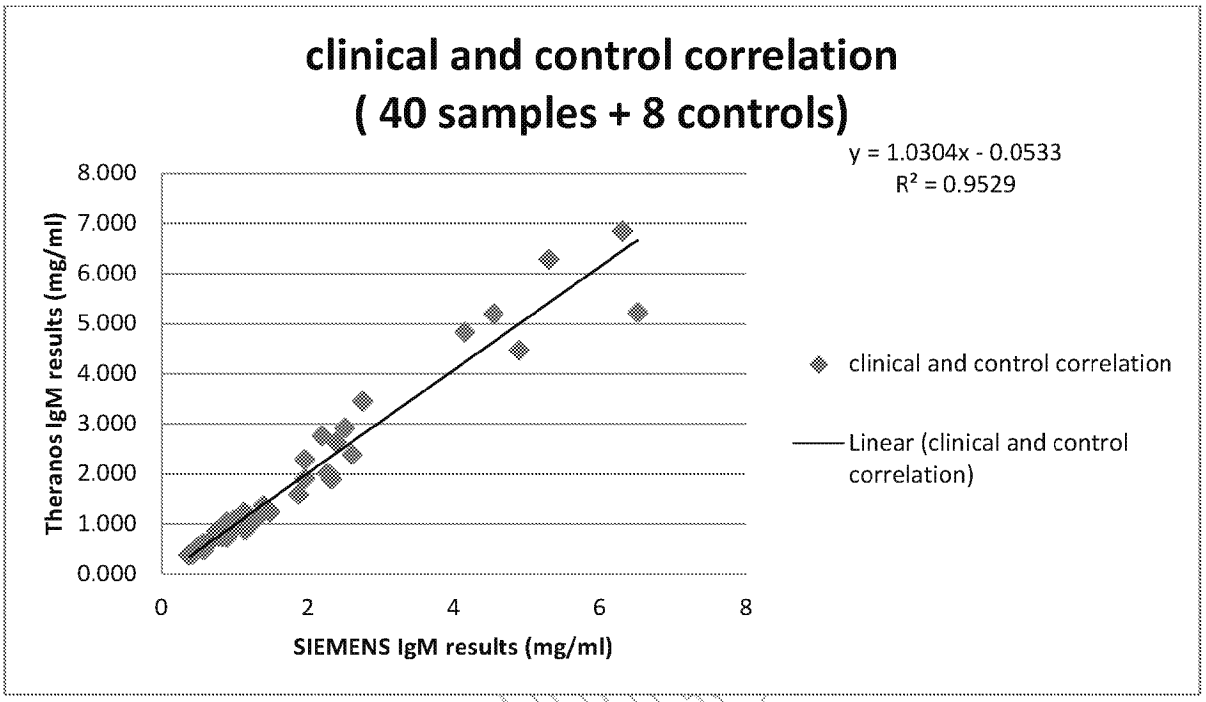

Figure 19: Clinical correlation of Theranos method vs. SIEMENS method

2.5 Stability

Assay stability monitoring is on-going with reagents and coated tips stored at 4C.