Human IL-10 Assay Feasibility Report

Theranos, Inc.

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1. ASSAY INFORMATION

1.1 Analyte Information

Interleukin-10 (IL-10) as an anti-inflammatory cytokine. In human, IL-10 is encoded by the IL 10 gene, IL-10 signals through a receptor complex consisting of the IL-10 receprot-1 and two IL-10 receptor 2 proteins. The mature human IL-10 is a protein with 160 amino acids and the functional IL-10 is a homodimer. IL-10 plays an important role in inflammatory and immune responses. The biological activities of IL-10 include both immunosuppressive and immuno-stimulatory effects. For example, IL-10 can suppress the production of pro-inflammatory cytokines by monocytes and neutrophils, and down regulates the expression of activating and co-stimulatory molecules on monocytes and dendritic cells. IL-10 also can improve the growth of B cells and mast cells, and inhibit or enhance the activities of T cells depending on their activation conditions. Healthy subjects range: 5 - 19.21 pg/mL. Severe burn with sepsis patients & other diseases like lymphoma had significantly higher serum levels of IL-10, range from 5 - over 1000 pg/mL.

1.2 Assay specification

This assay determines the concentration of IL-10 in human serum, plasma, and whole blood. The assay has a quantification range of 3.9pg/mL to 1000pg/mL.

1.3 Reference assay

The following assay was used as reference method:

1) R&D Human IL-10 Immunoassay, catalog #: D1000B

1.4 Material and method

A sandwich immunoassay using anti-human IL-10 antibodies was developed for the quantitative determination of human IL-10 in serum, plasma, and whole blood.

In this assay, a monoclonal mouse anti-human antibody was used as capture antibody of IL-10 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 on board with sample diluent and then co-incubated mouse monoclonal detection antibody. After the co-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. IL-10 concentration of unknown sample was calculated from calibration curve.

Table [SEQ Table * ARABIC]: IL-10 ASSAY METERIAL IN FINAL ASSAY PROCEDURE

Name	Supplier	Catalog number
IL-10 analyte	WHO NIBSC	93/722
Mab mouse anti human IL-10	Abnova	MAB3286
Mab mouse anti human IL-10	Abcam	AB13916
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	KPL	55-60-04
Biotin conjugation kit	Dojindo	LK10-10
AP conjugation kit	Dojindo	LK13-10
StabilZyme-AP	SurModics	SA01-1000

1.5 Raw data storage

Raw data of assay development were saved in Elog #629.

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 19 anti-human IL-10 antibodies were screened for binding activity on micro titer plate (MTP). All antibodies were labeled with Dojindo Biotin labeling kit-SH (cat: LK10-10) and Dojindo AP labeling kit-SH (cat LK13-10). Analyte used for initial antibody screening was R&D system recombinant human IL-10 protein baculovirus derived (cat: 217-IL).

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. IL-10 calibrators at 500pg/ml, 50pg/ml, 5pg/ml and 0pg/ml were incubated with coated antibodies. Then detection antibody-AP conjugates were diluted in the Surmodics StabilZyme AP conjugate stabilizer (cat: SA01) to 100ng/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, IL-10@ 0pg/ml).

Result:

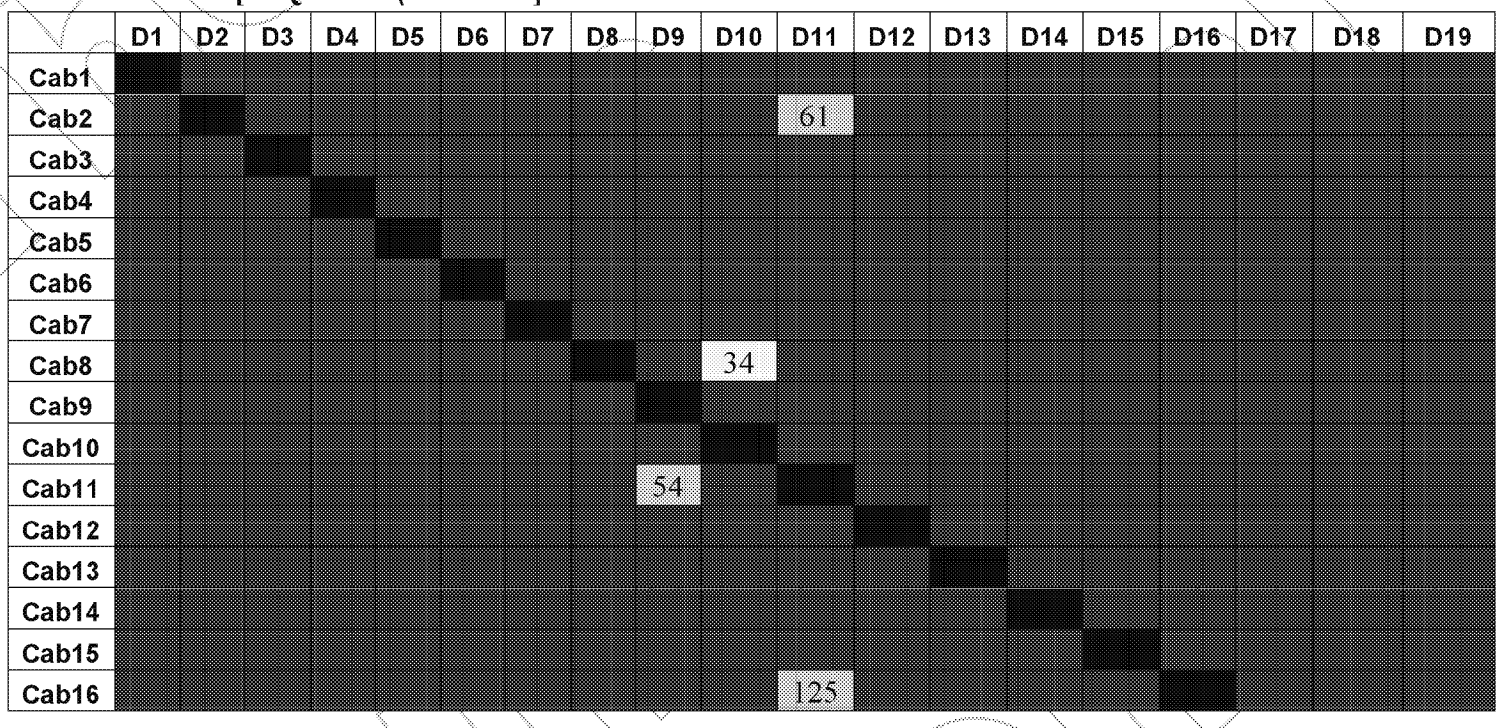
Out of the 19 X 19 antibody pairs screened, some antibody pairs showed variable degree of response. Only antibody pair C2/D11, C11/D9, and C16/D11 moved on the cross reactivity and interference tests.

Table [SEQ Table * ARABIC]: ANTIBODIES SCREENED ON MTP

	Company	target	function	cat #	Clone
1	Abcam	IL-10	Mouse Mab	AB10774	Clone 23738.11
2	Abcam	IL-10	Mouse Mab	AB22771	Clone B-S10
3	Abcam	IL-10	Mouse Mab	ab13916	Clone JES3-9D7
4	abnova	IL-10	Mouse Mab	MAB3286	Clone JES3-12G8
5	Abnova	IL-10	Mouse Mab	MAB2539	Clone SPM464
6	Abnova	Il-10	Mouse Mab	MAB3312	JES5-2A5
7	Abnova	IL-10	Mouse Mab	H00003586-M03	Clone 1C10

8	R&D	IL-10	Goat Pab	AB-217-NA	
9	R&D	IL-10	Mouse Mab	[HYPERLINK "http://www.rndsystems.com/pdf/mab2171.pdf" \t "_blank"]	Clone 25209
10	R&D	IL-10	Mouse Mab	[HYPERLINK "http://www.rndsystems.com/pdf/MAB2172.pdf" \t "_blank"]	Clone 127107
11	BD Biosciences	IL-10	Mouse Mab	554703	Clone JES3-19F1
12	Lifespan	IL-10	Mouse Mab	LS-C47137	Clone 3C12C12
13	Lifespan	IL-10	Mouse Mab	LS-C8076	Clone 5C68
14	Lifespan	IL-10	Mouse Mab	LS-C8066	Clone 5J47
15	Lifespan	IL-10	Mouse Mab	LS-C8068	Clone 5J48
16	Lifespan	IL-10	Mouse Mab	LS-C8079	NA
17	Abbiotec	IL-10	Rabbit Pab	250713	
18	Aniara	IL-10	Rabbit Pab	CT267	
19	Thermo Scientific	IL-10	Mouse Mab	OMA1-03353	Clone 945A5D11

Table [SEQ Table * ARABIC]: SUMMARY OF INITIAL ANTIBODY SCREENING ON MTP



Cab17	[redacted]
Cab18	
Cab19	

2.1.2 Cross reactivity and interference on MTP

Human IL-1a, IL-2, IL-4, IL-6, IL-8, IL-12, and INFg were tested for cross reactivity and interference for the three selected antibody pairs.

Table [SEQ Table * ARABIC]: PROTIENS TESTED FOR CROSS REACTIVITY AND INTERFERENCE

#	Protein	Normal conc. level[1]	Testing conc.
1	IL-1alpha	Less than 5pg/ml	Up to 250pg/ml
2	IL-2	Less than 12pg/ml	Up to 2000pg/ml
3	IL-4	Less than 5pg/ml	Up to 2000pg/ml
4	IL-6	Less than 5pg/ml	Up to 300pg/ml
5	IL-8	Less than 5pg/ml	Up to 2000pg/ml
6	IL-12	Less than 6pg/ml	Up to 500pg/ml
7	INFg	Less than 5pg/ml	Up to 1000pg/ml

1, data cited from Arup lab.

Method:

Previous method used in antibody screening was used here. However, for cross reactivity test, instead of using IL-10 calibrators, the above cross reactants were added as samples. In interference test, the above proteins which spiked into IL-10 calibrators of each concentration were tested as samples.

Results:

For all seven proteins tested, none of them showed cross reactivity with antibody pair C2/D11, C11/D9, or C16/D11. With antibody pair C16/D11, some interference was observed with IL-2, IL-4, IL-6, IL-8, IL-12 and INFg at low level of IL-10 6.2pg/ml, however, the interferent spiking level was much higher than the endogenous protein level, thus C11/D9 and C16/D11 moved to Theranos system for further testing.

Table [SEQ Table * ARABIC]: RESTULS OF CROSS REACTIVITY

C2/D11 Cross Reactivity					
Control IL-10 Conc. pg/mL	Recovered Conc. pg/mL	% Reactivity	IFNg Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
500.0	505.6	101	1000.0	OORL	0
166.7	153.0	92	333.3	OORL	0
55.6	52.8	95	111.1	OORL	0
18.5	20.0	108	37.0	OORL	0

6.2	11.7	189	12.3	OORL	0

IL-1a Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-2 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
250.0	OORL	0	2000.0	OORL	0
83.3	OORL	0	666.7	OORL	0
27.8	OORL	0	222.2	OORL	0
9.3	OORL	0	74.1	OORL	0
3.1	OORL	0	24.7	OORL	0

IL-4 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-6 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
2000.0	OORL	0	300.0	OORL	0
666.7	OORL	0	100.0	OORL	0
222.2	OORL	0	33.3	OORL	0
74.1	OORL	0	11.1	OORL	0
24.7	OORL	0	3.7	OORL	0

IL-8 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-12 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
2000	OORL	0	500.0	OORL	0
666.7	OORL	0	166.7	OORL	0
222.2	OORL	0	55.6	OORL	0
74.1	OORL	0	18.5	OORL	0
24.7	OORL	0	6.2	OORL	0

C11/D9 Cross Reactivity

Control IL-10 Conc. pg/mL	Recovered Conc. pg/mL	% Reactivity	IFNg Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
500.0	503.2	101	1000.0	OORL	0
166.7	155.7	93	333.3	OORL	0
55.6	54.0	97	111.1	OORL	0
18.5	18.6	100	37.0	OORL	0
6.2	6.4	104	12.3	OORL	0

IL-1a Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-2 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
250.0	OORL	0	2000.0	OORL	0
83.3	OORL	0	666.7	OORL	0
27.8	OORL	0	222.2	OORL	0
9.3	OORL	0	74.1	OORL	0
3.1	OORL	0	24.7	OORL	0

IL-4 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-6 Conc. pg/mL	Recovered Conc pg/mL	% Cross Reactivity
2000.0	OORL	0	300.0	OORL	0

666.7	OORL	0	100.0	OORL	0
222.2	OORL	0	33.3	OORL	0
74.1	OORL	0	11.1	OORL	0
24.7	OORL	0	3.7	OORL	0

IL-8 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-12 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
2000.0	OORL	0	500.0	OORL	0
666.7	OORL	0	166.7	OORL	0
222.2	OORL	0	55.6	OORL	0
74.1	OORL	0	18.5	OORL	0
24.7	OORL	0	6.2	OORL	0

C16/D11 Cross Reactivity

Control IL-10 Conc. pg/mL	Recovered Conc. pg/mL	% Reactivity	IFNg Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
500.0	498.8	100	1000.0	OORL	0
166.7	170.8	102	333.3	OORL	0
55.6	54.4	98	111.1	OORL	0
18.5	15.8	86	37.0	OORL	0
6.2	6.4	103	12.3	OORL	0

IL-1a Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-2 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
250.0	OORL	0	2000.0	OORL	0
83.3	OORL	0	666.7	OORL	0
27.8	OORL	0	222.2	OORL	0
9.3	OORL	0	74.1	OORL	0
3.1	OORL	0	24.7	OORL	0

IL-4 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-6 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
2000.0	OORL	0	300.0	OORL	0
666.7	OORL	0	100.0	OORL	0
222.2	OORL	0	33.3	OORL	0
74.1	OORL	0	11.1	OORL	0
24.7	OORL	0	3.7	OORL	0

IL-8 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity	IL-12 Conc. pg/mL	Recovered Conc. pg/mL	% Cross Reactivity
2000.0	OORL	0	500.0	OORL	0
666.7	OORL	0	166.7	OORL	0
222.2	OORL	0	55.6	OORL	0
74.1	OORL	0	18.5	OORL	0
24.7	OORL	0	6.2	OORL	0

Table [SEQ Table * ARABIC]: RESULTS OF INTERFERENCE

Interference test (Cab2/Dab11)								
Nominal IL-10 pg/mL	Control_IL10 only		IL-10 + IL-1a_1500 pg/mL		IL-10 + IL-2 _6000 pg/ml		IL-10 + IL-4_6000 pg/ml	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	503	101	469	94	416	83	412	82
166.7	163	98	151	91	138	83	130	78
55.6	50	90	53	95	48	86	42	76
18.5	20	109	18	99	17	94	17	90
6.2	9	152	8	130	9	140	6	94
Nominal IL-10 pg/mL	IL-10 + IL-6 _1500 pg/mL		IL-10 + IL-8_6000 pg/mL		IL-10 + IL-12_1500 pg/mL		IL-10 + IFNg_3000pg/mL	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	418	84	372	74	381	76	373	75
166.7	131	79	119	71	120	72	123	74
55.6	47	84	41	73	41	74	41	73
18.5	22	118	14	76	15	82	16	85
6.2	8	123	6	94	7	110	7	110

Interference test (Cab11/Dab9)								
Nominal IL-10 pg/mL	Control_IL10 only		IL-10 + IL-1a_1500 pg/mL		IL-10 + IL-2 _6000 pg/ml		IL-10 + IL-4_6000 pg/ml	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	500	100	461	92	430	86	407	81
166.7	166	100	159	95	144	86	144	86
55.6	54	98	50	89	46	83	45	81
18.5	18	95	25	135	15	81	14	78
6.2	8	125	6	91	6	94	6	105
Nominal IL-10 pg/mL	IL-10 + IL-6 _1500 pg/mL		IL-10 + IL-8_6000 pg/mL		IL-10 + IL-12_1500 pg/mL		IL-10 + IFNg_3000pg/mL	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	415	83	398	80	423	85	398	80
166.7	142	85	124	75	129	77	128	77
55.6	44	79	41	73	44	79	43	77
18.5	16	84	15	80	14	76	17	90
6.2	6	101	5	80	8	137	7	121

Interference test (Cab16/Dab11)								
Nominal IL-10 pg/mL	Control_IL10 only		IL-10 + IL-1a_1500 pg/mL		IL-10 + IL-2 _6000 pg/ml		IL-10 + IL-4_6000 pg/ml	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	501	100	461	92	469	94	431	86
166.7	167	100	159	95	159	96	145	87
55.6	58	105	50	89	51	92	46	82
18.5	17	94	25	135	16	87	16	85
6.2	7	117	6	91	4	62	3	46

Nominal IL-10 pg/mL	IL-10 + IL-6 _1500 pg/mL		IL-10 + IL-8_6000 pg/mL		IL-10 + IL-12_1500 pg/mL		IL-10 + IFNg_3000pg/mL	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500.0	442	88	420	84	420	84	405	81
166.7	147	88	139	83	132	79	136	81
55.6	47	85	48	86	45	81	43	77
18.5	18	100	14	73	12	64	13	68
6.2	4	65	1	24	2	25	1	23

2.2 Antibody screening on Theranos system

2.2.1 Theranos screening with three pairs of antibodies

Methods:

IL-10 calibrators prepared using R&D system human recombinant protein (cat: 217-IL) were tested with antibody pair C11/D9 and C16/D11. Edison co-incubation protocol 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. Starting block was used as assay diluent to diluent calibrators and detection antibody. Detection antibody prepared in StabliZyme at loading concentration of 1000ng/ml for a final concentration of 100ng/ml. The mixture co-incubated with reaction tip. All tips were first washed with wash buffer and incubated with AP substrate. RLU was measured for each tip. Calibrator recovery was calculated comparing the calculated concentration vs. nominal concentration. Different calibrator matrix were tested as well including 3% BSA blocking buffer, whole blood, and plasma to compare the recovery of calibrator in different matrix.

Results:

Antibody pair C11/D9 showed good assay range and assay sensitivity in blocking buffer calibrator, whereas whole blood and plasma calibrator had under recovery issue. C16/D11 blocking buffer calibrator had good assay range and assay sensitivity. Whereas whole blood calibrator had good recovery but over recovery with plasma calibrator.

Table [SEQ Table * ARABIC]: SUMMARY DATA OF THERANOS SCREENING

C11/D9															
1. Blocking buffer Calibrator			y = 0.003x - 5.099			2. Whole blood Calibrator					3. Plasma Calibrator				
IL-10 pg/mL	Inter Mean	Signal %CV	Inter mean	Conc. %CV	% Recovery	Inter Mean	Signal %CV	Inter mean	Conc. %CV	% Recovery	Inter Mean	Signal %CV	Conc. Inter mean	%CV	% Recovery
500.0	167521	13	497	13	99	98341	10	289.9	11	58	116991	10	345.9	10	69
166.7	60622	9	177	9	106	39996	6	114.9	7	69	42429	6	122.2	7	73
55.6	19110	4	52	5	94	11026	22	28.0	26	50	17192	9	46.5	10	84
18.5	8046	11	19	14	103	4967	7	9.8	11	53	5888	7	12.6	10	68
6.2	3346	15	5	30	80	1972	15	1.2	62	20	2366	9	2.0	32	32

0.0	852	15		842	14		604	25				

C16/D1 1														
1. Blocking buffer Calibrator			y = -4E-08x2 + 0.0117x - 6.666			2. Whole blood Calibrator				3. Plasma Calibrator				
IL-10 pg/mL	Inter Mean	Signal %CV	Inter mean	Conc. %CV	% Recovery	Inter Mean	Signal %CV	Inter mean	Conc. %CV	% Recovery	Inter Mean	Signal %CV	Inter mean	Conc. %CV	% Recovery
500.0	5189 2	9	492	7	98	5096 0	19	482. 4	16	96	7246 4	7	630. 1	5	126
166.7	1495 1	18	159	18	95	1680 0	20	178. 4	20	107	2343 1	5	245. 5	5	147
55.6	6455	11	67	12	121	5235	16	53.5	18	96	8978	8	95.1	8	171
18.5	2107	11	18	15	96	2346	15	20.6	19	111	3255	9	31.0	10	167
6.2	788	7	3	27	41	954	24	4.5	60	72	1095	16	6.1	34	99
0.0	326	29				322	19				301	13			

2.3 Assay optimization

2.3.1 Capture Antibody Titration

Methods:

Capture antibody titration was done by coating tips with Cab11 in 3% BSA blocking buffer at 20ug/ml, 10ug/ml, 5ug/ml, and 2.5ug/ml. Detection antibody was kept at 100ng/ml final concentration in StabilZyme-AP. Edison co-incubation protocol was used for capture titration.

Results:

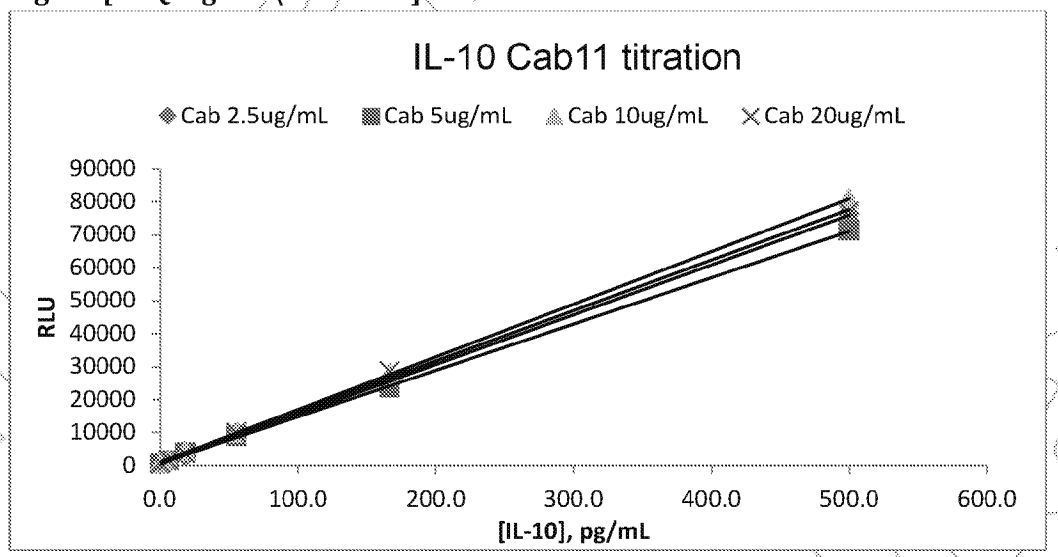
With detection antibody at 100ng/ml, capture antibody at 5ug/ml in blocking buffer seemed to saturate the coating surface. Increasing the coating concentration to 10ug/ml didn't improve CV. Thus 5ug/ml coating concentration was chosen as the final concentration for low background, high overall modulation, P/P modulation, and reasonable %CV.

Table [SEQ Table * ARABIC]: CAPTURE ANTIBODY TITRATION

Cab11 ug/mL	IL-10 pg/mL	Mean RLU	Conc. %CV	% Recovery	S/B
2.5	500.0	76130	13	99.5	164
	166.7	24519	9	97.4	
	55.6	9849	6	113.8	
	18.5	2953	15	86.2	
	6.2	1383	9	83.7	
	0.0	463			
5	500.0	71272	8	100.2	149
	166.7	23492	6	97.1	
	55.6	8827	7	104.0	

	18.5	3904	17	123.3	
	6.2	1362	41	77.5	
	0.0	479			
10	500.0	84669	11	95	143
	166.7	27861	15	99	
	55.6	10027	13	102	
	18.5	4328	6	116	
	6.2	1551	22	71	
	0.0	592			
20	500.0	76930	8	96	173
	166.7	28592	10	98	
	55.6	10011	10	97	
	18.5	3509	11	94	
	6.2	1432	30	97	
	0.0	444			

Figure [SEQ Figure * ARABIC]: CAPTURE TITRATION CALIBRATOION CURVE



2.3.2 DETECTION CONJUGATE TITRATION

Methods:

With capture antibody coating concentration at 5ug/ml and Edison protocol EPD-preincubation svn-4086, detection conjugate were prepared in 150ng/ml, 100ng/ml, 50ng/ml, and 25ng/ml in StabilZyme-AP. Calibration curve was run with each concentration of detection, and the results were used to compare to original 100ng/ml detection conjugate.

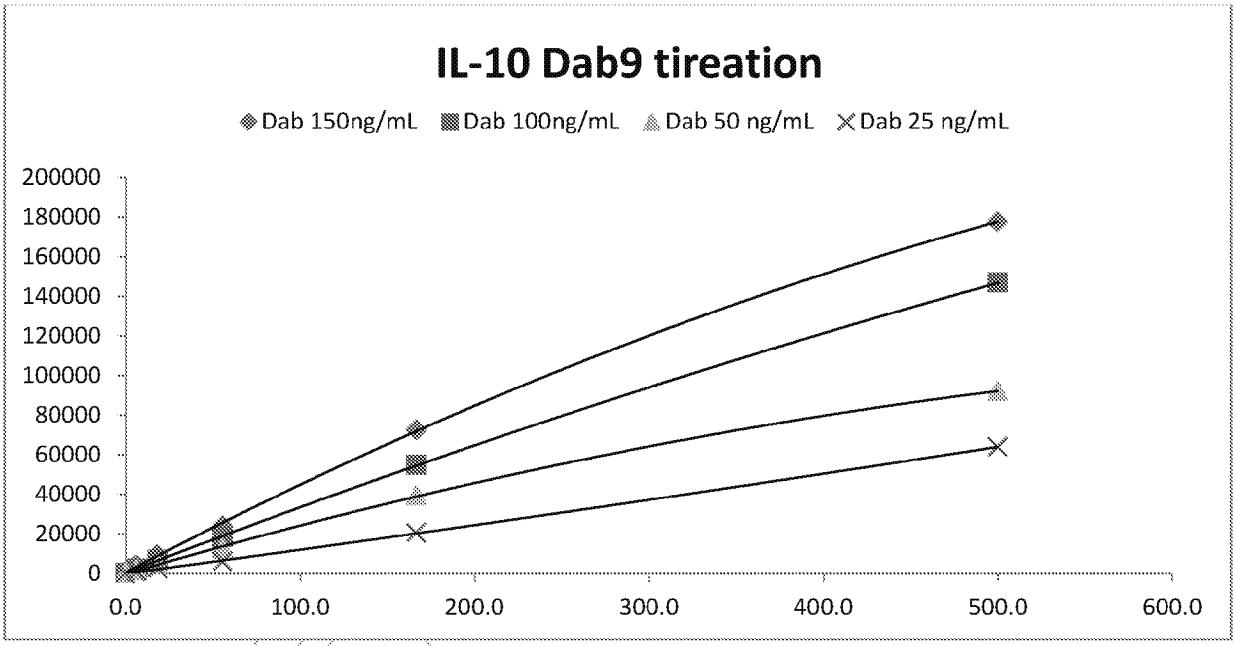
Results:

Detection conjugate at 25ng/ml in SurModics StabilZyme-AP gave the best overall modulation, whereas 50ng/ml showed better sensitivity. Thus detection concentration at 50ng/ml in StabilZyme-AP was selected as the final choice.

Table [SEQ Table * ARABIC]: DETECTION ANTIBODY TITRATION

Dab ng/mL	IL-10 pg/mL	Mean RLU	Conc. %CV	% Recovery	S/B
150	500.0	177825	5	102.7	230.6
	166.7	72664	8	102.8	94.2
	55.6	24300	5	91.9	31.5
	18.5	9414	5	101.4	12.2
	6.2	4158	7	128.2	5.4
	0.0	771	12		1.0
100	500.0	146875	17	102.7	290.8
	166.7	54907	9	102.2	108.7
	55.6	18474	10	96.9	36.6
	18.5	7014	6	105.0	13.9
	6.2	2690	15	109.1	5.3
	0.0	505	7		1.0
50	500.0	92312	8	97	259.1
	166.7	39527	5	100	110.9
	55.6	12691	7	87	35.6
	18.5	4545	4	95	12.8
	6.2	1888	13	133	5.3
	0.0	356	10		1.0
25	500.0	64118	13	100	334.8
	166.7	20586	8	101	107.5
	55.6	6299	13	92	32.9
	18.5	2630	13	113	13.7
	6.2	898	7	103	4.7
	0.0	191	15		1.0

Figure [SEQ Figure * ARABIC]: DETECTION TITRATION CALIBRATION CURVE



2.3.3 Selection of Assay Buffer

Methods:

With capture antibody kept at 5ug/ml in 3% BSA blocking buffer and detection conjugate was prepared at 100ng/ml in StabilZyme-AP. Edison co-incubation protocol was used to test the effect of different assay diluents including Starting block and 3% BSA blocking buffer.

Results:

When 3% BSA blocking buffer was used as assay diluent, buffer calibration curve didn't show a good assay range nor a decent assay sensitivity. Spiked whole blood calibration curve recovery ranged from 51% - 92% and spiked plasma calibration curve recovery ranged from 71% - 102%. Whereas Starting block assay diluent produced a good assay range and sensitivity in buffer calibrator. Spiked whole blood calibration curve recovered between 71% - 111% and spiked plasma calibration curve recovered between 99% - 171%.

Table [SEQ Table * ARABIC]: SUMMARY DATA OF CALIBRATION CURVE WITH 3% BSA BLOCKING BUFFER

	Buffer calibrator			Spiked Whole Blood Calibrator		Spiked Plasma Calibrator	
IL-10 pg/mL	S/B	Conc mean	% Recovery	Conc. mean	% Recovery	Conc. mean	% Recovery
500.0	37.5	504	101	410.2	82	512.5	102

166.7	18.3	167	100	125.5	75	159.0	95
55.6	7.5	49	88	28.1	51	39.6	71
18.5	2.6	16	84	12.6	68	13.3	72
6.2	1.4	7	110	5.7	92	5.0	80
0.0	1						

Table [SEQ Table * ARABIC]: SUMMARY DATA OF CALIBRATION CURVE WITH STARTING BLOCK BUFFER

	Buffer calibrator			Spiked Whole Blood Calibrator		Spiked Plasma Calibrator	
IL-10 pg/mL	S/B	Conc mean	% Recovery	Conc. mean	% Recovery	Conc. mean	% Recovery
500.0	159.3	492	98	482.4	96	630.1	126
166.7	45.9	159	95	178.4	107	245.5	147
55.6	20.4	67	121	53.5	96	95.1	171
18.5	6.5	18	96	20.6	111	31.0	167
6.2	2.4	3	41	4.5	72	6.1	99
0.0	1.0						

2.3.4 %CV

Method:

%CV was tested by choosing a mid-level calibrator 125pg/ml and ran it over 24 different Theranos instruments. Capture antibody was coated at 5ug/ml in blocking buffer on tips, detection antibody was prepared at 400ng/ml in StabliZyme loading concentration and final concentration of 50ng/ml. Assay diluent was starting block and Edsion protocol for testing %CV was ABA2_ver3 svn-5318.

Results:

Within the 24 Theranos instruments, intra signal %CV was between 0-11percent with inter signal CV at 11%. Intra observed concentration %CV was between 0-15percent with inter observed concentration %CV at 11%.

Table [SEQ Table * ARABIC]: SUMMARY DATA OF %CV TESTS

IL-10 125pg/mL $y = 2E\text{-}08x^2 + 0.0081x - 2.5197$

	RLU		Intra		Intra	Observed		Intra		Intra Conc.
Cartridge	Tip 1	Tip 2	Mean	Stdev	%CV	Tip 1	Tip 2	mean	StDev	%CV
1	16174	15330	15752	597	4	133.7	126.4	130.0	5	4
2	12092	14136	13114	1445	11	98.3	116.0	107.2	12	12
3	13740	14564	14152	582	4	112.6	119.7	116.1	5	4
4	12888	13566	13227	480	4	105.2	111.0	108.1	4	4

5	15998	16104	16051	75	0	132.2	133.1	132.6	1	0
6	12629	13516	13072	627	5	103.0	110.6	106.8	5	5
7	12974	14975	13974	1415	10	105.9	123.3	114.6	12	11
8	13890	13564	13727	230	2	113.8	111.0	112.4	2	2
9	16286	19117	17701	2002	11	134.7	159.6	147.2	18	12
10	17435	17192	17313	172	1	144.8	142.6	143.7	2	1
11	16770	17849	17310	763	4	138.9	148.4	143.7	7	5
12	16178	15158	15668	721	5	133.8	124.9	129.3	6	5
13	14738	15233	14985	350	2	121.2	125.5	123.4	3	2
14	14084	15196	14640	787	5	115.5	125.2	120.4	7	6
15	15176	16925	16051	1236	8	125.0	140.3	132.7	11	8
16	15157	15085	15121	51	0	124.8	124.2	124.5	0	0
17	16399	16289	16344	78	0	135.7	134.7	135.2	1	1
18	15676	18177	16926	1768	10	129.4	151.3	140.3	16	11
19	18216	18915	18565	494	3	151.7	157.8	154.8	4	3
20	17603	18471	18037	614	3	146.3	153.9	150.1	5	4
21	17309	17693	17501	271	2	143.7	147.1	145.4	2	2
22	15347	14795	15071	390	3	126.5	121.7	124.1	3	3
23	13927	17004	15466	2175	14	114.2	141.0	127.6	19	15
24	15358	16477	15917	791	5	126.6	136.4	131.5	7	5

Inter Mean	Inter Stdev	Inter %CV
15654	1736	11

Inter Mean	Inter Conc. Stdev	Inter Conc. %CV
129.2	14	11

2.3.5 Precision Test

Methods:
3 lots of coated tips were tested on Theranos system, intra and inter assay results with standard curve and 5 QC.

Results:

Over three lots of coated tips, the intra and inter %CV for standard curve and QC levels were all under 20%.

Table [SEQ Table * ARABIC]: SUMMARY DATA OF STANDARD CURVE PRECISION TEST INTRA LOT %CV

Lot1_1/20/11						
IL-10		RLU				
pg/mL	Cartridge	Tip 1	Tip 2	Intra RLU	Stdev	Intra Lot CV%
1000.0	1	54133	56037	56259	5841	10

	2	65143	60917			
	3	49493	51829			
500.0	1	30923	33659	33141	1956	6
	2	34012	35661			
	3	30652	33938			
250.0	1	13984	16141	14560	1787	12
	2	14501	17130			
	3	13214	12389			
125.0	1	7930	8127	7662	491	6
	2	6850	7788			
	3	3352	7614			
62.5	1	4348	5642	4418	698	16
	2	4571	4458			
	3	3705	3783			
31.3	1	2146	2407	1991	240	12
	2	1789	1830			
	3	1916	1858			
15.6	1	1196	1292	1152	109	9
	2	1024	1224			
	3	1025	1150			
7.8	1	732	924	797	77	10
	2	851	743			
	3	735	795			
3.9	1	523	518	505	55	11
	2	446	430			
	3	549	566			
0.0	1	246	206	217	20	9
	2	206	208			
	3					

Lot2_1/21/11

IL-10		RLU				
pg/mL	Cartridge	Tip 1	Tip 2	Mean RLU	Stdev	Intra Lot CV%
1000.0	1	51998	59426	58030	4189	7
	2	60647	63152			
	3	54080	58879			
500.0	1	38970	34653	35890	3228	9
	2	34724	40264			
	3	35343	31386			

250.0	1	14177	14724	15415	944	6
	2	15865	15387			
	3	15424	16911			
125.0	1	7991	7540	7701	291	4
	2	7631	8128			
	3	7402	7511			
62.5	1	4154	4250	4807	597	12
	2	5232	5730			
	3	4720	4759			
31.3	1	2887	2994	2890	168	6
	2	3073	2995			
	3	2623	2769			
15.6	1	1423	1567	1454	260	18
	2	1627	1787			
	3	1091	1227			
7.8	1	880	875	789	78	10
	2	800	769			
	3	709	699			
3.9	1	534	503	470	60	13
	2	532	404			
	3	427	417			
0.0	1	397	247	314	59	19
	2	309	253			
	3	359	319			

Lot3

IL-10		RLU				
pg/mL	Cartridge	Tip 1	Tip 2	Mean RLU	Stdev	Intra Lot CV%
1000.0	1	54174	51936	55803	3027	5
	2	59490	57381			
	3	53460	58378			
500.0	1	29269	31124	29955	859	3
	2	29351	30076			
	3	24158	27093			
250.0	1	12398	13631	15174	2544	17
	2	17370	17297			
	3	13108	14433			
125.0	1	6720	6844	6859	104	2
	2	6919	6955			

	3	8070	7203			
62.5	1	4750	4793	4604	611	13
	2	5145	3727			
	3	5118	4850			
31.3	1	1959	2172	2012	154	8
	2	2095	1823			
	3	2183	2229			
15.6	1	1930	1069	1076	10	1
	2	1491	1083			
	3	1413	1131			
7.8	1	848	728	789	50	6
	2	802	779			
	3	724	730			
3.9	1	449	550	470	45	10
	2	1064	459			
	3	440	452			
0.0	1	225	201	189	30	16
	2	170	159			
	3	226	219			

Table [SEQ Table * ARABIC]: SUMMARY DATA OF STANDARD CURVE PRECISION TEST INTER-LOT %CV

Std	IL-10 pg/mL	Cartridge	RLU Lot1 Tip 1	Lot1 Tip 2	Lot2 Tip 1	Lot2 Tip 2	Lot3 Tip 1	Lot3 Tip 2	Signal Mean	Signal Stdev	Signal %CV
1	1000.0	1	54133	56037	51998	59426	54174	51936	56697	4344	8
		2	65143	60917	60647	63152	59490	57381			
		3	49493	51829	54080	58879	53460	58378			
2	500.0	1	30923	33659	38970	34653	29269	31124	32514	3982	12
		2	34012	35661	34724	40264	29351	30076			
		3	30652	33933	35342	31386	24152	27093			
3	250.0	1	13984	16141	14177	14724	12398	13631	14894	1642	11
		2	14501	17130	15865	15387	17370	17297			
		3	13211	12381	15421	16911	13100	14431			

			4	9	4	1	8	3			
4	125.0	1	7930	8127	7991	7540	6720	6844	7254	1084	15
		2	6850	7788	7631	8128	6919	6955			
		3	3352	7614	7402	7511	8070	7203			
5	62.5	1	4348	5642	4154	4250	4750	4793	4652	598	13
		2	4571	4458	5232	5730	5145	3727			
		3	3705	3783	4720	4759	5118	4850			
6	31.3	1	2146	2407	2887	2994	1959	2172	2319	454	20
		2	1789	1830	3073	2995	2095	1823			
		3	1916	1858	2623	2769	2183	2229			
7	15.6	1	1196	1292	1423	1567	1930	1069	1320	270	20
		2	1024	1224	1627	1787	1491	1083			
		3	1025	1150	1091	1227	1413	1131			
8	7.8	1	732	924	880	875	848	728	785	67	9
		2	851	743	800	769	802	779			
		3	735	795	709	699	724	730			
9	3.9	1	523	518	534	503	449	550	482	54	11
		2	446	430	532	404	1064	459			
		3	549	566	427	417	440	452			
10	0.0	1	246	206	397	247	225	201	247	67	27
		2	206	208	309	253	170	159			
		3			359	319	226	219			

Table [SEQ Table * ARABIC]: SUMMARY DATA OF QC LEVEL INTRA AND INTER %CV

			RLU				
	IL-10		Lot1				Signal
QC samples	pg/mL	Cartridge	Tip 1	Tip 2	Mean	Stdev	%CV
ULOQ	1000.0	1	43674	55266	52373	4915	9
		2	49379	54393			
		3	55159	56365			
High QC	800.0	1	42413	41336	39131	2399	6
		2	37342	39628			
		3	37705	36361			
Mid QC	200.0	1	10635	11252	10477	698	7
		2	9422	11064			
		3	10594	9896			
Low QC	25.0	1	1499	1478	1671	171	10
		2	1849	1641			
		3	1674	1887			
LLOQ	5.0	1	19	112	524	40	8
		2	533	485			
		3	575	502			

2.3.6 Matrix effect

Methods:

To study matrix effect, calibrator material from R&D system (cat: 271-IL) was spiked into icteric serum, lipemic serum, and hemolyzed serum. Then these different sets of calibrators were tested on Theranos method. Concentration recovery was comparing to the nominal value.

Results:

Icteric and lipemic matrixes samples didn't show significant interference on IL-10 calibrator recovery in Theranos system when comparing to nominal value. However, severely hemolyzed sample could affect the recovery of IL-10.

Table [SEQ Table * ARABIC]: MATRIX EFFECT CALIBRATOR RECOVERY

Spike into Lipemic serum

	Signal [RLU]			Recovered IL-10, [pg/mL]			
IL-10 pg/mL	Mean RLU	Stdev	%CV	Mean Conc.	StDev	%CV	% Recovery
1000.0	81074	4044	5	786	46	6	79
500.0	44017	5065	12	393	50	13	79
250.0	22746	1244	5	192	11	6	77
125.0	12985	1619	12	106	14	13	85
62.5	6596	588	9	52	5	10	83
31.3	3500	278	8	26	2	9	83
15.6	2408	113	5	17	1	5	109
7.8	1425	65	5	9	1	6	116
3.9	888	100	11	5	1	17	120
0.0	469	81	17				
					Ave. % Recovery		**92**
					Ave. Conc. %CV		**9**

Spike into Icteric serum

	Signal [RLU]			Recovered IL-10, [pg/mL]			
IL-10 pg/mL	Mean RLU	Stdev	%CV	Mean Conc.	StDev	%CV	% Recovery
1000.0	88141	4358	5	867	51	6	87
500.0	45135	8542	19	405	84	21	81
250.0	23037	1887	8	195	17	9	78
125.0	15071	905	6	124	8	6	99
62.5	7277	595	8	57	5	9	92
31.3	3596	253	7	27	2	8	86
15.6	2163	479	22	17	2	12	110
7.8	1533	56	4	10	0	5	127
3.9	1178	104	9	7	1	11	179

0.0	477	95	20		
				Ave. % Recovery	**104**
				Ave. Conc. %CV	**10**

Spike into Hemolyzed serum

	Signal [RLU]			Recovered IL-10, [pg/mL]			
IL-10 pg/mL	**Mean RLU**	**Stdev**	**%CV**	**Mean Conc.**	**StDev**	**%CV**	**% Recovery**
1000.0	66815	6974	10	629	75	12	63
500.0	34688	2144	6	303	20	7	61
250.0	17192	1590	9	143	14	10	57
125.0	10456	1528	15	84	13	15	68
62.5	5713	357	6	44	3	7	71
15.6	2104	118	6	15	1	7	94
7.8	1326	103	8	8	1	10	106
3.9	980	147	15	5	1	22	139
0.0	624	136	22				
					Ave. % Recovery		**82**
					Ave. Conc %CV		**11**

2.3.7 Hematocrit effect and anticoagulant effect

Methods:

To study hematocrit effect, calibrator material from R&D system (cat: 271-IL) was spiked into whole blood and calibration curve was ran. Then this set of whole blood calibrator was spun down and plasma was saved. This set of plasma calibrator from spiked whole blood was also ran. Hematocrit effect was evaluated by comparing IL-10 recovery concentration in whole blood vs. EDTA plasma from the same donor.

Results:

Whole blood calibrator set and plasma calibrator from the spiked whole blood were analyzed. Hematocrit factor was calculated to be 1.63.

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

Whole blood calibrator						Plasma from the spiked whole blood calibrator					
IL-10 pg/mL	**Inter Signal Mean**	**%CV**	**Calc. conc.**	**Conc. %CV**	**% Recovery**	**Inter Signal Mean**	**%CV**	**Calc. conc.**	**Conc. %CV**	**% Recovery**	**Hematocrit Effect**
1000.0	97511	10	978.9	11	98	155737	5	1744.9	6	174	1.78
500.0	41362	4	366.8	4	73	85216	4	833.1	5	167	2.27
250.0	21390	10	180.0	11	72	38219	9	336.5	9	135	1.87
125.0	12147	15	98.9	16	79	22572	14	190.7	15	153	1.93
62.5	7128	8	56.2	9	90	11360	21	92.2	22	147	1.64
31.3	3565	11	26.6	13	85	5428	10	42.0	11	135	1.58

15.6	2519	17	18.0	20	115	2974	11	21.7	12	139	1.21
7.8	1685	26	11.2	32	143	2022	13	13.9	15	178	1.25
3.9	999	11	5.6	16	143	1146	24	6.7	30	170	1.19
0.0	595	19				460	17				**mean**
											1.63

2.3.8 LOQ/ULOQ and Extended Range

Theranos IL-10 has an assay range of 0, 3.9-1000pg/ml. LLOQ/ULOQ testing is to run calibrators 2X above ULOQ and ½ below LLOQ to define assay limits. Extended range testing is to run calibrators 4X ab ove ULOQ to test linearity and does response of standard curve. Assay condition was capture antibody C11 5ug/ml in 3% BSA blocking buffer, detection conjugate 50ng/ml in SurModics StabilZyme-AP final concentration (loading concentration 400ng/ml), Starting block buffer as assay diluent. Calibration curve was generated under this assay condition with Edison protocol ABA2_ver3 svn-5318 and data was analyzed with Dexter.

TABLE 19: EXTENDED CALIBRATION CURVE

IL-10 pg/mL	RLU Tip 1	Tip 2	Intra Mean	Stdev	Intra %CV	Inter Mean	Stdev	Signal %CV
4000.0	305181	341933	323557	25988	8	323557	13442	4
4X ULOQ	312626	309185	310906	2433	1			
	311535	322673	317104	7876	2			
2000.0	169078	172731	170905	2583	2	170905	12185	7
2X ULOQ	140535	151736	146136	7920	5			
	151994	162741	157368	7599	5			
1000.0	88473	91348	89910	2033	2	89910	3672	4
ULOQ	83882	90336	87109	4564	5			
	84487	92803	88645	5881	7			
500.0	44718	51949	48334	5113	11	48334	4716	10
	53840	58585	56212	3355	6			
	54845	55571	55208	513	1			
250.0	27313	29513	28413	1556	5	28413	1921	7
	24982	25382	25182	282	1			
	24358	27260	25809	2052	8			
125.0	12879	10590	11734	1619	14	11734	1474	13
	11936	12006	11971	50	0			
	14164	14482	14323	225	2			
62.5	6847	6757	6802	64	1	6802	723	11
	8236	8503	8370	189	2			
	7686	7247	7466	310	4			
31.3	4160	3758	3959	284	7	3959	195	5
	3609	3888	3748	197	5			
	3899	4030	3965	93	2			
15.6	2519	2296	2407	158	7	2407	247	10
	1858	1978	1918	85	4			

	2273	2351	2312	55	2			
7.8	1192	1219	1206	19	2	1206	60	5
	1208	1265	1236	41	3			
	1091	1239	1165	105	9			
3.9	1536	936	936	#DIV/0!	#DIV/0!	936	118	13
LLOQ	1094	1136	1115	30	3			
	872	911	891	28	3			
1.95	542	551	546	6	1	546	59	11
1/2 LLOQ	702	603	652	70	11			
	583	632	607	35	6			
0.0	2	2				496	112	23
	385	420	402	25	6			
	555	624	589	48	8			

Figure [SEQ Figure * ARABIC]: EXTENDED RANGE CALIBRATION CURVE BY DEXTER

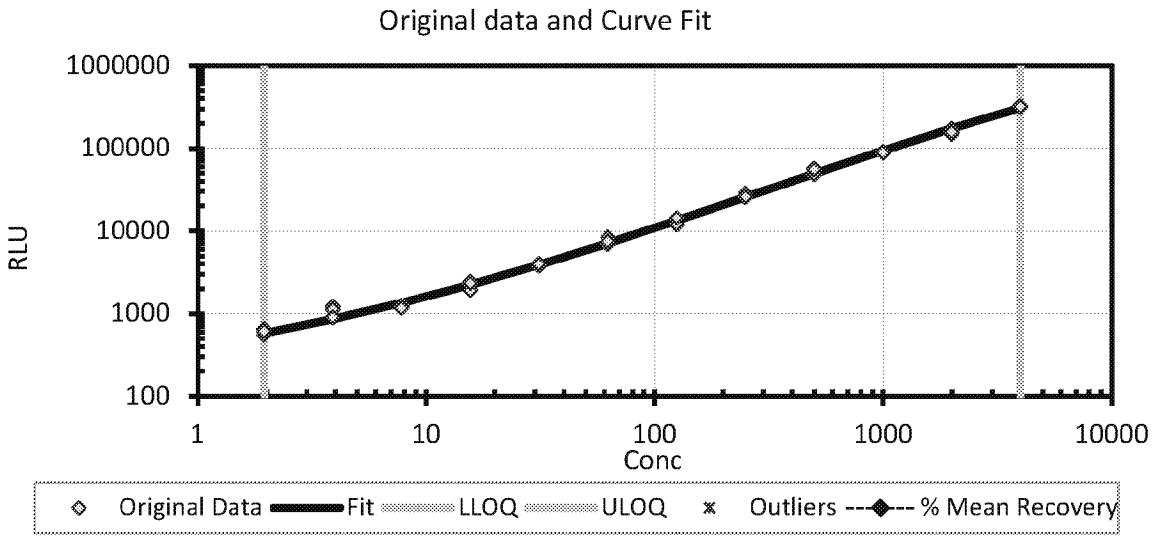


Table [SEQ Table * ARABIC]: EXTENDED RANGE CALIBRATION CURVE PARAMETERS

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	1.889
b2	7.056
b3	292.809
b4	-0.318
LLOQ	1.95 PG/ML
ULOQ	4000 PG/ML
LLOQ accuracy	109%
LLOQ precision	16%

ULOQ accuracy	103%
ULOQ precision	2.4%

2.3.9 Dilution Linearity

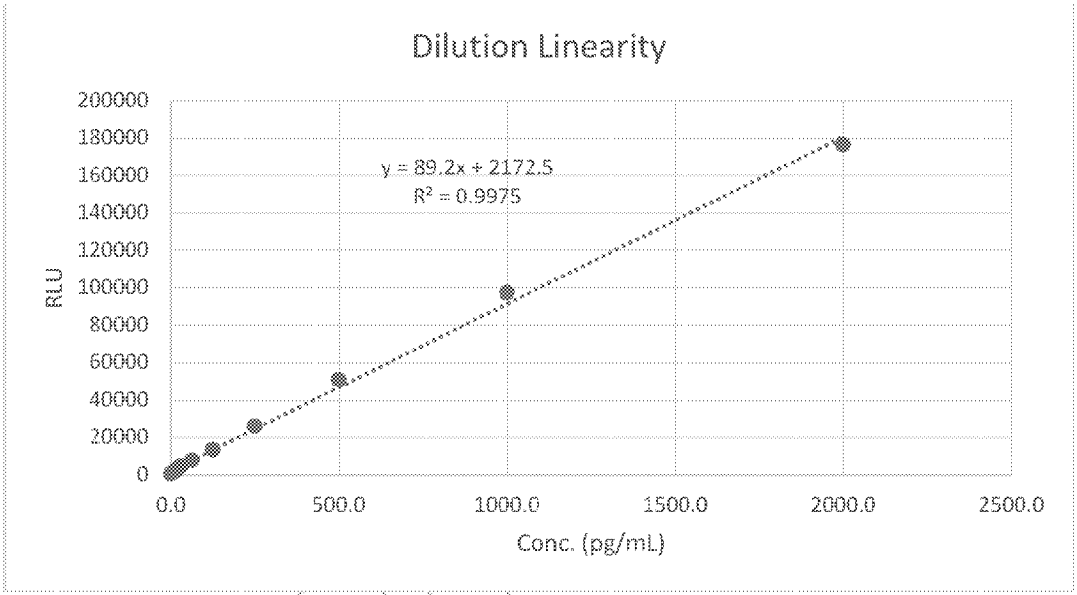
Methods:
Dilution linearity was tested by spiking 2000pg/ml of IL-10 analyte into a low endogounous serum sample, then serially diluted the sample by ½.

Results:

Table [SEQ Table * ARABIC]: SUMMARY DATA OF DILUTION LINEARITY

IL-10	Inter		Signal	Conc.			
pg/mL	Mean	Stdev	%CV	Inter mean	StDev	%CV	% Recovery
2000.0	176439	4533	3	2189	72	3	109
1000.0	97298	12420	13	1054	159	15	105
500.0	50787	2362	5	500	26	5	100
250.0	25871	1936	7	240	19	8	96
125.0	13534	375	3	120	4	3	96
62.5	7740	597	8	66	6	8	106
31.3	4807	486	10	39	4	11	126
15.6	2498	238	10	18	2	12	117
7.8	1425	139	10	8.72	1	14	112
3.9	1099	246	22	5.81	2	38	149
2.0	698	57	8	2.22	1	23	114
0.0	499	115	23				

Figure [SEQ Figure * ARABIC]: SPIKED SERUM SAMPLE DILUTION LINEARITY



2.3.10 Selectivity

Method:

Calibrator analyte from R&D system (cat: 271-IL) was spiked at three different levels into whole blood of 10 different individuals. Spiked analyte recovery was calculated comparing to nominal level if endogenous level was minimal. Capture antibody was coated on tips at 5ug/ml in blocking buffer, detection antibody was prepared at loading concentration of 400ng/ml and final working concentration of 50ng/ml in StabilZyme-AP. Starting block was used as assay diluent and Edison protocol ABA2_ver5 svn-5601 was used on Theranos system.

Results:

With analyte spiked at three different concentrations into 10 different individuals, all samples recovered well.

Table [SEQ Table * ARABIC]: SUMMARY DATA OF SELECTIVITY TEST

Blood ID	Spiked [IL-10] pg/mL	mean RLU	inter CV	Observed IL-10 [pg/mL]	Spiked [IL-10] pg/mL	Inter mean	Conc. %CV	% Recovery
W07051111020000	**0**	552	31	0.9	**15.6**	21.1	10	135
W07051110003500	**0**	734	12	2.5	**15.6**	18.6	17	119
W07051111019900	**0**	625	9	1.6	**15.6**	18.5	4	119
W07051110003800	**0**	633	24	1.6	**15.6**	12.9	6	82
W07051110003400	**0**	668	5	2.0	**15.6**	14.5	7	93
W07051110003600	**0**	597	9	1.3	**15.6**	15.7	20	101

W07051111019400	**0**	483	20	0.3	**15.6**	15.7	12	101
W07051111018200	**0**	567	8	1.1	**15.6**	15.6	16	100
W07051111018900	**0**	730	27	2.5	**15.6**	14.4	11	92
W07051111019600	**0**	551	8	0.9	**15.6**	16.7	19	107

Blood ID	Spiked [IL-10] pg/mL	Inter mean	Conc. %CV	% Recovery	Spiked [IL-10] pg/mL	Inter mean	Conc. %CV	% Recovery
W07051111020000	**62.5**	66.0	21	106	**125**	112.1	16	90
W07051110003500	**62.5**	59.9	6	96	**125**	120.6	8	96
W07051111019900	**62.5**	61.8	21	99	**125**	123.8	20	99
W07051110003800	**62.5**	57.4	6	92	**125**	116.4	13	93
W07051110003400	**62.5**	60.4	7	97	**125**	119.1	18	95
W07051110003600	**62.5**	63.3	5	101	**125**	117.8	5	94
W07051111019400	**62.5**	54.6	19	87	**125**	114.4	12	92
W07051111018200	**62.5**	48.3	12	77	**125**	88.0	7	70
W07051111018900	**62.5**	65.9	12	105	**125**	134.1	7	107
W07051111019600	**62.5**	71.2	15	114	**125**	109.9	7	88

2.3.11 Stability

2.3.11.1 Capture stability

Capture antibody stability was done by testing coated tips stored at 4C and RT over 24 weeks of time. Other reagents needed were prepared fresh on the day of testing.

Figure [SEQ Figure * ARABIC]: CAPTURE ANTIBODY STABILITY_4C

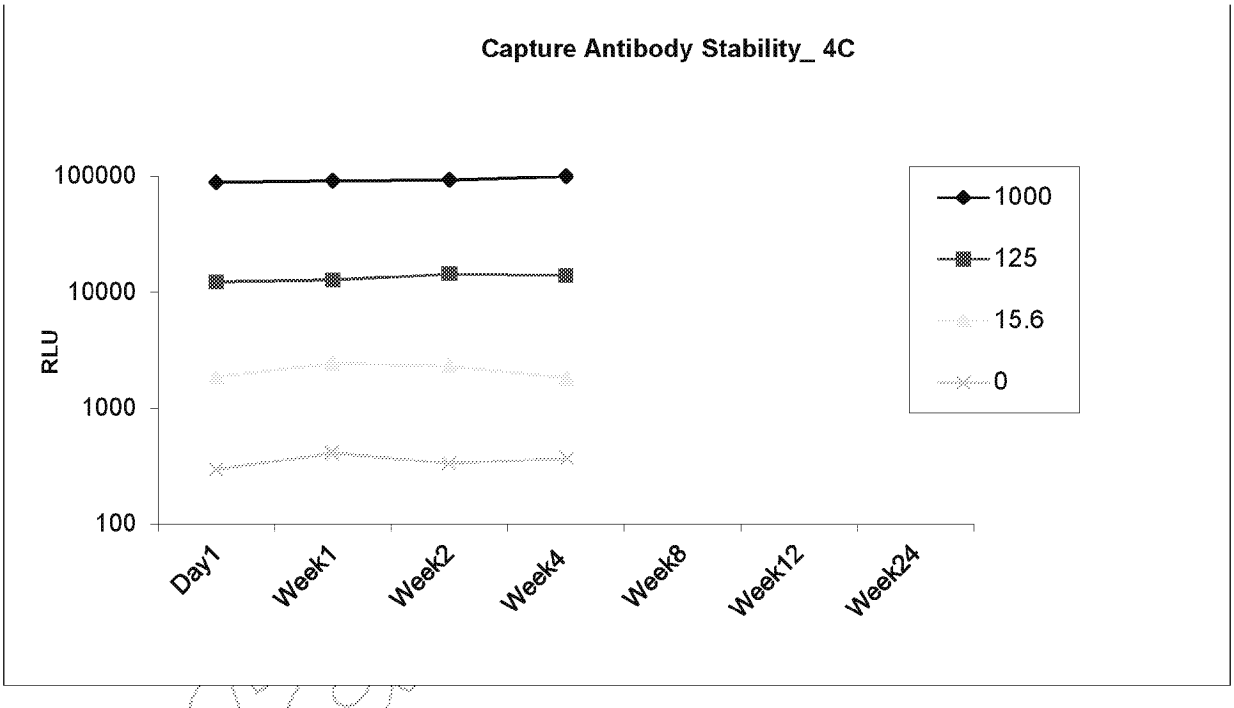
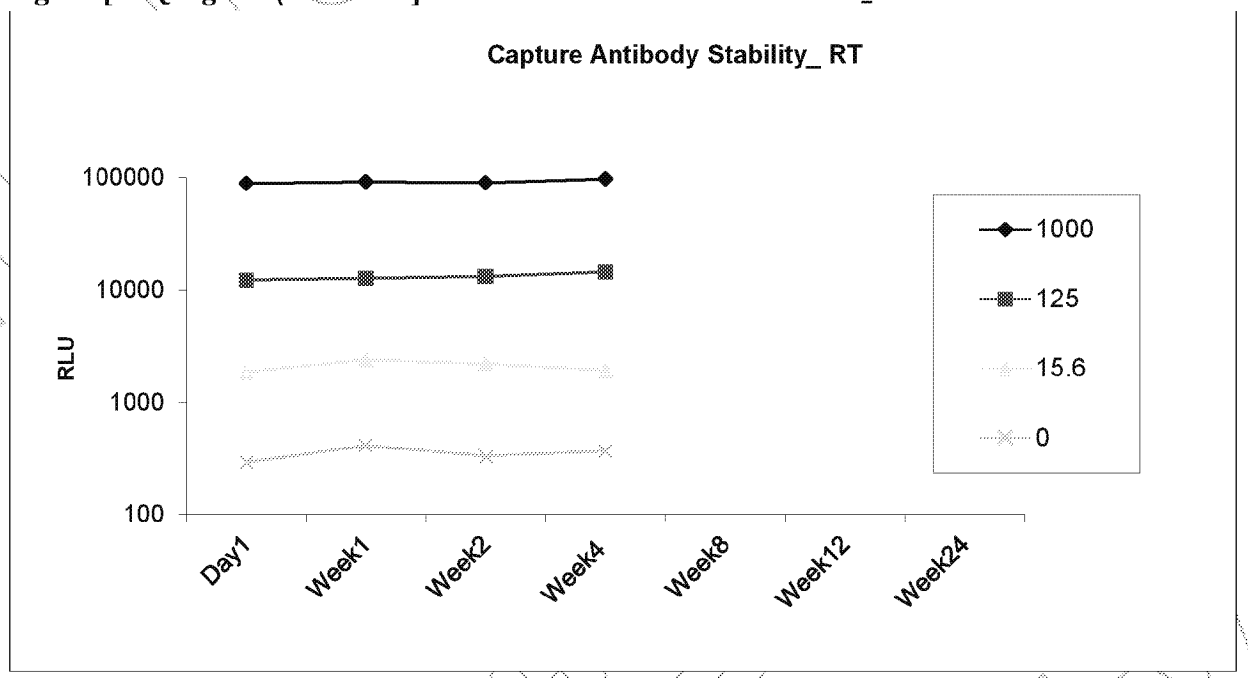


Figure [SEQ Figure * ARABIC]: CAPTURE ANTIBODY STABILITY_RT



2.3.11.2 Detection stability

Detection antibody stability was done by testing detection working diluent stored at 4C and RT over 24 weeks on MTP plate. Other reagents needed for the MTP plate were prepared fresh on the day of testing.

Figure [SEQ Figure * ARABIC]: DETECTION ANTIBODY STABILITY_4C

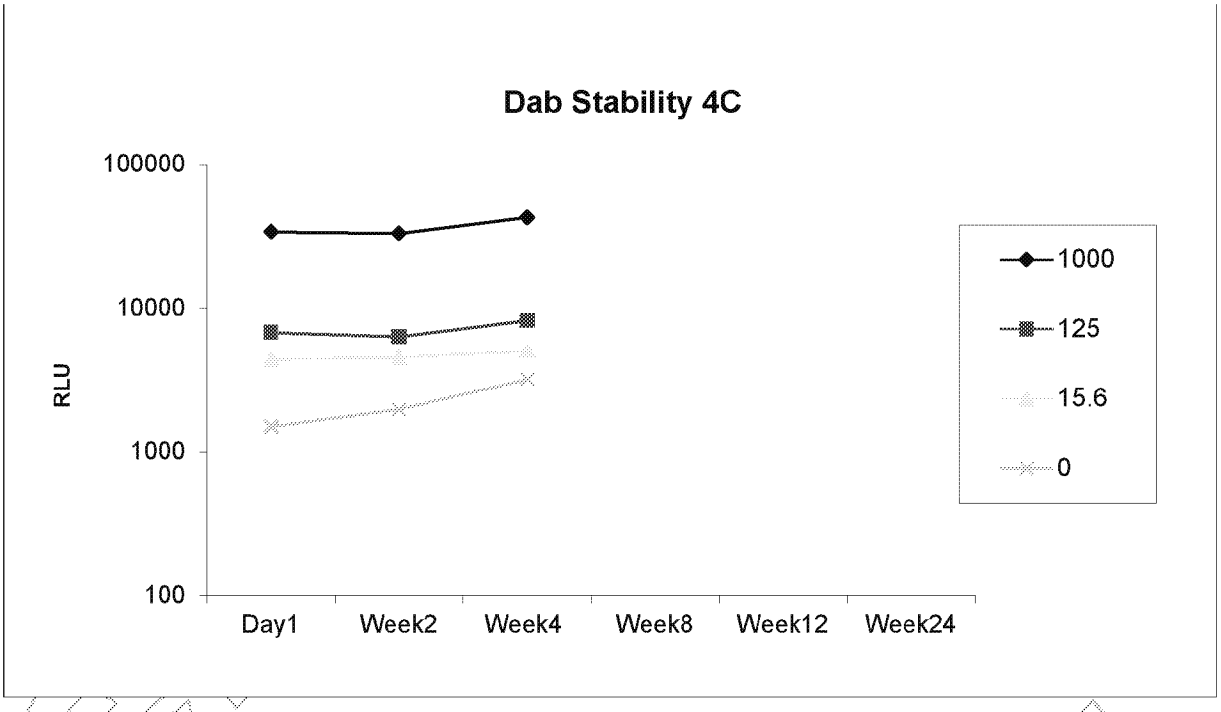
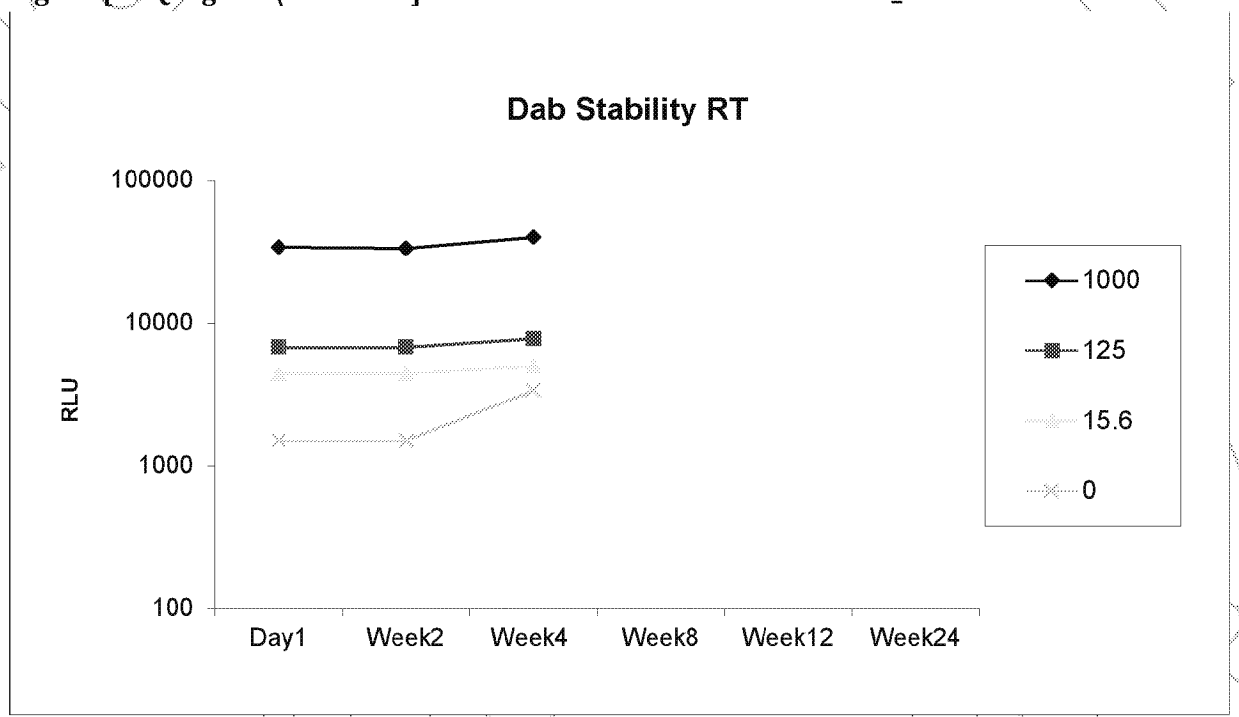


Figure [SEQ Figure * ARABIC]: DETECTION ANTIBODY STABILITY_RT



2.3.11.3 Reference Tips Stability

Reference tips stability was tested by coated the tips on TomTec with 20ug/ml UA in carb-bicarb buffer, then 5ug/ml capture antibody in blocking buffer. Let the tips dry in McDry 48hours, then pouch and store at 4C and RT.

Figure [SEQ Figure * ARABIC]: REFERENCE TIPS STABILITY_4C

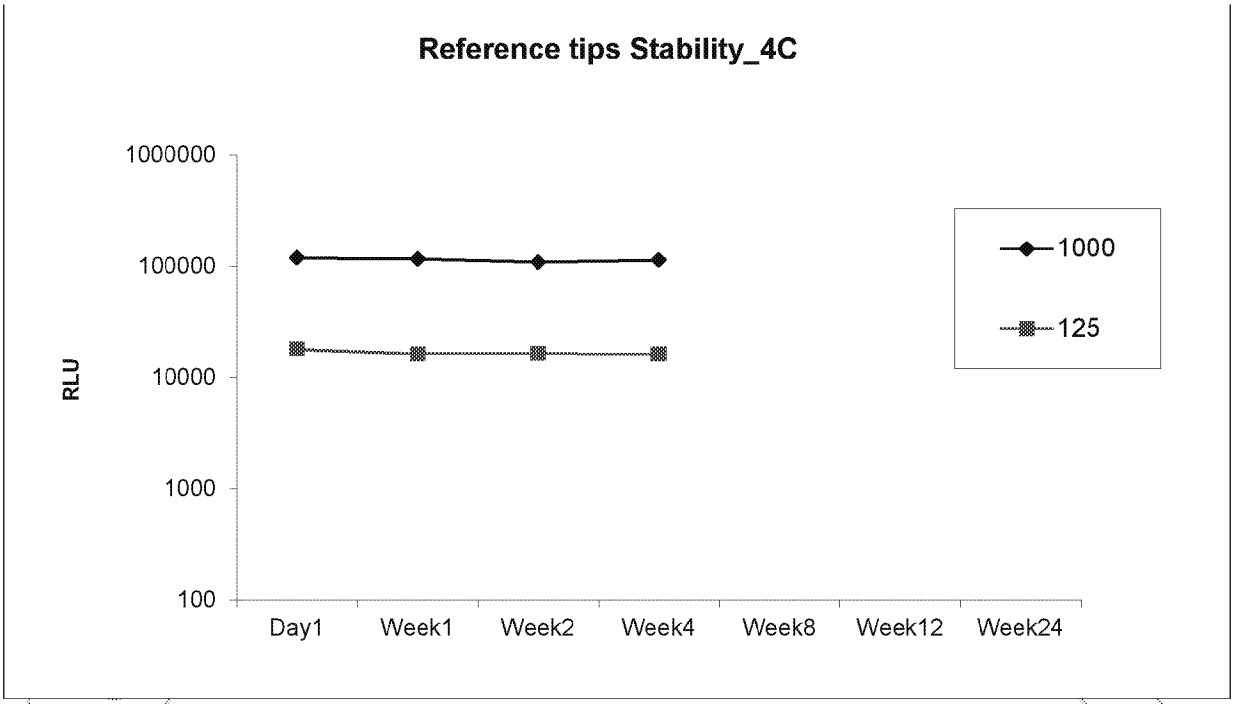
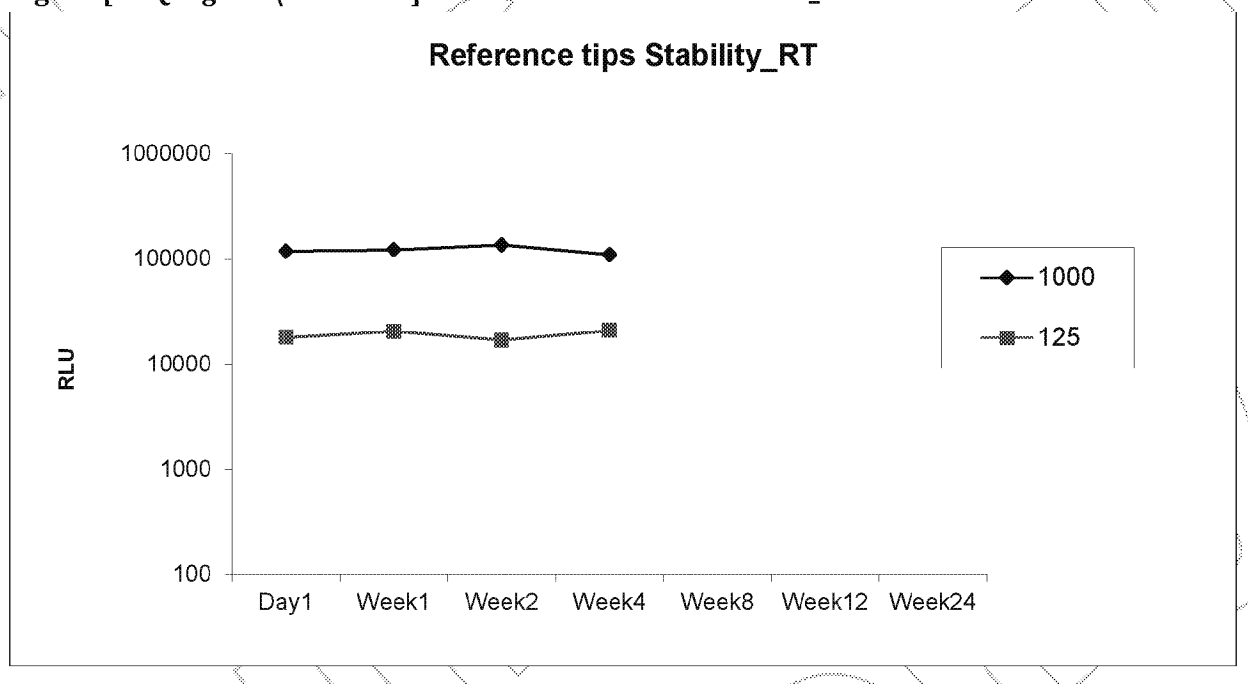


Figure [SEQ Figure * ARABIC]: REFERENCE TIPS STABILITY_RT



2.3.12 Calibrator Comparison

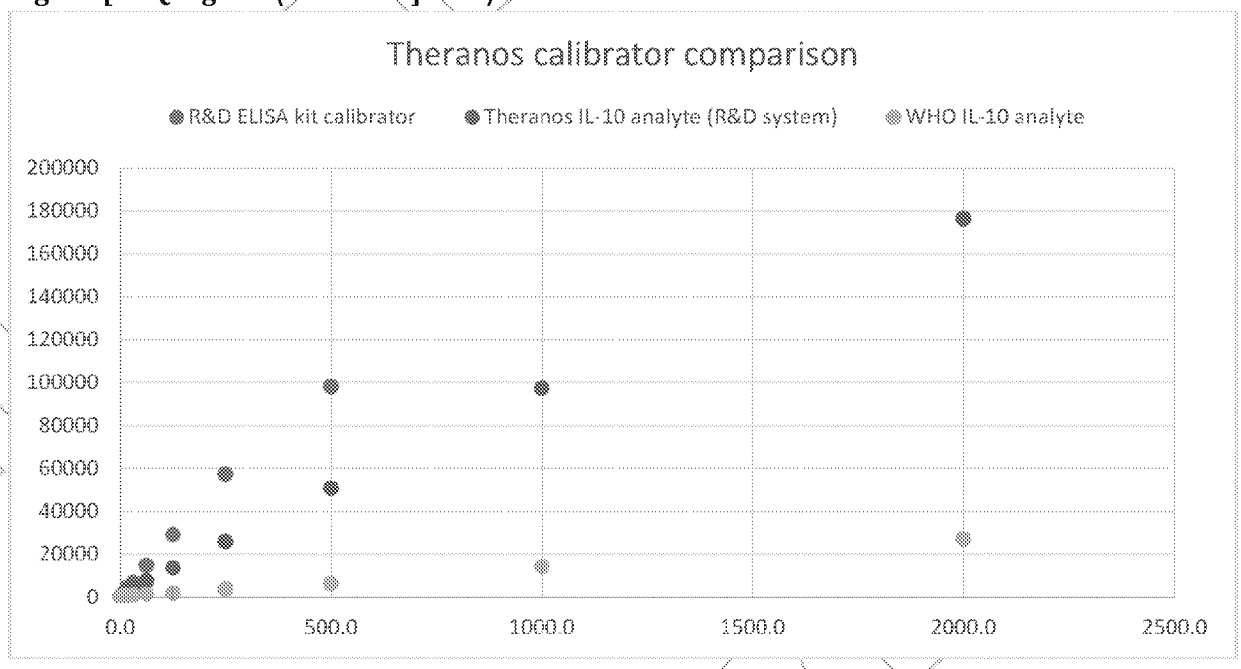
Methods:

Calibration material from R&D system (cat: 271-IL), WHO IL-10 and R&D system IL-10 ELISA kit was compared on Theranos system.

Results:

Theranos antibody pair C11/D9 recognized R&D IL-10 ELISA kit calibrator the best with S/B at 160fold from 0 to 500pg/ml, the second is R&D system IL-10 recombinant cat: 271-IL with modulation of 102 fold from 0 to 500pg/ml. however, within the same calibration range, WHO analyte only showed an overall modulation of 12 fold. Since WHO control is the international standard, all antibodies need to be rescreened with WHO analyte and assay need to be optimized again with the new pair of antibody.

Figure [SEQ Figure * ARABIC]: C11/D9 CALIBRATOR MATERIAL COMPARISON



2.4 ANTIBODY RESCREENING ON MTP

2.4.1 Antibody screening on MTP

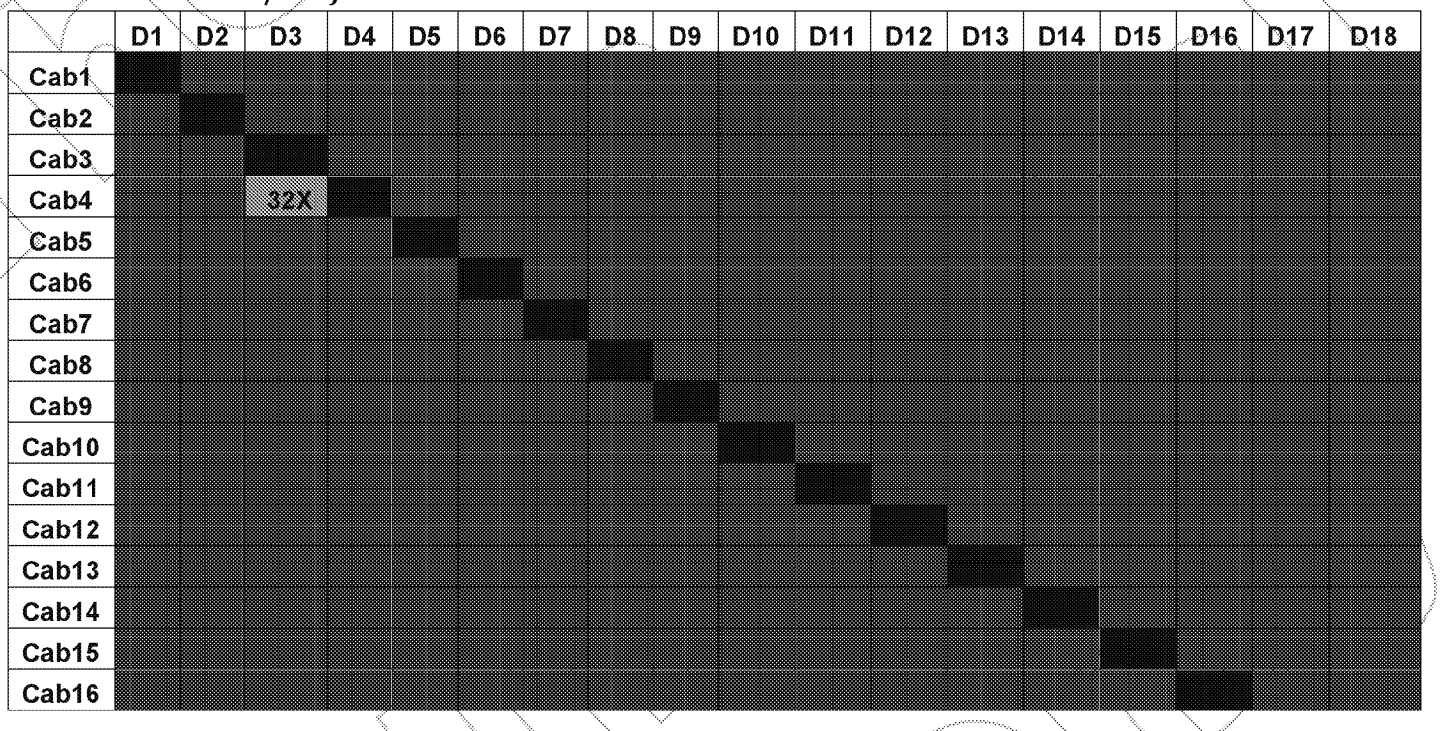
During the second round of antibody rescreening, a total of 18 anti-human IL-10 antibodies were screened for binding activity on micro titer plate (MTP). All antibodies were labeled with Dojindo Biotin labeling kit-SH (cat: LK10-10) and Dojindo AP labeling kit-SH (cat LK13-10). Analyte used for antibody screening was WHO IL-10 analyte (NIBSC code: 93/722)
Method:

The MTP was first coated with UltraAvidin (UA) at 20ug/ml in carb-bicarb coating buffer, followed by Biotinylated antibody at 5ug/ml in 3% BSA blocking buffer. IL-10 calibrators at 500pg/ml, 50pg/ml, 5pg/ml and 0pg/ml were incubated with coated antibodies. Then detection antibody-AP conjugates were diluted in the Surmodics StabilZyme AP conjugate stabilizer (cat: SA01) to 100ng/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, IL-10@ 0pg/ml).

Result:

Out of the 18x18 antibody pairs screened, only C4/D3 showed decent modulation and moved on to cross reactivity and interference tests.

Table [SEQ Table * ARABIC]: Antibody screening Summary with IL-10 WHO analyte (NIBSC code: 93/722)



2.4.2 Cross Reactivity and Interference

Human IL-4, IL-6, IL-8, IL-12, TNFa and INFg were tested for cross reactivity and interference with antibody pair C4/D3.

Method:

Previous method used in antibody screening was used here. However, for cross reactivity test, instead of using IL-10 calibrators, the above cross reactants were added as samples. In interference test, the above proteins which spiked into IL-10 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 C4/D3.

Table [SEQ Table * ARABIC]: C4/D3 CROSS REACTIVITY

Control_IL10								
IL-10 pg/mL	Mean Value	CV%	IL-4 pg/mL	Mean Value	Recovery	IL-6 pg/mL	Mean Value	Recovery
500.0	91993	0	2000.0	142	OORL	2000.0	180	OORL
166.7	34492	5	666.7	152	OORL	666.7	122	OORL
55.6	11994	0	222.2	172	OORL	222.2	120	OORL
18.5	4415	3	74.1	110	OORL	74.1	144	OORL
6.2	1673	6	24.7	164	OORL	24.7	176	OORL
0	146	25	0	124	OORL	0	156	OORL
			IL-8 pg/mL	Mean Value	Recovery	IL-12 pg/mL	Mean Value	Recovery
			2000.0	174	OORL	2000.0	230	OORL
			666.7	178	OORL	666.7	216	OORL
			222.2	128	OORL	222.2	160	OORL
			74.1	122	OORL	74.1	150	OORL
			24.7	188	OORL	24.7	182	OORL
			0	232	OORL	0	263	OORL
			IFNg pg/mL	Mean Value	Recovery	TNFa pg/ml	Mean Value	Recovery
			2000.0	196	OORL	2000.0	162	OORL
			666.7	110	OORL	666.7	156	OORL
			222.2	140	OORL	222.2	146	OORL
			74.1	142	OORL	74.1	164	OORL
			24.7	118	OORL	24.7	140	OORL
			0	130	OORL	0	130	OORL

Table [SEQ Table * ARABIC]: C4/D3 INTERFERENCE TEST

Interference test (Cab4/Dab3)

Nominal IL-10 pg/mL	Control_IL10 only		IL-10 + IL-4_2000 pg/mL		IL-10 + IL-6 _500 pg/ml		IL-10 + IL-8_1000 pg/ml	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500	499	100	476	95	514	103	499	100
166.7	167	100	160	96	181	109	169	102
55.6	54	97	52	94	58	105	52	94
18.5	20	109	18	100	21	116	19	105
6.2	6	101	6	96	7	120	5	78

Nominal IL-10 pg/mL	Control_IL10 only		IL-10 + IFNg _1000 pg/mL		IL-10 + TNFa_500 pg/mL	
	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %	Calc. IL-10 pg/mL	Recovery %
500	499	100	492	98	437	87
166.7	167	100	177	106	194	116
55.6	54	97	55	99	49	88
18.5	20	109	21	111	22	121
6.2	6	101	6	96	5	83

2.5 C4/D3 ON THERANOS SYSTEM

Method:

Buffer calibrator was prepared with WHO IL-10 analyte at 1000, 250, 62.5, 15.6, 3.9 and 0pg/ml. Tips were coated with 5ug/ml capture antibody C4 in blocking buffer, detection antibody was prepared at 400ng/ml loading concentration in StabliZyme-AP and final working concentration was 50ng/ml. Edison protocol ABA2_ver5 was used to run calibration curve on Theranos system.

Table [SEQ Table * ARABIC]: IL-10 STANDARD CURVE ON THERANOS SYSTEM WHO ANAYLTE

Nominal	Mean		Total Signal		Observed	Conc.	
[IL-10], pg/mL	RLU	Stdev	%CV	S/B	[IL-10], pg/mL	%CV	% Recovery
1000.0	37196	1271	3	95.1	1002	3	100
250.0	8712	1733	20	22.3	250	20	100
62.5	2443	388	16	6.2	62	19	100
15.6	903	188	21	2.3	15	38	98
3.9	561	53	9	1.4	5	34	121
0.0	391	127	33	1.0			

2.6 TRAINING SET

Method:

A total of 14 clinical samples, 10 from ProMedDx and 4 from Sunnylab were tested both on Theranos system and Predicate ELISA kit (R&D system). Recovery comparison was made between the two methods. Due to the limited number of clinical samples, WHO analyte was also used to spike into 5 plasma samples at three different concentration levels.

Results:

Comparing the results of R&D system IL-10 ELISA kit and Theranos method, a reasonable recovery was observed. In addition, all spiked plasma samples showed good recovery.

Table [SEQ Table * ARABIC]: TRAINING SET: THERANOS VS R&D ELISA KIT

			R&D ELISA	C4/D3
	Sample	Sample ID	IL-10 Cal. pg/mL	IL-10 Cal. pg/mL
1	1	1810884	11	9
2	2	1830123	3	8
3	4	1650366	310	514
4	5	1697635	3	7
5	6	1849062	15	13
6	12	11	4	12
7	14	18	16	210
8	15	20	16	18
9	19	46	7	3
10	24	1	12	4
11	1	Sepsis	33	78
12	2	Sepsis	2	2
13	3	Sepsis	17	18
14	4	Sepsis	14	11

Table [SEQ Table * ARABIC]: WHO ANALYTE SPIKED PLASMA RECOVERY ON THERANOS SYSTEM

Sample	IL-10 pg/mL	Conc. Inter mean	StDev	%CV	% Recovery
1	125.0	143	16	11	114
2	125.0	155	29	19	124
3	125.0	111	9	8	89
4	125.0	153	18	12	122
5	125.0	117	10	8	93

1	31.3	44	23	53	139
2	31.3	43	5	13	136
3	31.3	29	5	17	91
4	31.3	51	5	10	163
5	31.3	32	5	15	102
1	7.8	12	7	53	158
2	7.8	9	4	45	111
3	7.8	1	1	68	13
4	7.8	22	5	21	276
5	7.8	8	3	33	100

2.7 CALIBRATOR COMPARISON

Methods:

Three different sets of calibrator analyte material were prepared and run on both R&D ELISA kit and Theranos system. The calibrator sets are R&D ELISA kit calibrator, WHO IL-10 analyte, and R&D recombinant human IL-10 analyte used in part 1 of assay development.

Results:

R&D ELISA kit recognized the WHO analyte most strongly, and response to its own analyte and recombinant human IL-10 protein about the same. Whereas Theranos system recognized R&D ELISA kit calibrator most strongly, followed by human recombinant IL-10 protein, then WHO analyte the least. However WHO is an international reference material, we still decided to chose WHO analyte as calibrator material.

Figure [SEQ Figure * ARABIC]: DIFFERENT ANAYLTE TESTED ON R&D ELISA KIT

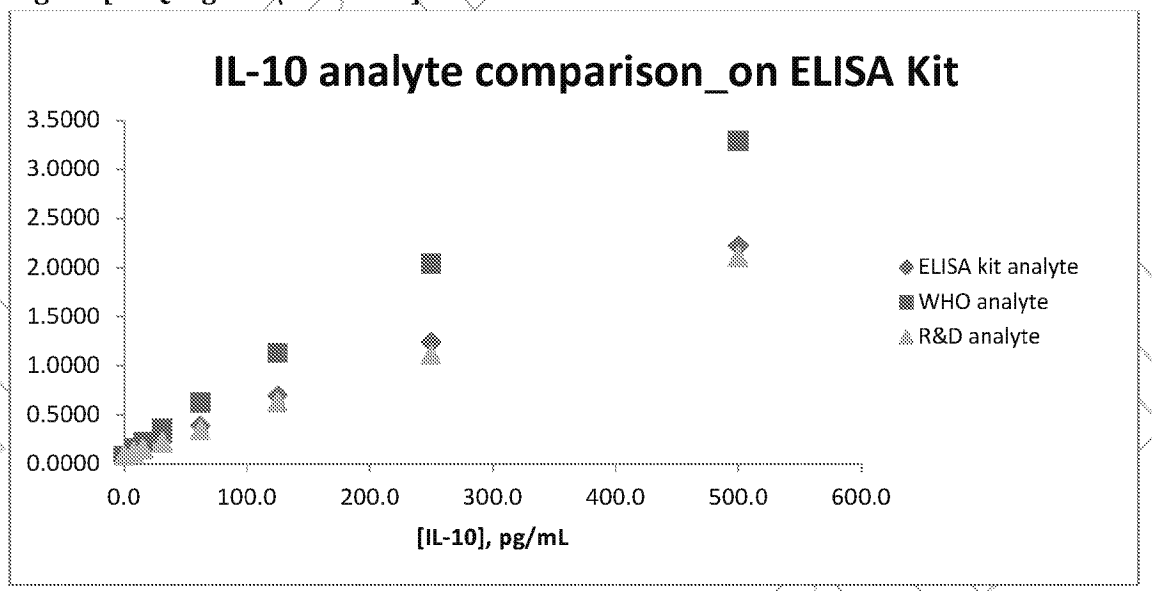
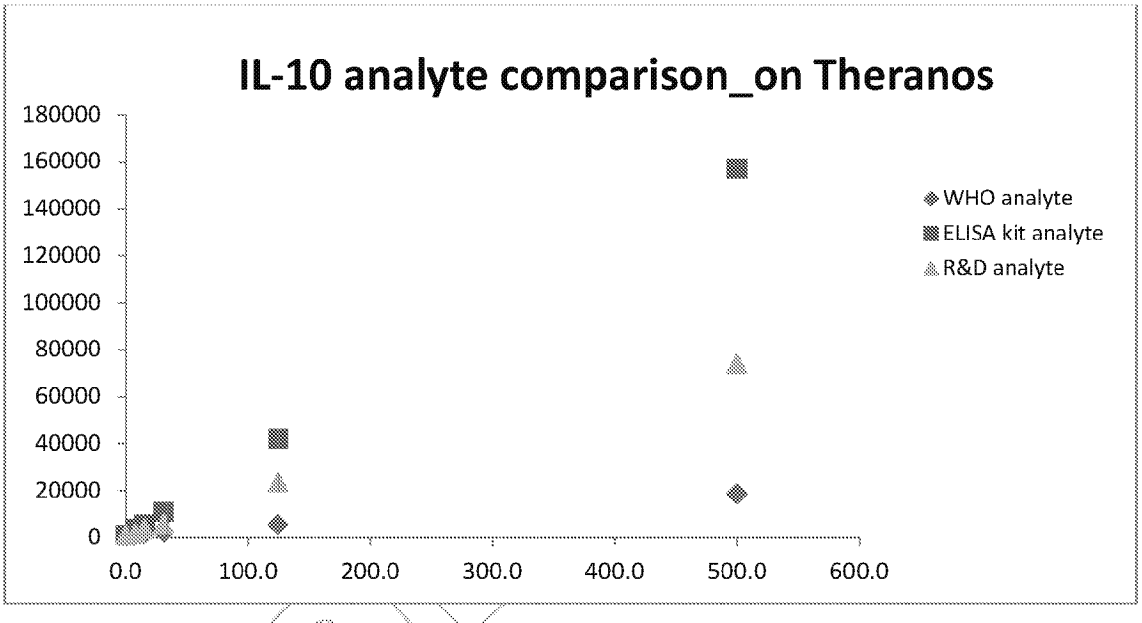


Figure [SEQ Figure * ARABIC]: DIFFERENT ANAYLTE TESTED ON THERANOS SYSTEM



2.8 SPIKED PLASMA, WHOLE BLOOD AND HEMATOCRIT EFFECT

Methods:

WHO IL-10 analyte was spiked into plasma, whole blood and then tested on Theranos system. Plasma from the spiked whole blood was also saved and tested on Theranos method for hematocrit effect.

Results:

Analyte spiked into plasma and whole blood recovered well comparing to buffer calibrator. The hematocrit effect for this assay was 1.85.

Table [SEQ Table * ARABIC]: SPIKED PLASMA AND WHOLE BLOOD

Nominal [IL-10] pg/mL	Spiked serum sampes			Spiked plasma sampes		
	Mean RLU	Cal. pg/mL	%Recovery	Mean RLU	Cal. pg/mL	%Recovery
1000.0	32870	961.8	96	35497	1049.2	105
250.0	9628	249.1	100	8979	230.8	92
62.5	2594	54.8	88	3150	69.8	112
15.6	1161	16.4	105	1242	18.5	119
3.9	726	4.8	123	839	7.8	200
0.0	696			736		

Nominal [IL-10] pg/mL	Spiked whole blood sampes			Plasma from the spiked whole blood			
	Mean RLU	Cal. pg/mL	%Recovery	Mean RLU	Cal. pg/mL	%Recovery	Hematocrit
1000.0	35355	1044.8	104	61377	1983.3	198	1.90
250.0	8359	213.3	85	14665	394.3	158	1.85
62.5	2694	57.4	90	4152	97.0	155	1.69
15.6	963	13.7	82	1459	24.3	156	1.78
3.9	794	5.2	107	943	10.6	271	2.02
0.0	531			663			mean
							1.85

3. ASSAY OPTIMIZATION

3.1 Capture Antibody titration

Methods:

The method mentioned in section 2.3.1 was used here.

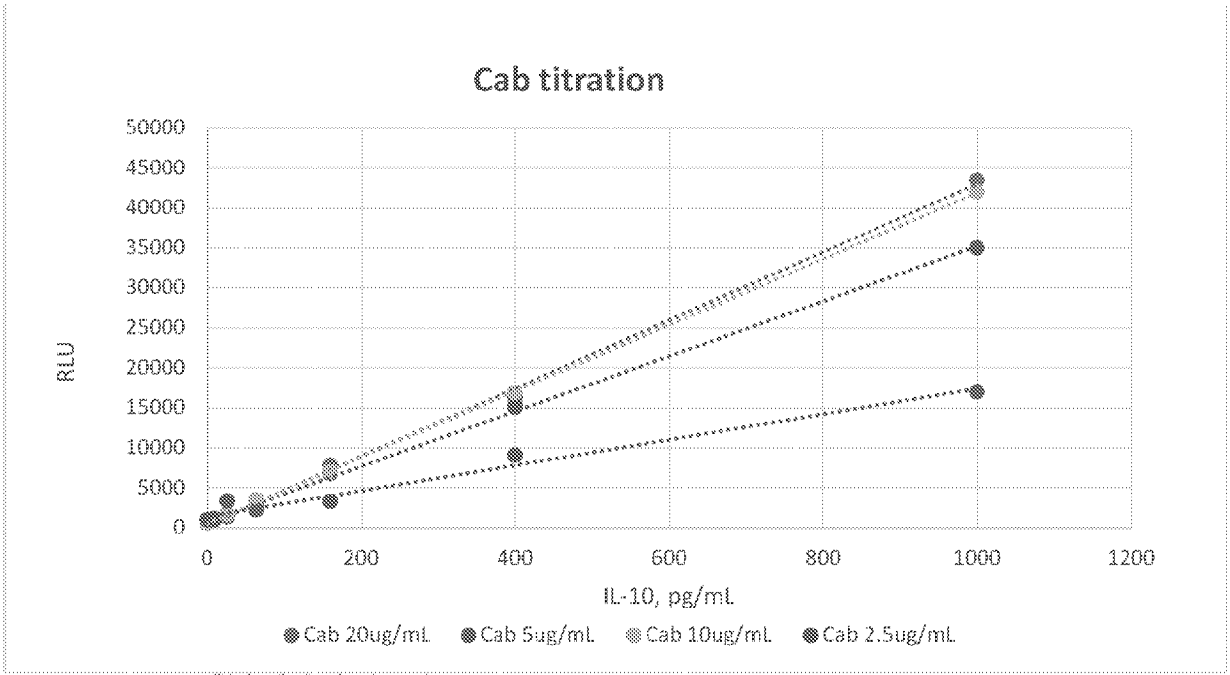
Results:

Capture antibody at 10ug/ml gave the best overall modulation, sensitivity and CV, thus it was chosen as the final assay capture concentration.

Table [SEQ Table * ARABIC]: CAPTURE ANTIBODY TITRATION

	Cab 20ug/mL		Cab 10ug/mL		Cab 5ug/mL		Cab 2.5ug/mL	
IL-10 pg/mL	Mean RLU	Signal %CV	Mean RLU	Signal %CV	Mean RLU	Signal %CV	Mean RLU	Signal %CV
1000	43427	7	41997	5	34960	5	16987	50
400	16166	4	16908	7	15100	5	9085	8
160	7807	6	6990	3	6777	18	3299	12
64	3039	8	3477	18	2710	13	2230	13
26.0	1381	11	1638	10	1648	4	3346	114
10.0	1017	10	1002	14	1118	23	1017	43
4.0	766	5	716	11	807	17	1009	60
0	548	22	568	15	630	6	961	63
S/B	79		74		55		18	

Figure [SEQ Figure * ARABIC]: CAPTURE ANTIBODY TITRATION CALIBRATION CURVE



3.2 Detection Conjugate Stabilizer

Method:

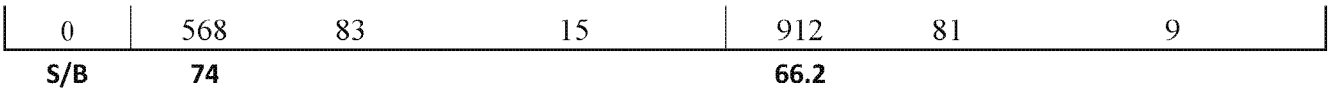
With other assay conditions kept the same, detection antibody was prepared in both StabilZyme-AP and BioStable AP conjugate stabilizer.

Results:

StabliZyme-AP gave a better overall modulation and lower background, thus it was chosen as the detection stabilizer.

Table [SEQ Table * ARABIC]: DETECTION CONJUGATE AP STABILIZER

	Stabilzyme AP stabilyzer _Control			BioStable AP conjugate stabilizer		
IL-10 pg/mL	Mean RLU	Stdev	Total Signal %CV	Mean RLU	Stdev	Total Signal %CV
1000	41997	2074	5	60348	3977	7
400	16908	1250	7	24645	2761	11
160	6990	232	3	10428	528	5
64	3477	628	18	4907	189	4
26.0	1638	169	10	2033	146	7
10.0	1002	137	14	1408	64	5
4.0	716	81	11	962	49	5



3.3 ASSAY DILUENT TESTING

Methods:

Different combination of assay diluents was tested for better modulation, CV, and sensitivity. Meanwhile capture antibody was kept at 10ug/ml and detection antibody was prepared at 400ng/ml loading concentration, and Edison protocol ABA_ver5 was used.

Results:
Superblock blocking buffer was chosen for further assay optimization since it gave the best overall modulation and lower %CV.

Table [SEQ Table * ARABIC]: ASSAY DILUENTS COMPARISON

Assay Diluents Combination	S/B
1. Control *Coating: In house blocking buffer coating* *Diluent: Starting Block Blocking Buffer*	70
2. Starting Block Blocking Buffer *Coating: Starting Block Blocking Buffer* *Diluent: Starting Block Blocking Buffer*	69
3. Super blocking Blocking buffer *Coating: Super blocking Blocking buffer* *Diluent: Super blocking Blocking buffer*	78
4. Blocker Casein in TBS_Pierce *Coating: Blocker Casein in TBS_Pierce* *Diluent: Blocker Casein in TBS_Pierce*	79
5. Sea Block Blocking buffer *Coating: Sea Block Blocking buffer* *Diluent: Sea Block Blocking buffer*	61

3.4 DETECTION TITRATION

Methods:
Capture antibody was coated on tips at 10ug/ml in blocking buffer, superblock blocking buffer was used as assay diluent, and detection antibody was prepared at different concentrations in StabliZyme-AP with Edison protocol ABA2_ver5.

Results:
The best condition was when detection antibody at 100ng/ml, thus it was chosen as the final assay condition.

Table [SEQ Table * ARABIC]: DETECTION ANTIOBDY TITRATION

	Dab 100 ng/mL		Dab 50 ng/mL			Dab 25ng/ml		
IL-10 pg/mL	Inter Mean	Total Signal %CV	Inter Mean	Stdev	Total Signal %CV	Inter Mean	Stdev	Total Signal %CV
1000	72957	6	48483	1865	4	23058	7836	34
400	27051	17	17209	3833	22	11808	203	2
160	12447	8	7735	874	11	4710	569	12
64	5462	5	3606	205	6	2347	95	4
26.0	3247	29	1797	387	22	1130	210	19
10.0	1537	7	1290	92	7	779	89	11
4.0	1284	8	1004	117	12	627	91	14
0	979	12	848	183	22	471	42	9
S/B	74.5		57.2			49.0		

4. CONCLUSION

We have successfully screened and optimized reagents for an immunoassay to detect IL-10 in human serum. The assay conditions generated in this report satisfy the feasibility requirement for the assay. Further testing will need to be done with clinical samples.