Human Total IgG Assay Development Report

Theranos, Inc.

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

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

Human Immunoglobulin G (IgG) is one of the five antibody isotypes that produced by plasma cell. It is the most abundant antibody isotypes in the circulation, representing about 75% among all immunoglobulin in human serum. The molecular weight of this complex glycoprotein is 150kDa which composed of four chains- two identical gamma heavy chains (50kDa each) and two identical light chains (25kDa each). IgG differentiates into four subclasses IgG1, IgG2, IgG3, and IgG4 based on the number of disulfide bonds and length of the hinge region.

IgG is an important component in immune system. It activates the classical pathway of the complement system which triggers the amplifying cascade of pathogen elimination. IgG is a good opsonin. By preparing the antigen for eating by phagocytic cells including macrophages, monocytes, polymorphonuclear neutrophils and some lymphocytes, it enhances phagocytosis. IgG is the only isotype that can cross human placenta, hence providing protection to the fetus.

Normal adult serum concentration of IgG is from 7mg/ml to 16mg/ml. Depressed level of IgG is normally associated with macroglobulinemia, chronic lymphocytic leukemia, lymphoid aplasia. On the other hand, elevated IgG level is related to hepatitis, rheumatoid arthritis, IgG myeloma, and acquired immunodeficiency syndrome.

1.2 Assay specifications

This assay determines the concentration of total IgG in human serum, plasma and whole blood. The assay has a quantification range of 0.78mg/mL to 50mg/mL (78 mg/dL to 5000 mg/dL).

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

The following assay was used as reference method:

SIEMENS ADVIA 1800

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

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

In this assay, a mouse anti-human monoclonal antibody was used as capture agent for IgG determination. Reaction tips were coated with Ultra-avidin first, then followed by coating of biotinylated capture antibody. Serum, plasma or whole blood samples were diluted 100,000 folds with sample diluent and incubated with capture antibody coated tips. A mouse anti-human IgG monoclonal antibody was conjugate with alkaline phosphatase and used as detection antibody. Detection antibody conjugate was incubated with reaction tips after sample incubation. 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. IgG concentration of unknown sample was calculated from calibration curve.

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

Name	Supplier	Catalog number
Human IgG	Sigma	I4506
Mouse anti-human IgG monoclonal antibody	Novus	NBP1-51523
Mouse anti-human IgG monoclonal antibody (gamma chain specific)	Southern Biotech	9040-01
Tris buffer (powder)	Sigma	T6664
Bovine serum albumin	Sigma	A3059
Sucrose	Sigma	S5016
5% Sodium Azide solution	Teknova	S0208
Carbonate-bicarbonate buffer	Sigma	C3041
1M Magnesium chloride solution	Sigma	M1028
0.1M Zinc Chloride solution	Sigma	39059
Wash buffer (20x concentrate)	Enzo Life Science	80-1351
UltraAvidin	Leinco	A110
AP substrate	In house	Current Lot 11102012-A
In house biotin labeling kit	In house	Current Lot 081412
AP conjugation kit	Dojindo	LK13

1.5 Raw data storage

Raw data of assay development were stored in Elog #811 and Theranos notebook #404.

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

2.1 Initial antibody screening on MTP

2.1.1 Initial antibody screening on MTP with calibrator

During initial assay development stage, twelve anti-IgG antibodies were screened for binding of human IgG on micro titer plate (MTP).

All the antibodies were labeled with Biotin using Dojindo Biotin labeling kit-SH (cat LK10). All these antibodies were also conjugated with alkaline phosphatase using Dojindo AP labeling kit-SH (cat LK13). All biotin conjugates and AP conjugates were paired with each other for initial screening.

Methods:

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 IgG calibrators at 0mg/ml, 0.5mg/ml, 5mg/ml, and 50mg/ml were hand diluted in blocking buffer 100,000 folds and incubated with coated antibodies. Then, detection antibody-AP conjugates were diluted in blocking buffer to 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 were calculated using RLU of each sample concentration level divided by the RLU of background (buffer blank, no IgG).

Results:

Many antibody pairs showed good modulations. Based on the overall response during cross reactivity and interference study, three pairs were selected to move forward to Theranos readers for further selection.

Table [SEQ Table * ARABIC]: Antibody screened on MTP

anti-IgG antibody #	Name	Supplier	Cat#	Lot#	Clone#
1	Mouse anti-human IgG Mab (Fc)	Southern Biotech	9040-01	L0810-XH21	JDC-10
2	Mouse anti-human IgG ab (Fc)	Southern Biotech	9042-01	G0608-S769F	H2
3	Mouse anti-human IgG Mab (gamma chain specific)	Sigma aldrich	I5885	052M4821	GG-5
4	Mouse anti-human IgG Mab	Novus	NBP1-51523	07172012	4D2D9G8
5	Rat anti-human IgG Mab	Novus	NBP1-96170	11012830316	KT-48
6	Mouse anti-human IgG Mab	Novus	NBP1-96169	1110317516	KT-47
7	Mouse anti-human IgG Mab	AbD serotec	MCA2477G		8A4
8	Mouse anti-human IgG (Fab)2 specific	Pierce	SA1-19255		4A11
9	Mouse anti-Human IgG (Fab specific)	Novus	NB110-8346		2A11
G4	Goat (Fab2) anti-human IgG (gamma chain specific)	Southern Biotech	2042-01	C5711-SG21	
G8	Rabbit anti-human IgG (Fab2) Pab	Novus	NBP1-72785	21376	
G11	Goat anti-human IgG	Fitzgerald	41C-CJ0118	455589	

Table [SEQ Table * ARABIC]: Results of initial screen on MTP

		#1	#2	#3	#4	#5	#6	#7	#8	#9	#G4	#G8	#G11
Capture antibody	#1												
	#2												
	#3												
	#4												
	#5												
	#6												
	#7												
	#8												
	#9												
	#G4												

	Excellent modulation(>200)
	Good modulation (>50)
	No or poor modulation

2.1.2 Cross reactivity and interference tests

Human IgA, IgD, IgE and IgM were tested for cross reactivity and interference on MTP for all seventeen pairs of antibodies which showed modulation >50 in the initial screening. These samples were prepared at 3X of the highest concentration in normal adult serum[-2].

Table 4: 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	10	0.68-3.78
Human IgD	Abcam	Ab91022	GR84036-1	0.5	< or = 0.153
Human IgE	Abbiotec	250202		0.002	< or = 0.0007
Human IgM (myeloma), whole molecule	Jackson Lab	009-000-012	104252	10	0.6-2.63

Methods:
Previous methods used in initial antibody screening were used here. However in cross reactivity test instead of using IgG calibrator, the above cross reactants which hand diluted 100,000 folds in blocking buffer were added as samples. For interference test, the above cross reactants that spiked into IgG calibrators at each concentration were used as samples.

Result:
Comparing the modulation, cross reactivity, and interference of all seventeen antibody pairs, three pairs of antibodies were selected for further evaluation on Theranos reader.

Table 5: Results of cross reactivity and interference

Antibody pair C4/D1

Cross reactivity										
IgG			IgA (10mg/ml)		IgD (0.5mg/ml)		IgE (0.002mg/ml)		IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.
0	4918	1	4419	1	3529	1	5034	1	4313	1
0.5	13582	3	3977	1	4148	1	5132	1	4164	1
5	85651	17	3546	1	3041	1	5310	1	3675	1
50	592045	120	4740	1	3454	1	5239	1	3308	1
Interference										
IgG			IgG+IgA (10mg/ml)		IgG+IgD (0.5mg/ml)		IgG+IgE (0.002mg/ml)		IgG+IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery
0	4941	1	4091	83	4760	96	4546	92	5812	118
0.5	12609	3	13320	106	16542	131	12483	99	12303	98
5	72605	16	68924	95	69134	95	100839	137	73213	101
50	570772	116	502728	88	494097	87	50228	88	503952	88

Antibody pair C3/D2

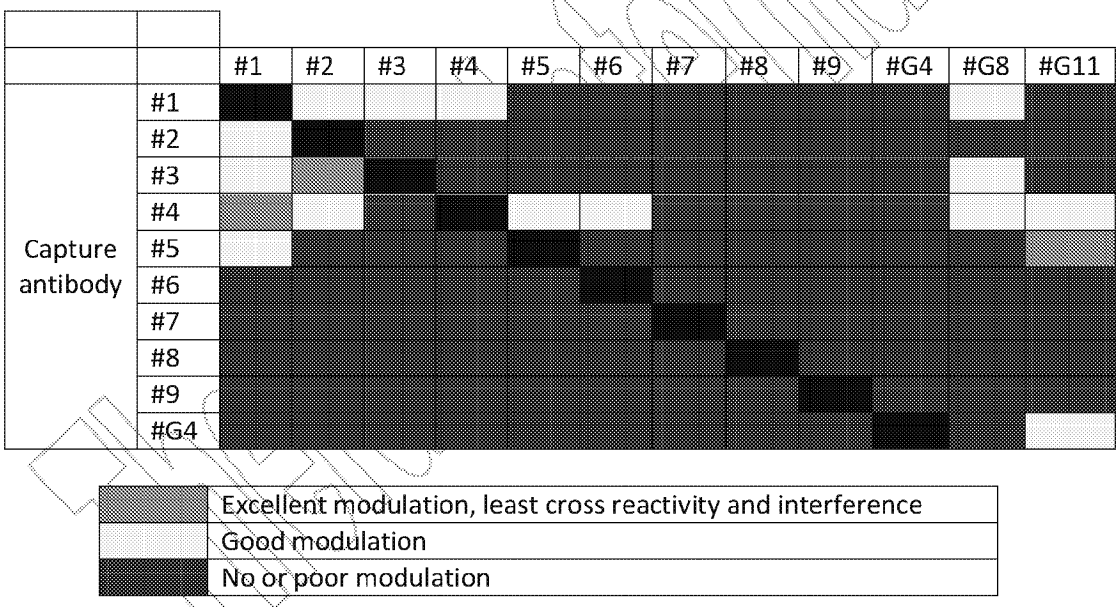
Cross reactivity										
IgG			IgA (10mg/ml)		IgD (0.5mg/ml)		IgE (0.002mg/ml)		IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.
0	16653	1	15366	1	15700	1	17828	1	16391	1
0.5	21988	1.3	14580	1	15483	1	17808	1	15569	1
5	62502	4	14700	1	16035	1	17165	1	16336	1
50	287483	17	15630	1	18264	1	18701	1	16433	1
Interference										
IgG			IgG+IgA (10mg/ml)		IgG+IgD (0.5mg/ml)		IgG+IgE (0.002mg/ml)		IgG+IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery
0	16653	1	14926	90	16724	100	22403	135	14436	87
0.5	21988	1.3	18037	82	23593	107	24419	111	18701	85
5	62502	4	43649	70	48421	77	71581	115	48573	78
50	287483	17	230323	80	23340	81	288643	100	236146	82

Antibody pair C5/D-G11

Cross reactivity										
IgG			IgA (10mg/ml)		IgD (0.5mg/ml)		IgE (0.002mg/ml)		IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.	Mean RLU	Mod.
0	14357	1	7187	1	6780	1	11517	1	6245	1

0.5	92125	6	8931	1	8575	1	10599	1	7059	1
5	530608	37	6642	1	7385	1	11739	1	5718	1
50	1939786	135	6961	1	8091	1	12183	1	7549	1
Interference										
IgG			IgG+IgA (10mg/ml)		IgG+IgD (0.5mg/ml)		IgG+IgE (0.002mg/ml)		IgG+IgM (10mg/ml)	
Conc. (mg/ml)	Mean RLU	Mod.	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery	Mean RLU	% recovery
0	14357	1	16689	116	14361	100	13065	91	19056	133
0.5	92125	6	75535	82	103575	112	81991	89	76670	83
5	530608	37	547125	103	464368	88	62081	117	506050	95
50	1939786	135	1813435	93	1832133	94	1804001	93	1877590	97

Table 6: Results of final screen on MTP



2.2 Antibody screening on readers

2.2.1 Antibody pairs screening with human IgG calibrators

From MTP screening, three pairs of antibodies were chosen to screen on readers.

Methods:

Edison protocol Human total IgG 100000X_5-5-5 was used for first round Edison screening. In summary, reaction tips were coated with UA at 20ug/ml in coating buffer and then Biotin labeled antibodies at 5ug/ml in blocking buffer. Human IgG calibrators were loaded to cartridge to incubate with coated tips for 5min. Detection antibody-AP conjugates were diluted to 50ng/ml

and incubated after sample incubation for 5min. Tips were then washed and incubated with AP substrate for 5min. RLU was measured for each tip.

Results:

All selected pairs showed good modulations on Edison. Signal modulations, curve regression and signal background were compared among all antibody pairs. C5/D-G11 was eliminated since it showed saturation towards the higher concentration of IgG calibrators. C3/D2 and C4/D1 were selected for further evaluation by testing with controls and clinical samples.

Table 7: Antibody screen with human IgG calibrators on Edison

	IgG	C3/D2			C4/D1			C5/D-G11		
Sample	Conc. (mg/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	50	1023650	16.3	129	893329	18.5	380	1673286	16	301
2	25	703777	5.4	89	570931	17.6	243	1495785	11	269
3	12.5	429296	18.1	54	267288	25.4	114	874750	17	157
4	6.25	284030	37.2	36	129432	25.3	55	590313	5	106
5	3.125	112774	26.5	14	42675	7.8	18	365965	8	66
6	1.56	87279	18.0	11	20257	8.9	9	206290	17	37
7	0.78	39713	17.2	5	12353	6.0	5	103515	28	19
8	0	7910	16.5	1	2350	4.8	1	5599	7	1

2.2.2 Training set with two final pairs

Methods:

"Training set" of control samples and clinical samples were tested with two final pairs of antibody using the same procedure. IgG concentration of each sample was calculated from calibration curves 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 12 clinical samples: 5 plasma (EDTA) and 5 plasma samples (lithium-heparin) from healthy donors of Stanford Blood Bank, 5 serum samples from Bioreclamation.

Three control reference materials were also included as control sample:

- IRMM reference material, catalog # ERM-DA470K/IFCC, certified IgG value: 9.17mg/ml, uncertainty: 0.18mg/ml.
- WHO international standard IgG, IgA, IgM, human serum, NIBSC code: 67/086. Each ampoule contains 100 units of activity of each of the three immunoglobulin. Theoretical value of IgG per unit is $80.4ug^{-1}$.

- Antibodies online Human standard serum with assigned values of IgG subclasses. Cat#: ABIN458853. Total IgG value 9.55mg/ml.

Results:

Eighteen control and clinical samples were measured with the two "final pairs" of antibody. Between these two pairs, C4/D1 (Ab#4 as capture antibody and Ab#1 as detection antibody) gave the best correlation and recovery comparing with reference method. C4/D1 was selected as the final pair to be used in assay development for further optimization.

Table 8: Training set of clinical samples measured by C4/D1

			C4/D1			
Samples	Sample lot#	Human IgG conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	Theranos IgG Conc. (mg/ml)	%recovery
Bioreclamation Serum samples	BRH267204	**23.99**	470489	8.3	**23.08**	***96***
	BRH267206	**2.76**	21666	23.9	**1.45**	***52***
	BRH267215	**2.85**	25802	5.9	**1.69**	***59***
	BRH267218	**6.94**	80343	17.1	**4.31**	***62***
	BRH267223	**16.30**	205848	16.7	**10.56**	***65***
Stanford blood bank plasma samples (EDTA and Lithium-Heparin)	EDTA01	**5.95**	68607	10.2	**3.65**	***61***
	EDTA03	**9.39**	142305	18.6	**7.51**	***80***
	EDTA04	**10.48**	183982	10.2	**9.52**	***91***
	EDTA07	**8.16**	62751	13.3	**3.60**	***44***
	EDTA10	**7.19**	52802	29.6	**3.10**	***43***
	LI-HEP01	**6.09**	74275	7.9	**4.19**	***69***
	LI-HEP03	**9.20**	108515	26.1	**5.87**	***64***
	LI-HEP04	**10.53**	123545	15.1	**6.86**	***65***
	LI-HEP07	**8.21**	93045	34.9	**4.97**	***61***
	LI-HEP10	**7.31**	81950	4.9	**4.62**	***63***
IRMM control	ERM-DA470K	**8.79**	100107	8.6	***5.59***	***64***
WHO control	NIBSC code: 67/086	**7.61**	127580	16.8	***6.56***	***86***
Antibodies online	ABIN458853	**8.18**	83578	27.9	***4.65***	***57***

Table 9: Training set of clinical samples measured by C3/D2

			C3/D2

		Human IgG conc. by SIEMENS ADVIA (mg/ml)	Mean RLU	%CV	Theranos IgG conc. (mg/ml)	%recovery
Bioreclamation Serum samples	BRH267204	**23.99**	746471	6.1	**28.93**	***121***
	BRH267206	**2.76**	100510	36.5	**2.08**	***75***
	BRH267215	**2.85**	65164	8.2	**1.31**	***46***
	BRH267218	**6.94**	294772	29.0	**7.14**	***103***
	BRH267223	**16.30**	373476	16.4	**11.07**	***68***
Stanford blood bank plasma samples (EDTA and Lithium-Heparin)	EDTA01	**5.95**	183099	20.1	**4.16**	***70***
	EDTA03	**9.39**	311239	21.4	**8.01**	***85***
	EDTA04	**10.48**	125285	38.6	**2.68**	***26***
	EDTA07	**8.16**	207609	18.5	**5.08**	***62***
	EDTA10	**7.19**	127488	47	**2.76**	***38***
	LI-HEP01	**6.09**	124513	7.3	**2.73**	***45***
	LI-HEP03	**9.2**	190637	4.1	**4.31**	***47***
	LI-HEP04	**10.53**	153350	23.6	**3.35**	***32***
	LI-HEP07	**8.21**	168179	30.5	**3.96**	***48***
	LI-HEP10	**7.31**	153111	28.6	**3.56**	***49***
IRMM control	ERM-DA470K	**8.79**	158924	17.2	***3.56***	***40***
WHO control	NIBSC code: 67/086	**7.61**	155358	16.9	***3.41***	***45***
Antibodies online	ABIN458853	**8.18**	148474	12.2	***3.12***	***38***

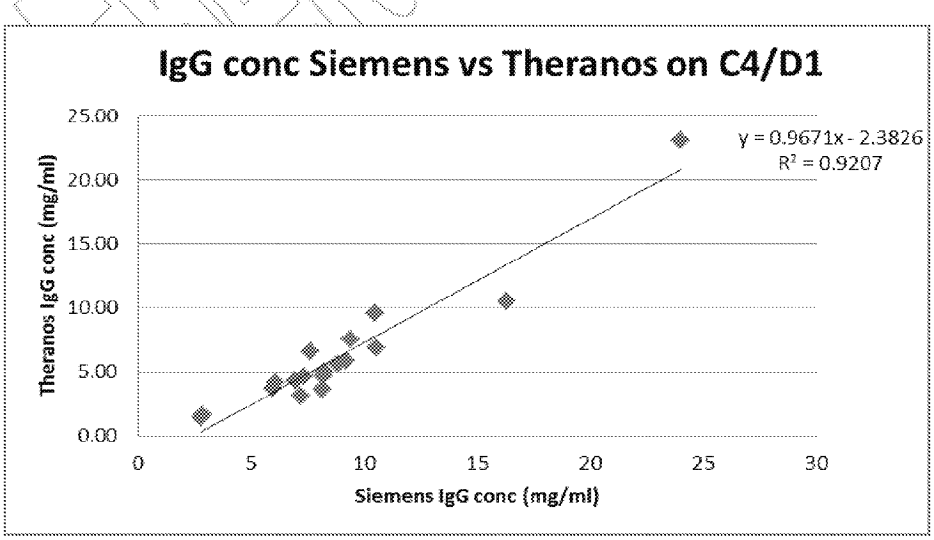


Figure [SEQ Figure * ARABIC]: Clinical sample correlation of C4/D1

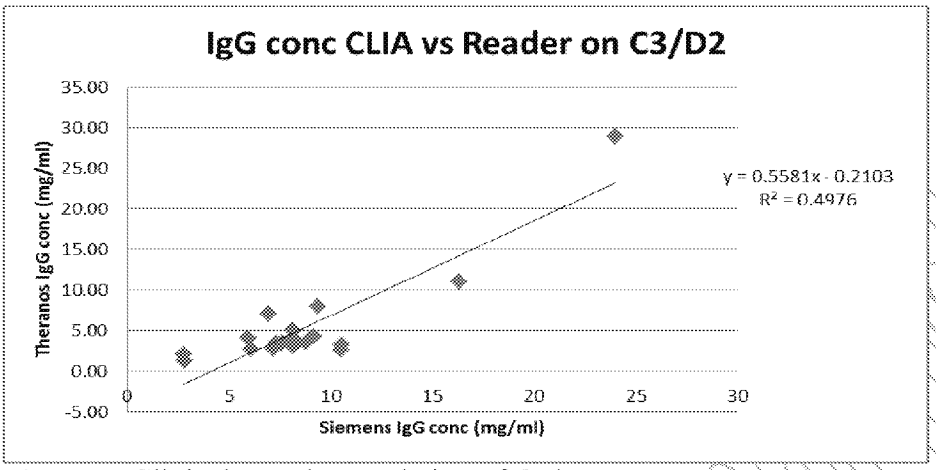


Figure 2: Clinical sample correlation of C3/D2

2.3 Theranos in house IgG binder capture antibody evaluation

Two IgG binder biotinylated capture antibodies were released from binder group: H2 and A9.

2.3.1 Detection antibody screening

Methods:

The MTP was first coated with UltraAvidin (UA) at 20ug/ml in coating buffer and then coated with Biotin labeled H2 capture antibody at 5ug/ml in blocking buffer. Human IgG calibrators at 0mg/ml, 0.5mg/ml, 5mg/ml, and 50mg/ml were hand diluted in blocking buffer 10,000 folds and incubated with coated antibodies. Then, detection antibody-AP conjugates were diluted in blocking buffer to 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 were calculated using RLU of each sample concentration level divided by the RLU of background (buffer blank, no IgG).

Results:

Comparing the modulation of commercial antibody pair C4/D1, binder capture antibodies were not able to give a high modulation, even with the best paring detection antibody respectively. However H2/D1, H2/D2, and H2/D6 were selected for further evaluation of binder H2; A9/D6, A9/D13 and A9/D14 were chosen for further evaluation of binder A9.

Table 10: Detection antibody screen for in house binder capture antibodies on MTP

		Detection antibody												
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D13	D14	D-G8	D-G11

Binder capture antibody	H2	55					52							
	A9						10				6	13		

2.3.1.1 Training set of Theranos in house binder H2

Method:

Edison protocol Generic2_10000X was used for first round Edison screening. In summary, reaction tips were coated with UA at 20ug/ml in coating buffer and then Biotin labeled antibodies at 5ug/ml in blocking buffer. Samples were loaded to cartridge to incubate with coated tips for 10min. Detection antibody-AP conjugates were diluted to 50ng/ml and incubated after sample incubation for 10min. Tips were then washed and incubated with AP substrate for 10min. RLU was measured for each tip.

A calibration curve was first run for each antibody pair. Then twenty one clinical samples were tested with two pairs of antibody using the same procedure. IgG concentration of each sample was calculated from calibration curves obtained from two pairs of antibody respectively. Percentage of recovery was calculated as concentration measured by Theranos method vs. SIEMENS ADVIA method.

Training set contained total 21 clinical samples: 11 plasma samples from healthy donors of Stanford Blood Bank, 10 serum samples from Bioreclamation.

Result:

The recovery of IgG seemed to be fine on calibrators. However, low recoveries were seen though out all clinical samples when compare to Siemens results for both two pairs of antibodies.

Table 11: Training set results of H2/D1

Samples	Sample lot#	Siemens IgG conc. (mg/ml)	Mean RLU	%CV	Theranos IgG conc. (mg/ml)	% recovery
Bioreclamation Serum samples	BRH353930	11	4695	5	1.60	15
	BRH353929	7	1942	13	0.46	7
	BRH402219	14	3879	4	1.22	9
	BRH402218	14	4773	14	1.64	12
	BRH353907	10	3594	38	1.10	11
	BRH402221	11	3407	23	1.02	9
	BRH353922	11	2636	23	0.71	6
	BRH353923	12	4267	15	1.40	12
	BRH353914	9	2461	22	0.64	7
	BRH402216	12	3750	20	1.16	10
Stanford blood bank plasma samples	W07051220082200	7	4604	32	0.36	5
	W07051200101700	11	3244	13	0.24	2
	W07051220093600	7	3693	35	0.28	4

	W07051200094300	7	3946	59	0.30	4
	W07051220093600	7	3815	7	0.29	4
	W07051220106000	12	4561	25	0.35	3
	W07051200094200	9	3521	29	0.27	3
	W07051200101900	11	4680	27	0.36	3
	W07051220098900	9	2384	6	0.18	2
	W07051200121900	5	1875	20	0.14	3
	W07051200107700	10	1501	10	0.11	1

Table 12: Training set results of H2/D6

Samples	Sample lot#	Siemens IgG conc. (mg/ml)	Mean RLU	%CV	Theranos IgG conc. (mg/ml)	% recovery
Bioreclamation Serum samples	BRH353930	11	15839	16	0.92	8
	BRH353929	7	8312	22	0.69	10
	BRH402219	14	17935	28	0.99	7
	BRH402218	14	34432	30	1.55	77
	BRH353907	10	9553	11	0.72	7
	BRH402221	11	18472	31	1.01	9
	BRH353922	11	19891	21	1.05	10
	BRH353923	12	23865	13	1.18	10
	BRH353914	9	15823	49	0.92	10
	BRH402216	12	21859	10	1.12	9
Stanford blood bank plasma samples	W07051220082200	7	15283	7	0.90	13
	W07051200101700	11	12991	23	0.83	8
	W07051220093600	7	12846	16	0.82	12
	W07051200094300	7	17916	35	0.99	14
	W07051220093600	7	23116	16	1.16	17
	W07051220106000	12	20067	14	1.06	9
	W07051200094200	9	17259	9	0.97	11
	W07051200101900	11	20965	24	1.09	10
	W07051220098900	9	7436	7	0.66	7
	W07051200121900	5	7345	27	0.66	13
	W07051200107700	10	7079	22	0.65	6

2.3.1.2 Training set of Theranos in house binder A9

Methods:

The method used here was the same as for the binder H2 training set. However, only three clinical samples: two plasma samples from healthy donors of Stanford Blood Bank, and one serum samples from Bioreclamation.

Results:

Even though all three pairs of antibody showed good recovery of calibrator IgG, all pairs of antibody A9/D6, A9/D13, and A9/D14 failed to respond to clinical samples with low recovery when compared to Siemens method.

Table 13: Training set results of A9/D6, A9/D13, and A9/D14

		A9/D6			
Sample lot#	Siemens IgG conc. (mg/ml)	Mean RLU	%CV	Theranos IgG conc. (mg/ml)	% recovery
BRH402219	14	21259	13	2.51	18
W07051200094300	7	14475	4	10.7	15
W07051200107700	10	19354	21	2.05	20
		A9/D13			
BRH402219	14	7306	41	1.95	14
W07051200094300	7	6260	10	1.53	22
W07051200107700	10	7042	29	1.84	18
		A9/D14			
BRH402219	14	183031	6	3.81	27
W07051200094300	7	117427	13	2.09	30
W07051200107700	10	125164	27	2.28	23

2.4 Method development with final pair of antibodies

2.4.1 Titration of capture antibody

Methods:

Capture concentration titration was done by coating with C4 in blocking buffer at 1ug/ml, 2.5ug/ml, 5ug/ml, and 10ug/ml respectively. Sample dilution was kept at 1:100000 and detection antibody concentration was kept at 50ng/ml. Edison protocol Human total IgG_100000x_5_5_5 was used for capture titration.

Results:

With detection antibody at 50ng/ml, capture antibody at 2.5ug/ml seemed to saturate the capture surface. Capture concentration higher than 2.5ug/ml also made the background go up. 2.5ug/ml of capture antibody was chose for other assay condition optimizations.

Table 14: Results of C4 capture titration

		coating 1ug/ml			coating 2.5 ug/ml		
Sample	Conc.(mg/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	50	40626	11	77.4	70727	11	81.0
2	25	8333	17	15.9	26580	28	30.5

3	**12.5**	4842	7	9.2	12357	5	14.2
4	**6.25**	2865	25	5.5	6412	15	7.3
5	**3.12**	1963		3.7	3213	2	3.7
7	**0.78**	660	11	1.3	1454	16	1.7
8	**0**	525	24	1.0	873	5	1.0

		coating 5ug/ml			coating 10ug/ml		
Sample	**Conc.(mg/ml)**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1	**50**	78672	19	38.5	75867	22	35.1
2	**25**	37053	8	18.1	35109	18	16.2
3	**12.5**	13314	36	6.5	20097	10	9.3
4	**6.25**	7056	5	3.5	7088	6	3.3
5	**3.12**	3995	9	2.0	4603	16	2.1
7	**0.78**	2268	8	1.1	2208	4	1.0
8	**0**	2042	9	1.0	2164	10	1.0

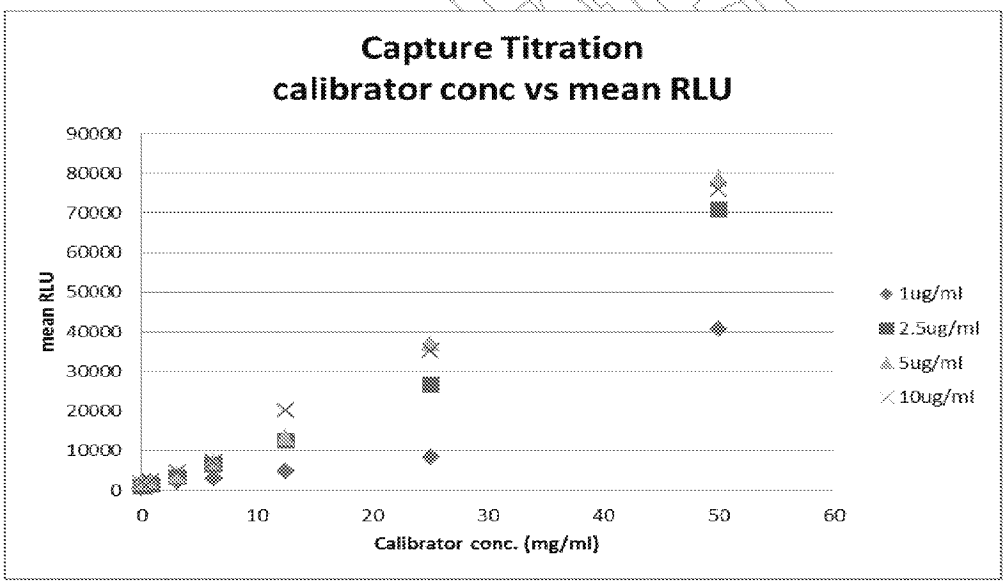


Figure 3: Results of capture titration

2.4.2 Selection of detection conjugate stabilizer

Methods:

With capture antibody at 2.5ug/ml in blocking buffer, detection conjugate was prepared at 50ng/ml in SurModics StabilZyme-AP stabilizer, Fluka Biostab AP conjugate stabilizer, and Theranos in-house AP stabilizer. All conditions were tested with protocol Human total IgG_100000x_5_5_5 to compare the effect of AP stabilizers.

Results:

Among three AP stabilizers, SurModics StabilZyme-AP gave lowest signal and modulations. Theranos in-house gave a good modulation. Fluka Biostab AP conjugate stabilizer gave the best modulation and clearest background. Thus Fluka Biostab AP conjugate stabilizer was chosen as the final stabilizer.

Table 15: Results of detection antibody stabilizer comparison

		D1 50ng/ml in BioStab			D1 50ng/ml in StabilZyme			D1 50ng/ml in in-house AP stabilizer		
Sample	**Conc. (mg/ml)**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1	**50**	890530	7	763	205315	13	75	595051	27	214
2	**25**	463227	24	397	91370	8	33	195827	24	70
3	**12.5**	257280	2	221	37776	7	14	125163	36	45
4	**6.25**	118855	9	102	19316	33	7	50337	22	18
5	**3.12**	64262	14	55	9142	6	3	28029	17	10
6	**1.56**	43893	21	38	6616	23	2.4	16463	17	6
7	**0.78**	17923	9	15	4453	37	1.6	8108	30	3
8	**0**	1167	4	1	2732	10	1	2780	5	1

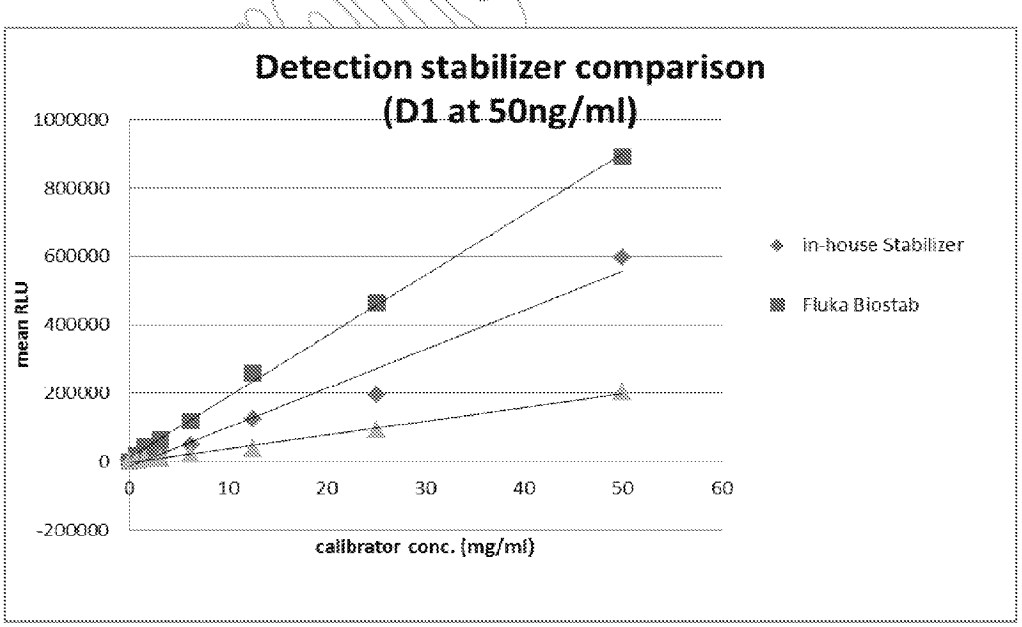


Figure 4: Results of detection antibody stabilizer comparison

2.4

2.4.1

2.4.2

2.4.3 Titration of detection antibody

2.4.3.1 Fluka Biostab AP stabilizer

Methods:

Titration of detection conjugate concentration was done by preparing detection conjugate in Fluka Biostab AP stabilizer at 5ng/ml, 10ng/ml and 25ng/ml respectively. Capture antibody was kept at 2.5ug/ml and the same Edison protocol human total IgG_100000x_5_5_5 was used.

Results:

Detection solutions at each concentration were prepared on day 1. The experiment was done on day 2; however, the RLU for detection concentration at 10ng/ml went up 10 times higher than expected. The same experiment was repeated with newly prepared detection at 5ng/ml, 10ng/ml and 25ng/ml. When tested on a later day, the RLU values went up again for 10ng/ml. This might due to instability of detection antibody in Fluka Biostab.

Table 16: Results of detection antibody titration- Fluka Biostab AP stabilizer

		D1 5ng/ml			D1 10ng/ml			D1 25ng/ml		
Sample	Conc. (mg/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	50	130374	9	62	230004	13	8.1	553433	10	193
2	25	60187	18	28	146748	12	5.1	251260	30	87
3	12.5	37695	15	18	92215	15	3.2	157593	11	55
4	6.25	18602	25	9	60662	2	2.1	80862	4	28
5	3.125	12620	17	6	54469	18	1.9	53773	23	19
6	1.56	7176	16	3	42831	3	1.5	22880	16	8
7	0.78	5592	9	3	34113	27	1.2	15585	20	5
8	0	2115	20	1	28552	12	1.0	2874	16	1

2.4.3.2 Theranos in-house AP stabilizer

Methods:

Titration of detection conjugate concentration was done by preparing detection conjugate in Theranos in-house AP stabilizer at 5ng/ml, 10ng/ml and 25ng/ml respectively. Capture antibody was kept at 2.5ug/ml and the same Edison protocol human total IgG_100000x_5_5_5 was used.

Results:

Based on the background, modulation, and saturation at 5ng/ml, 10ng/ml and 25ng/ml, the final condition for detection antibody was 5ng/ml in Theranos in-house stabilizer.

Table 17: Results of detection antibody titration-Theranos in-house stabilizer

		D1 5ng/ml			D1 10ng/ml			D1 25ng/ml		
Sample	Conc. (mg/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
1	50	156426	10	1241	306241	10	1825	640314	18	1661
2	25	101821	1	808	187476	6	1117	446524	13	1158
3	12.5	48389	15	384	100447	16	598	267080	14	693
4	6.25	23872	20	189	50859	17	303	113872	30	295
5	3.125	11298	5	90	24167	20	144	55627	5	144
6	1.56	6019	12	48	14178	7	84	38638	13	100
7	0.78	1897	16	15	5264	27	31	14088	9	37
8	0	126	19	1	168	7	1	386	15	1

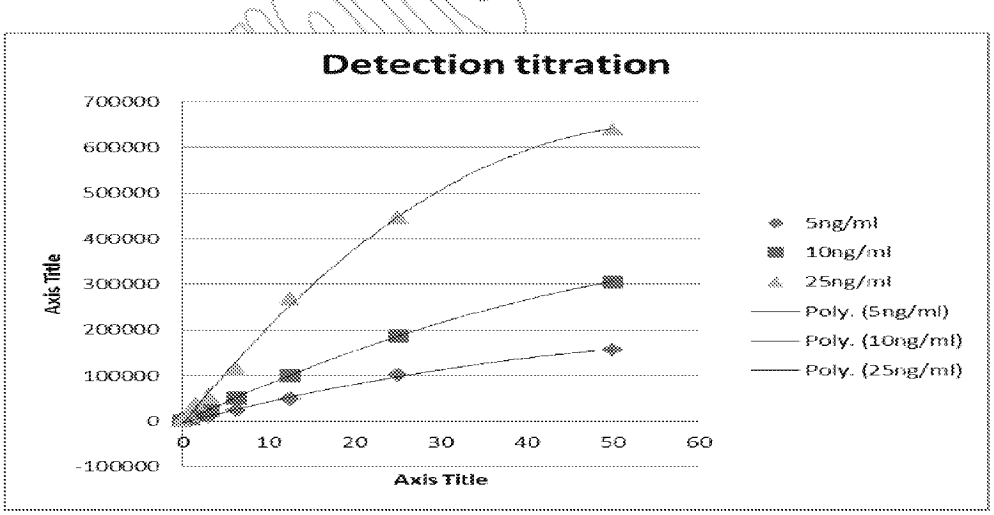


Figure 5: Results of detection antibody titration

2.4.4 Comparison of capture coating buffer

Methods:

Capture antibody was prepared at 2.5ug/ml in different buffers for comparison of capture coating buffer. Beside in-house blocking buffer (3%BSA-TBS, pH8.0), Starting block and Superblock from PIERCE were also used to prepare capture solution. Detection antibody was kept at 5ng/ml and the same Edison protocol Human total IgG_100000x_5_5_5 was used.

Results:

Pierce Superblock and Starting block tested for coating gave similar results. In-house blocking buffer showed the highest signal, best modulation and lowest background. In-house blocking buffer was kept unchanged to be used as capture coating buffer.

Table 18: Results of coating buffer comparison

		C4 in Blocking buffer			C4 in Starting block			C4 in Superblock		
Sample	Conc. (mg/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	**50**	156426	10	1241	119354	25	163	105033	13	286
2	**25**	101821	1	808	72290	23	99	69843	11	190
3	**12.5**	48389	15	384	44119	6	60	34851	20	95
4	**6.25**	23872	20	189						
5	**3.125**	11298	5	90	11835	10	16	9359	11	25
6	**1.56**	6019	12	48	4999	26	7	3834	41	10
7	**0.78**	1897	16	15	2687	52	4	2701	64	7
8	**0**	126	19	1	733	9	1	367	10	1

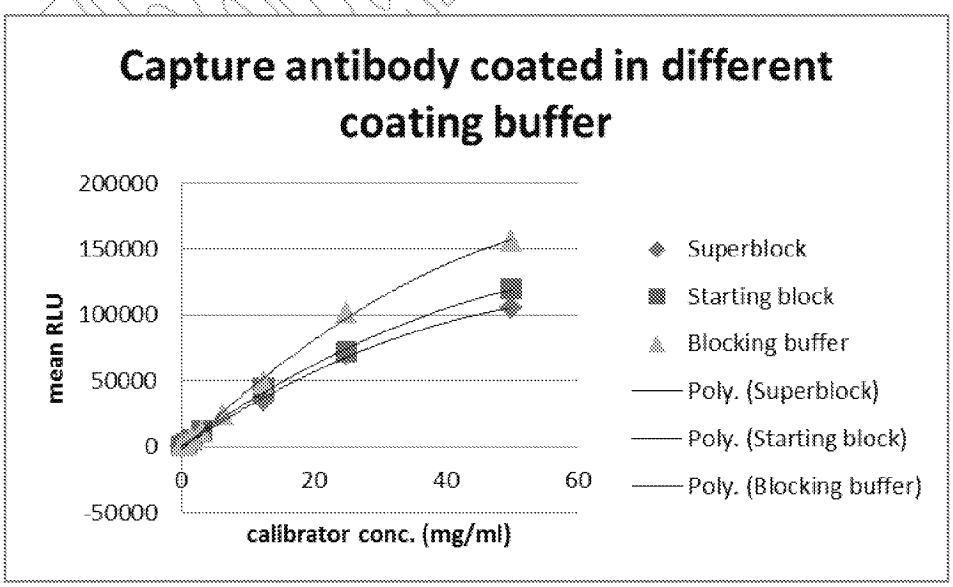


Figure 6: Results of coating buffer comparison

2.4.5 Incubation time testing

Methods:

With final condition of all reagents, protocol with incubation time of 2_2_1 was tested to check the effect of incubation time at each incubation step. Protocol Human total IgG_100000X_2_2_1 was used for the change of incubation time.

Results:

Shorter incubation time resulted with lower RLU signal and higher CV overall. The modulation of dose response, and calibration curve regression were still shown. With current reagents condition, incubation time 2_2_1 was not long enough to give the best modulation. However, if shorter incubation time is preferred, this assay would work after minor optimization of reagent conditions.

Table 19: Calibration curve with incubation time 2_2_1

Sample	Conc. (mg/ml)	Mean RLU	%CV	Modulation
1	50	14162	27	125
2	25	7700	22	68
3	12.5	4227	20	37
4	6.25	2078	23	18
5	3.125	1363	18	12
6	1.56	590	27	5
7	0.78	341	21	3
8	0	114	16	1

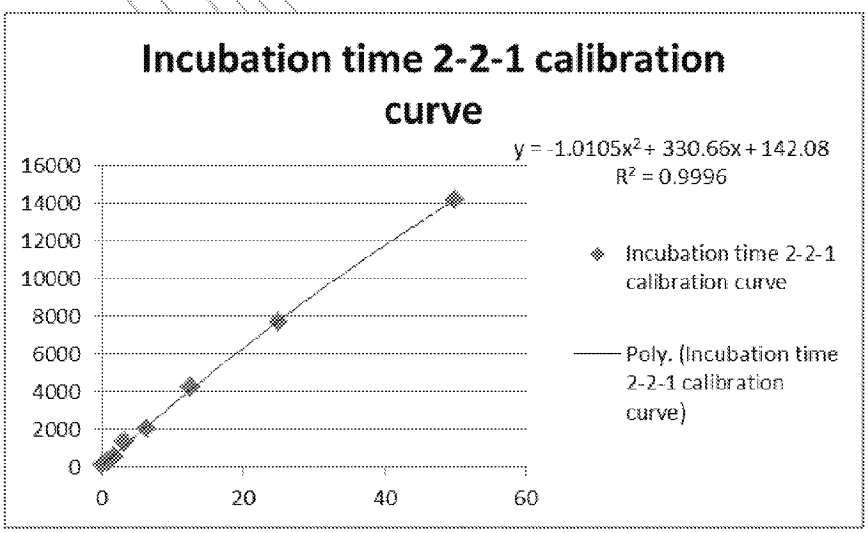


Figure 7: Calibration curve with incubation time 2_2_1

2.4.6 Effect of positive HAMA and RF samples

Five HAMA positive serum samples and five RF positive samples from PromedDx were analyzed with final assay condition. All samples were also tested for IgG level on SIEMENS Advia. Because the total IgG concentrations of most samples were within a narrow range, results correlation was not calculated by plotting Theranos data vs. SIEMENS data for linear regression analysis. Instead, difference between Theranos results and SIEMENS results was calculated to evaluate method compatibility with HAMA and RF positive samples.

From comparison of the difference between Theranos data and reference method, the results showed that HAMA positive status didn't affect the IgG analysis. On the other hand, RF positive samples showed severely affect the IgG analysis.

Table 20: Results of analysis of HAMA positive samples and RF positive samples

Sample	ProMedDx Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff
HAMA1	10580279	34824	10	9.70	11.20	-13
HAMA2	10580285	21539	20	6.17	7.58	-19
HAMA3	10580286	54189	12	14.73	17.49	-16
HAMA4	10580291	32164	19	9.00	11.63	-23
HAMA5	10580293	19199	10	5.53	7.54	-27
RF1	11745854	14768	5	4.32	8.68	-50
RF2	11745855	31982	26	8.95	13.19	-32
RF3	11745857	7682	5	2.33	5.56	-58
RF4	11745860	22490	9	6.42	9.63	-33
RF5	11745863	13466	14	3.96	10.44	-62

2.4.6.1 Trouble shooting for RF positive

Methods:

Due to low recovery level of IgG in RF positive samples, Scantibodies HBR-1 (heterophilic blocking reagent) was added to sample diluent at 400ug/ml. All the other assay conditions were kept the same, and the protocol was still Human total IgG_100000X_5_5_5. New calibration curve was run with 400ug/ml HBR-1 added to samples diluent, then the IgG concentrations in the RF positive samples were back calculated based on this new calibration curve.

Result:

With addition of 400ug/ml HBR-1 in sample diluent, recovery of IgG in RF positive samples improved when compare to Siemens result.

Table 21: Results of analysis of RF positive samples with 400ug/ml HBR-1 added

Sample	ProMedDx Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff
RF1	11745854	27194	6	6.81	8.68	-22
RF2	11745855	33286	6	8.41	13.19	-36
RF3	11745857	20644	12	5.11	5.56	-8
RF4	11745860	37861	15	9.62	9.63	0
RF5	11745863	32350	6	8.16	10.44	-22

2.4.6.2 Titration of HBR-1 in sample diluent

Since improvement of IgG recovery in RF positive samples was observed with the addition of 400ug/ml HBR-1 in sample diluent. HBR-1 was titrated down to 200ug/ml and 100ug/ml.

Methods:

Same assay method in 2.5.1.1 was applied here. With each condition, new calibration curve was run and IgG concentration in RF positive samples was back calculated with new calibration curve.

Result:

With 100ug/ml HBR-1 added to sample diluent, the recovery rate of IgG in RF positive samples decreased. Good recovery percentage was observed with addition of 200ug/ml HBR-1 in sample diluent, thus 200ug/ml HBR-1 was kept for the final assay condition.

Table 22: Results of analysis of RF positive samples with 100ug/ml and 200ug/ml HBR-1

	Sample	ProMedDx Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff
200ug/ml HBR-1	RF1	11745854	11597	11	6.43	8.68	-26
	RF2	11745855	18049	10	10.56	13.19	-20
	RF3	11745857	10742	16	5.90	5.56	6
100ug/ml HBR-1	RF1	11745854	19911	22	4.17	8.68	-52
	RF2	11745855	32194	26	6.66	13.19	-49

	RF3	11745857	3669	14	0.80	5.56	-86

2.4.7 Re-titration of capture antibody

The first batch of capture antibody conjugated with Dojindo Biotin-SH was used up. Theranos in house Biotin-SH labeling kit was later used to conjugate the capture antibody.

2.4.7.1 Tip comparison

Methods:

Tips with 2.5ug/ml capture antibodies conjugated with Dojindo Biotin-SH kit and Theranos in house Biotin-SH kit were coated. Three level calibrations were run to compare the activity of Dojindo tips and Theranos in house tips. The other assay condition was kept unchanged using the Human total IgG_100000X_5_5_5 protocol.

Result:

With capture antibody conjugated with Theranos in house Biotin-SH labeling kit, the RLU went down for high concentration calibrator, the background went up for blank, modulation also decreased. Thus it's necessary to re-titration the capture antibody concentration.

Table 23: Tip comparison of capture antibody conjugated with Dojindo kit and Theranos kit

		Capture antibody with Dojindo Biotin-SH kit			Capture antibody with Theranos in house Biotin-SH kit		
sample	Conc.(mg/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	50	313	10	1	745	10	1.0
7	0.78	3866	6	12	1032	20	1.4
8	0	83570	11	267	23733	15	31.9

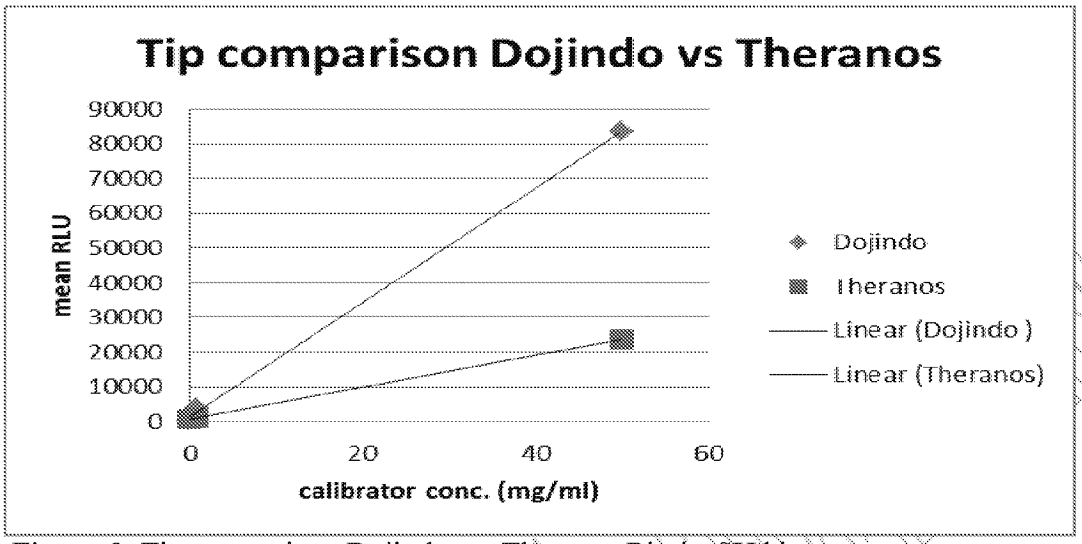


Figure 8: Tip comparison Dojindo vs. Theranos Biotin-SH kit

2.4.7.2 Re-titration of capture antibody concentration

Methods:

Capture concentration titration was done by coating with C4 in blocking buffer at 2.5ug/ml, 5ug/ml, and 10ug/ml respectively. Sample dilution was kept at 1:100000 and detection antibody concentration was kept at 50ng/ml. Edison protocol Human total IgG_100000x_5_5_5 was used for capture titration.

Results:

With detection antibody at 50ng/ml, capture antibody at 10ug/ml seemed to saturate the capture surface. Thus 5ug/ml of capture antibody was chose for other assay condition optimizations.

Table 24: Results of C4 capture re-titration

		C4 2.5ug/ml			C4 5ug/ml			C4 10ug/ml		
sample	Conc. (mg/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
1	50	89089	16	236	255619	21	350	278338	24	379
2	25	35407	45	94	130572	12	179	182359	7	248
4	6.25	10576	95	28	29515	5	40	39610	15	54
6	1.56	1927	41	5	7127	8	10	7798	4	11
7	0.78	822	29	2	2876	7	4	3321	20	5
8	0	377	14	1	731	19	1	735	2	1

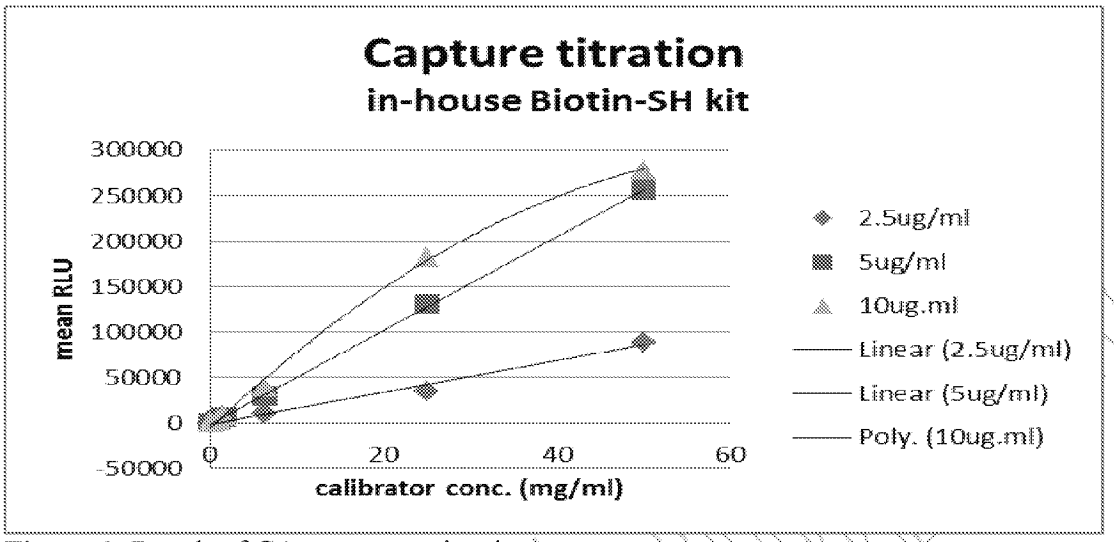


Figure 9: Result of C4 capture re-titration

2.4.8 Re-titration of detection antibody concentration

Since the C4 capture antibody was re-titration to 5ug/ml, it was necessary to re-titrate D1 detection antibody for assay optimization prupose.

Methods:

Titration of detection conjugate concentration was done by preparing detection conjugate in Theranos in-house AP stabilizer at 10ng/ml, 25ng/ml and 50ng/ml respectively. Capture antibody was kept at 5ug/ml and the same Edison protocol human total IgG_100000x_5_5_5 was used. Two controls and six clinical samples were also tested for IgG recovery at each detection concentration.

Results:

Based on the IgG recovery difference between Siemens and Theranos at 10ng/ml, 25ng/ml and 50ng/ml, the final condition for detection antibody is 50ng/ml in Theranos in-house stabilizer.

Table 25: Results of detection antibody re-titration

		D1 10ng/ml			D1 25ng/ml			D1 50ng/ml		
Sample	Conc. (mg/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
1	**50**	66062	6	366	162485	8	397	384625	11	588
3	**12.5**	12640	20	70	41223	11	101	102515	20	157
4	**6.25**	7300	9	40	19120	8	47	47146	4	72
5	**3.125**	3836	5	21	9806	4	24	23145	1	35
7	**0.78**	692	28	4	2359	29	6	4941	6	8

8	0	181	6	1	409	25	1	654	13	1

Table 26: Results of clinical samples recovery with D1 at 10ng/ml and 50ng/ml

			D1 10ng/ml			D1 50ng/ml		
	Sample	**Siemens IgG (mg/ml)**	**Mean RLU**	**Theranos IgG (mg/ml)**	**% diff.**	**Mean RLU**	**Theranos IgG (mg/ml)**	**% diff.**
Controls	IRMM	8.79	1443	1.48	-83	55812	9.26	5
	WHO	7.61	2971	2.88	-62	41426	6.71	-12
Bioreclamation serum samples	BRH627204	23.99	14311	12.32	-49	123630	21.84	-9
	BRH627206	2.76	598	0.65	-76	14147	2.11	-24
	BRH627218	6.94	3880	3.68	-47	40605	6.57	-5
	BRH627223	16.30	10673	9.39	-42	114270	20.06	23
Stanford blood bank plasma samples	W07051200237600	9.20	1366	1.40	-85	49749	8.18	-11
	W07051200238100	10.53	3755	3.56	-66	63227	10.59	1

2.4.9 Effect of HAMA and RF positive samples

Based on previous testing under **2.4.6** with HAMA and RF positive samples, RF positive samples would interfere the assay. Optimization of sample diluent was required.

Methods:

Total of seven different diluents were tried as samples diluent. All the other assay conditions were kept the same with C4 capture antibody at 5ug/ml and D1 detection antibody at 50ng/ml, and the protocol was the Human total IgG_100000X_5_5_5. New calibration curve was run for each samples diluent condition respectively, and then the IgG concentrations in all eighteen samples were back calculated based on this new calibration curve and compared to Siemens results.

Table 27: Different diluents used as sample diluent

	Diluent	**Supplier**	**Cat#**
1	400ug/ml HBR-1 in 3% BSA	Scantibodies	3KC533
2	200ug/ml HBR-1 in 3% BSA	Scantibodies	3KC533
3	200ug/ml HBR-3 in 3% BSA	Scantibodies	3KC701
4	RF-absorbance in 3% BSA	IBL international	KIRF561
5	Low cross buffer	Candor	100500

6	Protein free (TBS) buffer	Pierce	
7	Assay diluent (protein free)	SurModics	

Result:

Base on the recovery of IgG level in all nineteen samples, including five control, four clinical samples from Bioreclamation, five HAMA positive serum samples, and five RF positive serum samples, sample diluents of 400ug/ml HBR-1 in 3% BSA and 200ug/ml HBR-1 in 3% BSA gave the overall best recovery when comparing to Siemens system. 400ug/ml HBR-1 in 3% BSA was chosen as the final condition.

Table 28: Result of different sample diluents effecting HAMA and RF positive samples

			400ug/ml HBR-1 in 3% BSA		200ug/ml HBR-1 in 3% BSA	
	Samples	Siemens IgG conc. (mg/ml)	Theranos IgG conc. (mg/ml)	% diff.	Theranos IgG conc. (mg/ml)	% diff.
Controls	IRMM	8.79	7.62	-13	9.86	12
	WHO	7.61	6.46	-15	6.32	-17
	Biorad level 1	4.09	3.69	-10	3.03	-26
	Biorad level 2	8.44	7.45	-12	7.96	-6
	Antibody online	8.18	6.72	-18	6.27	-23
Bioreclamation serum samples	Biorec 204	23.99	28.58	19	18.63	-22
	Biorec 206	2.76	2.18	-21	2.08	-25
	Biorec 215	2.85	2.56	10	3.44	21
	Biorec 223	16.30	14.12	-13	13.25	-19
HAMA positive serum samples	HAMA 79	11.20	9.66	-14	12.81	14
	HAMA 85	7.58	6.71	-11		
	HAMA 86	17.49	19.25	10	16.54	-5
	HAMA 91	11.63	13.03	12		
	HAMA 93	7.54	6.93	-8		
RF positive serum samples	RF 54	8.68	6.74	-22	8.18	-6
	RF 55	13.19	13.69	4	14.18	8
	RF 57	5.56	2.02	-64	6.43	16
	RF 60	9.63	7.35	-24	8.51	-12
	RF 63	10.44	12.64	21	9.71	-7

2.4.10 Matrix effect

2.4.10.1 Hemolyzed serum samples

Five hemolyzed serum samples from Zeptometrix were analyzed. IgG concentrations from Theranos method of all samples had the recovery within 80% to 120% of the results from SIEMENS method. Hemolyzed matrix didn't show matrix effect in this assay.

Table 29: Results of hemolyzed serum samples

samples	Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff
Hemo 4	0107-027-00706	20501	16	7.63	9.66	-21
Hemo 5	0107-027-00705	18202	12	6.70	7.73	-13
Hemo 6	0107-027-00708	17806	14	6.54	8.22	-20
Hemo 7	0107-027-00709	54126	31	21.99	18.25	21
Hemo 8	0107-027-00703	37256	6	14.64	16.77	-13

2.4.10.2 Icteric serum samples

Five icteric serum samples from PromedDx were analyzed. IgG results from Theranos method showed acceptable recovery comparing to results from SIEMENS method. The matrix effect of icteric samples was not significant.

Table 30: Results of icteric serum samples

samples	ProMedDx Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff
Icteric1	1899979	24613	20	9.31	9.66	-4
Icteric2	1900426	19755	38	7.33	10.00	-27
Icteric3	11585302	22320	15	8.37	10.80	-22
Icteric4	11585447	23529	13	8.87	10.73	-17
Icteric5	11583525	34615	4	13.51	10.84	25

2.4.10.3 Lipemic serum samples

Five lipemic serum samples from Zeptometrix and four lipemic samples from ProMedDx were analyzed. IgG results of samples with higher than 600mg/dL triglycerides level showed lower recovery from Theranos method comparing to SIEMENS results. The results indicated that samples with high triglyceride level should be rejected for this assay, or lipemic samples should be run via another reliable method for confirmation.

Table 31: Results of lipemic serum samples

samples	Zeptometrix Lot#	Mean RLU	%CV	Theranos result (mg/ml)	SIEMENS result (mg/ml)	%Diff	Triglycerides conc. (mg/dL) by CLIA lab
Lip1	0107-027-00684	54652	21	22.23	21.25	5	461
Lip2	0107-027-00685	25943	11	9.86	8.03	23	530
Lip3	0107-027-00687	10511	49	3.68	16.14	-77	809
Lip4	0107-027-00688	12173	30	4.32	11.12	-61	650
Lip5	0107-027-00689	5774	58	2.00	10.25	-80	1325
	ProMedDx lot#						
Lip6	11211784	16726	13	8.12	8.21	-1	
Lip7	11211816	14678	42	7.21	6.84	5	
Lip8	11211940	17963	15	8.66	8.91	-3	
Lip9	11212185	15978	21	7.79	7.55	3	

2.4.11 Hematocrit effect and anticoagulant effect

Methods:

Whole blood, EDTA plasma, and heparin plasma samples from ten donors (5 male and 5 female) were obtained in pairs from Stanford Blood Center. All samples were analyzed with final assay procedure. Hematocrit effect was evaluated by comparing IgG results in whole blood versus in EDTA plasma samples from the same donor. EDTA plasma and heparin plasma from the same donor were also analyzed to compare the effect of anticoagulant. Since the IgG levels obtained from these ten donors were in a narrow range, linear regression was not plotted for EDTA vs heparin plasma. Instead the difference of IgG concentration between the Theranos system and Siemens system was compare in between each matching pairs.

Results:

Samples from ten donors collected in pairs of whole blood, EDA plasma, and heparin plasma were analyzed. Hematocrit factor was calculated to be 1.66 from the slope of plotting IgG results from EDTA plasma vs. results from whole blood.

The IgG results from EDTA plasma and heparin plasma correlate well with Siemens result without showing significant different in between each anticoagulant. This method could be used to analyze whole blood, EDTA plasma and heparin plasma.

Table 32: IgG results of whole blood and plasma matching pairs

		Whole blood			EDTA plasma			Heparin plasma		
Sample #	Blood Center ID	Mean RLU	%CV	Calc conc (mg/ml)	Mean RLU	%CV	Calc conc	Mean RLU	%CV	Calc conc

							(mg/ml)			(mg/ml)
M1	W07051200306800	10251	3	5.20	13482	8	6.67	14802	12	7.26
M2	W07051200306600	11081	34	5.58	16850	30	8.17	20107	7	9.60
M3	W07051200306700	12057	16	6.03	19442	14	9.31	20044	4	9.57
M4	W07051200306400	14124	8	6.96	24664	5	11.56	23931	24	11.25
M5	W07051200306500	11359	0	5.71	17808	7	8.60	17371	12	8.40
F1	W07051200306200	12517	2	6.23	19833	10	9.48	17579	16	8.49
F2	W07051200307000	12212	14	6.10	16593	9	8.06	23087	7	10.89
F3	W07051200306300	9714	11	4.95	14087	22	6.94	14560	23	7.16
F4	W07051200307500	13474	7	6.67	16616	19	8.07	25009	7	11.71
F5	W07051200307600	8669	9	4.46	12879	13	6.40	14635	27	7.19

Table 33: Theranos vs Siemens IgG results of EDTA and Heparin plasma matching pairs

		EDTA plasma			**Heparin plasma**		
Sample #	**Blood center ID**	**Siemens IgG (mg/ml)**	**Theranos IgG (mg/ml)**	**% diff.**	**Siemens IgG (mg/ml)**	**Theranos IgG (mg/ml)**	**% diff.**
M1	W07051200306800	8.33	6.67	-20	8.35	7.26	-13
M2	W07051200306600	9.37	8.17	-13	9.19	9.60	4
M3	W07051200306700	10.63	9.31	-12	10.44	9.57	-8
M4	W07051200306400	12.39	11.56	-7	12.32	11.25	-9
M5	W07051200306500	8.73	8.60	-1	8.63	8.40	-3
F1	W07051200306200	10.40	9.48	-9	10.58	8.49	-20
F2	W07051200307000	9.45	8.06	-15	9.38	10.89	16
F3	W07051200306300	8.05	6.94	-14	7.95	7.16	-10
F4	W07051200307500	9.72	8.07	-17	9.53	11.71	23
F5	W07051200307600	7.45	6.40	-14	7.47	7.19	-4

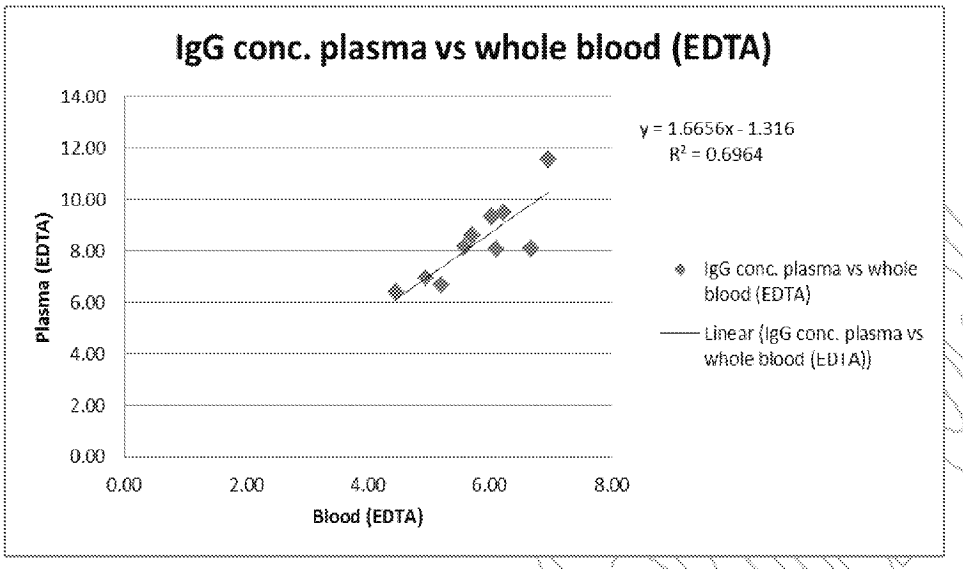


Figure 10: Results of hematocrit effect

2.5 Clinical sample analysis with final protocol

2.5.1 Calibration curve run with final assay condition

Final assay condition C4 capture antibody conjugated with Theranos in house Biotin-SH kit coated at 5ug/ml in 3% BSA blocking buffer, D1 detection antibody at 50ng/ml in Theranos in house AP stabilizer, 3% BSA blocking buffer with 400ug/ml HBR-1 as sample diluent. Calibration curve was generated under this assay condition with final protocol of Human total IgG_100000X_5_5_5 and data was analyzed by Dexter.

Table 34: IgG final calibration curve

Sample	Conc. (mg/ml)	Mean RLU	%CV	Modulation	Cal from Dexter (mg/ml)	%Accuracy
1	50	108986	3	303	53.27	107
2	25	57702	13	161	24.82	99
3	12.5	25434	7	71	11.46	92
4	6.25	15025	22	42	7.36	118
5	3.125	5166	13	14	3.05	98
6	1.56	2942	7	8	1.82	116
7	0.78	1188	9	3	0.70	90
8	0	359	13	1		

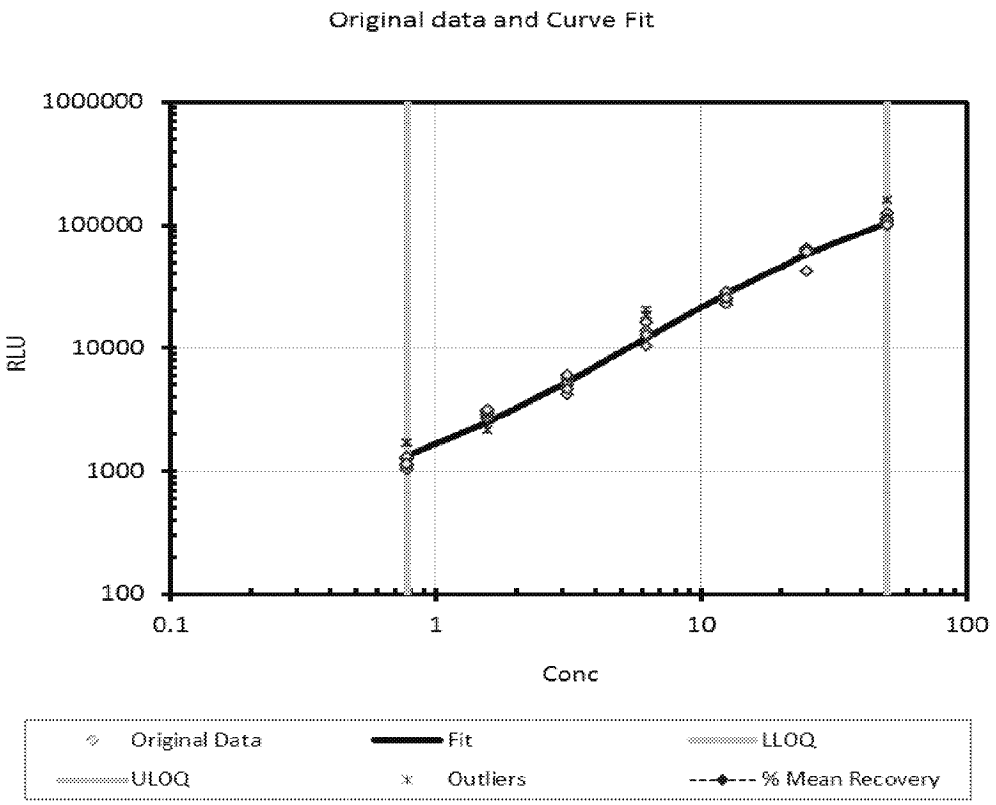


Figure 11: Calibration curve from Dexter analysis

Table 35: 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	2.545
b2	5.562
b3	5.853
b4	-0.707
LLOQ	0.78 mg/ml
ULOQ	50 mg/ml
LLOQ accuracy	84%
LLOQ precision	13.3%
ULOQ accuracy	107%
ULOQ precision	4.8%

2.5.2 Clinical sample analysis

2.5.2.1 Serum samples of different diseases from Bioreclamation

Twenty serum samples from different patients were obtained from Bioreclamation. Samples were analyzed by Theranos method and SIEMENS method for total IgG level. The result difference between Theranos method and SIEMENS method was calculated. Data from two methods correlated well.

Table 36: Results of diseased serum samples from Bioreclamation

Major disease	Sample ID	Mean RLU	%CV	Theranos IgG conc (mg/ml)	Siemens IgG conc (mg/ml)	% difference
MM	BRH627204	46226	10	20.49	23.99	-15
	BRH627205	16331	8	7.94	8.51	-7
	BRH627206	3630	26	2.36	2.76	-14
MS	BRH627207	22608	11	10.68	12.62	-15
	BRH627208	19491	32	10.65	12.53	-15
	BRH627209	25325	8	11.85	14.22	-17
SLE	BRH627210	22349	20	10.57	10.74	-2
	BRH627211	17493	33	8.46	10.11	-16
	BRH627212	27974	34	12.97	15.80	-18
	BRH641648	40440	61	18.14	17.98	1
Leukemia	BRH627215	3881	22	2.21	2.85	-22
	BRH627216	20170	15	9.63	8.86	9
	BRH627217	20373	27	9.72	10.63	-9
	BRH627218	13333	16	6.60	6.94	-5
	BRH627219	15155	14	7.42	8.04	-8
Kidney	BRH627220	20234	42	11.31	10.61	7
	BRH627221	19162	13	9.19	9.24	-1
	BRH627222	20635	8	9.83	9.63	2
	BRH627223	34923	13	15.88	16.30	-3
Otitis media	BRH641653	25638	17	11.98	12.05	-1

2.5.2.2 Serum samples of normal patients from Bioreclamation

Ten serum samples from normal patients were obtained from Bioreclamation. Samples were tested by Theranos method and Siemens method of total IgG level. The result difference between Theranos and Siemens method was calculated. Data from two sources correlated well.

Table 37: Results of normal serum samples from Bioreclamation

Sample#	Sample ID	mean RLU	%CV	Theranos IgG conc (mg/ml)	Siemens IgG conc (mg/ml)	% difference

B07	BRH353930	17196	9	8.33	11	-24
B09	BRH353929	11189	14	5.63	7	-20
B14	BRH402219	23696	3	11.15	14	-20
B16	BRH402218	26842	4	12.49	14	-11
B19	BRH353907	24320	13	11.42	10	14
B23	BRH402221	17776	19	8.58	11	-22
B24	BRH353922	20598	22	9.81	11	-11
B25	BRH353923	17949	34	8.66	12	-28
B26	BRH353914	17119	40	8.29	9	-8
B30	BRH402216	20167	14	9.63	12	-20

2.5.2.3 Plasma samples of normal donors from Stanford blood bank

Ten plasma samples of normal donors obtained from Stanford blood bank were tested on Theranos system and Siemens system. The result difference between the two was calculated. Data showed that results from these sources correlated well.

Table 38: Results of normal plasma samples from Stanford blood bank

Sample#	Sample ID	mean RLU	%CV	Theranos IgG conc (mg/ml)	Siemens IgG conc (mg/ml)	% difference
S1	W07051220082200	12360	14	6.16	7	-12
S2	W07051200101700	18850	23	9.05	11	-18
S3	W07051220093600	14937	20	7.32	7	5
S4	W07051200094300	12931	10	6.42	7	-8
S6	W07051220093600	13839	17	6.83	7	-2
S7	W07051220106000	23656	23	11.13	12	-7
S8	W07051200094200	15353	18	7.51	9	-17
S9	W07051200101900	22817	15	10.77	11	-2
S10	W07051220098900	25872	15	12.08	9	34
S11	W07051200121900	5344	21	2.87	5	-43

2.5.2.4 Paired serum, EDTA plasma and Heparin plasma samples of healthy donors from Stanford Blood Center

Paired serum, EDTA plasma and heparin plasma samples were obtained from ten healthy donors (5 male and 5 female donors) at Stanford Blood Center. All samples were analyzed by both Theranos and SIEMENS methods. Data from two methods correlated well. No significant difference was observed among serum, EDTA plasma and heparin plasma samples.

Table 39: Results of Stanford healthy donor serum sample analysis

Sample#	Lot #	Mean RLU	%CV	Theranos	Siemens	%

				IgG (mg/ml)	IgG (mg/ml)	difference
MS1	W07051200306800	16801	11	8.15	8.47	-4
MS2	W07051200306600	23123	11	10.90	9.25	18
MS3	W07051200306700	23754	12	11.18	10.66	5
MS4	W07051200306400	20149	13	9.62	12.32	-22
MS5	W07051200306500	18014	5	8.69	8.77	-1
FS1	W07051200306200	17761	32	8.58	10.57	-19
FS2	W07051200307000	13995	6	6.90	9.43	-27
FS3	W07051200306300	13230	3	6.56	8.03	-18
FS4	W07051200307500	16036	5	7.81	9.61	-19
FS5	W07051200307600	11874	12	5.94	7.53	-21

Table 40: Results of Stanford healthy donor EDTA plasma sample analysis

Sample#	Lot #	Mean RLU	%CV	Theranos IgG (mg/ml)	Siemens IgG (mg/ml)	% diff.
ME1	W07051200306800	13482	8	6.67	8.33	-20
ME2	W07051200306600	16850	30	8.17	9.37	-13
ME3	W07051200306700	19442	14	9.31	10.63	-12
ME4	W07051200306400	24664	5	11.56	12.39	-7
ME5	W07051200306500	17808	7	8.60	8.73	-1
FE1	W07051200306200	19833	10	9.48	10.40	-9
FE2	W07051200307000	16593	9	8.06	9.45	-15
FE3	W07051200306300	14087	22	6.94	8.05	-14
FE4	W07051200307500	16616	19	8.07	9.72	-17
FE5	W07051200307600	12879	13	6.40	7.45	-14

Table 41: Results of Stanford healthy donor Heparin plasma sample analysis

Sample#	Lot #	Mean RLU	%CV	Theranos IgG (mg/ml)	Siemens IgG (mg/ml)	% diff.
M1	W07051200306800	14802	12	7.26	8.35	-13
M2	W07051200306600	20107	7	9.60	9.19	4
M3	W07051200306700	20044	4	9.57	10.44	-8
M4	W07051200306400	23931	24	11.25	12.32	-9
M5	W07051200306500	17371	12	8.40	8.63	-3
F1	W07051200306200	17579	16	8.49	10.58	-20
F2	W07051200307000	23087	7	10.89	9.38	16
F3	W07051200306300	14560	23	7.16	7.95	-10

F4	W07051200307500	25009	7	11.71	9.53	23
F5	W07051200307600	14635	27	7.19	7.47	-4

2.5.2.5 Summary of clinical sample analysis

Total 70 serum and plasma samples were analyzed by Theranos method final protocol. Overall, the results from Theranos method correlated well with SIEMENS method.

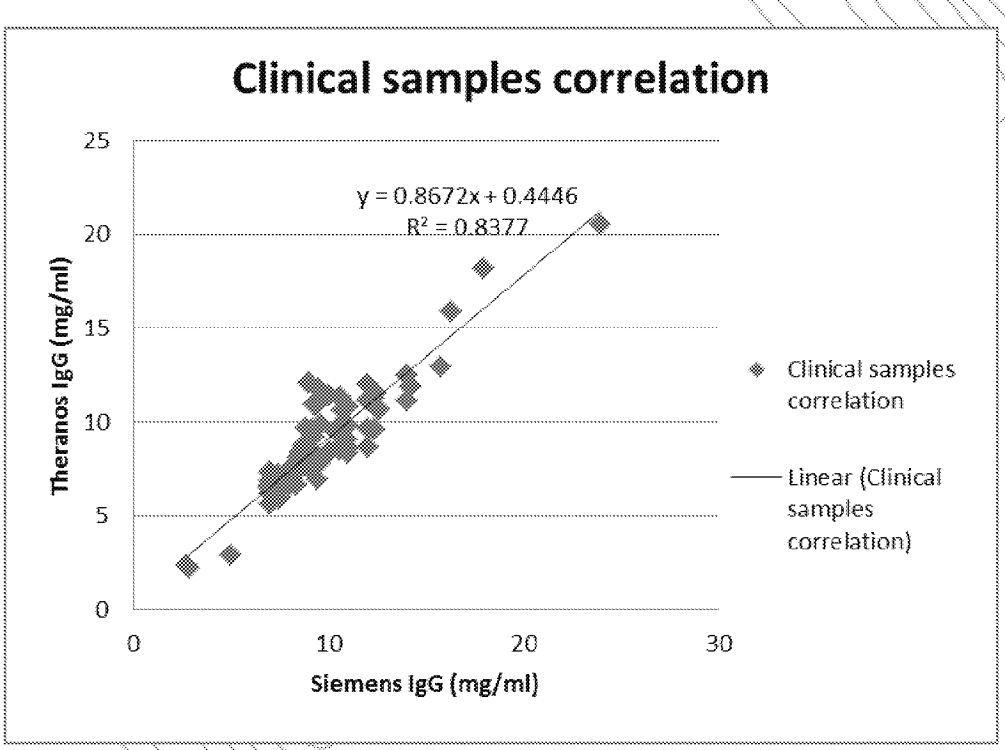


Figure 12: Correlation of Theranos method vs. SIEMENS method

2.6 Stability

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