Human IgD 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 D (IgD) is one of the five antibody isotypes that produced by plasma cell. It represents less than 1% among total plasma immunoglobulin. The molecular weight IgD is 185KDa. IgD is produced as a monomeric antibody with two heavy delta chains and two light chains. It has a short half-life of 2.8 days and it's highly sensitive to proteolysis.

IgD presents on the surface of many B lymphocytes, indicated the readiness of virgin B cells to be primed by antigen. The precise role of IgD remains unknown.

Normal adult serum concentration of IgD is less than 153ug/ml. Placenta is an effective barrier for IgD, thus the concentration of IgD is low or undetectable in newborns. IgD concentration progressively increases during childhood. Elevated IgD level is associate with hyper immunoglobulinemia syndrome (HIDS) and allergic disorders. HIDS is an autosomal recessive disorder with clinical features consists of recurrent attacks of fever, abdominal pain, and diarrhea. Diagnosis of HIDS is based on clinical criteria and elevated serum IgD level greater than 100 IU/ml (1IU/ml=1.412ug/ml). Very high level of IgD is found in IgD myeloma patients. IgD myeloma is a rare disease which only counts for 2% among all myeloma patients.

1.2 Assay specifications

This assay determines the concentration of IgD in human serum, plasma and whole blood. The assay has a quantification range of 3.125ug/mL to 400ug/mL (3.215mg/L to 400mg/L).

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

The following assay was used as reference method:

Instrument: SIEMENS BN™ II Nephelometry
Reagent kit: Binding Site Group Human IgD liquid reagent kit

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

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

In this assay, a biotinylated anti-human IgD Fab raised in E. coli was used as capture agent for IgD 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 15,000 folds with sample diluent and incubated with capture antibody coated tips. A goat $F(ab)'^2$ anti-human antibody was conjugate 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. IgD concentration of unknown sample was calculated from calibration curve.

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

Name	Supplier	Catalog number
Human IgD (myeloma plasma, kappa)	Athens Research	16-16-090704-M
Biotinylated IgD Fab	In house	F6
Goat F(ab)'2 anti-human IgD, delta chain	Southern Biotech	2032-01
Assay diluent (Protein free)	SurModics	SM01-100
Tris buffer (powder)	Sigma	T6664
TBST pH 8.0 (powder)	Sigma	T9039
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
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 #812 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, eight commercial anti-IgD antibodies and two in house Fab binders were screened for binding of human IgD on micro titer plate (MTP).

All the commercial antibodies were labeled with Biotin using in house Biotin-SH kit (lot 081412). 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. The two in house Fab binders came in the biotinylated forms and both two were screened against the eight commercial antibodies labeled with AP.

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 IgD calibrators at 0ug/ml, 4ug/ml, 40ug/ml, and 400ug/ml were hand diluted in blocking buffer 1000 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 IgD).

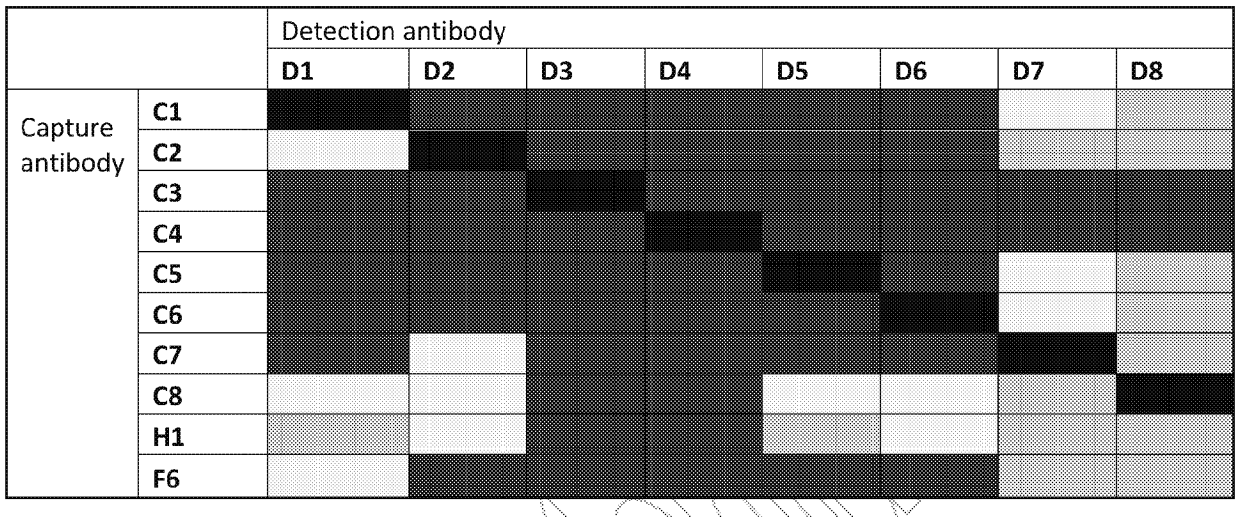
Results:

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-IgD antibody #	Name	Supplier	Cat#	Lot#	Clone#
1	purified mouse anti human IgD	Biolegend	348202		IA6-2
2	monoclonal mouse anti human IgD	antibodies-online	ABIN567689		NI158
3	goat anti human IgD, polyclonal, C terminus	Santa Cruz biotech	sc-34656		D-17
4	goat anti human IgD, polyclonal, N terminus	Santa Cruz biotech	sc-34567		E-13
5	mouse anti human IgD, monoclonal	US bio	I1895-03		10D231
6	monoclonal mouse anti human IgD, delta chain	Southern Biotech	9030-01		IADB6
7	Goat anti human IgD, delta chain	Southern biotech	2030-01		
8	Goat F(ab)'2 anti-human IgD, delta chain	Southern biotech	2032-01		
F6	Biotinylated Fab	Theranos		IgDF6102512	
H1	Biotinylated Fab	Theranos		IgDH1102512	

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



2.1.2 Cross reactivity and interference tests

Human IgA, IgE, IgG and IgM were tested for cross reactivity and interference on MTP for all twenty-five pairs of antibodies which showed modulation >50 in the initial screening. These samples were prepared at higher concentration than 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)
IgA from human colostrum	Sigma	I1010	SLBC0508	4	0.68-3.78
Human IgE	Abbiotec	250202		0.002	< or = 0.0007
Human IgM (myeloma), whole molecule	Jackson Lab	009-000-012	104252	4	0.6-2.63
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 IgD calibrator, the above cross reactants which hand diluted 1000 folds in blocking buffer were added as samples. For interference test, the above cross reactants that spiked into IgD calibrators at each concentration were used as samples.

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

Table 5: Results of H1/D7 F6/D7 F6/D8 cross reactivity

Cross reactivity											
H1/D7				**F6/D7**				**F6/D8**			
analyte	conc.	mean RLU	Mod.	analyte	conc.	mean RLU	Mod.	analyte	conc.	mean RLU	Mod.
IgD	0ug/ml	1660	1	IgD	0ug/ml	1547	1	IgD	0ug/ml	3633	1
IgA	4mg/ml	3349	2	IgA	4mg/ml	1644	1	IgA	4mg/ml	3447	1
IgE	0.002ug/ml	1405	1	IgE	0.002ug/ml	1375	1	IgE	0.002ug/ml	3213	1
IgM	4mg/ml	1142	1	IgM	4mg/ml	1782	1	IgM	4mg/ml	3130	1
IgG	20mg/ml	1058	1	IgG	20mg/ml	1293	1	IgG	20mg/ml	3676	1

Table 6: Results of H1/D7 F6/D7 F6/D8 interference

Interference										
IgD with H1/D7 antibody pair			IgD+IgA 4mg/ml		IgD+IgE 0.002ug/ml		IgD +IgM 4mg/ml		IgD+IgG 20mg/ml	
conc. (ug/ml)	mean RLU	modulation	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery
0	779	1	811	104	734	94	825	106	978	126
4	23943	31	18078	76	18991	79	18093	76	20769	87
40	174753	224	141227	81	127404	73	139424	80	127455	73
400	698721	897	591518	85	610949	87	581238	83	664861	95
IgD with F6/D7 antibody pair			IgD+IgA 4mg/ml		IgD+IgE 0.002ug/ml		IgD +IgM 4mg/ml		IgD+IgG 20mg/ml	
conc. (ug/ml)	mean RLU	modulation	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery
0	1281	1	1336	104	1374	107	1669	130	2299	179
4	21353	17	17202	81	19078	89	15888	74	20703	97
40	155401	121	135049	87	123638	80	103790	67	122366	79
400	511401	399	484025	95	539147	105	420804	82	457018	89
IgD with F6/D8 antibody pair			IgD+IgA 4mg/ml		IgD+IgE 0.002ug/ml		IgD +IgM 4mg/ml		IgD+IgG 20mg/ml	
conc. (ug/ml)	mean RLU	modulation	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery	mean RLU	% recovery
0	1820	1	1968	108	2047	112	2360	130	2505	138

4	18665	10	15476	83	18057	97	18127	97	16474	88
40	195319	107	144890	74	135424	69	121851	62	124006	63
400	778621	428	648337	83	556712	71	562940	72	544427	70

Table 7: Results of final screen on MTP

		Detection antibody							
		D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**
Capture antibody	**C1**								
	C2								
	C3								
	C4								
	C5								
	C6								
	C7								
	C8								
	H1								
	F6								

	Excellent modulation (>400), least cross reactivity and interference
	Good modulation (100-300)
	fair modulation (>50)
	No or poor modulation

2.2 Antibody screening on readers

2.2.1 Antibody pairs screening with human IgD calibrators

From MTP screening, antibody pairs H1/D7, F6/D7 and F6/D8 were chosen to screen on readers. Since both capture antibodies H1 and F6 were Theranos in house binder Fab, a commercial capture antibody C2 with detection antibody D8 was also selected to be tested on readers. This commercial antibody pair C2/D8 showed a signal/background modulation above 100X with minimum cross reactivity and interference against other immunoglobulin.

2.2.1.1 Calibration dilution determination

In order to get the best calibration curve during the Theranos screening process and eliminate saturation at high concentration of calibrator, different dilution factors were first carried out.

2.2.1.1.1 2000X calibrator dilution

Methods:

First all IgD calibrators were hand diluted 2000X in blocking buffer, then Edison protocol Generic2_ND 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. Hand diluted human IgD calibrators 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.

Results:

All selected pairs showed good modulations on Edison. Signal modulations, curve regression and signal background were compared among all antibody pairs. Commercial antibody pair C2/D8 was removed since it showed about 200X modulation whereas all remaining three antibody pairs showed more than 600X signal/background modulation. H1/D7, F6/D7 and F6/D8 moved on with further calibration dilution since high dilution could potentially eliminate the matrix effects if there was any.

Table 8: Antibody screen with human IgD calibrators on Edison (2000X calibrator dilution)

		C2/D8			H1/D7			F6/D7			F6/D8		
IgD Calibrator	**Conc. (ug/ml)**	**Mean RLU**	**%CV**	**Mod.**	**Mean RLU**	**%CV**	**Mod.**	**Mean RLU**	**%CV**	**Mod.**	**Mean RLU**	**%CV**	**Mod.**
1	**500**	392985	27.8	232	1060010	11.4	1429	928970	1	919	969124	20.8	602
2	**250**	229361	12.8	135	740053	21.6	997	652627	16	646	754942	5.4	469
3	**125**	149231	16.8	88	497418	22	670	449891	4.4	445	489684	13.1	304
4	**62.5**	123851	6.8	73	354639	15.7	478	173216	12.9	171	231812	33.5	144
5	**15.6**	31153	22	18	128883	19.9	174	95645	17.5	95	73239	7	45
6	**7.8**	15713	13.2	9	64735	20.8	87	43355	12.6	43	44260	0.5	27
7	**3.9**	9677	45.1	6	36780	9	50	28215	16.5	28	24286	13.7	15
8	**0**	1697	6.8	1	742	13.8	1	1011	15.3	1	1611	15.1	1

2.2.1.1.2 5000X calibrator dilution

Methods:

The same technique used in 2.2.1.1.1 was used here, except that instead of 2000X hand dilution of IgD calibrators, a 5000X hand dilution of calibrators in blocking buffer were performed.

Results:

H1/F7, F6/D7 and F6/D8 all performed well with great signal/background modulation, curve regression, signal background, and % recovery at each calibrator level. Thus all three pairs moved forward to Training set with clinical samples.

Table 9: Antibody screen with human IgD calibrators on Edison (5000X calibrator dilution)

		H1/D7			F6/D7			F6/D8		
IgD Calibrator	**Conc. (ug/ml)**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1	**500**	528947	10.8	1071	792862	5.8	748	719058	17	337
2	**250**	349080	18.8	707	502272	13.2	474	477530	0	224
3	**125**	189930	16.5	384	263358	10	248	325581	6	153
4	**62.5**	122080	20.4	247	218154	5.2	206	200952	9	94
5	**15.6**	36578	8.4	74	51787	6	49	67148	15	31
6	**7.8**	12259	22.1	25	19017	8	18	25021	17	12
7	**3.9**	6735	11.8	14	13092	19.1	12	14749	15	7
8	**0**	494	11.4	1	1060	3.8	1	2132	34	1

2.2.2 Training set with three final pairs

2.2.2.1 5000X sample dilution training set

Methods:

"Training set" of control samples and clinical samples were tested with three final pairs of antibody using the same procedure as **2.2.1.1.2** with control and clinical samples hand diluted 1:5000 in blocking buffer. IgD 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 BN™ II).

Training set contained total 20 clinical serum samples from ZeptoMetrix.

One control reference material was also included as control sample:

- Non WHO reference material IgD serum, Human, NIBSC code: 67/037. Each ampoule contains 100 units of activity of IgD. The total volume of the standard reconstituted in 1.0ml distilled water has been recalculated to be 1.06ml. The reconstituted standard will therefore contain 94.3 units of IgD in $1.0ml^{-1}$. Theoretical result of 1 unit containing $1.41ug$ of IgD^{-2}.

Results:

Twenty ZeptoMetrix clinical serum samples and one control were tested with the final three pairs of antibodies. With all three pairs, the recovery of IgD of the control samples was good

comparing to the reported value. However, all 20 clinical samples had an over recovery of the IgD level when comparing to reference method (SIEMENS BN™ II). These results suggested that Theranos IgD calibrator was calibrated against the non WHO IgD serum (code: 67/037). However, matrix effect could interference the recovery of IgD in fresh human serum samples. Increase the dilution factor could help to eliminate the matrix effect of clinical samples.

Table 10: Training set of clinical samples measured by H1/D7 (1:5000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinical serum samples	0001-027-02304	132	376543	19.5	268	203
	0001-027-02305	5	18822	23.9	10	209
	0001-027-02306	7	24756	21.0	14	201
	0001-027-02307	106	278756	18.0	193	182
	0001-027-02309	40	123059	9.5	80	199
	0001-027-02310	52	182028	6.8	122	234
	0001-027-02313	9	20911	17.8	12	130
	0001-027-02314	111	392904	28.2	280	252
	0001-027-02315	104	322286	23.6	226	217
	0001-027-02316	10	36834	11.9	22	216
	0001-027-02320	103	183515	2.0	123	119
	0001-027-02321	164	304910	6.5	213	130
	0001-027-02323	18	28844	12.5	17	92
	0001-027-02324	61	122778	12.7	80	130
	0001-027-02326	30	94723	32.6	60	200
	0001-027-02327	110	266157	22.4	184	167
	0001-027-02328	16	31498	21.3	18	114
	0001-027-02329	97	229401	8.3	157	161
	0001-027-02330	7	32224	16.0	19	267
	0001-027-02331	47	202527	3.3	137	291
WHO control	NIBSC code:67/037	133	167728	20.3	112	84

Table 11: Training set of clinical samples measured by F6/D7 (1:5000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinical serum	0001-027-02304	132	640733	15.2	315	239
	0001-027-02305	5	32431	7.5	11	220
	0001-027-02306	7	57775	15.5	21	301

samples	0001-027-02307	106	468150	19.3	222	209
	0001-027-02309	40	179567	20.3	75	188
	0001-027-02310	52	335173	16.5	152	293
	0001-027-02313	9	52119	17.7	19	208
	0001-027-02314	111	591709	11.5	288	260
	0001-027-02315	104	569579	8.3	276	266
	0001-027-02316	10	81800	14.6	31	311
	0001-027-02320	103	263109	7.4	116	113
	0001-027-02321	164	519761	20.9	249	152
	0001-027-02323	18	59448	23.8	22	121
	0001-027-02324	61	181084	18.7	76	125
	0001-027-02326	30	156133	1.1	64	215
	0001-027-02327	110	520887	16.4	250	227
	0001-027-02328	16	51685	7.5	19	116
	0001-027-02329	97	266665	8.2	118	121
	0001-027-02330	7	41839	23.0	15	209
	0001-027-02331	47	299995	17.9	134	286
WHO control	NIBSC code:67/037	133	315471	29.5	143	108

Table 12: Training set of clinical samples measured by F6/D8 (1:5000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinical serum samples	0001-027-02304	132	685380	5.3	353	267
	0001-027-02305	5	41612	23.4	12	239
	0001-027-02306	7	74609	13.4	24	346
	0001-027-02307	106	581522	12.0	289	273
	0001-027-02309	40	188892	8.3	74	186
	0001-027-02310	52	371694	8.8	168	324
	0001-027-02313	9	53597	2.0	16	181
	0001-027-02314	111	620230	24.9	312	282
	0001-027-02315	104	608626	10.8	305	294
	0001-027-02316	10	80821	14.8	27	267
	0001-027-02320	103	256251	10.2	107	104
	0001-027-02321	164	586116	8.8	292	178
	0001-027-02323	18	67186	9.8	21	119
	0001-027-02324	61	175166	15.3	68	111
	0001-027-02326	30	227386	7.8	93	310
	0001-027-02327	110	539338	6.5	264	240
	0001-027-02328	16	76642	11.6	25	156
	0001-027-02329	97	343058	6.7	153	158

	0001-027-02330	7	47309	16.4	14	200
	0001-027-02331	47	267052	7.6	113	240
WHO control	NIBSC code:67/037	133	293684	5.9	129.28	97

2.2.2.2 25000X sample dilution training set

Methods:

Since over recovery of clinical samples was observed at 5000X sample dilution, dilution factor of samples were increased to 25000X. The same method used previously was adopted here, the only difference was that all calibrators, control, and clinical samples were hand diluted 1:25000 in blocking buffer. IgD 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 BN™ II).

Training set contained total nine clinical serum samples from ZeptoMetrix.

One control reference material was also included as control sample:

- Non WHO reference material IgD serum, Human, NIBSC code: 67/037. Each ampoule contains 100 units of activity of IgD. The total volume of the standard reconstituted in 1.0ml distilled water has been recalculated to be 1.06ml. The reconstituted standard will therefore contain 94.3 units of IgD in $1.0ml^{-1}$. Theoretical result of 1 unit containing $1.41ug$ of IgD^{-2}.

Results:

Nine ZeptoMetrix clinical serum samples and one control were tested with the final three pairs of antibodies. With all three pairs, the recovery of IgD of the control samples was good comparing to the reported value. However, all nine clinical samples had an under recovery of the IgD level when comparing to reference method (SIEMENS BN™ II).

Table 13: Training set of clinical samples measured by H1/D7 (1:25000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinical serum samples	0001-027-02304	132	28507	7.7	138	104
	0001-027-02305	5	1521	18.3	4	87
	0001-027-02306	7	2069	17.2	6	89
	0001-027-02307	106	12802	7.2	54	51
	0001-027-02309	40	4523	11.1	16	39
	0001-027-02316	10	2825	42.8	9	90
	0001-027-02321	164	22012	5.8	101	62
	0001-027-02323	18	2706	24.8	9	48
	0001-027-02326	30	4497	12.8	16	52

WHO control	NIBSC code:67/037	133	25593	5.3	121	91

Table 14: Training set of clinical samples measured by F6/D7 (1:25000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinicial serum samples	0001-027-02304	132	65222	3.0	93	70
	0001-027-02305	5	3540	4.9	4	73
	0001-027-02306	7	3922	7.7	4	58
	0001-027-02307	106	45575	18.3	62	59
	0001-027-02309	40	14198	20.2	17	43
	0001-027-02316	10	7288	14.8	8	81
	0001-027-02321	164	52152	7.9	72	44
	0001-027-02323	18	7583	15.9	8	47
	0001-027-02326	30	17838	19.1	22	73
WHO control	NIBSC code:67/037	133	86988	8.3	128	96

Table 15: Training set of clinical samples measured by F6/D8 (1:25000)

Samples	Sample lot#	IgD conc. by Siemens BN ™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
ZeptoMetrix clinical serum samples	0001-027-02304	132	58920	15.6	111	84
	0001-027-02305	5	4431	15.9	4	81
	0001-027-02306	7	6436	21.2	7	93
	0001-027-02307	106	35606	6.1	58	55
	0001-027-02309	40	17122	18.7	23	57
	0001-027-02316	10	8279	9.0	9	90
	0001-027-02321	164	52529	12.2	96	58
	0001-027-02323	18	9133	11.1	10	57
	0001-027-02326	30	14944	9.8	19	64
WHO control	NIBSC code:67/037	133	65039	12.5	126	95

2.2.2.3 15000X sample dilution training set

Methods:

In order to achieve the best clinical sample recovery and finalize the best antibody pair to move on for assay optimization, 15000X sample dilution training set test was performed with IgD antibody pairs H1/D7 and F6/D8. The method used here was the same as previous. IgD calibrators, control, and twenty clinical samples from Zeptometrix were all hand diluted 25000X in blocking buffer. IgD concentration of control and all clinical samples were calculated from the calibration curve of each antibody pair respectively. Percentage of recovery was calculated as concentration measured by Theranos method vs. Reference method (Siemens BN™ II).

Training set contained total twenty clinical serum samples from ZeptoMetrix.

One control reference material was also included as control sample:

- Non WHO reference material IgD serum, Human, NIBSC code: 67/037. Each ampoule contains 100 units of activity of IgD. The total volume of the standard reconstituted in 1.0ml distilled water has been recalculated to be 1.06ml. The reconstituted standard will therefore contain 94.3 units of IgD in $1.0ml^{-1}$. Theoretical result of 1 unit containing $1.41ug$ of IgD^{-2}.

Results:

Twenty ZeptoMetrix clinical serum samples and one control were tested with the final two pairs of antibody H1/D7 and F6/D8. The correlation between Theranos result and Siemens result was good for both pairs. However, F6/D8 pair out performed H1/D7 pair when comparing percentage recovery of each clinical samples. Thus, F6/D8 was selected as the final assay pair for further assay optimization.

Table 16: Training set of clinical samples with H1/D7 (1:15000)

Samples	Sample lot#	IgD conc. by Siemens BN™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
WHO control	NIBSC code:67/037	133	44058	4.1	106	80
ZeptoMetrix clinical serum samples	0001-027-02304	132	49710	35.7	122	92
	0001-027-02305	5	2073	86.9	3	57
	0001-027-02306	7	4787	31.0	8	110
	0001-027-02307	106	33226	22.9	76	71
	0001-027-02309	40	12509	3.3	24	60
	0001-027-02321	164	51680	17.3	128	78
	0001-027-02310	52	21296	20.4	45	86
	0001-027-02313	9	5855	3.3	10	108
	0001-027-02315	104	40133	43.1	95	91
	0001-027-02316	10	6232	0.7	10	105
	0001-027-02320	103	36283	1.1	84	82
	0001-027-02319	28	6687	4.9	11	41
	0001-027-02323	18	3167	15.8	5	26
	0001-027-02324	61	20808	0.4	44	71

	0001-027-02326	30	5800	8.0	10	32
	0001-027-02327	110	52629	2.5	131	119
	0001-027-02328	16	7527	27.1	13	82
	0001-027-02329	97	43121	31.5	103	106

Table 17: Training set of clinical samples with F6/D8 (1:15000)

Samples	Sample lot#	IgD conc. by Siemens BN™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
WHO control	WHO IgD	133	132324	6.1	115	87
ZeptoMetrix clinical serum samples	0001-027-02304	132	132115	9.6	115	87
	0001-027-02305	5	4875	7.6	5	90
	0001-027-02306	7	6910	33.4	6	91
	0001-027-02307	106	93252	7.7	82	77
	0001-027-02309	40	38957	21.4	35	87
	0001-027-02321	164	151863	7.5	132	81
	0001-027-02310	52	65476	15.3	58	111
	0001-027-02313	9	10732	3.9	10	109
	0001-027-02314	111	108562	6.7	95	86
	0001-027-02315	104	150226	3.9	131	126
	0001-027-02316	10	13206	7.9	12	120
	0001-027-02320	103	112179	4.2	98	95
	0001-027-02319	28	36863	15.0	33	118
	0001-027-02323	18	21122	15.7	19	106
	0001-027-02324	61	63467	10.7	56	92
	0001-027-02326	30	35103	17.8	31	105
	0001-027-02327	110	92012	1.7	81	73
	0001-027-02328	16	19812	0.3	18	112
	0001-027-02329	97	95913	13.2	84	87

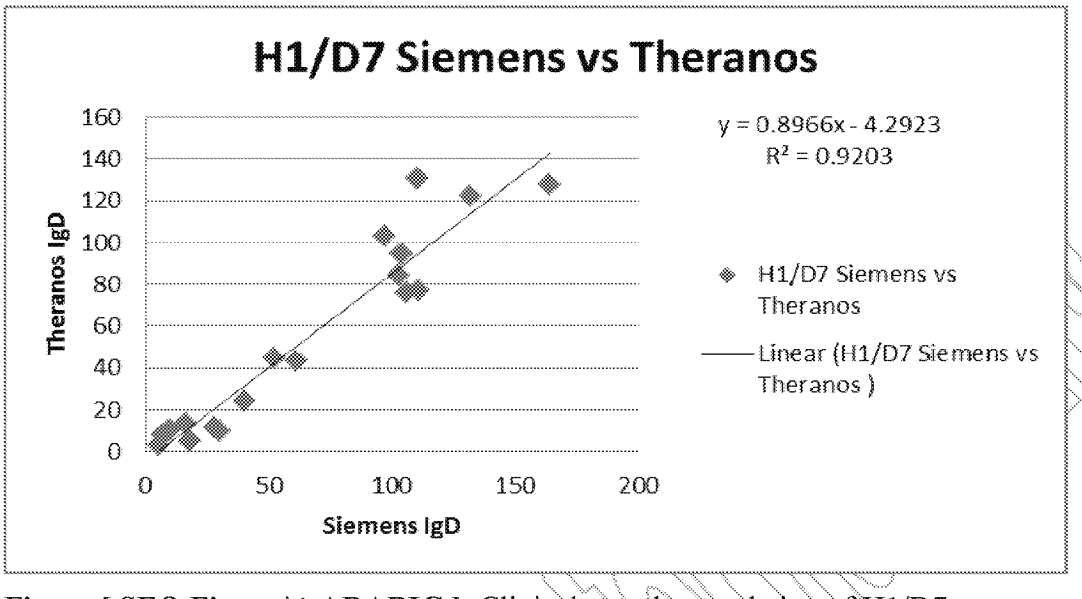


Figure [SEQ Figure * ARABIC]: Clinical sample correlation of H1/D7

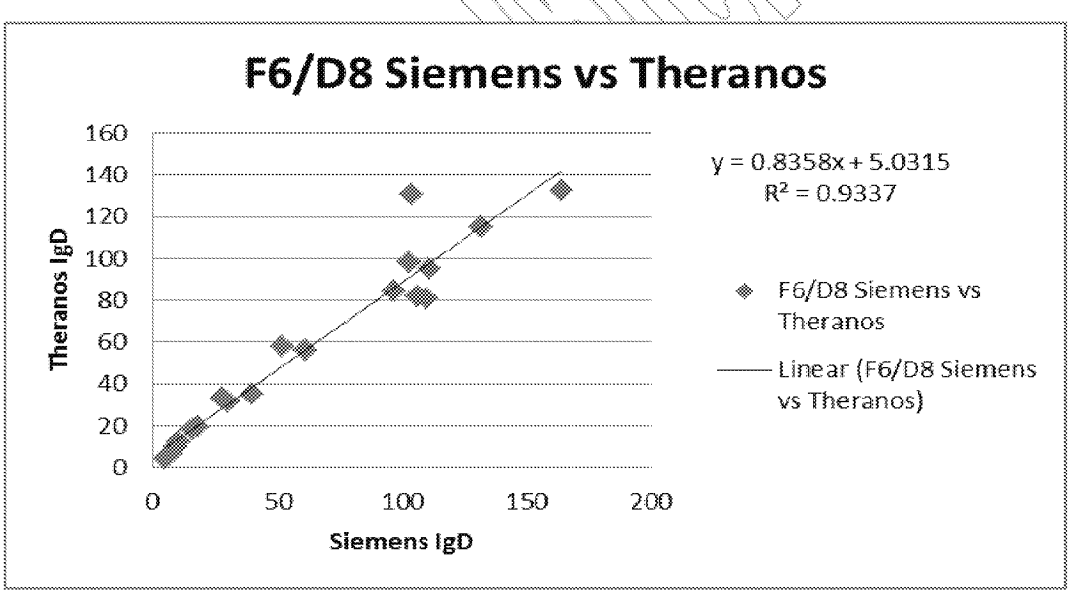


Figure 2: Clinical sample correlation of F6/D8

2.3 Method development with final pair of antibodies

2.3.1 Selection of capture coating buffer

Methods:

Capture antibody F6 was prepared at 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 50ng/m in blocking buffer. IgD calibrators were first hand diluted 1:3 with blocking buffer and the Edison protocol Generic2_5000X was used to achieve a final dilution of 15000X. Meanwhile, new Edison protocol Generic2_15000X was being prepared.

Results:

Overall three different coating buffers gave good signal/background modulation. Pierce Superblock showed the highest average inter-cartridge %CV of 28.3, and failed to differentiate calibrator 3 (6.25ug/ml) and calibrator 4 (12.5ug/ml). Starting block showed good modulation in between each calibrator; however, background was higher than Theranos in house blocking buffer. As a result, Theranos in house blocking buffer was kept as the capture coating buffer.

Table 18: Results of coating buffer comparison

		F6 in Blocking buffer			F6 in Starting block			F6 in Superblock		
Calibrator	**Conc. (ug/ml)**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1	**400**	629359	14.7	254.5	514018	2.4	145.1	413092	20.9	192.9
2	**200**	369028	12.3	149.2	390602	17.8	110.3	276739	24.2	129.2
3	**100**	127211	22.6	51.4	92579	26.3	26.1	111235	42.4	51.9
4	**50**	61293	27.6	24.8	52442	7.9	14.8	30229	32.0	14.1
6	**12.5**	24329	16.4	9.8	25036	5.6	7.1	14231	27.0	6.6
7	**6.25**	10567	7.3	4.3	13623	16.5	3.8	16350	18.8	7.6
8	**3.125**	6455	21.8	2.6	9007	22.0	2.5	4565	22.3	2.1
9	**0**	2473	3.0	1.0	3542	33.0	1.0	2142	38.8	1.0
		average %CV:15.7			average %CV: 16.4			average %CV: 28.3		

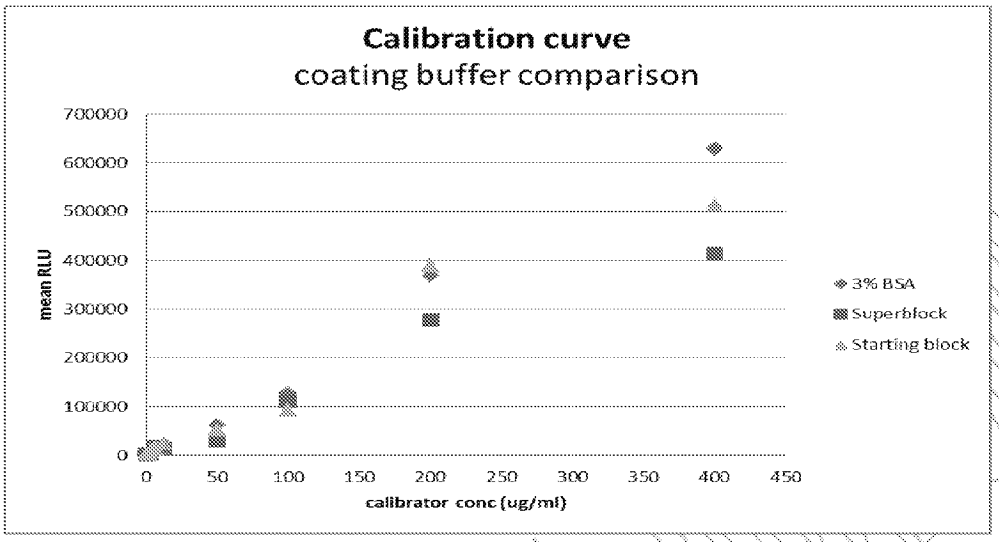


Figure 3: Results of coating buffer comparison

2.3.2 Titration of capture antibody

Methods:

Capture concentration titration was done by coating with F6 in blocking buffer at 2.5ug/ml, 5ug/ml, and 10ug/ml respectively. Sample dilution was kept at 1:15000 with 1:3 hand dilution in blocking buffer and then Edison protocol Generic2_5000X was used. Detection antibody concentration was kept at 50ng/ml in blocking buffer.

Results:

With detection antibody at 50ng/ml, capture antibody at 10ug/ml resulted in an overall high RLU and low signal/background modulation. Capture antibody at 2.5ug/ml and 5ug/ml performed comparably well. 2.5ug/ml showed lowest background, good sensitivity (2.9X), and good signal/background modulation of 112.5X. 5ug/ml had best sensitivity (3.2X) and signal/background modulation of 164.5X. 5ug/ml of capture antibody was chose for further assay condition optimizations.

Table 19: Results of capture titration

		coating 2.5ug/ml			coating 5 ug/ml			coating 10ug/ml		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	**400**	429394	19.8	112.5	660831	18.0	164.5	868299	24.9	71.5
2	**200**	224137	28.9	58.7	303045	17.5	75.4	452570	11.2	37.3
3	**100**	145731	3.5	38.2	209820	0.4	52.2	321396	3.1	26.5
4	**50**	57364	12.3	15.0	101597	23.5	25.3	157646	42.5	13.0

6	**12.5**	24360	1.8	6.4	33523	8.2	8.3	44316	24.2	3.7
7	**6.25**	13615	12.7	3.6	22018	13.9	5.5	40515	23.0	3.3
8	**3.125**	11052	15.3	2.9	12771	20.3	3.2	17478	16.9	1.4
9	**0**	3816	8.2	1.0	4018	8.4	1.0	12136	13.5	1.0

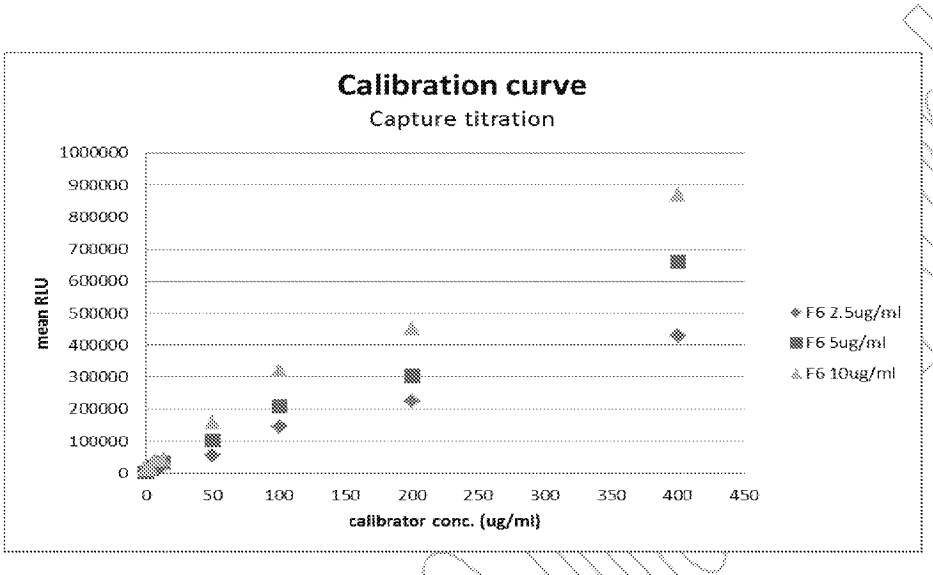


Figure 4: Results of capture titration

2.3.3 Selection of detection conjugate stabilizer

Methods:

With capture antibody at 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 first hand dilution of samples 1:3 in blocking buffer and then protocol Generic2_5000X to compare the effect of AP stabilizers.

Results:

Among three AP stabilizers, Fluka Biostab AP conjugate stabilizer gave the lowest modulation (19.5X), highest background and highest signal. SurModics StabilZyme-AP gave the lowest background, but low signal and a modulation of 86.2X. Theranos in house AP stabilizer performed the best with highest signal/background modulation and good sensitivity, thus Theranos in house AP stabilizer was chosen as the final stabilizer.

Table 20: Results of detection antibody stabilizer comparison

		D8 50ng/ml in BioStab			D8 50ng/ml in StabilZyme			D8 50ng/ml in in-house AP stabilizer		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation

1	**400**	282233	23.2	19.5	24619	15.2	86.2	98834	23.0	137.7
3	**100**	137153	8.2	9.5	5416	13.0	19.0	34971	9.9	48.7
5	**25**	62829	15.9	4.3	1342	12.4	4.7	11807	6.9	16.5
7	**6.25**	38990	32.7	2.7	771	16.6	2.7	3547	21.9	4.9
8	**3.125**	25307	18.9	1.8	624	16.4	2.2	1927	20.3	2.7
9	**0**	14458	15.2	1.0	286	31.1	1.0	718	17.5	1.0

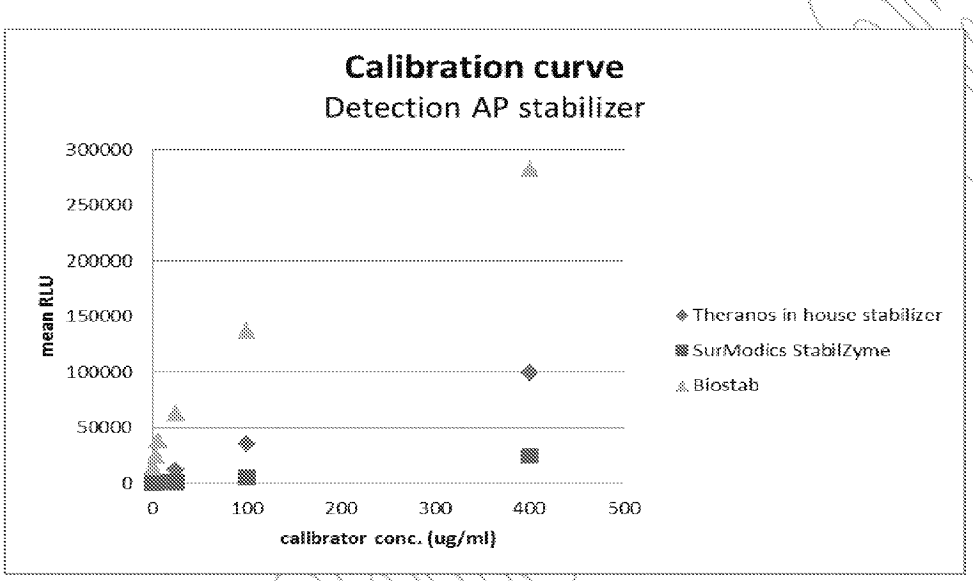


Figure 5: Results of detection antibody stabilizer comparison

2.4

2.4.1

2.4.2

2.3.4 Titration of detection antibody

Methods:

Titration of detection conjugate concentration was done by preparing detection conjugate in Theranos in-house AP stabilizer at 25ng/ml, 50ng/ml and 100ng/ml respectively. Capture antibody was kept at 5ug/ml. calibrators were first hand diluted in blocking buffer 1:3 then Edison protocol Generic2_5000X was used.

Results:

Based on the background, modulation, and saturation at 25ng/ml, 50ng/ml and 100ng/ml, detection antibody was 25ng/ml in Theranos in-house stabilizer was chosen.

Table 21: Results of detection antibody titration

		D8 25ng/ml			D8 50ng/ml			D8 100ng/ml		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
1	400	455064	22.7	335.5	837316	19.5	196.6	1505092	8.4	98.8
3	100	181792	9.2	134.0	362692	4.0	85.2	412709	10.7	27.1
5	25	38788	6.2	28.6	98280	10.7	23.1	160670	15.9	10.5
7	6.25	12768	7.8	9.4	21957	6.0	5.2	54398	14.2	3.6
8	3.125	8403	15.5	6.2	14246	5.4	3.3	32975	29.2	2.2
9	0	1356	39.9	1.0	4259	36.2	1.0	15234	4.8	1.0

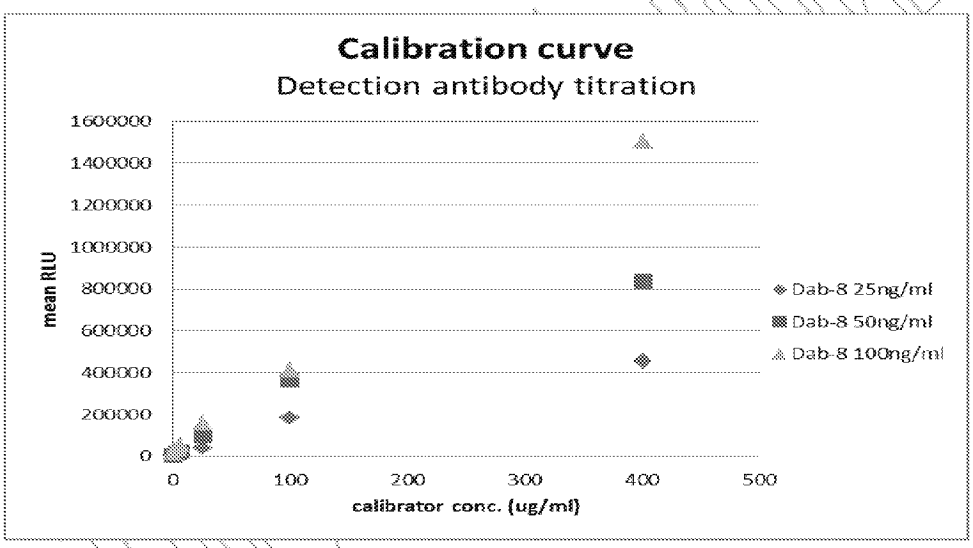


Figure 6: Results of detection antibody titration

2.3.4.1 Clinical samples with detection antibody concentration 25ng/ml

Before moving forward for more assay optimization, Eight ZeptoMetrix clinical serum samples and one control were tested for IgD recovery with current assay conditions. IgD capture antibody F6 5ug/ml in Theranos in house blocking buffer, detection antibody D8 25ng/ml in Theranos in house AP stabilizer.

Methods:

Capture antibody F6 were coated 5ug/ml in Theranos in house blocking buffer, detection antibody D8 were prepared in Theranos in house AP stabilizer at 25ng/ml. Edison protocol Generic2_15000X was used. IgD value of each clinical samples and control were calculated

from the calibration curve ran under the same condition. Percentage of recovery was obtained by comparing Theranos method vs. Reference method (Siemens BN™ II)

Results:

IgD calibrators showed an acceptable recovery comparing to theoretical value. However, control material non WHO IgD serum NIBSC code 67/037 had a low recovery as long as the eight ZeptoMetrix clinical serum samples.

Table 22: Clinical samples and control recovery with detection antibody 25ng/ml

Samples	Sample lot#	IgD conc. by Siemens BN™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery
WHO control	NIBSC code:67/037	133	43609	12.7	59.08	44
ZeptoMetrix clinical serum samples	0001-027-02304	132	95840	15.3	137.36	104
	0001-027-02305	5	5606	20.4	4.97	99
	0001-027-02306	7	3874	4.1	3.05	44
	0001-027-02307	106	40180	10.6	53.93	51
	0001-027-02309	40	12153	25.4	13.26	33
	0001-027-02310	52	23417	36.5	29.11	56
	0001-027-02313	9	6402	12.8	5.91	66
	0001-027-02314	111	34552	24.8	45.52	41

2.3.4.2 Clinical samples with detection antibody concentration 50ng/ml

Since clinical samples and control had low IgD recovery at 25ng/ml detection concentration. 50ng/ml detection antibody concentration was chosen. More clinical samples were tested for IgD recovery before moving to the next step.

Methods:

The same method used previously applied here with the only difference being 50ng/ml detection antibody in Theranos in house AP stabilizer. Edison protocol Generic2_15000X was used.

Results:

Clinical samples showed good recovery when comparing to reference method. Thus 50ng/ml detection antibody in Theranos in house AP stabilizer was chosen as the final assay condition.

Table 23: Clinical samples and control recovery with detection antibody 50ng/ml

Samples	Sample lot#	IgD conc. by Siemens BN™ II (ug/ml)	Mean RLU	%CV	IgD conc. by Theranos (ug/ml)	% recovery

ZeptoMetrix clinical serum samples	0001-027-02304	132	137969	13.5	107.40	81
	0001-027-02305	5	14328	18.7	4.01	80
	0001-027-02307	106	126464	10.8	97.56	92
	0001-027-02309	40	50584	8.8	30.63	77

2.3.5 Selection of sample diluent

Sample diluent comparison was done by using Candor low cross buffer, Theranos in house low BSA buffer, SurModics assay diluent (protein free) as sample diluent, and comparing the result including background, signal, modulation, sensitivity, and CV to original condition where Theranos in house blocking buffer was first used as sample diluent.

Methods:

Total four different diluents were tried as samples diluent: Candor low cross buffer, Theranos in house low BSA buffer, SurModics assay diluent (Protein free), and Theranos in house blocking buffer. All the other assay conditions were kept the same with F6 capture antibody at 5ug/ml in Theranos in house blocking buffer and D8 detection antibody at 50ng/ml in Theranos in house AP stabilizer, and the protocol was the Generic2_15000X. Calibration curve was run for each samples diluent condition respectively.

Results:

Among all four sample diluent, SurModics assay diluent (protein free) gave the best result with lowest background, and highest modulation. Thus SurMidics assay diluent (protein free) was chosen as the final assay condition.

Table 24: Results of sample diluent comparison

		blocking buffer		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Mod.
1	400	576362	15.3	155.9
2	200	315925	10.9	85.5
3	100	117145	4.9	31.7
4	50	73883	4.2	20.0
8	3.125	12590	36.5	3.4
9	0	3697	32.0	1.0

		SurModics assay diluent (protein free)			Candor low cross buffer			low BSA blocking buffer		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.

1	**400**	220755	18.0	191.8	237735	40.2	72.7	665425	22.5	127.9
3	**100**	82200	21.8	71.4	26932	32.8	8.2	238968	25.5	45.9
4	**50**	48469	27.8	42.1	18261	24.4	5.6	120466	4.3	23.1
7	**6.25**	5297	16.5	4.6	9121	13.7	2.8	24701	28.6	4.7
8	**3.125**	3298	11.9	2.9	5511	33.1	1.7	13599	13.5	2.6
9	**0**	1151	30.1	1.0	3269	14.8	1.0	5204	39.3	1.0

2.3.6 Edison protocol optimization

Based on the existing Edison protocol Generic2_15000X, a new protocol Edison protocol Generic2_15000X_PSW was generated. New protocol was tested in the purpose of improving background, CV, modulation, and clinical sample recovery.

Methods:

First two set of IgD calibrators were run under these two protocol: Generic2_15000X and Generic2_15000X_PSW. The tips were coated at 5ug/ml in Theranos blocking buffer, and detection antibody was prepared at 50ng/ml in Theranos in house AP stabilizer, and the sample diluent was SurModics assay diluent (Protein free). Then total of nine ZeptoMetrix clinical serum samples were run under the two protocols respectively. Percentage of recovery was calculated by comparing Theranos result vs. Reference method (Siemens BN™ II).

Results:

The addition of post sample washes improved recovery of IgD in clinical samples, thus the correlation between Theranos result and Siemens result got improved. As a result, new protocol Generic2_15000X_PSW was chosen as the final assay protocol.

Table 25: ZeptoMetrix clinical sample recovery comparison between two protocols

		Generic2_15000X				**Generic2_15000X_PSW**			
Sample lot #	**IgD conc. by Siemens BN ™ II (ug/ml)**	**Mean RLU**	**%CV**	**IgD conc. by Theranos (ug/ml)**	**% recovery**	**Mean RLU**	**%CV**	**IgD conc. by Theranos (ug/ml)**	**% recovery**
0001-027-02303	28	31830	28.4	35.81	128	32394	36.2	31.46	112
0001-027-02304	132	88154	11.6	108.78	82	103138	4.3	120.53	91
0001-027-02305	5	5444	18.3	6.21	124	6149	18.7	5.09	102
0001-027-02306	7	7054	28.1	7.92	113	8862	6.6	7.52	107
0001-027-02307	106	70653	33.0	84.92	80	93804	14.5	107.73	102
0001-027-02309	40	18639	42.9	20.57	51	25155	21.1	23.65	59
0001-027-02310	52	32332	20.4	36.41	70	62194	12.8	66.54	128

0001-027-02311	33	21132	45.1	23.39	71	29854	11.8	28.68	87
0001-027-02312	40	32776	7.8	36.94	92	34560	13.3	33.86	85

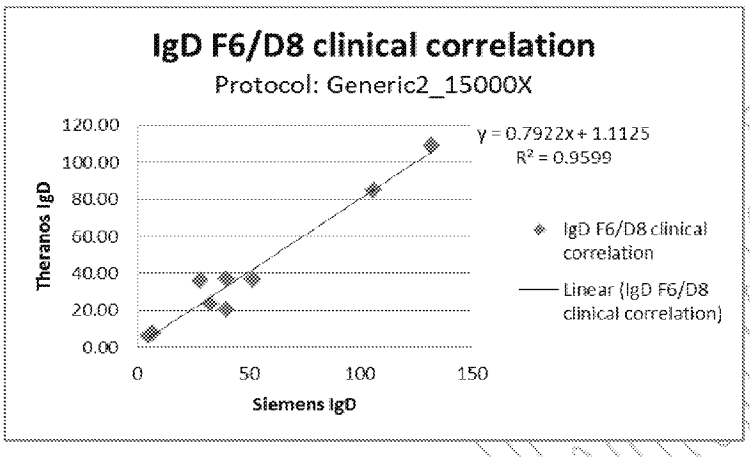


Figure 7: ZeptoMetrix clinical samples correlation (Protocol: Generic2_15000X)

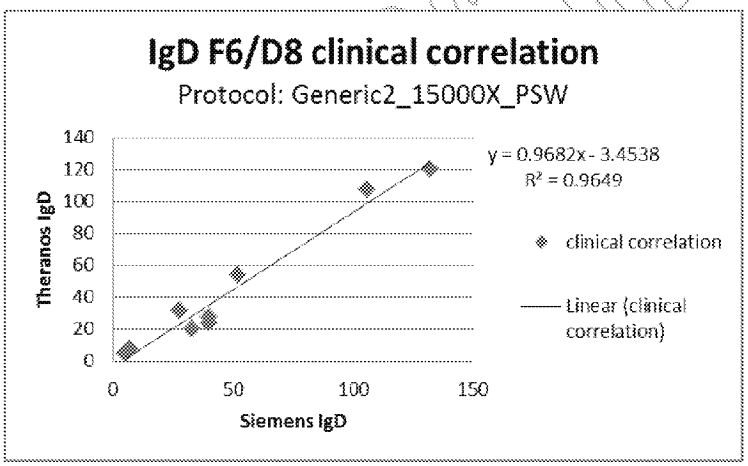


Figure 8: ZeptoMetrix clinical sampes correlation (protocol: Generic2_15000X_PSW)

2.3.7 Effect of positive HAMA and RF samples

HAMA and RF positive samples were not tested for this assay. According to the SIEMENS BN™ II product information, samples containing RF or other circulating immune complexes are not suitable for IgD measurement due to the unpredictable degree of non-specific scatter these samples types may generate. As a result, there was no reliable reference data used for comparison for HAMA and RF positive samples.

2.3.8 Matrix effect

Lipemic, icteric, and hemolyzed samples were not tested for this assay. According to the SIEMENS BN™ II product information, lipemic or hemolyzed samples are not suitable for IgD measurement due to the unpredictable degree of non-specific scatter these samples types may generate. As a result, there was no reliable reference data used for comparison for matrix effect.

2.3.9 Hematocrit effect

Methods:

Ten tubes of EDTA blood (5 obtained from Stanford blood center, 5 obtained from in house study) were analyzed with the final assay condition. Hematocrit effect was evaluated by comparing IgD results in whole blood (EDTA) vs. in plasma (EDTA). A linear regression was plotted for whole blood (EDTA) against plasma (EDTA).

Results:

Samples form the ten donors were analyzed. Hematocrit factor was calculated to be 1.51 for the slope of plotting IgD results from EDTA plasma vs. results from whole blood.

Table 26: IgD results of whole blood and EDTA plasma

	whole blood results		EDTA plasma result	
Sample Id	mean RLU	%CV	mean RLU	%CV
W07051310000600	2850	8.9	4885	13.0
W07051300001600	1192	4.6	1677	6.3
W07051300001300	13746	7.6	22023	19.8
W07051300001500	8180	8.5	11464	2.9
W07051300001700	53167	23.3	81250	3.8
in house tube 204	4002	12.5	6423	20.3
in house tube 205	1288	11.9	2197	10.7
in house tube 206	1306	21.2	1918	7.5
in house tube 209	19799	21.5	27068	7.4
in house tube 210	1842	16.0	4116	12.1

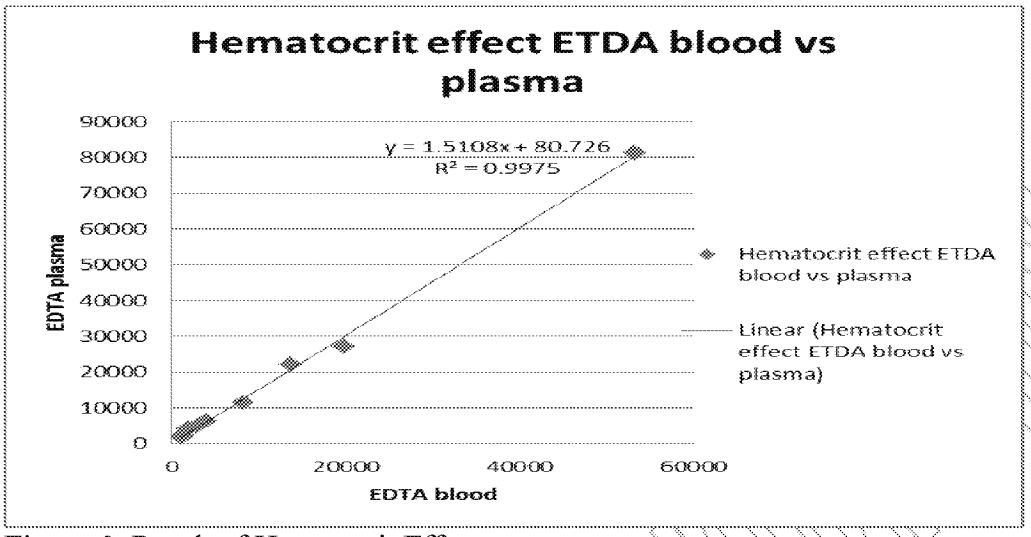


Figure 9: Result of Hematocrit Effect

2.3.10 Anticoagulant effect and serum/plasma effect

Methods:

EDTA plasma, lithium-heparin plasma, and serum 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. EDTA plasma and heparin plasma from the same donor were also analyzed to compare the effect of anticoagulant and a linear regression was plotted between the EDTA plasma vs. lithium-heparin plasma. Serum samples from these ten patients were also analyzed with the final protocol. The result was plotted against EDTA plasma and lithium-heparin plasma to analyze the effect of different matrix.

Results:

Samples from ten donors collected in pairs of EDTA plasma, lithium-heparin plasma and serum were analyzed. IgD results from EDTA plasma and lithium-heparin plasma correlated well with each other, demonstrating that different anticoagulant didn't have significant effect on testing result. IgD result from serum was also analyzed against EDTA plasma and lithium-heparin separately, no significant difference was observed across different matrix. Thus this assay can be used to test serum and plasma samples with no significant difference among results.

Table 27: IgD results of matching EDTA plasma, lithium-heparin plasma, and serum

	Lot #	Serum	Plasma (EDTA)	Plasma (Li-hep)
M1	W07051200306800	2.58	1.85	2
M2	W07051200306600	3.54	3.84	3.65
M3	W07051200306700	44.96	43.53	40.99
M4	W07051200306400	1.14	1.09	1.26

M5	W07051200306500	5.4	5.56	5.79
F1	W07051200306200	9.48	9.06	8.96
F2	W07051200307000	2.94	2.25	2.32
F3	W07051200306300	3.06	3.17	2.9
F4	W07051200307500	5.32	5.06	4.19
F5	W07051200307600	9.82	8.93	9.02

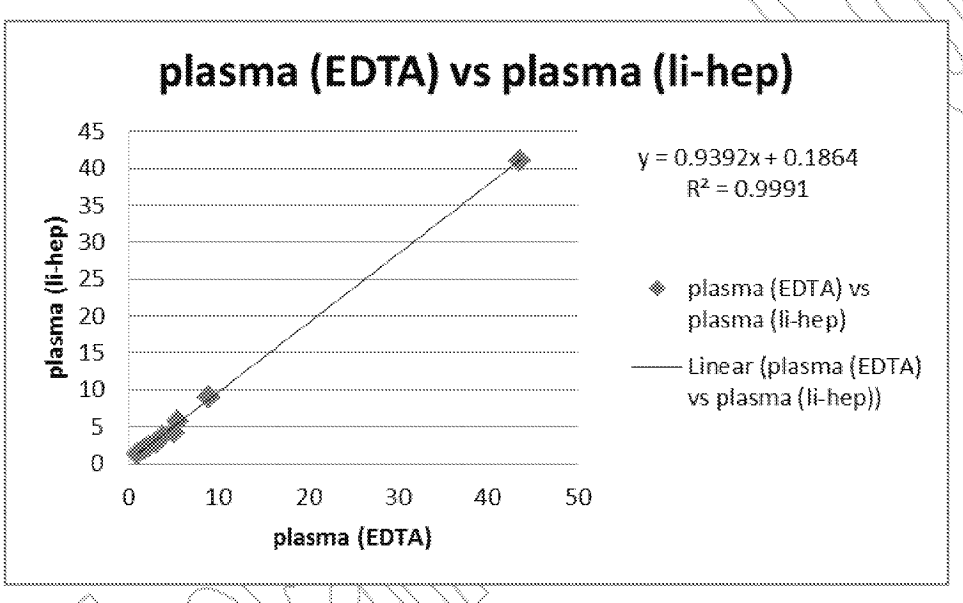


Figure 10: Results of EDTA plasma vs. lithium-heparin plasma

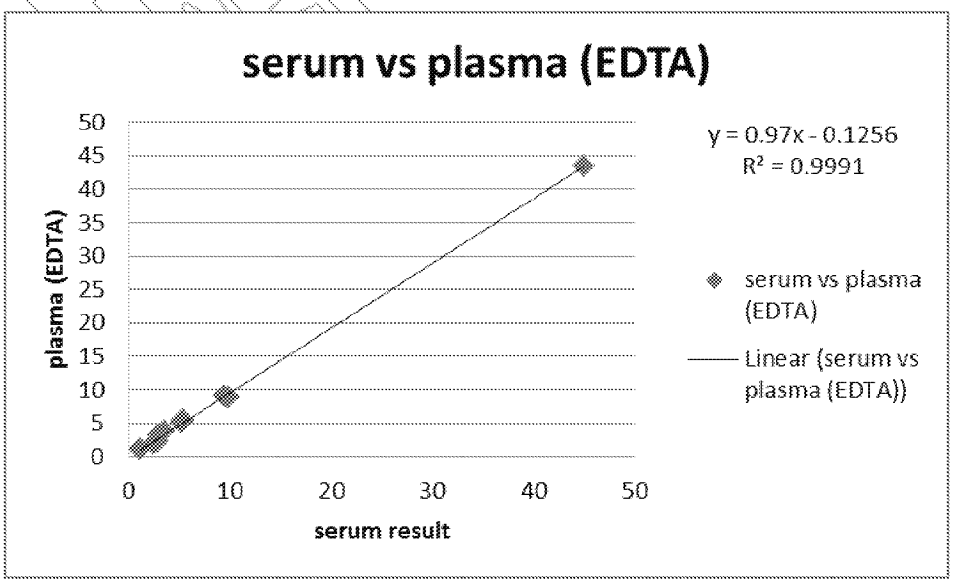


Figure 11: Results of serum vs. EDTA plasma

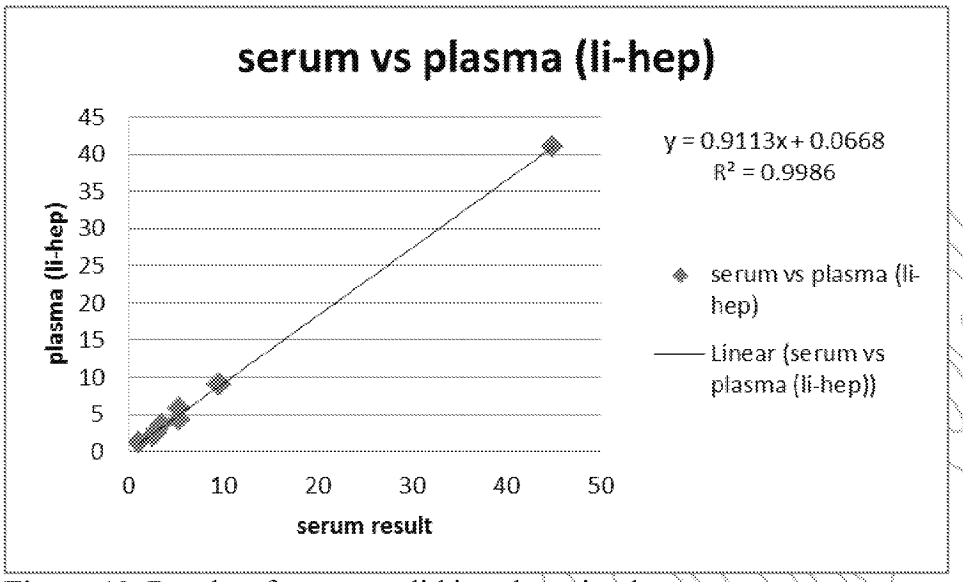


Figure 12: Results of serum vs. lithium-heparin plasma

2.4 Clinical sample analysis with final protocol

2.4.1 Calibration curve run with final assay condition

Final assay condition Biotinylated-F6 capture antibody coated at 5ug/ml in Theranos 3% BSA blocking buffer, D8 detection antibody at 50ng/ml in Theranos in house AP stabilizer, SurModics assay diluent (protein free) was used as sample diluent. Calibration curve was generated under this assay condition with final protocol of Generic2_15000X_PSW and data was analyzed by Dexter.

Table 28: IgD final calibration curve

Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Modulation	Calc. from Dexter (ug/ml)	%Accuracy
1	400	402780	6.3	302.5	423.67	106
2	200	199428	6.3	149.8	180.72	90
3	100	116735	19.5	87.7	100.97	101
4	50	66227	10.2	49.7	55.99	112
5	25	34113	15.9	25.6	28.08	112
6	12.5	15366	13.3	11.5	11.55	92
7	6.25	10284	17.7	7.7	6.99	112
8	3.125	5851	10.9	4.4	3.05	98
9	0	1332	17.8	1.0	0.00	

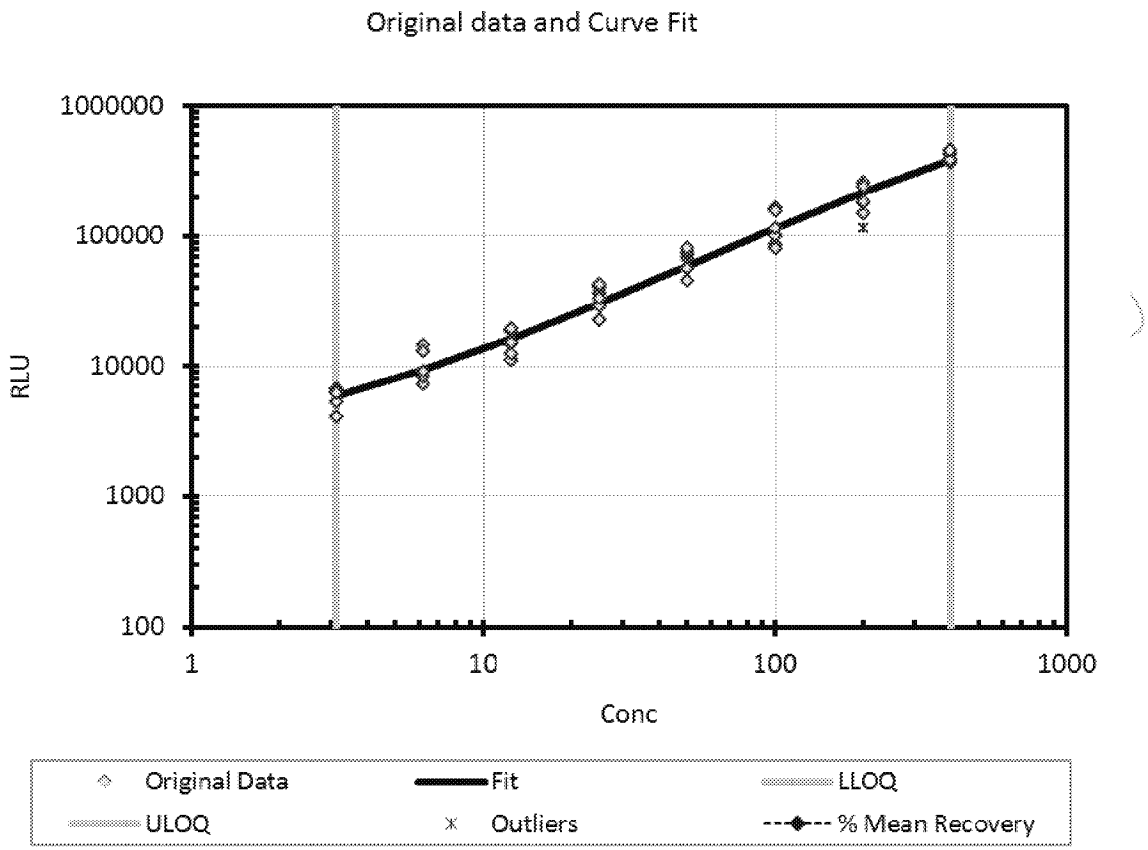


Figure 13: Calibration curve from Dexter analysis

Table 29: 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	3.110
b2	6.505
b3	54.218
b4	-0.496
LLOQ	3.13 ug/ml
ULOQ	400 ug/ml
LLOQ accuracy	98%
LLOQ precision	17.5%
ULOQ accuracy	106%
ULOQ precision	8.4%

2.4.2 Control samples analysis

One control samples was analyzed with the final assay condition to ensure the assay calibrators were calibrated against this control

- Non WHO reference material IgD serum, Human, NIBSC code: 67/037. Each ampoule contains 100 units of activity of IgD. The total volume of the standard reconstituted in 1.0ml distilled water has been recalculated to be 1.06ml. The reconstituted standard will therefore contain 94.3 units of IgD in $1.0ml^{-1}$. Theoretical result of 1 unit containing $1.41ug$ of IgD^{-2}.

Table 30: Result of Non WHO reference material IgD serum

ZeptoMetrix sample ID	Mean RLU	%CV	Theranos IgD conc (ug/ml)	Siemens IgD conc (ug/ml)	% difference
NIBSC 67/037	144980	16.0	127	133	-4

2.4.3 Clinical sample analysis

Thirty serum samples were obtained from ZeptoMetrix. Samples were analyzed by Theranos method and SIEMENS method for IgD level. The result difference between Theranos method and SIEMENS method was calculated. Data from two methods correlated well.

Table 31: Results of thirty serum samples from ZeptoMetrix

ZeptoMetrix sample ID	Mean RLU	%CV	Theranos IgD conc. (ug/ml)	Siemens IgD conc. (ug/ml)	% difference
0001-027-02293	33860	18.5	28	31	-10
0001-027-02296	15203	22.3	11	13	-12
0001-027-02300	41644	24.7	35	32	8
0001-027-02302	94332	12.6	81	90	-10
0001-027-02303	39996	17.4	33	28	19
0001-027-02304	151025	3.8	133	132	1
0001-027-02305	7815	16.6	5	5	-4
0001-027-02306	10520	16.4	7	7	3
0001-027-02307	128365	15.0	112	106	5
0001-027-02309	43229	9.8	36	40	-10
0001-027-02310	70366	18.1	60	52	15
0001-027-02311	36208	14.7	30	33	-9
0001-027-02312	39569	13.6	33	40	-18
0001-027-02313	10567	6.9	7	9	-20

0001-027-02314	121865	18.8	106	111	-5
0001-027-02315	115134	10.8	100	104	-4
0001-027-02316	13406	0.2	10	10	-2
0001-027-02317	33898	4.2	28	32	-13
0001-027-02318	17119	4.7	13	15	-13
0001-027-02319	27507	9.5	22	28	-20
0001-027-02320	107180	17.4	92	103	-10
0001-027-02321	168109	5.3	149	164	-9
0001-027-02323	19543	18.4	15	18	-15
0001-027-02324	58873	10.2	50	61	-19
0001-027-02326	31920	22.2	26	30	-13
0001-027-02327	108594	0.7	94	110	-15
0001-027-02328	19829	11.6	16	16	-3
0001-027-02329	104743	14.8	90	97	-7
0001-027-02330	9079	13.4	6	7	-16
0001-027-02331	60167	16.0	51	47	8

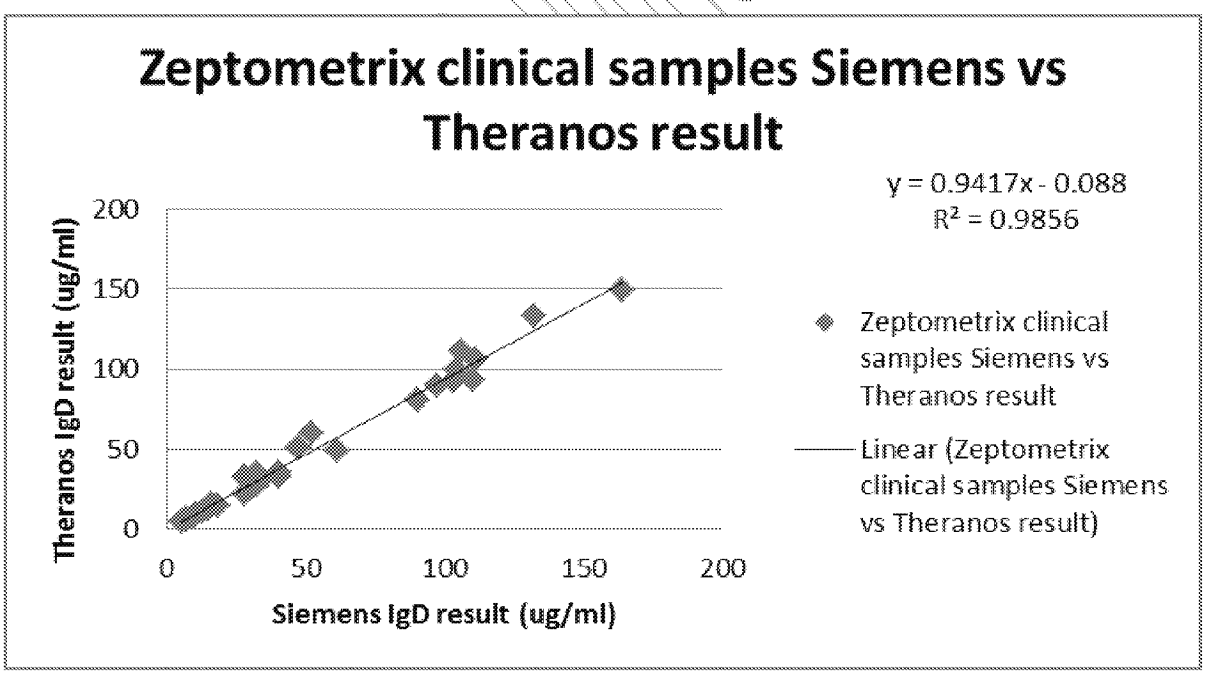


Figure 14: Correlation of Theranos method vs. SIEMENS method

2.5 Stability

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

2.6 IgD binder capture batch to batch comparison

Methods:

Binder group release second batch of biotinylated IgD binder capture antibody lot: IgDF6012213. Along with the original batch lot: IgDF6102512, these two batches of capture antibody were coated at 5ug/ml in Theranos blocking buffer. Calibration curve were run with these two batches of tips under the final assay condition.

Results:

IgD binder capture antibody batch 2 was not as active as batch 1 with overall lower RLU; however, signal/background modulation was good for the new batch comparing to old batch.

Table 32: IgD binger capture antibody batch to batch comparison

		Batch 1 binder Cab-F6			Batch 2 binder Cab-F6		
Calibrator	Conc. (ug/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
1	400	307592	8.6	267.2	215632	3.9	342.9
2	200	196307	10.9	170.6	147703	9.0	234.9
3	100	90325	6.5	78.5	72713	7.4	115.6
4	50	36529	7.6	31.7	39726	11.4	63.2
5	25	21270	13.4	18.5	20157	14.4	32.1
6	12.5	12598	8.6	10.9	11630	21.2	18.5
7	6.25	8701	10.6	7.6	5147	18.4	8.2
8	3.125	4321	19.3	3.8	2950	14.5	4.7
9	0	1151	25.8	1.0	629	10.3	1.0

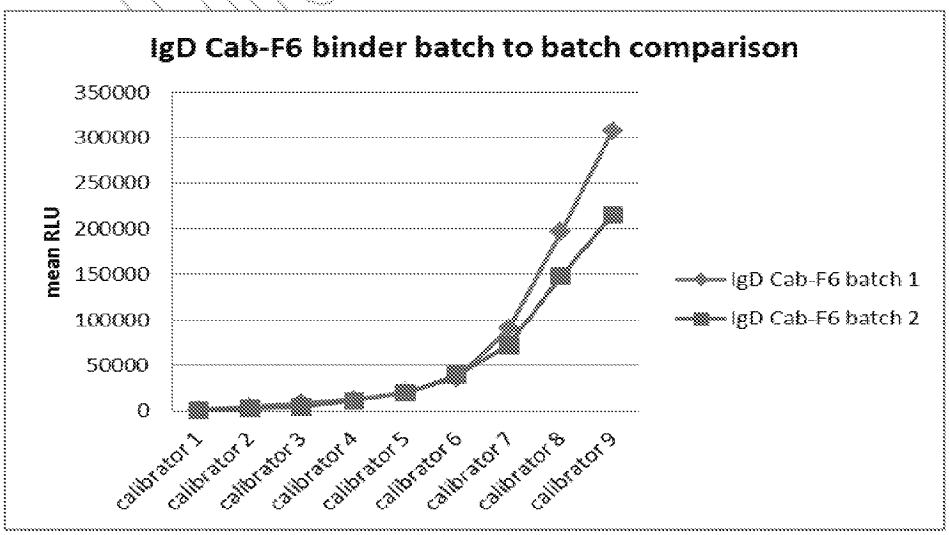


Figure 15: IgD binder capture antibody batch to batch comparison

2.7 References

1. NIBSC Non WHO reference material Immunoglobulin D (IgD) serum, Human NIBSC code: 67/037 Instructions for use (Version 6.0, Dated 30-01-2013) [HYPERLINK "http://www.nibsc.ac.uk/documents/ifu/67-037.pdf"]
2. Rowe, D.S, Anderson, S.G, and Tachett, L (1970) A research Standard for Human Serum Immunoglobulin D. Bull. Wld Hlth Org. 43, 607-609.