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Human CCL11/Eotaxin Assay Development Report

Theranos, Inc.

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1. Analyte background

CCL11 is a potent eosinophil chemo attractant that was originally purified from Broncho alveolar lavage fluid of guinea pigs sensitized by aerosol challenge with ovalbumin. Micro sequencing of the purified protein revealed the guinea pig CCL11 to be a member of the beta (CC) chemokine family of inflammatory and immune regulatory cytokines. cDNA clones for guinea pig, mouse and human CCL11 have recently been isolated. Human CCL11 cDNA encodes a 97 amino acid residue precursor protein from which the amino-terminal 23 amino acid residues are cleaved to generate the 74 amino acid residue mature human CCL11. At the protein sequence level, mature human CCL11 is approximately 60% identical to mature mouse and guinea pig CCL11. In addition, human CCL11 also shows high amino acid sequence identity to human MCP-1, 2 and 3. Human CCL11 is chemo tactic for eosinophils, but not mononuclear cells or neutrophils. The CC chemokine receptor 3 (CCR3) has now been identified to be a specific human CCL11 receptor.

2. Assay specifications

The Theranos assay is a sandwich ELISA designed to measure Eotaxin in buffer, whole blood, plasma and serum with a reportable range of 1000 to 25 pg/mL. This assay is specific for recombinant and native human Eotaxin and no cross-reactivity to other chemokines is expected.

3. Assay Sensitivity

The limit of detection for this immunoassay is 10 pg/mL. LOD was calculated using the formula (2 x (Average SD of bottom 2 standard points) /(Slope of bottom 2 standard points)). Calibration was run with an instrument protocol designed to run the assay in a multiplex with other asthma biomarkers.

4. Reference assays

R&D Eotaxin Elisa Kit: Catalog Number DTX00

This kit is a sandwich ELISA with an assay range of 1000 – 15.6 pg/mL with sensitivity of 5 pg/mL.

5. Antibody Screening

Antibody pairs were screened to obtain the pair with best dose response for the assay.

	CAB1	CAB2	CAB3	CAB4	CAB5	CAB6
DAb1						
DAb2			**PAIR B**			
DAb3		**PAIR A**				
DAb4						
DAb5						
DAb6						

Number of Capture antibody tested: 6

Number of Detection antibody tested: 6

Total Number of antibody pairs tested: 12

Good dose response

Poor or no modulation

The antibody pair A was selected for the Theranos assay. The criteria were good dose response for the range of the assay and highest sensitivity for the final assay conditions.

Table [SEQ Table * ARABIC]: Response of Antibody pair A and B on Theranos system.

	Conc.	**Pair A**			**Pair B**		
	pg/mL	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**
1	2000	440846	41197	9	248697	2018	1
2	667	184371	15446	8	83241	4132	5
3	222	78428	3444	4	30542	1921	6

4	74	35876	386	1	9834	2208	22
5	25	17862	1481	8	4230	99	2
6	0	8288	1439	17	1075	66	6
	S/B	53			231		
	Slope	372			118		
	Avg Stdev	1102			791		
	LOD, pg/mL	6			13		

6. Capture Antibody Titration

Capture antibody was tested at 2.5, 5, 10ug/mL. A concentration of 10ug/mL was determined to be optimum

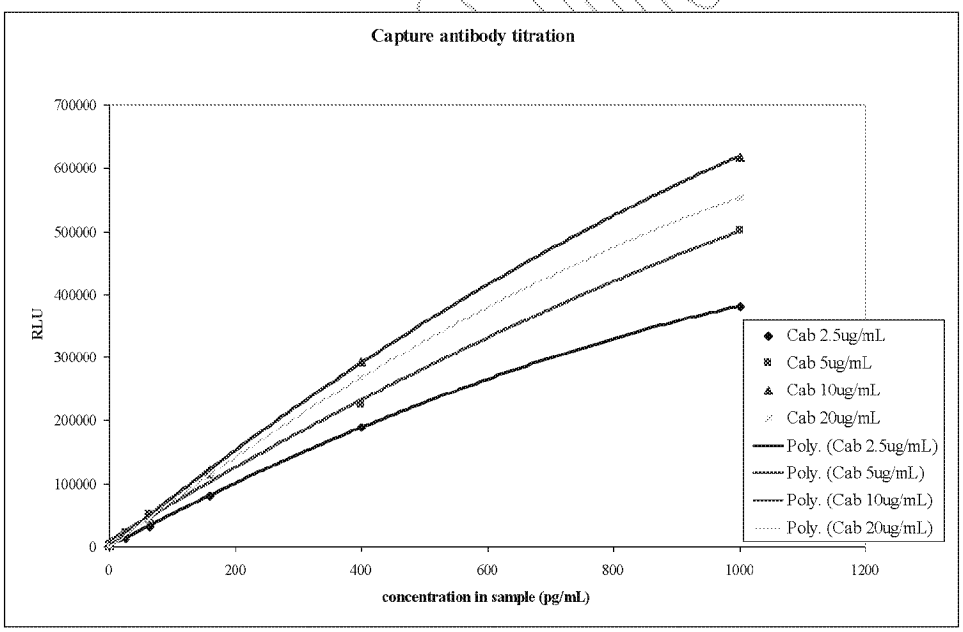


Figure [SEQ Figure * ARABIC]: Capture antibody titration plot

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

	20ul/ml			10ug/ml			5ug/ml			2.5ug/ml		
Analyte	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**
pg/mL												
1000	553721	21924	4	618117	45068	7	500467	68526	14	500467	68526	14
400	268728	29114	11	292162	21501	7	225863	27379	12	225863	27379	12
160	115937	16197	14	121336	14378	12	112214	14739	13	112214	14739	13
64	42509	4607	11	44517	5988	13	50292	3812	8	50292	3812	8
26	18577	1026	6	20686	1833	9	21716	1473	7	21716	1473	7
0	2415	281	12	2570	464	18	3015	348	12	3015	348	12
	S/B_Std 1/6		229			240			166			206
	S/B_Std 5/6		7.7			8.0			7.2			6.9
	AvgCV		9			11			11			11
	Slope		626			653			739			483
	Avg stdev		1971			2761			1878			2216
	LOD, pg/mL		6.30			8.46			5.08			9.18

7. Detection Antibody Titration

Detection antibody was tested at 25ng/ml, 50ng/ml, 100ng/ml in alkaline phosphatase stabilizing buffer. A concentration of 50ng/mL was determined to be optimum.

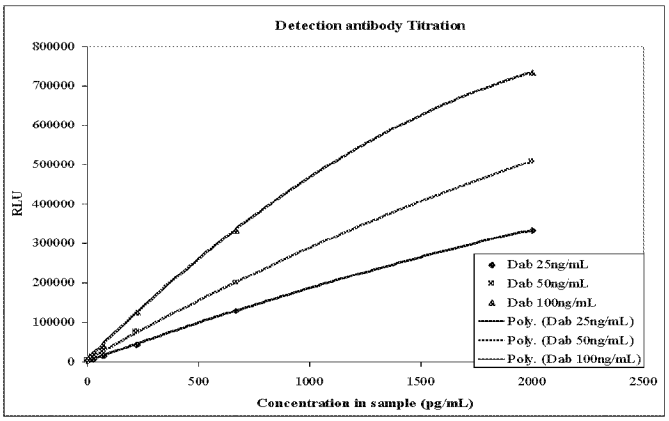


Figure [SEQ Figure * ARABIC]: Detection antibody titration plot

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

	100 ng/mL			**50 ng/mL**			**25 ng/mL**		
Analyte pg/mL	Avg	Stdev	%CV	Avg	Stdev	%CV	Avg	Stdev	%CV
1000	974613	24901	3	792041	174937	22	603438	165794	27
400	556237	68790	12	393570	118998	30	290491	99463	34
160	234449	25840	11	138475	8686	6	141989	41686	29
64	93201	9276	10	57315	6790	12	55873	18619	33
26	60189	23441	39	22389	913	4	25416	9050	36
0	10872	7588	70	2899	374	13	2730	2375	87
	S/B_Std 1/6	90			273			221	
	S/B_Std 5/6	5.5			7.7			9.3	
	AvgCV	24			15			41	
	Slope	1253			855			827	
	Avg stdev	13435			2692			10015	
	LOD, pg/mL	21.45			6.30			24.21	

8. Whole blood screen

Numerous whole blood samples were screened to check for endogenous Eotaxin levels. A subset of samples was spun down to obtain plasma. The endogenous analyte levels were calculated from an assay buffer standard curve.

Table [SEQ Table * ARABIC]: Whole blood screen

Sample	Whole blood	Plasma
	pg/mL	pg/mL
1	334	48
2	665	125
3	243	73
4	524	85
5	516	174
6	272	42
7	753	90
8	646	94
9	672	
10	1070	
11	473	
12	878	
13	692	
14	750	
15	879	
16	912	
17	1029	
18	818	
19	1164	
20	1044	
21	857	
22	540	
23	941	
24	1092	

9. Spike recovery from Whole blood

Eotaxin was spiked at 5 levels ranging from 1000 – 25 ug/mL, into human whole blood that had an endogenous level of 116 pg/mL. The endogenous level of the sample and percent recovery of spiked sample was calculated from an assay buffer standard curve. The recovery ranged from 81 -100%, with an average of 91%.

Table [SEQ Table * ARABIC]: Eotaxin spiked into whole blood data

Spike into Whole blood						
Calibrator#	**Nominal pg/ml**	**Average**	**Stdev.**	**CV%**	**Calc. pg/ml**	**% Recovery**
1	1116	537951	30422	6	918	82
2	516	345678	29320	8	471	91
3	276	202024	12540	6	224	81
4	180	170973	20418	12	180	100
5	141	140211	2696	2	140	99
6	116	120584	11270	9		
					Average	91

10. Plasma from spiked whole blood

The spiked whole blood was spun down to obtain plasma and tested to estimate hematocrit effect. The recovery of the analyte in plasma is lower than whole blood indicating that the analyte remained in the fraction with the red blood cells.

Table [SEQ Table * ARABIC]: Plasma from spiked whole blood data

Plasma from spiked blood						
Calibrator#	**Nominal pg/ml**	**Average**	**Stdev.**	**CV%**	**Calc. pg/ml**	**% Recovery**
1	1116	328058	30755	9	436	39
2	516	199529	14810	7	220	43
3	276	103764	2121	2	97	35

4	180	85363	9574	11	78	43
5	141	78434	4773	6	71	50
6	116	64853	3203	5	57	49
					Average	42

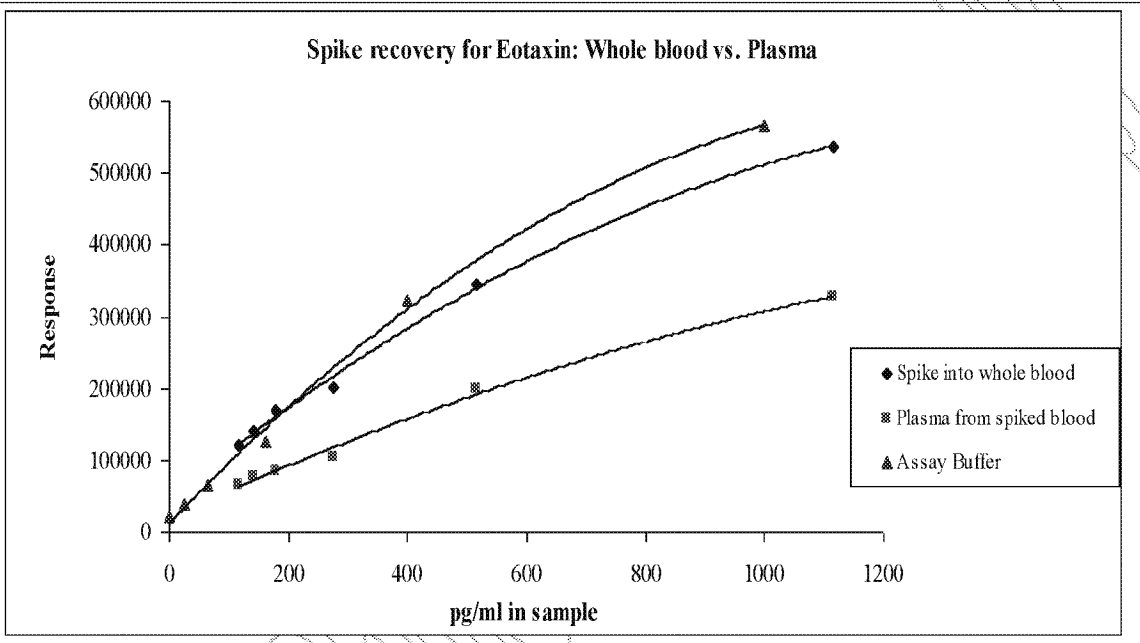


Figure [SEQ Figure * ARABIC]: Spiked recovery graph for whole blood and plasma sample

11. Eotaxin Spiked into Plasma

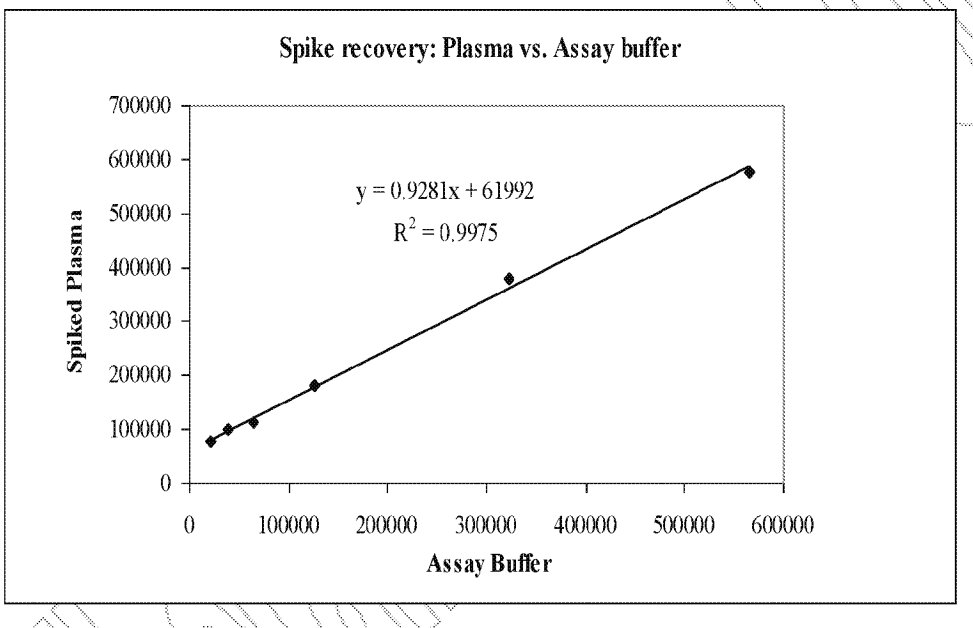
Eotaxin was spiked at 5 levels ranging from 1000 – 25 ug/mL, into plasma that had an endogenous level of 70 pg/mL. The endogenous level of the sample and percent recovery of spiked sample was calculated from an assay buffer standard curve. The recovery ranged from 80 -114%, with an average of 95%.

Table [SEQ Table * ARABIC] : Eotaxin spiked into plasma data

Spike into Plasma						
Calibrator#	**Nominal pg/ml**	**Average**	**Stdev.**	**CV%**	**Calc. pg/ml**	**% Recovery**
1	1070	577823	44640	8	1028	96
2	470	377673	20682	5	536	114
3	230	181616	5885	3	194	85

4	134	112396	7528	7	107	80
5	95	99774	9579	10	93	98
6	70	78776	6739	9		
					Average	95

Figure [SEQ Figure * ARABIC]: Eotaxin spike recovery data



12. Extended Assay range

High levels of Eotaxin beyond the regular range of the assay were spiked into whole blood (with a Eotaxin level of 211 pg/mL), and the recovery was measured to determine the upper range of the assay. Recoveries of spikes up to 6400 pg/mL were consistent with the normal assay range.

Nominal concentration = Endogenous level + spiked analyte.

Table [SEQ Table * ARABIC]: Assay buffer calibration curve data

Assay Buffer curve						
Std.	**Nominal pg/mL**	**Average**	**Stdev.**	**%CV**	**Calc. pg/mL**	**% Recovery**
1	6250	1870439	117166	6	6244	100
2	2500	1354727	71653	5	2523	101

3	1000	831232	26164	3	964	96
4	400	357429	46592	13	409	102
5	160	174072	21518	12	199	125
6	64	78925	10170	13	62	96
7	26	52823	2571	5	19	74
8	0	29154	3867	13	0	
					Average	99

Table [SEQ Table * ARABIC]: Analyte spiked into whole blood data

Spiked whole blood						
Std.	**Nominal pg/mL**	**Average**	**Stdev.**	**%CV**	**Calc. pg/mL**	**% Recovery**
1	6461	1882851	44953	2	6371	99
2	2711	1192745	114802	10	1861	69
3	1211	712997	28946	4	790	65
4	611	412427	29068	7	465	76
5	371	239660	5975	2	281	76
6	211	183178	7889	4	211	100
					Average	81

13. Analyte Validation

The analyte used in the Theranos assay was validated against a commercially available reference.

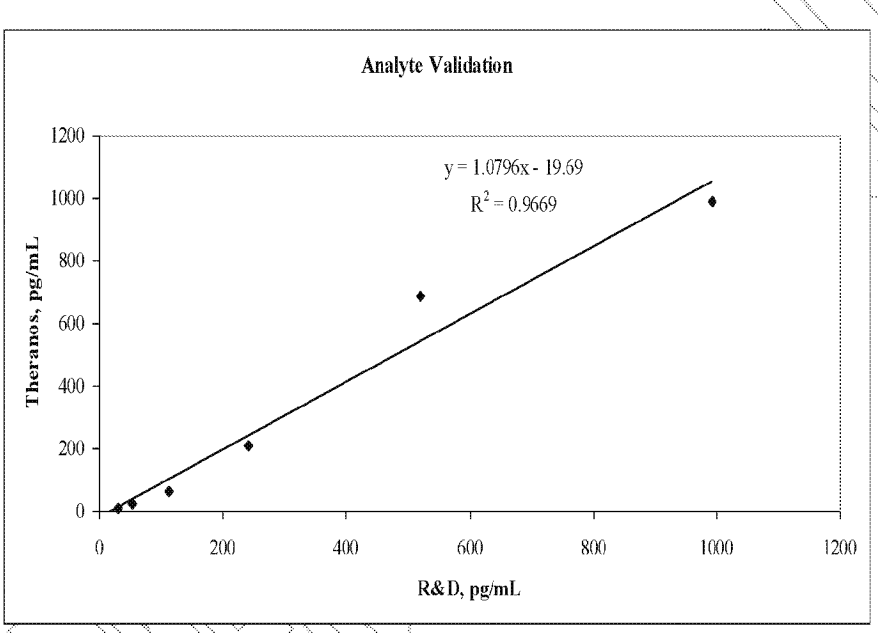


Figure [SEQ Figure * ARABIC]: Analyte correlation graph

14. Assay Validation

The assay was validated by testing Clinical asthma serum samples (N=10) on the Theranos system and the reference assay from R&D systems with the following results: Theranos (y) = 1.0563* Reference method (x) – 5.0385; R^2 = 0.842.

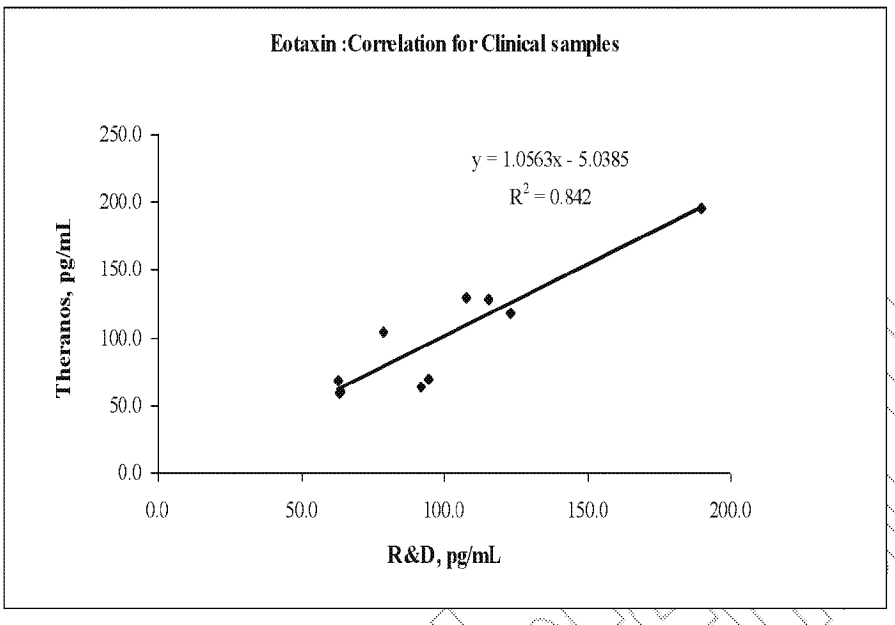


Figure [SEQ Figure * ARABIC]: Clinical samples correlation graph

15. Precision

A 6-point assay buffer standard curve was assayed on 12 instruments in replicate on each instrument for three reagent lots.

Ave. Intra CV: 3 %

Ave. Total CV: 26%

Ave. Day-Day CV: 12%

Mid-point of calibrator (160 pg/mL) was assayed on 24 cartridges across 24 instruments to determine the mid-range CV.

Ave. Intra CV: 7%

Ave. Total CV: 12 %

16. Dilution Linearity

Dilution linearity of the assay was tested by spiking into serum (with no detectable Eotaxin) at 1000 pg/mL and serially diluting the sample to yield sample concentrations within the dynamic range of the assay. Recovery ranged from 98 - 117 %. Percent recovery = 100*(calculated conc. of standard) / (calculated conc. of Std1* (Nominal conc. of Std.)/(Nominal conc. of Std1)).

Table [SEQ Table * ARABIC]: Dilution linearity data

Dilution Linearity						
Std.	**Nominal pg/ml**	**Average**	**Stdev.**	**CV%**	**Calc. pg/ml**	**% Recovery**
1	1000	744414	80809	11	1000	100
2	400	341865	7551	2	401	100
3	160	155568	16699	11	157	98
4	64	79630	2419	3	64	101
5	25.6	51404	3163	6	30	117
6	0	24725	957	4	0	
					Average	103

17. Matrix effects

17.1 Spike into Lipemic Plasma

Eotaxin was spiked at 5 levels ranging from 1000 – 25 pg/mL into pooled lipemic human serum with undetectable levels of analyte. The recovery was estimated with an assay buffer calibration curve. The recovery ranged from 67 – 105%, with an average of 90%.

Table [SEQ Table * ARABIC]: Matrix effects data

Calibrator	Nominal pg/mL	Average	Stdev	%CV	Calc. pg/mL	% Recovery
1	1000	1170426	8846	1	944.9	94
2	400	617053	28064	5	392.3	98
3	160	319631	20945	7	168.3	105
4	64	114071	12587	11	43.2	67
5	25.6	74167	4603	6	21.7	85
6	0	22004	4018	18	0.0	
					Average	90

18. Stability

18.3 Capture antibody surface stability

Stability will be tested for a period of 12 weeks for storage at 4°C and room temperature with a 4-point assay buffer curve. Analyte standards were pre-made for the entire study, aliquoted and flash frozen for single time use. A freshly made working concentration of detection antibody is made for each time point.

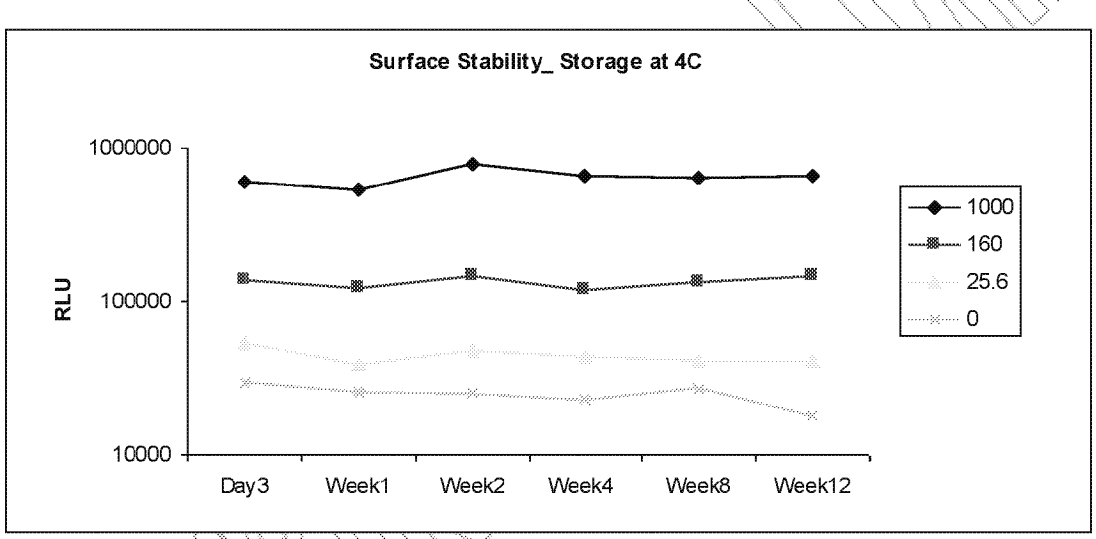


Figure [SEQ Figure * ARABIC]: Capture Antibody stability at 4C

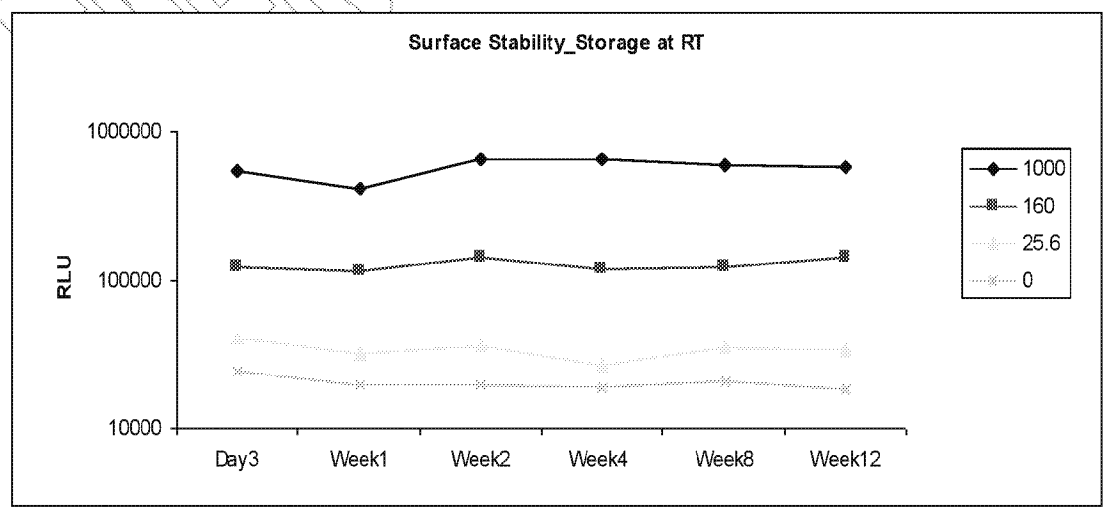


Figure [SEQ Figure * ARABIC]: Capture antibody stability at RT

18.4 Reference Surface Stability

Reference or on-board instrument assay controls stability will be tested for a period of 12 weeks for storage at 4°C and room temperature. Two levels of analyte, 400 and 64 pg/mL were used.

Table [SEQ Table * ARABIC]: Reference / Assay controls stability data

Temp	Eotaxin	Weeks		
	pg/mL	0.4	1	2
RT	400	1291674	1059796	1182002
	64	332032	280259	277864
4C	400	1254971	1052110	1246313
	64	333440	275469	306873

18.5 Detection antibody Stability

Detection antibody stability at working concentration will be tested for a period of 12 weeks for storage at 4°C and room temperature in appropriate Alkaline Phosphatase stabilizer, with a 4-point assay buffer curve. Analyte standards were pre-made for the entire study, aliquoted and flash frozen for single time use.

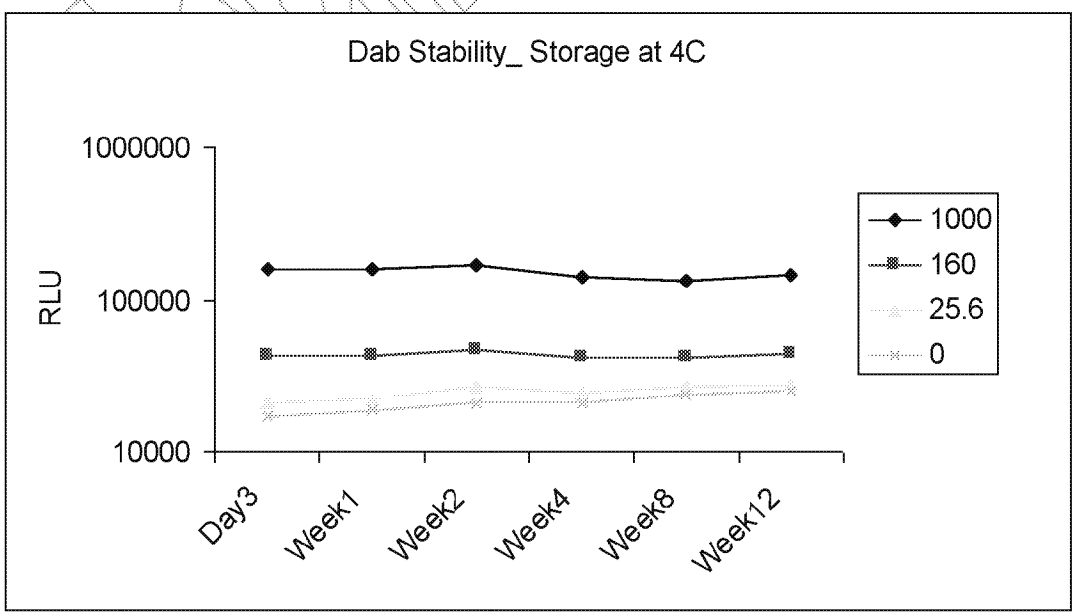


Figure [SEQ Figure * ARABIC]: Detection Antibody stability data at 4C

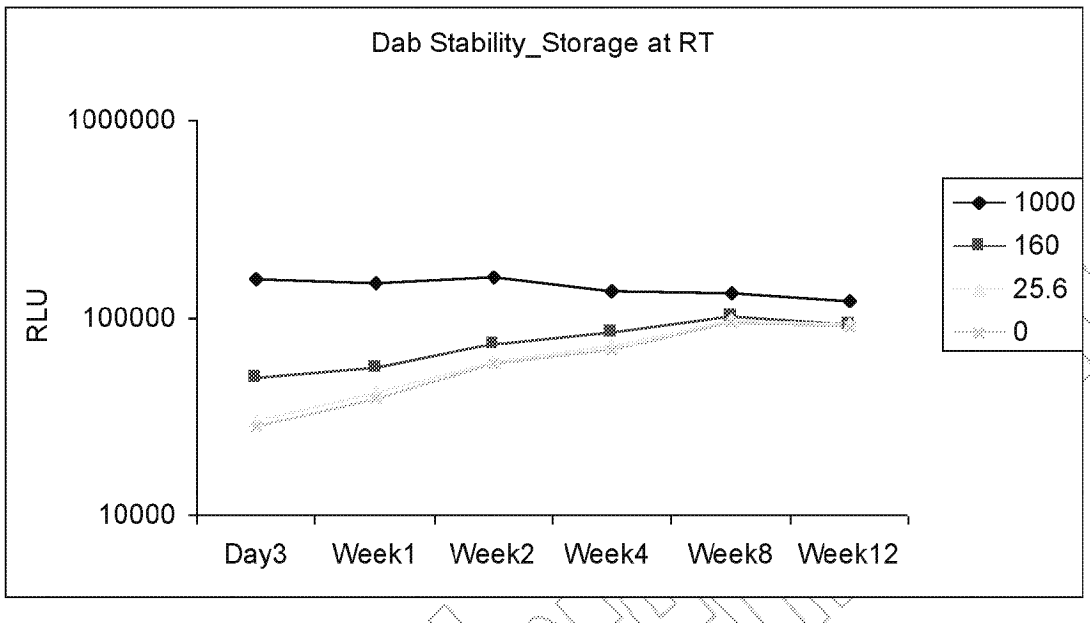


Figure [SEQ Figure * ARABIC]: Detection Antibody stability data at RT

19. Calibration

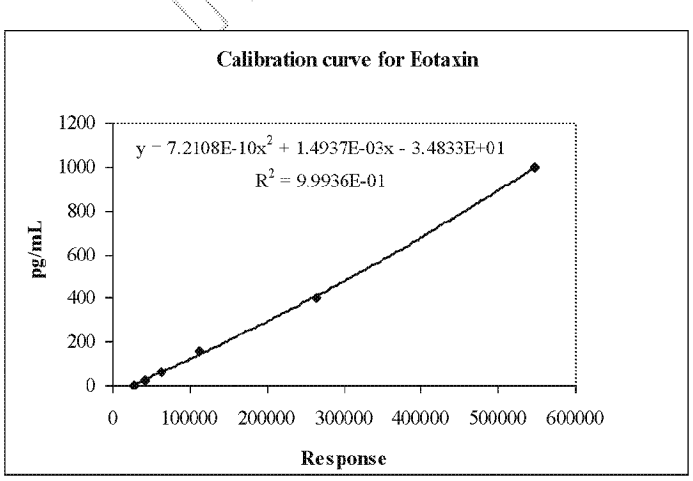
Calibration was carried out on a 6-point standard curve (1000-26 pg/ml in sample) in assay buffer on duplicate cartridges. Calibration for this exercise is traceable originally to a commercial source of Eotaxin, see section on Analyte validation.

Table [SEQ Table * ARABIC]: Calibration Curve data

Std	Nominal pg/ml	Average	Stdev.	%CV	Calc. pg/mL	% Recovery
1	1000	547208	54708	10	998	100
2	400	263944	6456	2	410	102
3	160	112550	8415	7	142	89
4	64	63378	4180	7	63	98
5	25.6	41688	1315	3	29	112
6	0	28034	4301	15		

Average %CV	18
Slope	533
Avg. Stdev	2808
LOD, pg/mL	10.5

Figure [SEQ Figure * ARABIC]: Calibration curve plot



20. Clinical samples for Customers

20 Customer samples were analyzed on the Theranos system with the Calibration specified above and the following results were obtained.

Table [SEQ Table * ARABIC]: Clinical samples for customers data

Sample #	1	2	1	2	Ave.	Stdev.	Total CV%	Theranos Calc. Conc.pg/ml
1	21097	27967	28694	29177	26734	3791	14	6
2	64164	63849	57183	57761	60739	3782	6	59
3	52746	49619	48184	50190	50185	1905	4	42
4	38401	38833	44875	38352	40115	3181	8	26
5	42340	44001	42283	39141	41941	2030	5	29
6	62192	68035	70677	72099	68250	4376	6	70
7	23164	21617	19785	20801	21342	1428	7	0
8	22290	20481	18487	16349	19402	2560	13	0
9	114686	120437	80687	104591	105100	17544	17	130
10	64194	74514	55400	56763	62718	8762	14	62
11	86714	93912	89823	94069	91129	3540	4	107
12	43912	44114	38123	38256	41101	3364	8	28
13	29973	36119	36473	36582	34787	3215	9	18
14	48570	42340	41539	45068	45455	4405	10	35
15	26611	26464	25590	23491	25539	1438	6	4
16	63179	62897	55742	68551	62592	5256	8	61
17	152384	152952	154222	156620	154044	1881	1	212
18	67446	65667	74355	79408	71719	6350	9	76
19	47146	48268	53664	46147	48806	3352	7	40
20	109599	113119	120407	125909	117258	7315	6	150

21. Conclusions

We have successfully developed an immunoassay to detect Eotaxin (CCL11) in human serum, plasma and whole blood.