Alpha-1-Antitrypsin (A1AT) Assay Development Report

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1.1 Analyte Information

Alpha-1-antitrypsin (A1AT) is a protease inhibitor belonging to the Serpin superfamily. Serpin are a group of proteins with similar structures that were first identified as a set of proteins able to inhibit protease. With molecular weight of 52kDa, A1AT is a single chain glycoprotein consisting of 394 AA in the mature form and exhibits a number of glycoforms. A1AT protects tissues from enzymes of inflammatory cells, especially neutrophil elastase. It has a reference range in blood of 1.5-3.5mg/mL. In the absent of A1AT, neutrophil elastase is free to break down elastin which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, chronic obstructive pulmonary disease (COPD) in adults, and cirrhosis in adults and children. The gene is located on the long arm of the 14th chromosome. A1AT deficiency is an autosomal recessive hereditary disorder in which a deficiency of A1AT leads to a chronic uninhibited tissue breakdown. This causes the degradation especially of lung tissues, and eventually leads to characteristic manifestations of pulmonary emphysema. Smoking can lead to oxidation of methionine 358 of A1AT, a residue essential for binding elastase; this is thought to be one of the primary mechanisms by which cigarette smoking (or second hand smoke) can lead to emphysema. Because A1AT is expressed in the liver, certain mutations in the gene encoding the protein can cause mis-folding and impaired secretion, which can lead to liver cirrhosis.

1.2 Assay specification

This assay determines the concentration of Alpha-1-antitrypsin (A1AT) in human serum, plasma (EDTA, lithium-heparin), and whole blood. The assay has a quantification range of 0.022mg/mL to 4.6mg/mL (2.2mg/dL to 460mg/dL).

1.3 Reference assay

The following assay was used as reference method:

SIEMENS ADVIA 1800

1.4 Material and method

A sandwich immunoassay using anti-human A1AT antibodies was developed for the quantitative determination of human A1AT in serum, plasma, and whole blood.

In this assay, a monoclonal mouse anti-human antibody was used as capture antibody of A1AT determination. Reaction tips were first coated with Ultra-Avidin, followed by a layer of biotinylated capture antibody. Calibrators, serum, plasma, or whole blood samples were diluted 25000 folds with sample diluent and incubated with capture antibody coated tips. A polyclonal

rabbit anti-human antibody was conjugated 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 incubation with AP substrate. The chemiluminescence results were measured and reported as Relative Light Unit (RLU). A calibration curve was generated by plotting the measured response (RLU) vs. concentration of each calibrator point. A1AT concentration of unknown sample was calculated from calibration curve.

TABLE 1: A1AT ASSAY MATERIAL IN FINAL ASSAY PROCEDURE

Name	Supplier	Catalog number
Alpha-1-antitrypsin	Athens Research	16-16-011609
Mab mouse anti human A1AT	Pierce	HYB191-01-02
Pab rabbit anti human A1AT	Novus	NBP1-78098
Tris buffer (powder)	Sigma	T6664
Bovine serum albumin	Sigma	A3059
Sucrose	Sigma	S5016
5% Sodium Azide solution	VWR	101320-516
Carbonate-bicarbonate buffer	Sigma	C3041
1M Magnesium chloride solution	Sigma	M1028
0.1M Zinc Chloride solution	Sigma	39059
TBST (powder)	Sigma	T9039
UltraAvidin	Leinco	A110
AP substrate	In house	Current Lot 11102012-A
In house biotin labeling kit	In house	N/A
AP conjugation kit	Dojindo	LK13
StabilZyme-AP	SurModics	SA01-1000

1.5 Raw data storage

Raw data of assay development were stored in Elog #917 and Notebook # 404.

2. ASSAY DEVELOPMENT

2.1 Antibody screening on MTP

2.1.1 Initial antibody screening on MTP

During initial assay development stage, a total of 15 anti-human A1AT antibodies were screened for binding activity on micro titer plate (MTP). All antibodies were labeled with Theranos in house Biotin-SH kits and Dojindo AP labeling kit-SH (cat LK13).

Method:

The MTP was first coated with UltraAvidin (UA) at 20ug/ml in carb-bicard coating buffer, followed by Biotinylated antibody at 5ug/ml in 3% BSA blocking buffer. A1AT calibrators at 5mg/ml, 0.5mg/ml, 0.05ng/ml and 0mg/ml were hand diluted in blocking buffer 25,000 folds and incubated with coated antibodies. Then detection antibody-AP conjugates were diluted in the blocking buffer to 50ng/ml and incubated after calibrator incubation. Finally AP substrate was added to each well and Relative Luminescence Unit (RLU) was measured by a plate reader. Modulations for each antibody pair were calculated using RLU of each calibrator concentration level divided by the RLU of background (Buffer blank, A1AT 0mg/ml).

Result:

Out of the 15 X 15 antibody pairs screened, some antibody pairs showed variable degree of response. Only antibody pair C6/D12, C6/D14, C12/D14, and C12/D2 moved on the cross reactivity and interference tests.

TABLE 2: ANTIBODIES SCREENED ON MTP

#	Vendor	Product	Isotype	Clone	Cat #
1	Millipore	Mab mouse anti human a1-antitrypsin	IgG_{2a}	TMF1#4B5	178260-1MG
2	Pierce	Mab mouse anti human a1-antitrypsin	IgG_{2a}	1AT10-105.10	MA1-10625
3	Pierce	Mab mouse anti human a1-antitrypsin	IgG_1	1AT10-209.3	MA1-10627
4	Pierce	Mab mouse anti human a1-antitrypsin	IgG_1	1AT10-213.5	MA1-10628
5	Pierce	Mab mouse anti human a1-antitrypsin	IgG_1	1AT11-210.1	MA1-10626
6	Pierce	Mab mouse anti human a1-antitrypsin	$IgG_{1, kappa}$	3B7	HYB191-01-02

8	Pierce	Mab mouse anti human a1-antitrypsin	IgG_1	704	MA1-91470
9	Novus	Pab goat anti human a1-antitrypsin	IgG		NB600-602
10	Novus	Pab goat anti human a1-antitrypsin			NBP1-50484
11	Pierce	Pab goat anti human a1-antitrypsin			PA1-26908
12	Novus	Pab rabbit anti human a1-antitrypsin			NBP1-78098
13	Novus	Pab rabbit anti human a1-antitrypsin			34720002
14	Novus	Pab rabbit anti human a1-antitrypsin			H00005265-D01P
16	Abcam	Pab goat anti-rabbit IgG (H+L) secondary antibody-AP conjugated			ab97048
17	Abcam	Pab rabbit anti goat IgG (H+L) secondary antibody-AP conjugated			ab6742

TABLE 3: SUMMARY OF INITIAL ANTIBODY SCREENING ON MTP

	D1	D2	D3	D4	D5	D6	D8	D9	D10	D11	D12	D13	D14	D16	D17
C1															
C2															
C3															
C4															
C5															
C6											84X		31X		
C8															
C9															
C10															
C11															
C12		26X	16X	12X	11X	67X	14X		11X				72X		
C13															
C14						45X					69X				

	Good modulation > 50 fold, move to next step
	Fair modulation >25 fold
	Little modulation > 10 fold
	No modulation

2.1.2 Cross reactivity and interference on MTP

Human serum albumin, Prealbumin, ferritin, transferrin, alpha-1-antichymotrypsin, alpha-1-acid glycoprotein were tested for cross reactivity and interference for the four selected antibody pairs.

Apha-1-antichymotrypsin is an alpha globulin glycoprotein which is also a member of the serpin superfamily. Alpha-1-acid glycoprotein is an acute phase plasma alpha globulin glycoprotein.

TABLE 4: PROTEINS TESTED FOR CROSS REACTIVITY AND INTERFERENCE

#	Protein	Normal conc. level	Testing conc.
1	Human serum albumin	34-54mg/ml	50mg/ml
2	Prealbumin	0.18-0.36mg/ml	0.5mg/ml
3	Ferritin	male 30-300ng/ml, female 15-200ng/ml	500ng/ml
4	Transferrin	2-4mg/ml	4mg/ml
5	Alpha-1-antichymotrypsin	0.6mg/ml	1mg/ml
6	Alpha-1-acid glycoprotein	0.6-1.2mg/ml	1.25mg/ml

Method:

Previous method used in antibody screening was used here. However, for cross reactivity test, instead of using A1AT calibrators, the above cross reactants which hand diluted 25,000 fold in blocking buffer were added as samples. In interference test, the above proteins which spiked into A1AT calibrators of each concentration were tested as samples.

Results:

For all six proteins tested, none of them showed cross reactivity or interference with antibody pair C6/D12, C6/D14, C12/D14, or C12/D2, thus all four pairs move to Theranos system for further testing.

TABLE 5: RESULTS OF CROSS REACTIVITY

C6/D12	Conc.	mean RLU	%CV	Modulation
A1AT	0 mg/ml	616	1	1
	0.05 mg/ml	1775	5	3
	0.5 mg/ml	10715	3	17
	5 mg/ml	41031	5	67
human serum albumin	50 mg/ml	1307	4	2
Prealbumin	0.5 mg/ml	618	9	1
ferritin	500 ng/ml	523	18	1
transferrin	4	834	7	1

C6/D14	Conc.	mean RLU	%CV	Modulation
A1AT	0 mg/ml	596	6	1
	0.05 mg/ml	821	18	1.4
	0.5 mg/ml	4267	8	7
	5 mg/ml	20940	2	35
human serum albumin	50 mg/ml	698	11	1
Prealbumin	0.5 mg/ml	600	3	1
ferritin	500 ng/ml	932	9	2
transferrin	4	607	9	1

	mg/ml			
A1ACT	1 mg/ml	722	3	1
A1AGP	1.25 mg/ml	679	15	1

	mg/ml			
A1ACT	1 mg/ml	696	1	1
A1AGP	1.25 mg/ml	696	0	1

C12/D14	Conc.	mean RLU	%CV	Modulation
A1AT	0 mg/ml	1534	2	1
	0.05 mg/ml	9773	6	6
	0.5 mg/ml	59218	4	39
	5 mg/ml	134767	3	88
human serum albumin	50 mg/ml	1781	3	1
Prealbumin	0.5 mg/ml	1564	3	1
ferritin	500 ng/ml	1324	2	1
transferrin	4 mg/ml	1327	9	1
A1ACT	1 mg/ml	1600	0	1
A1AGP	1.25 mg/ml	1582	6	1

C12/D2	Conc.	mean RLU	%CV	Modulation
A1AT	0 mg/ml	1496	2	1
	0.05 mg/ml	2100	10	1.4
	0.5 mg/ml	7373	0	5
	5 mg/ml	43002	5	29
human serum albumin	50 mg/ml	1465	9	1
Prealbumin	0.5 mg/ml	1390	6	1
ferritin	500 ng/ml	1361	7	1
transferrin	4 mg/ml	1433	1	1
A1ACT	1 mg/ml	1780	1	1
A1AGP	1.25 mg/ml	1922	2	1

TABLE 6: RESULTS OF INTERFERENCE

A1AT	C6/D12			A1AT	C6/D14		
conc. (mg/ml)	average	%CV	Modulation	conc. (mg/ml)	average	%CV	Modulation
0	1175	13	1	0	1451	5	1
0.05	4225	3	4	0.05	2029	15	1.4
0.5	26002	5	22	0.5	8880	2	6
5	68997	10	59	5	35025	1	24

Human serum albumin spike 50mg/ml	
mean	%difference
2602	122
4647	10
20190	-22

Human serum albumin spike 50mg/ml	
mean	%difference
1068	-26
1780	-12
6872	-23

61204	-11
Prealbumin spike 0.5mg/ml	
mean	%difference
1018	-13
3391	-20
19859	-24
59091	-14
Ferritin spike 500ng/ml	
mean	%difference
849	-28
3732	-12
20867	-20
64880	-6
Transferrin spike 4mg/ml	
mean	%difference
1026	-13
3542	-16
21046	-19
59940	-13
Alpha-1-antichymotrypsin spike 1mg/ml	
mean	%difference
1334	14
4297	2
22530	-13
67221	-3
Alpha-1-acid glycoprotein spike 1.25mg/ml	
mean	%difference
1040	-12
4667	10
23482	-10
69194	0

30343	-13
Prealbumin spike 0.5mg/ml	
mean	%difference
1072	-26
1747	-14
6959	-22
28505	-19
Ferritin spike 500ng/ml	
mean	%difference
1050	-28
1829	-10
7934	-11
32585	-7
Transferrin spike 4mg/ml	
mean	%difference
1189	-18
1720	-15
7706	-13
28433	-19
Alpha-1-antichymotrypsin spike 1mg/ml	
mean	%difference
1255	-14
2432	20
8211	-8
34906	0
Alpha-1-acid glycoprotein spike 1.25mg/ml	
mean	%difference
1869	29
2695	33
8602	-3
34572	-1

A1AT	**C12/D14**		
conc. (mg/ml)	average	%CV	Modulation
0	1045	4	1

A1AT	**C12/D2**		
conc. (mg/ml)	average	%CV	Modulation
0	1096	3	1

0.05	15778	5	15	0.05	2100	10	2
0.5	79348	0	76	0.5	7373	0	7
5	141413	17	135	5	43002	5	39

Human serum albumin spike 50mg/ml	
mean	%difference
1072	3
12869	-18
68849	-13
124085	-12

Human serum albumin spike 50mg/ml	
mean	%difference
856	-22
1572	-25
7401	0
43014	0

Prealbumin spike 0.5mg/ml	
mean	%difference
1272	22
13107	-17
70611	-11
127474	-10

Prealbumin spike 0.5mg/ml	
mean	%difference
1043	-5
1713	-18
7240	-2
39816	-7

Ferritin spike 500ng/ml	
mean	%difference
956	-9
13607	-14
75262	-5
144346	2

Ferritin spike 500ng/ml	
mean	%difference
1087	-1
1717	-18
7882	7
44854	4

Transferrin spike 4mg/ml	
mean	%difference
905	-13
13905	-12
75067	-5
140950	0

Transferrin spike 4mg/ml	
mean	%difference
980	-11
1663	-21
7999	8
43282	1

Alpha-1-antichymotrypsin spike 1mg/ml	
mean	%difference
1344	29
14270	-10
76475	-4
147244	4

Alpha-1-antichymotrypsin spike 1mg/ml	
mean	%difference
950	-13
2100	0
7747	5
44716	4

Alpha-1-acid glycoprotein spike 1.25mg/ml	
mean	%difference

Alpha-1-acid glycoprotein spike 1.25mg/ml	
mean	%difference

1287	23
14758	-6
80518	1
146468	4

1188	8
1972	-6
7906	7
42012	-2

2.2 Antibody screening on Theranos system

2.2.1 Theranos screening with four pairs of antibodies

Methods:

Full sets of A1AT calibrators were tested with antibody pair C6/D12, C6/D14, C12/D14, and C12/D2. Edison protocol Generic2_25000X_5-5-5 was used for initial testing. The reaction tips were coated first with 20ug/ml UA in carb-bicarb buffer followed by 5ug/ml of capture antibody diluted in 3% BSA blocking buffer. Calibrators were first diluted with 3% BSA blocking buffer on board then incubated with reaction tips for 5 minutes. Detection antibody-AP conjugates were diluted to 100ng/ml in 3% BSA and incubated with reaction tip for 5 minutes after calibrator incubation. All tips were first washed with wash buffer and incubated with AP substrate for another 5 minutes. RLU was measured for each tip. Calibrator recovery was calculated comparing the calculated concentration vs. CLIA reported concentration (CLIA SIEMENS Advia 1800).

Results:

Antibody pair C6/D12, C6/D14, and C12/D14 showed satisfying results with reasonable CV, good signal/background modulation and point/point modulation. When comparing the calculated concentration to reported concentration, the % recovery was all within 80%-120%. However, C12/D2 had a poor overall modulation and sensitivity at low calibrator points. As a result, pair C6/D12, C6/D14, C12/D14 moved on to Theranos training set.

TABLE 7: SUMMARY DATA OF THERANOS SCREENING

C6/D12

Calibrator #	Nominal conc	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.	log RLU	log conc.	back-calc.	% accuracy
1	4.60	3.46	mg/ml	161463	5	306	1.7	5.208	0.539	3.06	89%
2	2.30	1.73	mg/ml	92586	8	175	1.3	4.967	0.238	1.58	92%
3	1.15	0.86	mg/ml	72756	9	138	2.3	4.862	-0.063	1.19	138%
4	0.575	0.43	mg/ml	31646	5	60	3.1	4.500	-0.364	0.44	103%
5	0.20	0.15	mg/ml	10092	7	19	1.9	4.004	-0.823	0.11	76%
6	0.067	0.05	mg/ml	5289	0	10	2.5	3.723	-1.298	0.05	106%
7	0.022	0.017	mg/ml	2076	4	4	3.9	3.317	-1.782	0.018	107%
8	0.00	0.00	mg/ml	528	16	1		2.722			

C6/D14

Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.	log RLU	log conc.	back-calc.	% accuracy
1	4.60	3.46	mg/ml	82107	22	202	1.7	4.914	0.539	3.72	108%
2	2.30	1.73	mg/ml	48589	6	120	1.6	4.687	0.238	1.56	91%
3	1.15	0.86	mg/ml	29568	7	73	2.4	4.471	-0.063	0.88	102%
4	0.575	0.43	mg/ml	12156	21	30	4.2	4.085	-0.364	0.46	106%
5	0.20	0.15	mg/ml	2871	17	7	2.3	3.458	-0.823	0.16	105%
6	0.067	0.05	mg/ml	1230	14	3	1.5	3.090	-1.298	0.04	87%
7	0.022	0.017	mg/ml	831	11	2	2.0	2.919	-1.782	0.018	110%
8	0.00	0.00	mg/ml	406	21	1		2.608			

C12/D14

Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.	log RLU	log conc.	back-calc.	% accuracy
1	4.60	3.46	mg/ml	105253	9	368	1.3	5.022	0.539	2.77	80%
2	2.30	1.73	mg/ml	81251	14	284	2.3	4.910	0.238	2.06	119%
3	1.15	0.86	mg/ml	36080	29	126	1.4	4.557	-0.063	0.81	94%
4	0.575	0.43	mg/ml	24993	12	87	3.1	4.398	-0.364	0.53	123%
5	0.20	0.15	mg/ml	8173	10	29	3.1	3.912	-0.823	0.15	98%
6	0.067	0.05	mg/ml	2673	29	9	2.0	3.427	-1.298	0.04	82%
7	0.022	0.017	mg/ml	1335	29	5	4.7	3.126	-1.782	0.019	112%
8	0.00	0.00	mg/ml	286	2	1		2.456			

C12/D2

Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	15880	42	39.1	2.1
2	2.30	1.73	mg/ml	7543	15	18.6	2.4
3	1.15	0.86	mg/ml	3150	9	7.8	2.2
4	0.575	0.43	mg/ml	1408	6	3.5	2.4
5	0.20	0.15	mg/ml	596	35	1.5	1.6
6	0.067	0.05	mg/ml	374	18	0.9	0.8
7	0.022	0.017	mg/ml	442	52	1.1	1.1
8	0.00	0.00	mg/ml	406	33	1.0	

2.2.2 Training sets with three pairs of antibodies

Methods:

First calibration curve was run with antibody pair C6/D12, C6/D14, and C12/D14 using Edison protocol Generic2_25000X_5-5-5. The procedure was kept the same as described previously in Theranos screening. Then control material, clinical samples, HAMA and RF positive samples were tested. A1AT concentration of each samples tested was calculated from the calibration

curve obtained from three pairs of antibody respectively. Percentage of recovery was calculated as concentration measured by Theranos method vs. reference method (CLIA SIEMENS)

Training set included total of eight emphysema patient serum samples, eight COPD patient serum samples, four HAMA positive serum samples, four RF positive serum samples, and five control materials.

Control material tested:

- Bio-Rad liquichek Immunology control level 1, 2, and 3. Ref # 590X, lot # 52460.
- NIBSC WHO 1st international standard for alpha-1-antitrypsin, plasma derived. NIBSC code: 05/162.
- IRMM reference material. Cat # ERM-DA470K/IFCC.

Results:

Total 24 clinical samples and 5 control materials were tested for the A1AT recovery with the final three pairs of antibodies. Among the three final pairs, C6/D14 and C12/D14 showed low recovery with HAMA positive serum samples which indicated that HAMA positive samples might interfere the recovery of A1AT with these two pairs of antibody combination. C6/D12 showed good recovery with control material, HAMA and RF positive samples, but low recovery with samples with high A1AT level. Further optimization of the assay could possibly solve this problem, thus C6/D12 was chosen as the final antibody pair for assay optimization.

TABLE 8: SUMMARY OF TRAINING SET

Emphysema serum sample			C6/D12			C6/D14			C12/D14		
Sample Id	CLIA Advia result	Units	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy
3	1.08	mg/ml	13	1.22	113%	11	1.01	93%	1	1.59	147%
4	0.94	mg/ml	16	0.92	98%	6	0.66	71%	21	1.13	120%
5	0.92	mg/ml	5	0.73	80%	1	0.61	66%	5	0.96	104%
6	1.70	mg/ml	4	1.26	74%	12	0.95	56%	12	1.41	83%
7	1.61	mg/ml	4	1.31	81%	16	1.16	72%	21	1.57	97%
8	1.58	mg/ml	18	1.25	79%	3	1.05	66%	33	1.15	73%
9	2.11	mg/ml	8	1.72	82%	11	1.21	57%	22	1.29	61%
10	1.31	mg/ml	5	1.44	110%	3	0.96	74%	13	1.45	111%
Reference material											
Sample Id	Theoretical conc.	Units	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy
WHO control	1.00	mg/ml	13	1.01	101%	13	0.69	69%	21	1.15	115%
WHO control	0.50	mg/ml	11	0.60	121%	9	0.51	103%	20	0.73	146%

IRMM	1.12	mg/ml	22	0.96	86%	24	0.99	88%	15	1.32	118%
Bio-Rad level 1	0.656	mg/ml	6	0.66	101%	14	0.50	76%	38	0.60	91%
Bio-Rad level 2	1.25	mg/ml	1	0.87	70%	8	0.65	52%	33	0.70	56%
Bio-Rad level 3	1.82	mg/ml	26	1.46	80%	9	0.85	46%	16	1.13	62%
COPD serum sample											
Theranos #	CLIA Advia	Units	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy
21	2.98	mg/ml	13	1.90	64%	18	2.62	88%	4	2.48	83%
22	1.82	mg/ml	10	1.07	59%	9	1.13	62%	14	1.54	84%
23	**3.60**	mg/ml	23	1.84	51%	16	2.65	74%	18	2.21	61%
24	1.86	mg/ml	12	1.32	71%	11	1.72	93%	2	2.03	109%
25	1.80	mg/ml	24	1.14	63%	23	1.35	75%	36	1.67	93%
26	**3.26**	mg/ml	2	1.88	58%	25	2.35	72%	16	2.76	85%
27	1.89	mg/ml	6	1.44	76%	18	1.32	70%	6	2.06	109%
28	2.99	mg/ml	21	1.87	62%	8	2.62	88%	32	2.51	84%
HAMA and RF serum samples											
Theranos #	CLIA Advia	Units	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy	inter-CV	back-calc.	% accuracy
HAMA 1	1.24	mg/ml	18	0.92	74%	9	0.47	38%	1	0.32	26%
HAMA 2	1.51	mg/ml	3	1.17	77%	8	0.84	55%	9	0.81	54%
HAMA 3	1.53	mg/ml	11	0.99	65%	10	0.51	34%	13	0.48	31%
HAMA 4	1.50	mg/ml	13	1.29	86%	10	0.86	57%	7	0.95	63%
RF 54	1.69	mg/ml	9	1.69	100%	7	1.56	92%	11	1.32	78%
RF 57	1.20	mg/ml	18	0.76	63%	7	0.67	56%	7	0.66	55%
RF 60	2.53	mg/ml	26	3.17	125%	22	4.09	162%	25	2.61	103%
RF 63	1.89	mg/ml	17	1.47	78%	9	1.15	61%	8	1.19	63%

2.3 Assay optimization

2.3.1 Selection of detection conjugate stabilizer

Methods:

With capture antibody kept at 5ug/ml in 3% BSA blocking buffer and blocking buffer used as assay diluent, detection conjugate was prepared at 100ng/ml in Theranos in-house AP stabilizer, SurModics StabilZyme-AP, and Sigma Biostab AP stabilizer. Edison protocol Generic2_25000X_5-5-5 was used to test the effect of different detection conjugate stabilizer.

Results:
Among the three AP stabilizers, Theranos in-house AP stabilizer and SurModics StabilZyme-AP showed the overall best overall modulation, acceptable %CV, and good P/P modulation. On the other hand, Sigma Biostab AP stabilizer had the high %CV, poor overall modulation, and no separation between the top calibration points.

TABLE 9: SELECTION OF DETECTION CONJUGATE STABILIZER DATA SUMMARY

C6/D12				Theranos in-house AP stabilizer			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	119174	13	102.6	1.6
2	2.30	1.73	mg/ml	76286	4	65.7	1.6
3	1.15	0.86	mg/ml	48144	10	41.4	1.6
4	0.575	0.43	mg/ml	29785	2	25.6	2.5
5	0.20	0.15	mg/ml	11827	19	10.2	3.0
6	0.067	0.05	mg/ml	3889	1	3.3	1.8
7	0.022	0.017	mg/ml	2172	21	1.9	1.9
8	0.00	0.00	mg/ml	1162	21	1.0	
				SurModics StabilZyme-AP			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	595076	13	191.1	2.0
2	2.30	1.73	mg/ml	304825	20	97.9	1.6
3	1.15	0.86	mg/ml	189305	18	60.8	1.9
4	0.575	0.43	mg/ml	100525	9	32.3	3.1
5	0.20	0.15	mg/ml	32146	10	10.3	3.3
6	0.067	0.05	mg/ml	9623	4	3.1	1.3
7	0.022	0.017	mg/ml	7622	22	2.4	2.4
8	0.00	0.00	mg/ml	3113	37	1.0	
				Sigma Biostab AP stabilizer			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	88919	13	13.2	1.0
2	2.30	1.73	mg/ml	89568	13	13.3	1.5
3	1.15	0.86	mg/ml	61188	18	9.1	1.3
4	0.575	0.43	mg/ml	45373	34	6.7	1.9
5	0.20	0.15	mg/ml	23574	16	3.5	1.8
6	0.067	0.05	mg/ml	13082	23	1.9	1.6
7	0.022	0.017	mg/ml	8327	20	1.2	1.2
8	0.00	0.00	mg/ml	6758	24	1.0	

2.3.2 Detection conjugate titration

2.3.2.1 Theranos In-house AP stabilizer

Methods:

With all the assay conditions kept the same including the Edison protocol, detection conjugate were prepared in 50ng/ml, 25ng/ml and 10ng/ml in Theranos in-house AP stabilizer. Calibration curve was run with each concentration of detection, and the results were used to compare to original 100ng/ml detection conjugate in Theranos in house AP stabilizer.

Results:

Though the overall %CV and modulation for each concentration of detections were good, saturation at the high calibrator points was observed at each detection level. This saturation could result in low recovery of serum samples with high A1AT levels.

TABLE 10: DETECTION TITRATION WITH THERANOS IN-HOUSE AP STABILIZER

C6/D12				Theranos in-house AP stabilizer 100ng/ml			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	119174	13	103	1.6
2	2.30	1.73	mg/ml	76286	4	66	1.6
3	1.15	0.86	mg/ml	48144	10	41	1.6
4	0.575	0.43	mg/ml	29785	2	26	2.5
5	0.20	0.15	mg/ml	11827	19	10	3.0
6	0.067	0.05	mg/ml	3889	1	3	1.8
7	0.022	0.017	mg/ml	2172	21	2	1.9
8	0.00	0.00	mg/ml	1162	21	1	
C6/D12				**Theranos in-house AP stabilizer 50ng/ml**			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	77038	3	107	1.3
2	2.30	1.73	mg/ml	58123	7	81	1.8
3	1.15	0.86	mg/ml	31641	6	44	1.6
4	0.575	0.43	mg/ml	19643	7	27	3.6
5	0.20	0.15	mg/ml	5420	7	8	1.9
6	0.067	0.05	mg/ml	2868	14	4	2.5
7	0.022	0.017	mg/ml	1134	13	2	1.6
8	0.00	0.00	mg/ml	721	11	1	
C6/D12				**Theranos in-house AP stabilizer 25ng/ml**			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	38107	8	115	1.6

2	2.30	1.73	mg/ml	23445	11	71	1.5
3	1.15	0.86	mg/ml	15562	11	47	1.8
4	0.575	0.43	mg/ml	8590	14	26	3.7
5	0.20	0.15	mg/ml	2305	9	7	1.5
6	0.067	0.05	mg/ml	1505	7	5	2.6
7	0.022	0.017	mg/ml	570	24	2	1.7
8	0.00	0.00	mg/ml	333	20	1	
C6/D12				**Theranos in-house AP stabilizer 10ng/ml**			
Calibrator #	Nominal conc.	CLIA reassigned	unit	Inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	13551	11	82	1.3
2	2.30	1.73	mg/ml	10825	10	66	2.1
3	1.15	0.86	mg/ml	5243	3	32	1.8
4	0.575	0.43	mg/ml	2985	12	18	2.5
5	0.20	0.15	mg/ml	1186	8	7	2.0
6	0.067	0.05	mg/ml	588	7	4	2.0
7	0.022	0.017	mg/ml	289	14	2	1.7
8	0.00	0.00	mg/ml	165	17	1	

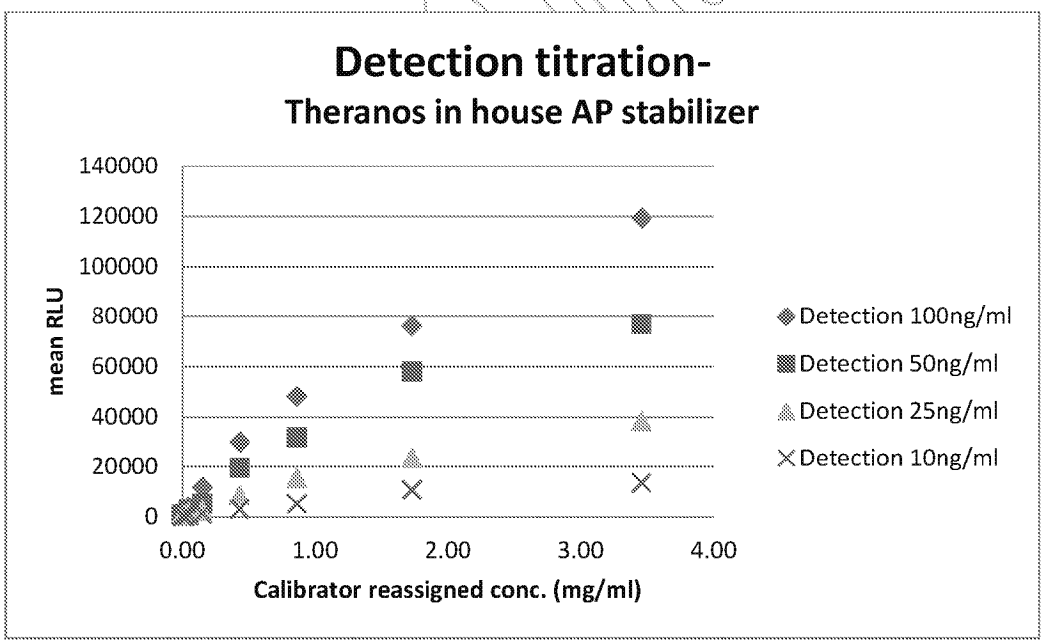

FIGURE 1: DETECTION TITRATION WITH THERANOS IN HOUSE AP STABILIZER CALIBRATION CURVE

2.3.2.2 SurModics StabilZyme-AP stabilizer

Since Theranos in-house AP stabilizer couldn't clearly separate the high level calibrators at any detection concentration, SurModics StabilZyme-AP stabilizer was then chosen as the AP stabilizer for detection titration.

Methods:

The procedure was the same as the previous detection titration with in house AP stabilizer; the only difference was using StabilZyme-AP to dilute the detection conjugate into 50ng/ml, 25ng/ml and 10ng/ml. The calibration results at three levels were then comparing to detection conjugate at 100ng/ml in StabilZyme-AP.

Results:

Detection conjugate at 50ng/ml in SurModics StabilZyme-AP gave the best overall modulation, whereas 25ng/ml showed almost identical S/B modulation and P/P modulation. Detection concentration at 25ng/ml in StabilZyme-AP was selected as the final choice.

TABLE 11: DETECTION TITRATION WITH SURMODICS STABILZYME-AP STABILIZER

C6/D12				SurModics StabilZyme-AP 100ng/mL			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	595076	13	191	2.0
2	2.30	1.73	mg/ml	304825	20	98	1.6
3	1.15	0.86	mg/ml	189305	18	61	1.9
4	0.575	0.43	mg/ml	100525	9	32	3.1
5	0.20	0.15	mg/ml	32146	10	10	3.3
6	0.067	0.05	mg/ml	9623	4	3	1.3
7	0.022	0.017	mg/ml	7622	22	2	2.4
8	0.00	0.00	mg/ml	3113	37	1	
C6/D12				SurModics StabilZyme-AP 50ng/mL			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	399997	11	236	1.7
2	2.30	1.73	mg/ml	230249	12	136	1.8
3	1.15	0.86	mg/ml	130618	9	77	2.1
4	0.575	0.43	mg/ml	61280	26	36	3.0
5	0.20	0.15	mg/ml	20171	23	12	3.7
6	0.067	0.05	mg/ml	5418	16	3	2.0
7	0.022	0.017	mg/ml	2765	14	2	1.6
8	0.00	0.00	mg/ml	1693	7	1	
C6/D12				SurModics StabilZyme-AP 25ng/mL			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	185657	28	213	1.8
2	2.30	1.73	mg/ml	103007	7	118	1.8
3	1.15	0.86	mg/ml	56137	4	64	2.3
4	0.575	0.43	mg/ml	24309	8	28	3.6

5	0.20	0.15	mg/ml	6757	14	8	2.1
6	0.067	0.05	mg/ml	3240	11	4	2.1
7	0.022	0.017	mg/ml	1546	24	2	1.8
8	0.00	0.00	mg/ml	873	5	1	
C6/D12				**SurModics StabilZyme-AP 10ng/mL**			
Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter-CV	B/S Mod.	P/P Mod.
1	4.60	3.46	mg/ml	62921	17	186	1.5
2	2.30	1.73	mg/ml	43256	16	128	2.0
3	1.15	0.86	mg/ml	22093	4	65	2.1
4	0.575	0.43	mg/ml	10761	11	32	3.4
5	0.20	0.15	mg/ml	3189	10	9	2.6
6	0.067	0.05	mg/ml	1227	11	4	2.0
7	0.022	0.017	mg/ml	628	19	2	1.9
8	0.00	0.00	mg/ml	338	20	1	

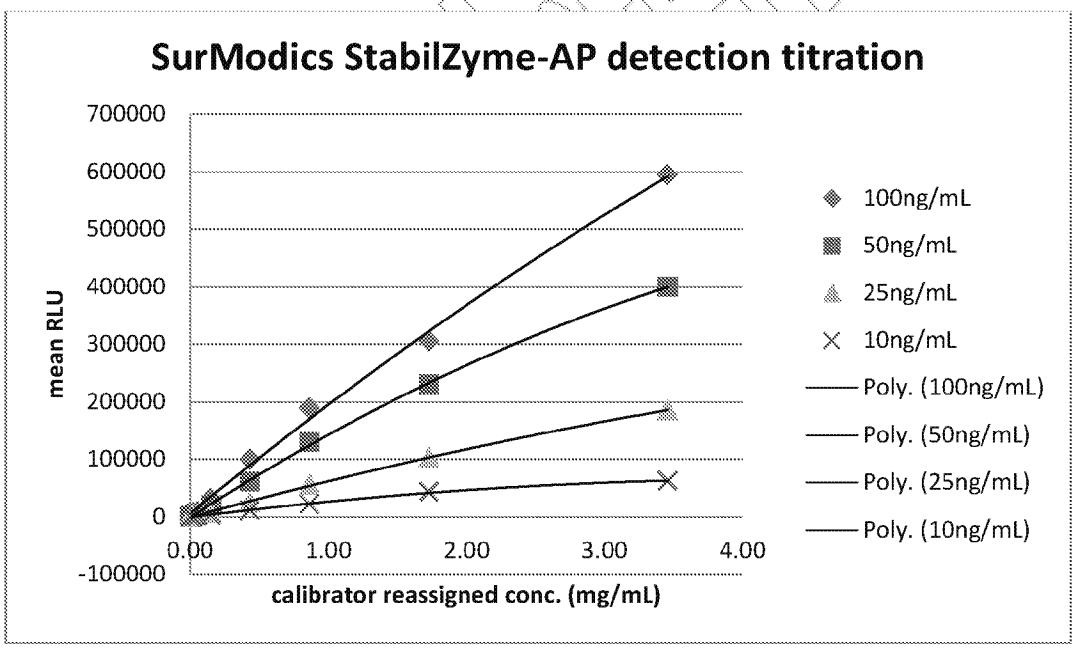

FIGURE 2: DETECTION TITRATION WITH SURMODICS STABLIZYME-AP CALIBRATION CURVE

2.3.3 Capture antibody titration

Methods:

Capture antibody titration was done by coating tips with C6 in 3% BSA blocking buffer at 10ug/ml, 5ug/ml, and 2.5ug/ml. detection antibody was kept at 25ng/ml in StabilZyme-AP. Edison protocol Generic2_25000X_5-5-5 was used for capture titration.

Results:

With detection antibody at 25ng/ml, capture antibody at 5ug/ml in blocking buffer seemed to saturate the coating surface. Increasing the coating concentration to 10ug/ml didn't improve CV. Thus 5ug/ml coating concentration was chosen as the final concentration for low background, high overall modulation, P/P modulation, and reasonable %CV.

TABLE 12: CAPTURE ANTIBODY TITRATION

C6/D12 capture titration				Cab 2.5ug/ml			
Calibrator	Nominal conc.	CLIA reassigned conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	70664	14	314.5	3.7
2	2.30	1.73	mg/ml	19083	11	84.9	2.0
3	1.15	0.86	mg/ml	9547	15	42.5	2.7
4	0.575	0.43	mg/ml	3495	8	15.6	3.4
5	0.20	0.15	mg/ml	1018	16	4.5	2.0
6	0.067	0.05	mg/ml	515	39	2.3	1.2
7	0.022	0.017	mg/ml	418	22	1.9	1.9
8	0.00	0.00	mg/ml	225	14	1.0	
C6/D12 capture titration				**Cab 5ug/ml**			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	185657	28	212.7	1.8
2	2.30	1.73	mg/ml	103007	7	118.0	1.8
3	1.15	0.86	mg/ml	56137	4	64.3	2.3
4	0.575	0.43	mg/ml	24309	8	27.9	3.6
5	0.20	0.15	mg/ml	6757	14	7.7	2.1
6	0.067	0.05	mg/ml	3240	11	3.7	2.1
7	0.022	0.017	mg/ml	1546	24	1.8	1.8
8	0.00	0.00	mg/ml	873	5	1.0	
C6/D12 capture titration				**Cab 10ug/ml**			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	217374	8	361.3	2.0
2	2.30	1.73	mg/ml	108708	18	180.7	1.7
3	1.15	0.86	mg/ml	63212	10	105.1	2.2
4	0.575	0.43	mg/ml	29239	3	48.6	3.9
5	0.20	0.15	mg/ml	7576	18	12.6	2.9
6	0.067	0.05	mg/ml	2655	2	4.4	1.8
7	0.022	0.017	mg/ml	1435	28	2.4	2.4
8	0.00	0.00	mg/ml	602	35	1.0	

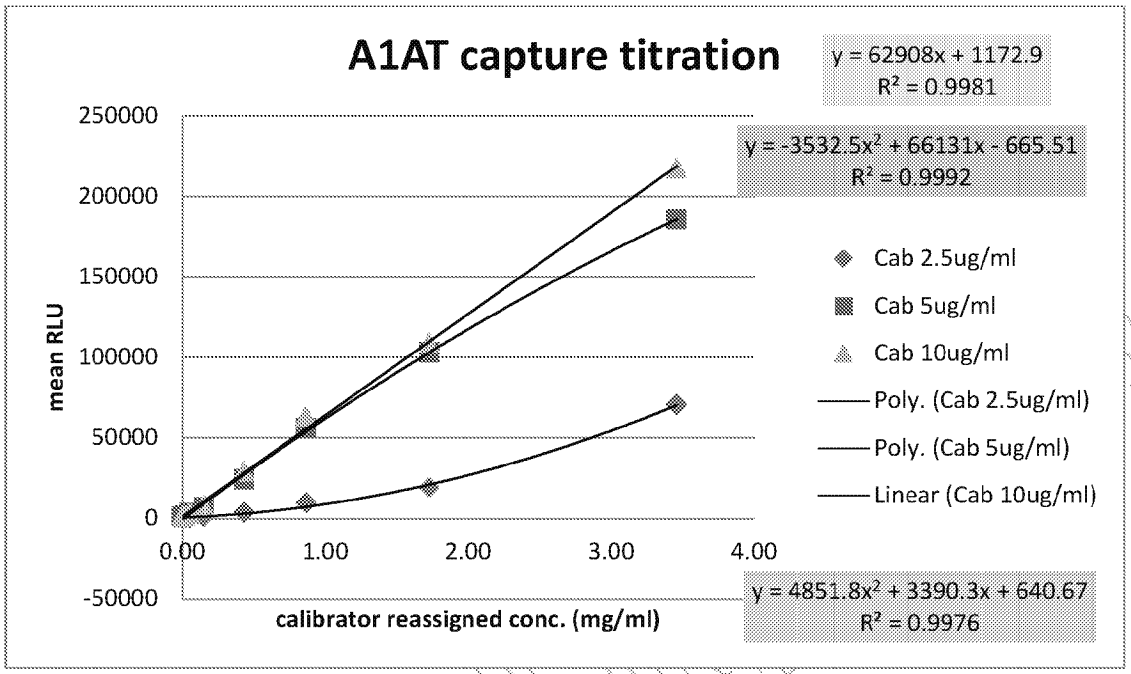

FIGURE 3: CAPTURE ANTIBODY TITRATION CALIBRATION CURVE

2.3.4 Effect of incubation time

Methods:

Capture antibody was coated on tips at 5ug/ml in 3% BSA blocking buffer, detection conjugate was prepared at 25ng/ml in SurModics StabliZyme-AP, 3% BSA blocking buffer was used as assay diluent, new calibration curve was run with Edison protocol Generic2_25000X_10-10-10. The result was compared to the control protocol Genenric2_25000X_5-5-5.

Results:

Increasing incubation time from 5-5-5 to 10-10-10 increased the overall RLU including the background, as a result decreased the S/B and P/P modulation. Thus Edison protocol Generic2_25000X_5-5-5 was kept as the final assay protocol.

TABLE 13: EFFECT OF INCUBATION TIME

Incubation time				Generic2_25000X_5-5-5			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	151235	6	96.3	1.7
2	2.30	1.73	mg/ml	89234	8	56.8	1.6
3	1.15	0.86	mg/ml	57104	11	36.4	2.2
4	0.575	0.43	mg/ml	25828	8	16.4	3.5
5	0.20	0.15	mg/ml	7427	21	4.7	1.6

6	0.067	0.05	mg/ml	4572	3	2.9	2.1
7	0.022	0.017	mg/ml	2127	11	1.4	1.4
8	0.00	0.00	mg/ml	1571	23	1.0	
Incubation time				**Generic2_25000X_10-10-10**			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	342112	16	86.0	1.4
2	2.30	1.73	mg/ml	245972	7	61.8	1.6
3	1.15	0.86	mg/ml	157465	6	39.6	3.0
4	0.575	0.43	mg/ml	52931	12	13.3	3.1
5	0.20	0.15	mg/ml	17328	8	4.4	2.6
6	0.067	0.05	mg/ml	6675	6	1.7	1.3
7	0.022	0.017	mg/ml	5097	14	1.3	1.3
8	0.00	0.00	mg/ml	3978	20	1.0	

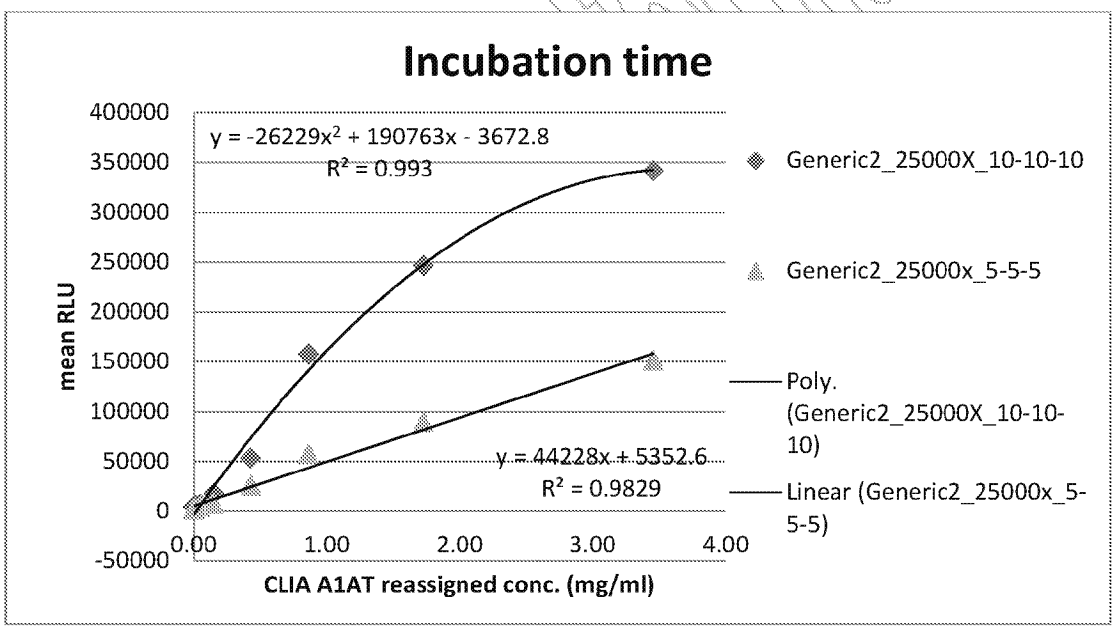

FIGURE 4: EFFECT OF INCUBATION TIME CALIBRATION CURVE

2.3.5 Edison protocol optimization

Methods:

Post sample wash (PSW) was added to the existing assay protocol Generic2_25000X_5-5-5 in the hope of lowering background, improving %CV and modulation. Calibrators were run under these two protocols: Genenric2_25000X_5-5-5 and Generic2_25000X_5-5-5_PSW. The tips were coated at 5ug/ml in blocking buffer; detection conjugate was prepared at 25ng/ml in StabilZyme-AP, and the sample diluent was 3% BSA blocking buffer.

Results:

Addition of PSW didn't decrease the background, nor did it help with %CV or modulation. Thus Edison protocol Generic2_25000X_5-5-5 was kept as the final assay protocol.

TABLE 14: EDISON PROTOCOL OPTIMIZATION

Incubation time				Generic2_25000X_5-5-5			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	151235	6	96.3	1.7
2	2.30	1.73	mg/ml	89234	8	56.8	1.6
3	1.15	0.86	mg/ml	57104	11	36.4	2.2
4	0.575	0.43	mg/ml	25828	8	16.4	3.5
5	0.20	0.15	mg/ml	7427	21	4.7	1.6
6	0.067	0.05	mg/ml	4572	3	2.9	2.1
7	0.022	0.017	mg/ml	2127	11	1.4	1.4
8	0.00	0.00	mg/ml	1571	23	1.0	
Incubation time				**Generic2_25000X_5-5-5_PSW**			
Calibrator	Nominal conc.	CLIA reassgiend conc.	unit	inter-mean	inter-%CV	S/B mod.	P/P mod.
1	4.60	3.46	mg/ml	137487	9	79.5	1.6
2	2.30	1.73	mg/ml	85516	12	49.4	1.6
3	1.15	0.86	mg/ml	54660	7	31.6	2.2
4	0.575	0.43	mg/ml	24525	10	14.2	3.3
5	0.20	0.15	mg/ml	7350	15	4.2	1.9
6	0.067	0.05	mg/ml	3956	3	2.3	1.6
7	0.022	0.017	mg/ml	2513	4	1.5	1.5
8	0.00	0.00	mg/ml	1730	11	1.0	

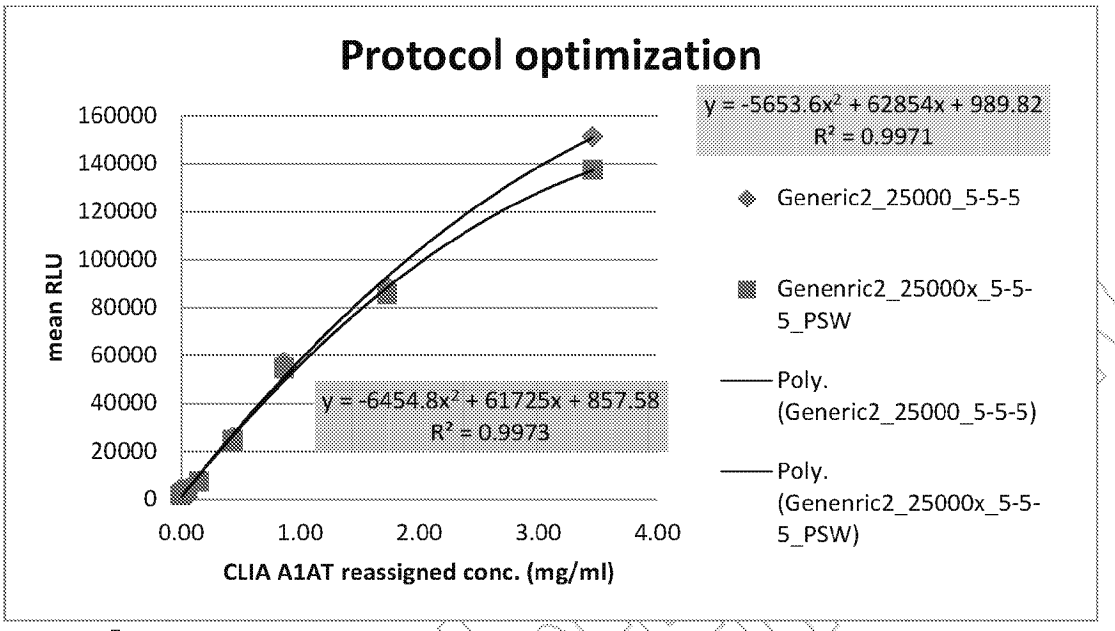

FIGURE 5: EDISON PROTOCOL OPTIMIZATION CALIBRATION CURVE

2.4 CLINICAL SAMPLE TESTING

2.4.1 HAMA and RF positive serum samples

2.4.1.1 HAMA and RF sample recovery with detection conjugate 25ng/ml

Methods:

Four HAMA and four RF positive serum samples were analyzed with final assay condition. All samples were also tested for A1AT level on SIMENS Advia 1800. Back calculation was based on the calibration curve which was run under the same assay condition. Capture antibody 5ug/ml in 3% BSA blocking buffer coated on tips, detection conjugate 25ng/ml in SurModics StabilZyme-AP, assay diluent 3% BSA blocking buffer, and Edison protocol Genenric2_25000X_5-5-5.

Results:

All HAMA and RF samples showed low recovery when comparing the back calculated A1AT concentration to SIEMENS Advia A1AT results.

TABLE 15: HAMA AND RF POSITIVE SERUM SAMPLE RECOVERY DETECTION 25NG/ML

HAMA and RF serum samples							Dab 25ng/ml
Sample Id	Concentration	Units	inter-mean	inter RLU-CV	back-calc.	inter conc.-CV	% recovery

HAMA 1	1.24	mg/ml	55175	13	0.83	11	67%
HAMA 2	1.51	mg/ml	59951	7	0.89	6	59%
HAMA 3	1.53	mg/ml	54196	6	0.82	5	54%
HAMA 4	1.50	mg/ml	73354	11	1.07	10	72%
RF 54	1.69	mg/ml	90771	13	1.36	12	80%
RF 57	1.20	mg/ml	21481	4	0.40	3	33%
RF 60	2.53	mg/ml	39629	31	0.63	25	25%
RF 63	1.89	mg/ml	71365	18	1.05	17	55%

2.4.1.2 HAMA and RF sample recovery with detection conjugate 50ng/ml

HAMA and RF showed a low recovery with detection conjugate at 25ng/ml in StabilZyme-AP, whereas during training set same set of HAMA and RF samples showed good clinical recovery with higher detection concentration. Detection conjugate concentration was adjusted to 50ng/ml instead of 25ng/ml, and same set of HAMA and RF samples were tested for recovery.

Methods:

Previous assay condition was kept unchanged with only increasing the detection conjugate concentration to 50ng/ml. first new calibration curve was run, then HAMA and RF positive serum samples was tested under the same assay condition. Clinical recovery was calculated comparing A1AT Theranos method vs. SIEMENS Advia result.

Results:

Increasing detection conjugate concentration to 50ng/ml improved the HAMA and RF positive samples clinical recovery. Thus the final assay condition was locked with detection antibody concentration at 50ng/ml in StabilZyme-AP.

TABLE 16: HAMA AND RF POSITIVE SERUM SAMPLE RECOVERY DETECTION 50NG/ML

HAMA and RF serum samples							Dab 50ng/ml
Sample Id	Concentration	Units	inter-mean	inter RLU-CV	back-calc.	inter conc.-CV	% recovery
HAMA 1	1.24	mg/ml	126254	10	1.21	9	97%
HAMA 2	1.51	mg/ml	163019	15	1.55	15	102%
HAMA 3	1.53	mg/ml	122692	12	1.17	11	77%
HAMA 4	1.50	mg/ml	141541	18	1.34	17	90%
RF 54	1.69	mg/ml	179757	14	1.71	14	101%
RF 57	1.20	mg/ml	112840	17	1.09	16	91%
RF 60	2.53	mg/ml	166131	6	1.57	6	62%
RF 63	1.89	mg/ml	184251	15	1.75	16	93%

2.4.2 Matrix effect

Methods:

Four hemolyzed, four icteric, and four lipemic serum samples were tested for A1AT recovery on Theranos system with the final assay conditions. Capture antibody coated on tips 5ug/ml in 3% BSA blocking buffer, detection conjugate at 50ng/ml in StabliZyme-AP, and 3% BSA blocking buffer as assay diluent. The A1AT concentration was back calculated based on the calibration curve which was run under the same condition.

Results:

Hemolyzed, icteric, and lipemic matrixes samples didn't show significant interference on A1AT recovery in Theranos system when comparing to SIEMENS Advia results. However, severely lipemic samples with triglyceride level greater than 10mg/ml could affect the recovery of A1AT.

TABLE 17: MATRIX EFFECT SAMPLE RECOVERY

Hemolyzed								
PromedDx	lot #	CLIA results	unit	inter-mean	inter-CV	Back calc.	inter-CV	% recovery
1	N/A	1.85	mg/mL	172901	17	1.60	17	86%
2	N/A	1.83	mg/mL	155224	9	1.47	9	80%
5	705	0.91	mg/mL	87634	5	0.87	4	96%
6	708	1.26	mg/mL	125482	13	1.20	12	95%
Lipemic								
ZeptoMetrix lot #	CLIA Triglyceride	CLIA A1AT	unit	inter-mean	inter-CV	Back calc.	inter-CV	% recovery
683	5.76	1.17	mg/mL	137361	11	1.31	11	112%
687	8.09	1.8	mg/mL	154839	3	1.46	3	81%
688	6.5	1.75	mg/mL	199558	6	1.90	6	109%
689	13.25	2.16	mg/mL	111901	11	1.08	10	50%
689 rerun	13.25	2.16	mg/mL	121524	19	1.17	17	54%
Icteric								
PromedDx	lot #	CLIA results	unit	inter-mean	inter-CV	Back calc.	inter-CV	% recovery
24		1.57	mg/mL	160974	8	1.52	8	97%
302		1.48	mg/mL	115212	6	1.11	5	75%
447		1.99	mg/mL	194399	6	1.85	6	93%
525		1.55	mg/mL	174795	14	1.66	15	107%

2.4.3 Hematocrit effect and anticoagulant effect

Methods:

Whole blood, serum, EDTA plasma, and Lithium-heparin plasma samples obtained from ten donors (5male, 5female). All samples were analyzed with final assay condition. Hematocrit effect was evaluated by comparing A1AT recovery concentration in whole blood vs. EDTA plasma from the same donor. EDTA plasma, lithium-heparin plasma and serum from the same donor were also analyzed to compare the effect of anticoagulant.

Results:

Samples from then donors collected in pairs of whole blood, EDTA plasma, and lithium-heparin plasma were analyzed. Hematocrit factor was calculated to be 1.69.

A1AT results from serum, EDTA, and lithium-heparin plasma matches with each other with difference less than 20%. Thus this assay could be used to analyzed whole blood (Hematocrit factor=1.69), EDTA plasma, lithium-heparin plasma, and serum with no significant differences in between each anticoagulant.

TABLE 18: A1AT THERANOS RESULTS OF WHOLE BLOOD, PLASMA, SERUM MATCHING PAIRS

Theranos A1AT (mg/ml)	EDTA	Li-Hep	Diff. Li-hep / EDTA	serum	Diff. serum / EDTA	Diff. serum / Li-hep	Whole blood (EDTA)	Hematocrit factor
M1	1.24	1.28	3%	1.22	-2%	-5%	0.95	1.31
M2	1.29	1.39	8%	1.41	9%	2%	0.63	2.05
M3	1.14	1.14	0%	1.29	13%	13%	0.74	1.54
M4	1.05	1.12	6%	1.19	13%	7%	0.58	1.80
M5	1.10	1.03	-6%	1.00	-9%	-3%	0.63	1.75
F1	1.22	1.32	8%	1.12	-8%	-15%	0.70	1.75
F2	1.22	1.15	-6%	1.35	10%	17%	0.83	1.47
F3	1.02	1.12	9%	1.15	13%	3%	0.54	1.89
F4	1.22	1.13	-7%	1.18	-3%	4%	0.75	1.62
F5	0.98	0.89	-9%	0.78	-20%	-12%	0.57	1.72
							average Hematocrit	1.69

2.4.4 Calibration curve with final assay conditions

Final assay condition was capture antibody C6 5ug/ml in 3% BSA blocking buffer, detection conjugate 50ng/ml in SurModics StabilZyme-AP, 3% BSA blocking buffer as assay diluent. Calibration curve was generated under this assay condition with final Edison protocol Generic2_25000X_5-5-5 and data was analyzed by Dexter.

TABLE 19: DATA OF A1AT FINAL CALIBRATION CURVE

Calibrator #	Nominal conc.	CLIA reassigned	unit	inter-mean	inter RLU-CV	B/S Mod.	P/P Mod.	back calc.	inter conc.-CV	% recovery

1	4.60	3.46	mg/ml	285956	15	416.2	1.6	2.93	20	85%
2	2.30	1.73	mg/ml	173727	7	252.9	1.8	1.65	7	95%
3	1.15	0.86	mg/ml	96954	10	141.1	2.7	0.95	8	110%
4	0.575	0.43	mg/ml	36290	5	52.8	5.3	0.43	4	100%
5	0.20	0.15	mg/ml	6824	5	9.9	2.3	0.11	4	76%
6	0.067	0.05	mg/ml	2951	18	4.3	1.9	0.05	22	96%
7	0.022	0.017	mg/ml	1582	9	2.3	2.3	0.02	18	117%
8	0.00	0.00	mg/ml	687	17	1.0				

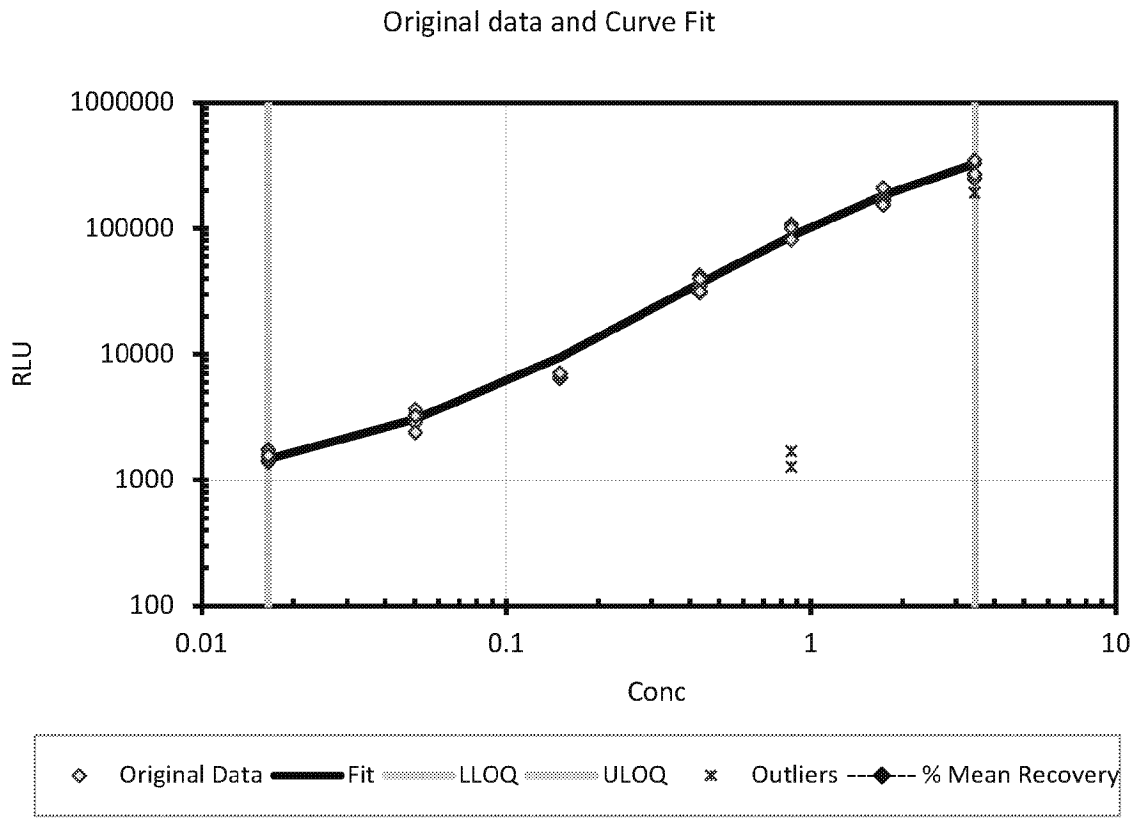

FIGURE 6: CALIBRATION CURVE GENERATED BY DEXTER

TABLE 20: 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	2.833
b2	6.035
b3	0.345
b4	-0.710

LLOQ	0.02 mg/ml
ULOQ	3.46 mg/ml
LLOQ accuracy	117%
LLOQ precision	17.90%
ULOQ accuracy	85%
ULOQ precision	19.60%

2.4.5 Clinical samples recovery with final assay condition

2.4.5.1 Plasma sample recovery with final assay condition

Ten EDTA plasma samples and ten matching Li-hep plasma samples were analyzed by Theranos method and SIEMENS Advia method for A1AT level. The results difference between the two methods was calculated. Data for Theranos methods matches well with results obtained from SIEMENS method.

TABLE 21: 20 PLASMA SAMPLES RECOVERY

EDTA plasma							
Sample #	CLIA result	inter-mean	inter RLU-CV	Theranos result	inter conc.-CV	% accuracy	% difference
M1	1.16	130616	11	1.24	10	107%	7%
M2	1.25	135945	9	1.29	8	103%	3%
M3	1.32	118384	4	1.14	3	86%	-14%
M4	1.14	108382	29	1.05	26	92%	-8%
M5	1.06	114333	12	1.10	11	104%	4%
F1	1.22	128123	12	1.22	12	100%	0%
F2	1.30	128228	8	1.22	8	94%	-6%
F3	1.19	104816	17	1.02	15	86%	-14%
F4	1.32	126865	18	1.22	17	92%	-8%
F5	0.85	100412	6	0.98	5	115%	15%
Lithium-heparin plasma							
Sample #	CLIA result	inter-mean	inter-CV	inter-ave	inter-CV	% accuracy	% difference
M1	1.40	134700	15	1.28	14	92%	-8%
M2	1.32	146966	3	1.39	3	105%	5%
M3	1.38	118668	2	1.14	2	83%	-17%
M4	1.14	116304	11	1.12	10	98%	-2%
M5	1.08	106336	18	1.03	16	96%	-4%
F1	1.31	138527	19	1.32	18	101%	1%
F2	1.26	120244	17	1.15	15	92%	-8%
F3	1.26	115781	21	1.12	19	89%	-11%
F4	1.28	117550	2	1.13	1	88%	-12%

F5	0.84	89501	3	0.89	3	106%	6%

2.4.5.2 Serum samples recovery with final assay condition

Total 40 clinical serum samples were tested for A1AT level both on Theranos system and SIEMENS Advia. The results difference between two methods was calculated. Data from Theranos methods matched well with results obtained from SIEMENS method.

TABLE 22: 40 SERUM SAMPLES RECOVERY

Zeptometrix Emphysema serum samples									
Theranos #	Lot Number	CLIA Advia	Units	inter-mean	inter RLU-CV	back calc.	inter conc.-CV	% accuracy	%difference
1	0302-077-00201	1.04	mg/mL	120542	5	1.15	5	111%	11%
2	0302-077-00203	1.19	mg/mL	108671	17	1.05	15	89%	-11%
3	0302-077-00205	1.08	mg/mL	128699	16	1.23	15	114%	14%
4	0302-077-00217	**0.94**	mg/mL	113626	6	1.09	5	116%	16%
5	0302-077-00218	**0.92**	mg/mL	93607	23	0.92	20	100%	0%
6	0302-077-00226	1.70	mg/mL	201344	18	1.93	19	114%	14%
7	0302-077-00227	1.61	mg/mL	147344	13	1.40	13	87%	-13%
8	0302-077-00230	1.58	mg/mL	161193	16	1.53	16	97%	-3%
9	0302-077-00248	2.11	mg/mL	193219	4	1.84	5	87%	-13%
10	0302-077-00275	1.31	mg/mL	155091	5	1.47	5	112%	12%
ZeptoMetrix Alcoholic Cirrohsis serum sample									
Theranos #	Lot Number	CLIA Advia	Units	inter-mean	inter RLU-CV	back calc.	inter conc.-CV	% accuracy	%difference
11	9712-027-0904	1.09	mg/mL	104900	14	1.02	12	94%	-6%
12	9712-027-0906	1.52	mg/mL	184944	1	1.75	1	115%	15%
13	9712-027-0908	1.91	mg/mL	202055	14	1.93	15	101%	1%
14	9712-027-0909	1.40	mg/mL	156688	23	1.49	22	106%	6%
15	9702-027-	2.03	mg/mL	177720	6	1.69	6	83%	-17%

16	1510 9712-027-0912	1.97	mg/mL	225957	3	2.18	4	111%	11%
17	9712-027-0924	1.68	mg/mL	180869	17	1.72	18	102%	2%
18	9712-027-0925	1.46	mg/mL	177218	6	1.68	6	115%	15%
19	9712-027-0945	2.02	mg/mL	177570	15	1.70	15	84%	-16%
20	9910-027-02453	1.48	mg/mL	155665	5	1.47	5	99%	-1%
ProMedDx COPD serum samples									
Theranos #	Lot Number	CLIA Advia	Units	inter-mean	inter RLU-CV	back calc.	inter conc.-CV	% accuracy	%difference
21	11762700	2.98	mg/mL	319606	7	3.37	10	113%	13%
22	11762702	1.82	mg/mL	194691	21	1.87	23	103%	3%
23	11762704	**3.60**	mg/mL	304247	4	3.15	5	87%	-13%
24	11791537	1.86	mg/mL	186295	3	1.77	4	95%	-5%
25	11791539	1.80	mg/mL	174515	10	1.66	10	92%	-8%
26	11791543	**3.26**	mg/mL	351486	5	3.89	9	119%	19%
27	11791554	1.89	mg/mL	186522	5	1.77	6	94%	-6%
28	11791560	2.99	mg/mL	291950	6	3.01	9	101%	1%
29	11785869	1.75	mg/mL	165165	8	1.56	8	89%	-11%
30	11762699	2.91	mg/mL	287037	7	2.95	8	101%	1%
serum samples from normal donors									
Theranos #	Sample #	CLIA result	Units	inter-mean	inter RLU-CV	back calc.	inter conc.-CV	% accuracy	%difference
31	M1	1.18	mg/mL	127602	18	1.22	17	103%	3%
32	M2	1.37	mg/mL	149180	8	1.41	8	103%	3%
33	M3	1.31	mg/mL	135849	16	1.29	15	99%	-1%
34	M4	1.16	mg/mL	124637	16	1.19	15	103%	3%
35	M5	1.16	mg/mL	102983	20	1.00	17	87%	-13%
36	F1	1.23	mg/mL	116464	12	1.12	11	91%	-9%
37	F2	1.37	mg/mL	141391	24	1.35	23	98%	-2%
38	F3	1.23	mg/mL	120115	18	1.15	16	94%	-6%
39	F4	1.25	mg/mL	122358	26	1.18	24	94%	-6%
40	F5	0.97	mg/mL	77060	11	0.78	9	81%	-19%

2.5 Stability

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