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Interleukin 12 (IL-12) Assay Feasibility Report

Theranos, Inc.

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1 ASSAY INFORMATION

1.1 Analyte Information

Interleukin 12 (IL-12) is an interleukin that is naturally produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation. It is also known as the natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF). The p40 subunit of IL-12 has been shown to have extensive amino acid sequence homology to the extracellular domain of the human IL-6 receptor while the p35 subunit shows distant but significant sequence similarity to IL-6, G-CSF, and chicken MGF. These observations have led to the suggestion that IL-12 might have evolved from a cytokine/soluble receptor complex. Human and murine IL-12 share 70% and 60% amino acid sequence homology in their p40 and p35 subunits, respectively. Current evidence indicates that IL-12, produced by macrophages in response to infectious agents, is a central mediator of the cell-mediated immune response by its actions on the development, proliferation, and activities of TH1 cells. In its role as the initiator of cell-mediated immunity, it has been suggested that IL-12 has therapeutic potential as a stimulator of cell-mediated immune responses to microbial pathogens, metastatic cancers, and viral infections such as AIDS.

1.2 Assay specification

The IL-12 Theranos immunoassay is designed to measure the concentration of IL-12 serum, plasma and whole blood. It is a quantitative assay and has the range of 2.5 to 2000pg/mL

1.3 Reference/Predicate method

The following kit was used as predicate/reference method:

R&D Systems IL-12 Quantikine ELISA Kit Cat# DY1270

1.4 Materials and methods

Table [SEQ Table * ARABIC]: Assay materials used in IL-12 assay in final conditions

Name	Supplier	Catalog number
Human IL-12 analyte	R&D Systems	219-IL
Human IL-12 p70 Antibody (Capture)	R&D Systems	MAB219
Human IL-12 p70 Antibody (Detection)	R&D Systems	MAB611
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
Starting Block in TBS	Pierce	37543
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
Human IL-12 Quantikine ELISA Kit	R&D Systems	DY1270

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 16 Interleukin 12 (IL-12) antibodies were screened for binding activity on micro titer plate (MTP). All antibodies were labeled with Dojindo Biotin labeling kit-SH (Cat#LK10 and Dojindo AP labeling kit-SH (Cat# LK13).

The MTP was first coated with UltraAvidin (UA) at 20ug/ml in carb-bicard coating buffer, followed by Biotinylated antibody at 10ug/ml in 3% BSA blocking buffer. IL-12 calibrators at 500pg/ml, 50pg/ml, 2.5pg/ml and 0pg/ml incubated with coated antibodies for 10minutes. Then detection antibody-AP conjugates were diluted in the stabilzyme buffer to 100ng/ml and incubated for 10 minutes. The surface is washed with TBS with 0.05%Tween for 3 times. The alkaline phosphatase substrate is incubated on the surface/well for 10 minutes, and the resulting chemiluminescence is read in Relative Light Units (RLU) by plate reader (M5).Modulations for each antibody pair were calculated using RLU of each calibrator concentration level divided by the RLU of background (Buffer blank, IL-12 at 0pg/ml).

Cab# 6 is biotin conjugates and hence were not screened as detection antibodies. Out of the 16 X 15 antibody pairs screened, some antibody pairs showed variable degree of response and some pairs had very good response (Table 4). Antibody pairs C6/D5, C7/D2, C11/D7 and C6/D7 are chosen for cross reactivity and interference tests because it has good modulation (Table 4).

Table [SEQ Table * ARABIC]: Antibodies screen on MTP

#	Vendor	Cat #	Clone #	Clonality/host/Isotype
1	Abcam	ab25105	polyclonal	rabbit polyclonal
2	Abcam	ab25036	Clone QS-12p70	mouse Monoclonal
3	Abcam	ab9992	polyclonal	Goat polyclonal
4	Abcam	ab37635	polyclonal	chicken polyclonal
5	Mabtech	3450-3-250	mouse monoclonal	mouse monoclonal
6	Mabtech	3450-8-250	biotinylated	mouse monoclonal
7	R&D Systems	MAB611	clone 24945	mouse monoclonal
8	R&D Systems	MAB1570	clone 27537	mouse monoclonal
9	R&D Systems	AB-219-NA	goat polyclonal	Goat polyclonal
10	R&D Systems	AF-219-NA	goat polyclonal	Goat polyclonal

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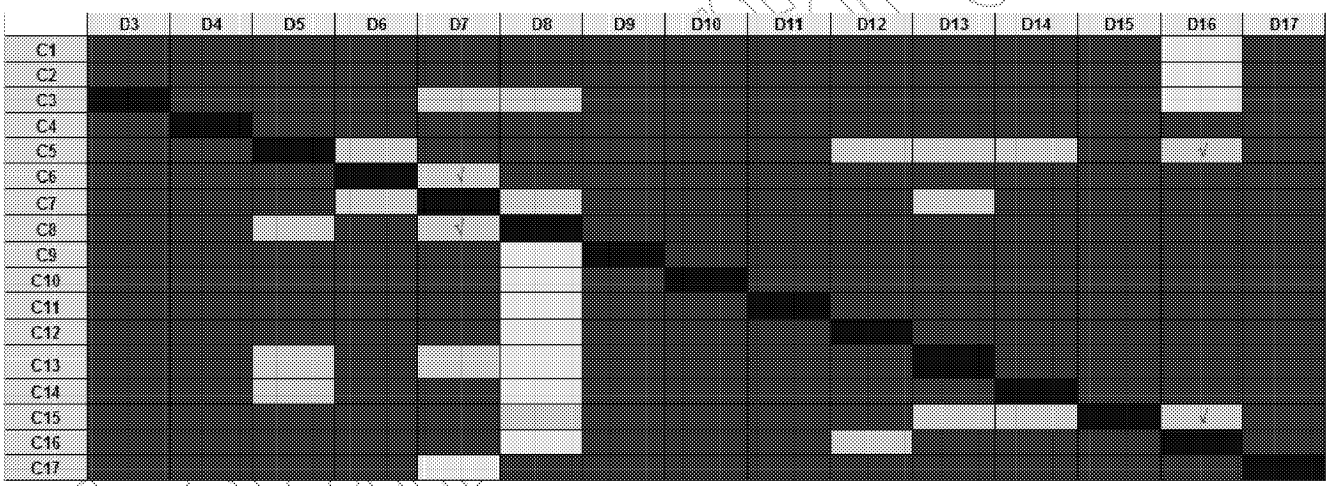
11	R&D Systems	MAB219	mouse monoclonal	mouse monoclonal
12	Enzo	ALX-804-874-C100		
13	Aniara	ACT273/CT273	Rabbit polyclonal	
14	eBioscience	14-7122-85	C18.2	
15	eBioscience	14-7128-82	B-T21	
16	eBioscience	16-7129-85	C8.6	

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



	Good modulation , move to next step
	Fair modulation
	No modulation

Table [SEQ Table * ARABIC]: Best Antibody pairs from intial screen on MTP

#	Cab	Dab	S/B	Notes
1	6	5	137	Both Abs are Mouse Ab
2	7	2	159	Both Abs are Mouse Ab
3	11	7	129	Both Abs are Mouse Ab
4	6	7	59	Both Abs are Mouse Ab

2.1.2 Cross reactivity and interference testing on MTP

4 different analytes which are chemokines (table 5) were tested for cross reactivity and interference for the four selected antibody pairs. IL-12, p70 is naturally produced by dendritic

cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation.

Table [SEQ Table * ARABIC]: Analytes tested for cross reactivity and interference

Analyte	Vendor	Cat#	Range (pg/mL)
IL-23	R&D	1290-IL	2500-39
IL-12 p35	R&D	499-ML	2000-25
rec. mouse IL-12/IL-23 p40 homodimer	R&D	309-IL	2000-31.2
rec. human IL-12/IL-23 p40 monomer	Abcam	ab53503	1000-31.2

Table [SEQ Table * ARABIC]: Analyte testing concentrations for interference

Analyte	Normal Range (pg/mL)	Testing conc (pg/mL)
IL-23	2500-39	7500
IL-12 p35	2000-25	6000
rec. mouse IL-12/IL-23 p40 homodimer	2000-31.2	6000
rec. human IL-12/IL-23 p40 monomer	1000-31.2	3000

2.1.2.1 Cross reactivity testing method

For each Capture/Detection antibody pair : 5 point IL-12 standard curve calibrators were used as controls and 5 point standard curve of cross reactant analyte (table5) were used as test samples to determine the cross reactivity. The test method was the same as mentioned in 2.1.1

Results for cross reactivity testing with 4 cab/dab pairs: For all four proteins tested, none of them showed major cross reactivity except at 2pg/mL (slight cross reactivity), thus all four pairs will be used for interference testing. Data is summarized is table7-10.

Table [SEQ Table * ARABIC]: Cross reactivity data for C6/D5

Capture Antibody # 6 / Detection Antibody # 5										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	483.8	96.8	1.0	0.2	1.5	0.3	1.4	0.3	0.9	0.2
100	107.1	107.1	0.9	0.9	1.4	1.4	0.9	0.9	0.5	0.5
50	51.5	103.0	1.4	2.8	2.0	4.1	2.3	4.7	1.3	2.6
10	8.8	87.9	3.0	29.5	1.8	18.3	0.9	9.4	1.3	12.6
2	2.1	106.7	0.7	35.1	0.6	30.1	0.9	47.2	0.8	39.0

Table [SEQ Table * ARABIC]: Cross reactivity data for C7/D2

Capture Antibody # 7/ Detection Antibody # 2										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	492.8	98.6	0.9	0.2	1.4	0.3	0.7	0.1	0.6	0.1
100	108.4	108.4	0.6	0.6	0.8	0.8	0.8	0.8	0.7	0.7
50	45.6	91.1	0.6	1.2	0.7	1.5	0.7	1.4	0.4	0.8
10	10.3	102.9	1.0	9.7	0.6	6.0	0.8	8.2	0.6	5.8
2	2.0	99.8	0.8	37.7	2.7	135.7	2.1	106.2	2.6	131.6

Table [SEQ Table * ARABIC]: Cross reactivity data for C11/D7

Capture Antibody # 11/ Detection Antibody # 7										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	477.4	95.5	1.0	0.2	1.7	0.3	1.8	0.4	1.0	0.2
100	114.3	114.3	0.8	0.8	1.2	1.2	1.1	1.1	0.9	0.9
50	50.3	100.5	0.5	1.0	1.4	2.7	0.9	1.9	0.8	1.6
10	7.9	79.2	0.8	7.9	1.0	10.5	0.7	7.3	0.9	8.6
2	2.3	115.2	1.0	49.1	1.1	55.6	0.9	47.2	0.7	35.6

Table [SEQ Table * ARABIC]: Cross reactivity data for C6/D7

Capture Antibody # 6/ Detection Antibody # 7										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	476.3	95.3	2.1	0.4	3.3	0.7	2.7	0.5	2.0	0.4
100	118.4	118.4	1.7	1.7	3.4	3.4	2.5	2.5	2.8	2.8
50	47.0	94.0	2.0	4.0	3.1	6.1	3.6	7.2	2.0	4.0
10	8.5	84.7	11.2	112.0	3.3	32.9	2.7	26.6	2.5	25.1
2	2.2	111.4	1.5	77.2	4.3	216.3	3.2	158.9	2.9	143.1

2.1.2.2 Interference testing method

For each Capture/Detection antibody pair : 5 point IL-12 standard curve calibrators were used as controls and each cross reactant (3x of highest level as shown in Table6) were spiked into the IL-12 calibrators (as test samples) to determine the interference of each cross reactant. The test method was the same as mentioned in 2.1.1

Results for interference with 4 cab/dab pairs: For all four proteins tested, C6/D7 showed major interference with the cross reactant IL-23 (Table 12). All the other three pairs did not have any major interference or cross reactivity. Two antibody pairs (C11/D7 and C6/D5) move to Theranos system for further optimization. Data is summarized in Table11 and 12.

Table [SEQ Table * ARABIC]: Interference data for C6/D5 and C7/D2

Capture Antibody # 6 / Detection Antibody # 5										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	524.9	105.0	484.9	97.0	466.9	93.4	463.8	92.8	481.9	96.4
100	72.4	72.4	64.6	64.6	58.8	58.8	58.6	58.6	61.8	61.8
50	59.6	119.1	53.0	106.0	46.0	91.9	45.3	90.6	55.1	110.1
10	11.8	118.2	12.1	121.0	11.3	113.3	8.3	83.1	10.9	108.6
2	1.9	93.7	2.1	106.0	2.4	118.4	2.0	98.3	1.9	96.0

Capture Antibody # 7/ Detection Antibody # 2										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	518.7	103.7	419.9	84.0	536.3	107.3	496.1	99.2	509.8	102.0
100	75.2	75.2	57.8	57.8	63.9	63.9	65.8	65.8	56.2	56.2
50	60.0	119.9	47.2	94.4	61.6	123.3	56.5	113.0	55.5	111.0
10	11.3	112.8	10.1	100.6	11.0	109.8	10.2	102.3	9.0	90.5
2	1.9	94.7	1.5	76.1	4.6	230.6	2.7	136.8	2.5	123.0

Table [SEQ Table * ARABIC]: Interference data for C11/D7 and C6/D7

Capture Antibody # 11/ Detection Antibody # 7										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	523.2	104.6	516.4	103.3	522.2	104.4	521.2	104.2	519.1	103.8
100	72.4	72.4	70.1	70.1	76.1	76.1	68.7	68.7	69.7	69.7
50	59.1	118.1	60.7	121.5	60.2	120.5	57.1	114.3	57.6	115.1
10	12.1	121.0	11.3	113.1	11.9	118.7	11.0	110.1	10.7	106.6
2	1.9	92.5	1.7	84.0	2.1	104.4	1.7	86.2	1.8	89.8

Capture Antibody # 6/ Detection Antibody # 7										
	IL-12 (Control)		IL-23		IL-12 p35		IL-12/23 p40 monomer		IL-12/23 p40 homodimer	
Nominal pg/mL	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery	Back cal. Conc	% Recovery
500	516.8	103.4	1042.3	208.5	467.3	93.5	474.4	94.9	489.2	97.8
100	75.1	75.1	201.9	201.9	61.4	61.4	59.0	59.0	64.2	64.2
50	60.2	120.4	177.4	354.9	48.3	96.5	51.3	102.6	55.0	109.9
10	11.4	113.8	36.4	364.3	9.3	93.4	9.0	90.5	8.9	88.5
2	1.9	94.2	5.4	270.2	1.6	80.2	1.4	70.9	1.3	66.8

2.2 Antibody screening on Theranos System

2.2.1 Theranos screen with two pairs of antibodies

Eight point calibration curve of IL-12 were tested with antibody pairs: C11/D7 and C6/D5. Theranos system protocol, [redacted] was used for initial testing. The samples were diluted on board to 5 fold using 3% BSA blocking buffer. The reaction surfaces were coated first with 20ug/ml UA in carb-bicarb buffer followed by 10ug/ml of capture antibody diluted in 3% BSA blocking buffer. Calibrators were first diluted with 3% BSA blocking buffer on board then incubated with dab for 10 minutes (pre incubation), Detection antibody-AP conjugates were diluted to 100ng/ml in stabilzyme AP. The sample + Dab mixture is the incubated with reaction surfaces (co-incubation).All surfaces were washed with wash buffer and incubated with AP substrate for another 10 minutes. RLU was measured for each tip. The recovery was calculated comparing the calculated concentration vs. nominal value. A whole blood sample with low endogenous level of IL-12 was chosen and spiked with high

concentrations IL-12 analyte and tested on Theranos system for IL-12 concentrations. These samples were then spun down and the plasma was tested on Theranos system for IL-12 concentrations.

Cab6/Dab5 and Cab11/Dab7 showed satisfying results with reasonable CV, good signal/background modulation. Cab6/Dab5 did not have very good recovery, Cab11/Dab7 had better recoveries for plasma from spiked whole blood samples. Matrix effects are pronounced and calibration will have to be done using spiked plasma calibrators. Recovery from whole blood is poor. Cab11/Dab7 was chosen for Theranos Training set experiment. Data is summarized from table 13-18.

Table [SEQ Table * ARABIC]: C11/D7 calibration curve

Nominal	RLU				
[IL-12] pg/mL	Mean	% CV	S/B	Back calculation	% Recovery
2000	156104	12	298	1981.1	**99**
500	43110	17	82	495.7	**99**
100	9672	4	18	102.0	**102**
50	5172	11	10	48.5	**97**
10	1444	15	3	9.5	**95**
5	1106	13	2	5.4	**108**
2.5	696	15	1	2.4	**97**
0	524	16			

Table [SEQ Table * ARABIC]: C11/D7 Whole Blood spiked recovery

Nominal	RLU			
[IL-12] pg/mL	Mean	% CV	Backcalculation	% Recovery
2000	81750	7	980.3	**49**
500	32276	13	369.6	**74**
50	1855	4	12.3	**25**
5	836	5	3.4	**68**
0	551	5	1.6	

Table [SEQ Table * ARABIC]: C11/D7 Plasma from spiked WB recovery

Nominal	RLU			
[IL-12] pg/mL	Mean	% CV	Backcalculation	% Recovery
2000	273873	5	3851.5	**193**
500	49374	6	577.4	**115**
50	7538	12	76.3	**153**
5	1221	14	6.4	**127**
0	456	8	1.1	

Table [SEQ Table * ARABIC]: C6/D5 calibration curve

Nominal	RLU				
[IL-12] pg/mL	Mean	% CV	S/B	Back calculation	% Recovery
2000	231542	7	869	2096.1	**105**
500	62663	10	235	460.9	**92**
100	13412	9	50	106.6	**107**
50	6645	10	25	54.2	**108**
10	1456	19	5	8.3	**83**
5	981	17	4	5.3	**106**
2.5	634	14	2	2.7	**106**
0	266	19			

Table [SEQ Table * ARABIC]: C6/D5 Whole Blood spiked recovery

Nominal	RLU			
[IL-12] pg/mL	Mean	% CV	Backcalculation	% Recovery
2000	109805	6	841.9	**42**
500	32511	18	241.1	**48**
50	3898	18	30.8	**62**
5	869	9	4.5	**89**
0	452	11	1.5	

Table [SEQ Table * ARABIC]: C6/D5 Plasma from spiked WB recovery

Nominal	RLU			
[IL-12] pg/mL	Mean	% CV	Backcalculation	% Recovery
2000	372749	11	4095.9	**205**
500	86973	3	651.9	**130**
50	8176	17	66.1	**132**
5	326	12	0.8	**16**
0	350	4	0.6	

2.3 Training set and whole blood screen with Cab11/Dab 7

Eight point calibration curve was tested with antibody pair C5/D16 using Theranos system protocol [redacted]. 46 normal serum and 24 disease state plasma were screened on the R&D IL-12 ELISA kit (Predicate) and all samples were < 7.8 pg/mL. Hence the only way for validating the current pair is to compare against spiked WHO standard calibrators. Spiked in WHO IL-12 international standard into serum and compare spike recoveries to that of the Theranos Il-12 spiked serum calibrators

9 whole blood samples and 9 spun down plasma from WB were tested for the analyte IL-12 recovery with C11/D7 pair. Il-12 endogenous levels in the whole blood of 9 normal donor samples shows a range of 2.5-3.9 pg/mL. The same blood samples were spun down and the plasma showed endogenous IL-12 levels ranging from 0.6-37.6 pg/mL.

This experiment confirms that the current Ab pair (Cab 5/Dab 16) will recognize clinical samples across the range of the assay and hence C11/D7 was chosen as the final antibody pair for assay optimization. (Data summarized in Table 19, 20/Figure 1)

Table [SEQ Table * ARABIC]: Theranos IL-12 Spiked serum calibrator

Nominal [IL-12] pg/mL	RLU Mean	% CV	S/B	Back calculation	% Recovery
2000	202216	3	394	1948.4	**97**
1000	121777	9	237	1045.0	**104**
500	53695	8	105	487.5	**97**
100	12617	18	25	109.8	**108**
50	4852	13	9	41.3	**80**
10	1487	12	3	11.8	**101**
5	642	9	1	4.7	**70**
0	513	27		3.5	

Table [SEQ Table * ARABIC]: WHO standard IL-12 Spiked serum calibrator

Nominal [IL-12] pg/mL	RLU Mean	% CV	S/B	Back calculation	% Recovery
2000	186150	4	455	1783.9	**89**
1000	93535	3	229	866.9	**86**
500	51433	18	126	461.2	**92**

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100	9868	7	24	86.3	**84**
50	4952	9	12	42.3	**80**
10	1357	6	3	10.7	**83**
5	719	13	2	5.5	**69**
0	409	19		2.8	

Figure [SEQ Figure * ARABIC]: IL-12 spiked calibrators (Theranos spiked calibrators vs WHO standard spiked calibrators)

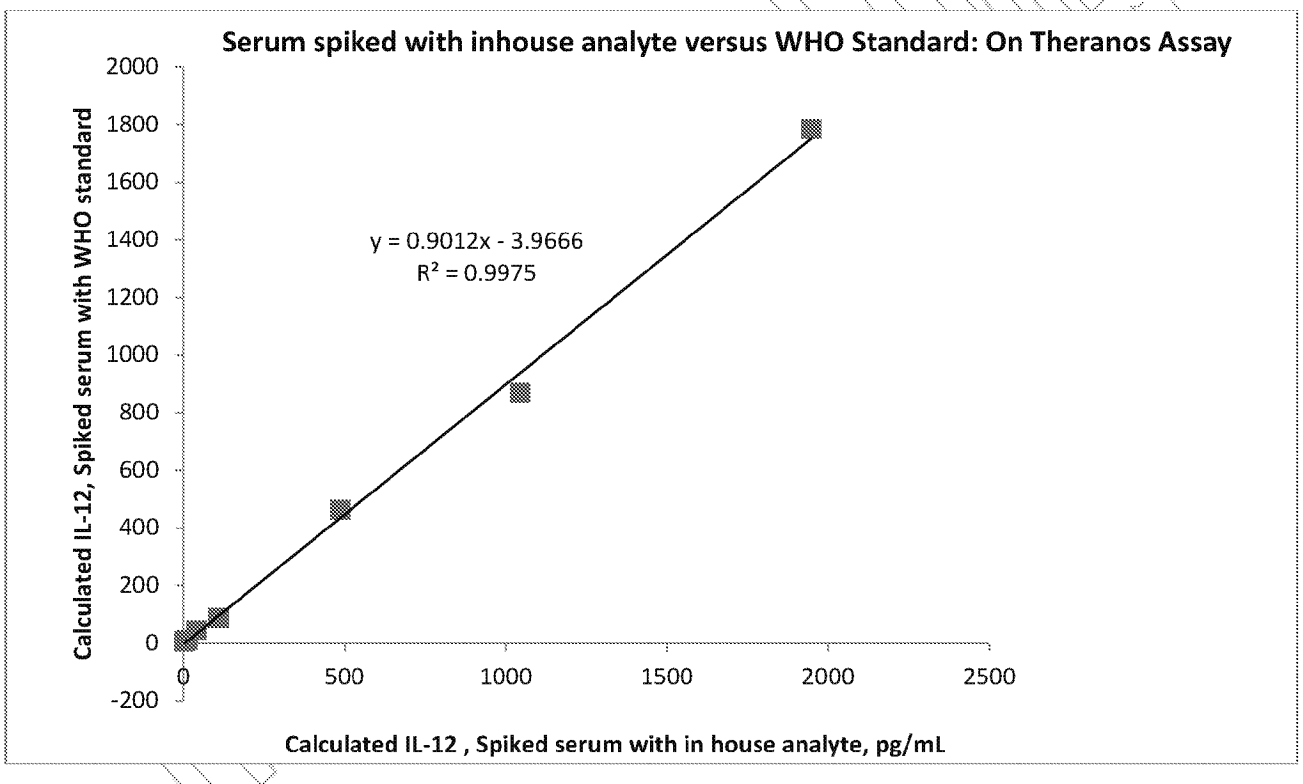

Table [SEQ Table * ARABIC]: Whole blood and Plasma screen for C11/D7 antibody pair

	IL-12 pg/mL	
Sample#	WB Screen	Plasma screen
1	2.8	37.6
2	2.8	2.5
3	3.0	0.6
4	2.9	4.0
5	3.0	3.2
6	3.3	3.2
7	3.7	4.4

8	3.9	7.7
9	2.5	7.8

3 ASSAY OPTIMIZATION

3.1 Capture antibody titration

The capture antibody concentration was titrated at levels: 20, 10 (control), 5 and 2.5 µg/mL. Table 22 summarizes the results of C11/D7 at 100ng/mL. Eight point pooled spiked serum calibrators were used for testing. Both 5µg/mL and 10 µg/mL give best modulation (S/B) and tight CVs. 5µg/mL of capture antibody concentration is chosen for further optimization to save antibody.

Table [SEQ Table * ARABIC]: Capture antibody titration summary

Cab conc	IL-12 conc (pg/mL) Nominal	Mean RLU	%CV	Back Cal	% Recovery	S/B
	2000.0	126242	8	1788.9	89.4	333.7
	1000.0	74548	8	1267.4	126.6	197.1
2.5	500.0	38141	12	444.4	88.7	100.8
ug/mL	100.0	8247	18	110.0	108.8	21.8
	50.0	3586	6	49.9	97.6	9.5
	10.0	1052	6	9.3	83.9	2.8
	5.0	769	14	5.3	87.3	2.0
	0.0	378	29	1.1		
	2000.0	191615	5	1981.8	99.0	437.2
	1000.0	102584	5	985.5	98.4	234.1
	500.0	54967	5	509.3	101.6	125.4
5	100.0	11256	15	98.7	97.6	25.7
ug/mL	50.0	5543	12	48.9	95.7	12.6
	10.0	1655	13	10.4	93.9	3.8
	5.0	959	9	4.9	79.9	2.2
	0.0	438	22	1.4	125.8	
	2000.0	184839	10	2046.9	102.3	512.4
	1000.0	111799	10	1000.7	100.0	309.9
	500.0	62894	9	500.4	99.9	174.3
10	100.0	11347	10	99.6	98.5	31.5
ug/mL	50.0	5625	8	52.2	102.1	15.6
	10.0	1186	9	9.3	84.0	3.1
	5.0	819	24	5.2	86.0	2.2
	0.0	361	12	1.2	111.8	

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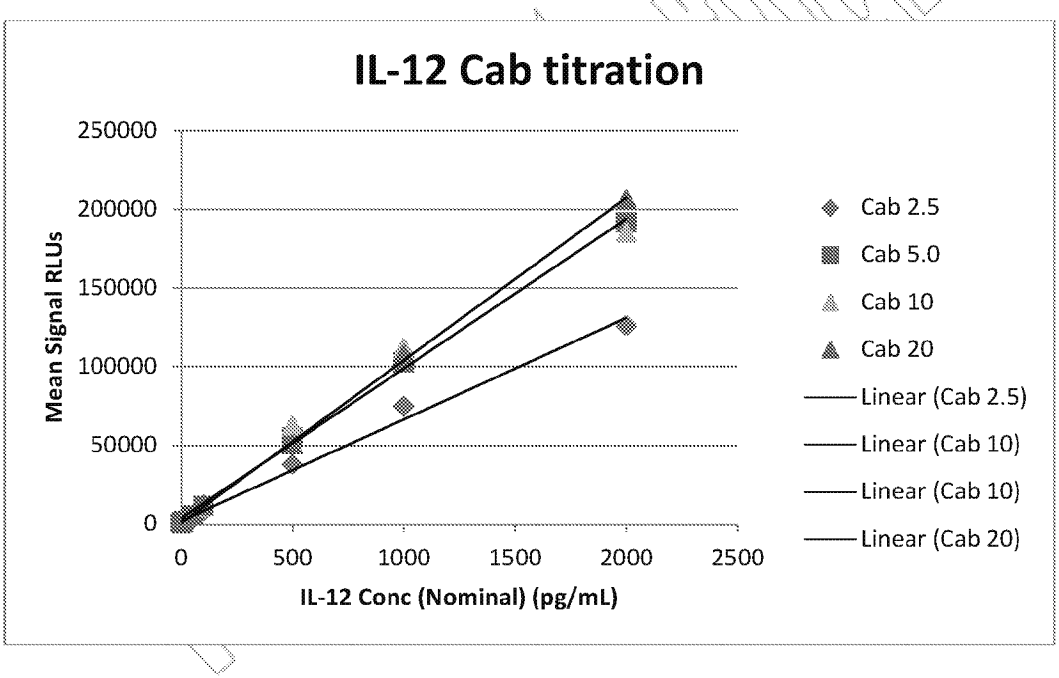
	2000.0	206156	7	2439.4	121.9	530.9
	1000.0	107336	13	944.5	94.3	276.4
	500.0	50729	10	396.7	79.2	130.6
20	100.0	11993	13	104.3	103.2	30.9
ug/mL	50.0	5578	17	51.4	100.6	14.4
	10.0	1386	14	11.5	103.2	3.6
	5.0	898	7	6.2	102.3	2.3
	0.0	388	17	1.4	128.0	

Figure [SEQ Figure * ARABIC]: Capture antibody titration summary



3.2 Detection Antibody Titration

[TC " Capture Antigen Surface Antigen " \f C \l "1"]

The AP conjugated detection antibody was titrated at levels 150, 100, 50 and 25 ng/mL. With all the assay conditions kept the same including the Theranos system protocol, calibration curve was run with each concentration of detection, and the results were used to compare to original 100ng/ml detection conjugate in Stabilzyme AP. All the four levels gave good modulation. 50ng/mL gave the best modulation (S/B), CV and most optimal recoveries and was hence chosen for further optimization. Data is summarized in Table 23

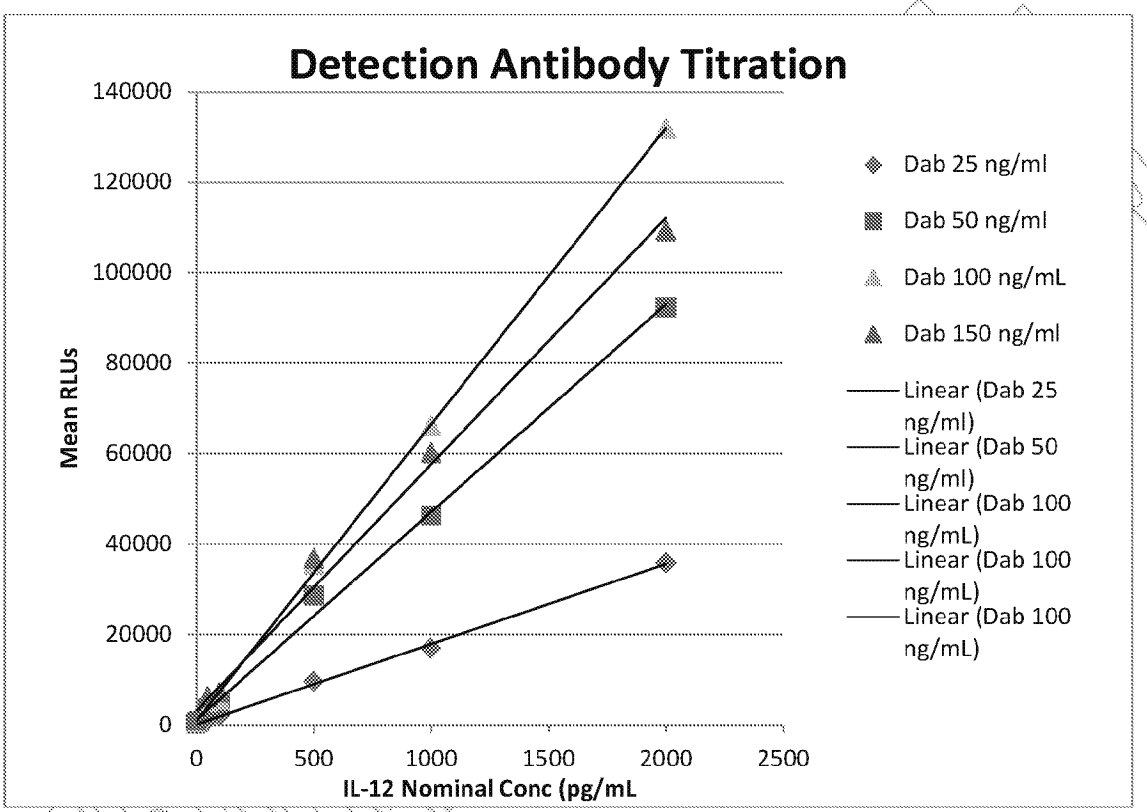
Table [SEQ Table * ARABIC]: Detection antibody titration summary

Dab Conc	IL-12 conc (pg/mL) Nominal	Mean RLU	%CV	Back Cal	% Recovery	S/B
	2000.0	35953	3	2213.8	110.6	212.6
	1000.0	16922	6	834.0	83.3	100.1
25	500.0	9650	6	487.4	97.3	57.1
ng/mL	100.0	2175	27	140.7	139.3	12.9
	50.0	1012	7	32.8	64.3	6.0
	10.0	374	9	11.6	105.7	2.2
	5.0	266	13	5.3	87.5	1.6
	0.0	169	19	1.4	142.8	
	2000.0	92293	10	2045.9	102.2	258.0
	1000.0	46266	10	913.8	91.2	129.3
	500.0	28625	8	546.3	108.8	80.0
50	100.0	4746	12	82.5	80.9	13.3
ng/mL	50.0	3646	3	61.3	117.9	10.2
	10.0	916	12	9.3	77.2	2.6
	5.0	649	9	5.2	74.9	1.8
	0.0	358	13	1.7	85.2	
	2000.0	131853	15	1981.6	99.0	186.8
	1000.0	66046	7	980.0	97.8	93.6
	500.0	35331	10	534.4	106.4	50.1
100	100.0	5539	6	71.2	69.6	7.8
ng/mL	50.0	5109	11	63.5	121.3	7.0
	10.0	1795	14	11.6	94.4	3.0
	5.0	1148	17	4.6	62.7	2.0
	0.0	706	15	1.4		
	2000.0	109257	11	2030.8	101.5	101.0
	1000.0	60168	15	919.0	91.8	55.6
	500.0	36857	7	529.9	105.8	34.1
150	100.0	7119	10	77.7	77.0	6.6
ng/mL	50.0	6276	4	65.1	127.6	5.8
	10.0	2034	10	7.6	69.4	2.9
	5.0	1886	15	6.3	104.4	2.7

[PAGE * MERGEFORMAT]

0.0	1082	10	1.4	135.4

Figure [SEQ Figure * ARABIC]: Detection antibody titration



3.3 Effect of Assay Diluent

Three commercially available blockers (StartingBlock, SeaBlock and SuperBlock) and one in-house blocking buffer (3% BSA in TBS) were tested as diluents for the assay. Data was compared to the control diluent which was the blocking buffer consisted of 3% BSA and 0.05% sodium azide in TBS. Starting Block used as assay diluent gave the best S/B and better modulation and was chosen for further optimization. . The data is summarized in Table 24.

Table [SEQ Table * ARABIC]: Effect of different blocker solutions

Blocker	IL-12 (pg/mL) Nominal	Mean RLU	%CV	Back Calc. (pg/mL)	% Recovery	S/B
Control 3% BSA blocking buffer	2000.0	109595	10	2084.3	104.2	287.8
	1000.0	44928	23	902.3	90.1	118.0
	500.0	27241	25	553.9	110.5	71.5
	100.0	5473	11	100.7	99.6	14.4
	50.0	3187	7	50.6	98.9	8.4
	10.0	959	16	9.5	86.0	2.5
	5.0	656	20	5.2	85.2	1.7
	0.0	381	20	3.8	85.2	
Starting block	2000.0	102949	5	2060.3	103.0	248.4
	1000.0	39778	10	920.9	92.0	96.0
	500.0	24640	30	549.3	109.6	59.4
	100.0	5240	12	91.9	90.9	12.6
	50.0	3541	11	55.6	108.8	8.5
	10.0	919	11	9.3	83.9	2.2
	5.0	611	30	5.2	85.7	1.5
	0.0	415	32	3.2		
Sea Block	2000.0	115043	6	2021.8	101.0	291.4
	1000.0	50334	13	933.7	93.3	127.5
	500.0	27152	8	532.4	106.2	68.8
	100.0	4700	24	83.5	82.6	11.9
	50.0	3552	9	60.5	118.4	9.0
	10.0	1008	8	9.1	82.2	2.6
	5.0	763	25	5.3	86.5	2.0
	0.0	395	8	1.4	131.2	
Super Block	2000.0	92043	3	1638.5	81.9	258.5
	1000.0	52041	17	957.9	95.7	146.2
	500.0	29651	21	569.2	113.6	83.3
	100.0	4084	25	70.4	69.6	11.5
	50.0	2689	7	41.9	81.9	7.6
	10.0	891	19	7.1	64.4	2.5
	5.0	594	7	3.4	55.8	1.7
	0.0	356	22	1.1	100.6	

3.4 Effect of HBR in diluent

Effect of HBR (Heterophile blocking reagent) in diluent was tested since both the capture and detection antibody are mouse monoclonals (to avoid interference with HAMA in the future with clinical smaples). IL-12 spiked serum calibrators were tested with two different diluents, starting block which will be the control and starting block with 400ug.mL HBR which will be the test diluent. The background is lower and the S/B (modulation) is slightly higher with HBR added, it does affect CVs either. Since there is not much of a difference between the two diluents, Starting block was chosen for further optimization.

Table [SEQ Table * ARABIC]: Effect of HBR in the diluent

Assay diluent	IL-12 conc Nominal pg/mL	Mean RLU	%CV	Mean Calculated	% Recovery	S/B
Starting block	2000	98492	6	2014.3	101	**308**
	1000	50483	7	975.6	97	**158**
	500	23882	8	476.0	95	**75**
	100.0	5374	6	114.0	112	**17**
	50.0	2446	11	45.1	88	**8**
	25	1565	5	24.4	92	**5**
	10	878	9	9.5	82	**3**
	5.0	680	25	5.6	87	**2**
	2.5	450	24	2.4	60	**1**
	0.00	319	7	1.5		
Starting block + 400 ug/mL HBR	2000	90036	13	1804.6	90	**317**
	1000	42904	11	827.1	83	**151**
	500	23890	9	476.0	95	**84**
	100.0	5362	7	113.7	113	**19**
	50.0	3012	3	59.0	116	**11**
	25	1795	6	29.7	115	**6**
	10	833	8	8.6	80	**3**
	5.0	618	25	4.7	81	**2**
	2.5	494	14	3.0	92	**2**
	0.00	284	14	0.8		

3.5 Theranos System Protocol optimization

Three different protocols were tested for IL-12 assay. The effect of sample dilution and post sample wash (PSW) were tested in this experiment. Post sample wash (PSW) and higher sample dilution were tested in the hope of lowering background, improving %CV and modulation. Calibrators were run under these two protocols: The surfaces were coated at 5ug/ml in blocking buffer; detection conjugate was prepared at 50ng/ml in StabilZyme-AP, and the sample diluent was Strating block. The three different protocols are as follows

a) [redacted]
b)
c)

Seven point IL-12 spiked pooled serum calibrators were tested on each of the three protocol. One whole blood sample was spiked with 5 levels of IL-12 (2000, 500, 100, 50 and 0 pg/mL). The samples were tested on each protocol and IL-12 concentrations were back calculated. The whole blood sample was then spun down and plasma samples (all 5 levels) were tested on each protocol and analyzed for IL-12 concentrations.

All protocol performed in a similar manner and protocol: [redacted] was chosen as the final protocol

Table [SEQ Table * ARABIC]: Theranos System Protocol optimization summary

Protocol : [redacted]

Whole Blood Spike Recovery

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	123370	14	1991.9	98
500	23850	12	403.1	75
100	7337	14	104.0	74
50	6533	2	84.1	92
0	3072	6	41.1	

Protocol : [redacted]

Plasma obtained from spiked Whole blood

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	228767	7	3167.6	154
500	72797	4	1261.9	226
100	16363	8	263.7	166
50	9363	10	145.3	134
0	4253	10	58.6	

Protocol : [redacted]

Whole Blood Spike Recovery

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	64161	15	1640.8	81
500	22984	19	595.2	114
100	4407	13	91.9	77
50	2993	15	59.0	84
0	1448	5	19.9	

Protocol: [redacted]

Plasma obtained from spiked Whole blood

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	163257	5	3660.6	180
500	44235	12	1160.2	217
100	10569	6	250.9	186
50	6231	9	118.4	140
0	2167	2	34.9	

[redacted]

Whole Blood Spike Recovery

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	68004	12	1867.2	92
500	25677	3	587.0	113
100	3656	23	82.7	68
50	2090	15	49.4	69
0	1448	21	21.2	

[redacted]

Plasma obtained from spiked Whole blood

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	134202	6	5676.7	281
500	38136	7	900.1	173
100	7579	19	187.1	154
50	6584	5	157.3	221
0	1945	17	34.5	

3.6 Hematocrit Effect

The purpose of the experiment is determine if there is any hematocrit effect on the assay. One whole blood sample was spiked with IL-12 analyte, which is then analyzed. The sample is the spun down and the plasma is tested to see if there is any hematocrit effect.

A correction factor of 1.5 needs to be applied to the serum calibration

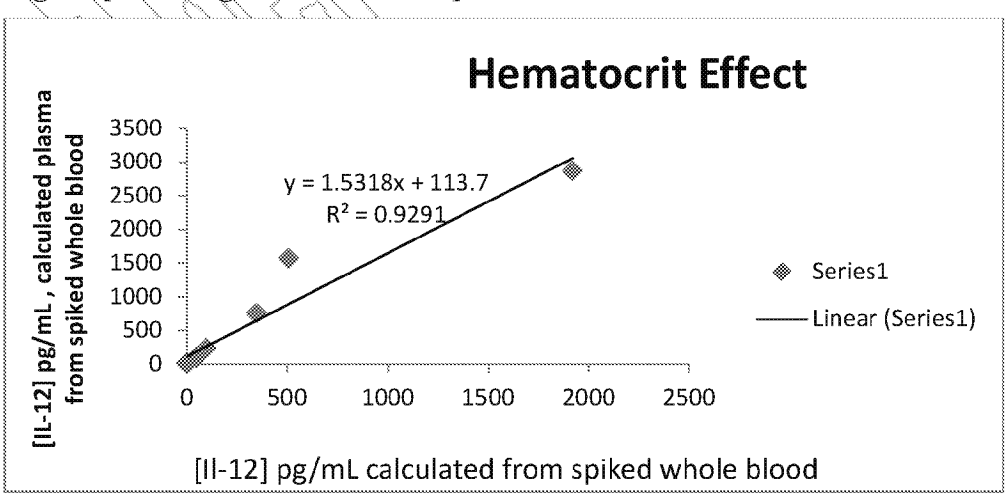
Table [SEQ Table * ARABIC]: Whole Blood Spike Recovery

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	79797	10	1922.0	96
1000	26389	11	658.2	66
500	14101	25	348.2	70
100	4423	8	100.2	99
50	2519	20	47.1	92
10	871	18	8.6	78
5	570	14	3.8	63
2.5	425	11	2.1	59
0	317	26	1.0	

Table [SEQ Table * ARABIC]: Plasma from Spiked WB

IL-12 pg/mL Nominal	Average	CV% RLU	Mean Calculated	% Recovery
2000	117497	8	2867.0	143
1000	51950	5	1263.4	126
500	30613	21	748.1	149
100	9456	22	232.6	230
50	4259	16	94.8	185
10	1056	27	11.8	104
5	676	15	5.3	85
2.5	552	15	3.5	93
0	1168	9	1.3	

Figure [SEQ Figure * ARABIC]: Hematocrit effect



4 CONCLUSION

We have successfully screened and optimized reagents for an immunoassay to detect IL-12 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.