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IgE (In-house Binder) Assay Development Report

Theranos, Inc.

May 1, 2013

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ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

1.1 Assay Specifications[TC "Assay Specifications" \f C \l "3"]

The assay is designed to quantitatively detect IgE in human whole blood, plasma, and serum, with a reportable range of 25-2025 ng/mL. This assay was previously developed using commercial antibodies. This report describes the re-development of the assay using a new in-house biotinylated Fab fragment as the capture.

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

The following commercial assay has been used in-house as a predicate method:

- Siemens IMMULITE® 2000 Total IgE

The originally developed Theranos assay with commercial capture antibody has also been used as a reference.

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

A biotin-labeled anti-IgE Fab fragment (produced in-house) is coated on an avidin surface and serves as the capture surface. The sample (whole blood, plasma or serum) is diluted and then incubated on the capture surface for 10 minutes, then an alkaline phosphatase(AP)-labeled anti-human IgG antibody is incubated on the surface for 10 minutes. After the detection antibody incubation, the surface is washed and the alkaline phosphatase substrate is incubated on the surface for 10 minutes. The resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Reagent Name	Supplier	Catalog #
Human Immunoglobulin E (IgE, kappa), Plasma	Scripps Laboratories	Cat# I0323, Part# 90469
Anti-Human IgE Biotinylated Fab (C-Ab)	In-house binders group	A10
Mouse Anti-Human IgE Antibody (D-Ab)	Advanced Immunochemicals	3-E1
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13-10
Phospho Glo Substrate	KPL	55-60-04
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
SeaBlock Blocking Buffer	Thermo Scientific	37527

ASSAY DEVELOPMENT
[TC "ASSAY OPTIMIZATION" \F C \L "2"]

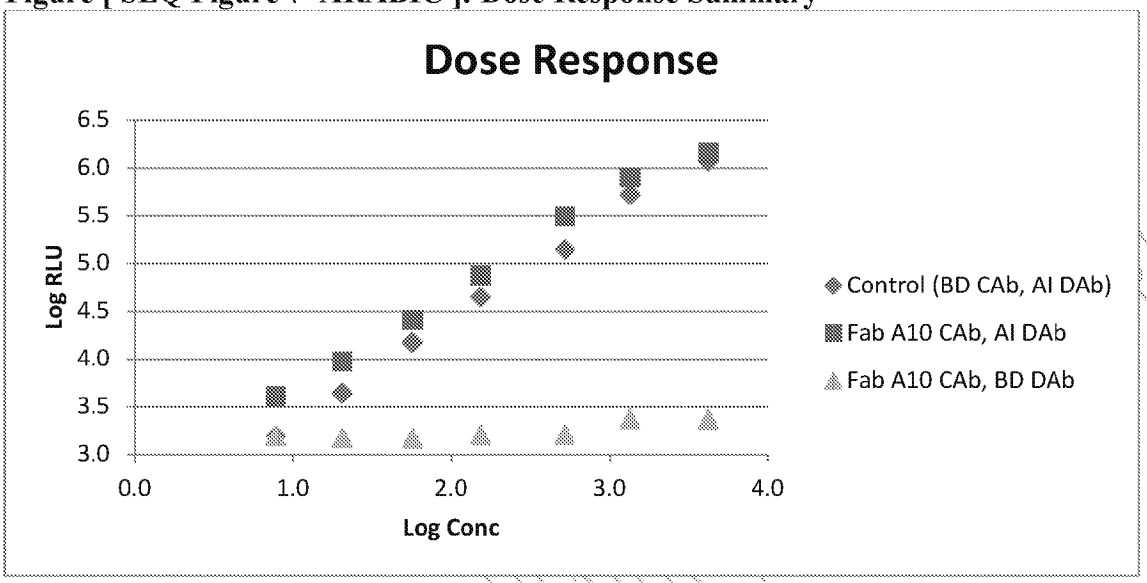
1.2 Initial Theranos Screen

Dose response of calibrators was tested on the Theranos system for the in-house Fab A10 in comparison to the commercial capture antibody. Fab A10 was tested with two different AP-conjugated detection antibodies: one from Advanced Immunochemicals (the originally selected detection for the assay) and one from BD Pharmingen (the originally selected capture for the assay). Aside from changing the antibodies, all conditions were set using the originally developed assay conditions, including the protocol (Generic2_50X). There was no response when using the BD Pharmingen detection antibody. However, the in-house Fab A10 showed good dose response when used with the Advanced Immunochemicals detection antibody.

Table [SEQ Table * ARABIC]: Dose Response

	Commercial CAb (AI DAb)			In-house Fab A10 (AI DAb)			Fab A10 (BD DAb)		
IgE (ng/mL)	Mean RLU	CV	Mod.	Mean RLU	CV	Mod.	Mean RLU	CV	Mod.
4221.6	1170465	13	3559	1432116	20	988	2326	28	1
1353.6	527216	10	1603	795159	19	549	2328	35	1
525.6	138521	7	421	311653	8	215	1610	31	1
153.6	43851	18	133	74397	4	51	1590	33	1
57.1	14683	14	45	25411	25	18	1477	40	1
20.6	4333	11	13	9364	25	6	1487	37	1
7.8	1570	14	5	4078	36	3	1568	37	1
0	329	26	1	1449	31	1	1858	38	1

Figure [SEQ Figure * ARABIC]: Dose Response Summary



[TC "Detection Antibody Conjugate Verification" \f C \l "1"]

1.3 Interference

Interference was tested with human immunoglobulins A, D, G, and M (IgA, IgD, IgG, IgM). The Fab and commercial control capture antibody were tested by spiking a high level of each immunoglobulin into IgE calibrators and running the spiked calibrators in the assay. Interference was minimal for IgA, IgD, and IgM and results were comparable to the commercial CAb control. (See tables 3 and 4). Some interference was seen with IgA in initial testing, however follow-up testing with a different, more pure IgA material showed no interference with IgA. (See table 5).

Table [SEQ Table * ARABIC]: Interference, Commercial CAb

	Ctrl No Spike		IgA 4.6 mg/mL			IgD 200 µg/mL			IgG 20 mg/mL			IgM 4.5 mg/mL		
IgE (ng/mL)	Mean RLU	CV	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl
1500	386113	30	281302	28	73	375922	20	97	410104	14	106	429079	14	111
500	123651	10	88990	13	72	142798	22	115	126857	6	103	147983	18	120
15	3426	38	3418	19	100	2730	75	80	3626	7	106	3985	18	116
0	440	42	1410	23	320	515	22	117	361	40	82	460	28	105

Table [SEQ Table * ARABIC]: Interference, In-house Fab A10

	Ctrl No Spike		IgA 4.6 mg/mL			IgD 200 µg/mL			IgG 20 mg/mL			IgM 4.5 mg/mL		
IgE (ng/mL)	Mean RLU	CV	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl	Mean RLU	CV	% of Ctrl
1500	711270	12	518170	9	73	676393	14	95	748194	4	105	748084	3	105
500	254400	20	169165	14	66	249747	17	98	267707	12	105	286049	10	112
15	6503	59	6201	33	95	4024	73	62	7235	17	111	8063	20	124
0	981	40	3106	29	317	1384	30	141	1053	28	107	956	18	97

Table [SEQ Table * ARABIC]: IgA Interference Follow-up, In-house Fab A10

	Ctrl No Spike		IgA 4 mg/mL		
IgE (ng/mL)	Mean RLU	CV	Mean RLU	CV	% of Ctrl
1500	551089	3	462478	13	84
500	119854	18	132541	6	111
15	4579	7	6182	0	135
0	971	16	1116	2	115

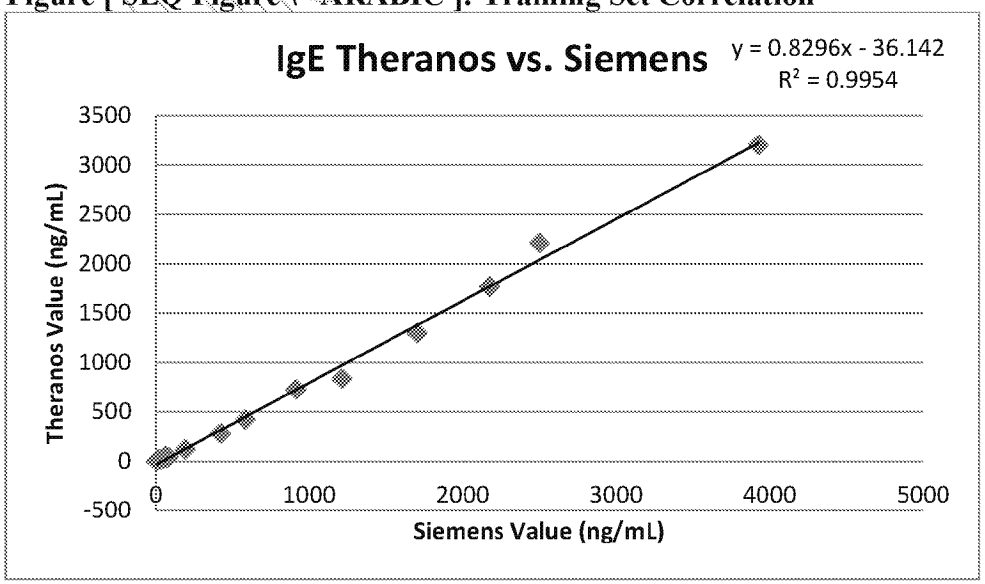
1.4 Training Set

Fifteen serum samples with varying levels of IgE across the range of the calibration curve were tested on the Siemens Immulite 2000 system and in the Theranos assay with Fab A10 as capture. Correlation between the assays was very good, with an R^2 value of 0.9954 and a slope of 0.8296.

Table [SEQ Table * ARABIC]: Training Set

Sample	Mean RLU	CV	Theranos Result (ng/mL)	Siemens Result (ng/mL)
1	1299628	2	3199	3926
2	29140	35	51	71
3	22639	24	40	82
4	180876	18	280	422
5	2324	17	OORL	5
6	1069862	11	2207	2501
7	8888	20	15	23
8	17278	21	30	61
9	9664	31	17	35
10	748124	12	1295	1704
11	931883	5	1768	2174
12	275745	23	422	586
13	73320	21	120	195
14	521160	12	831	1214
15	463012	13	728	914

Figure [SEQ Figure * ARABIC]: Training Set Correlation



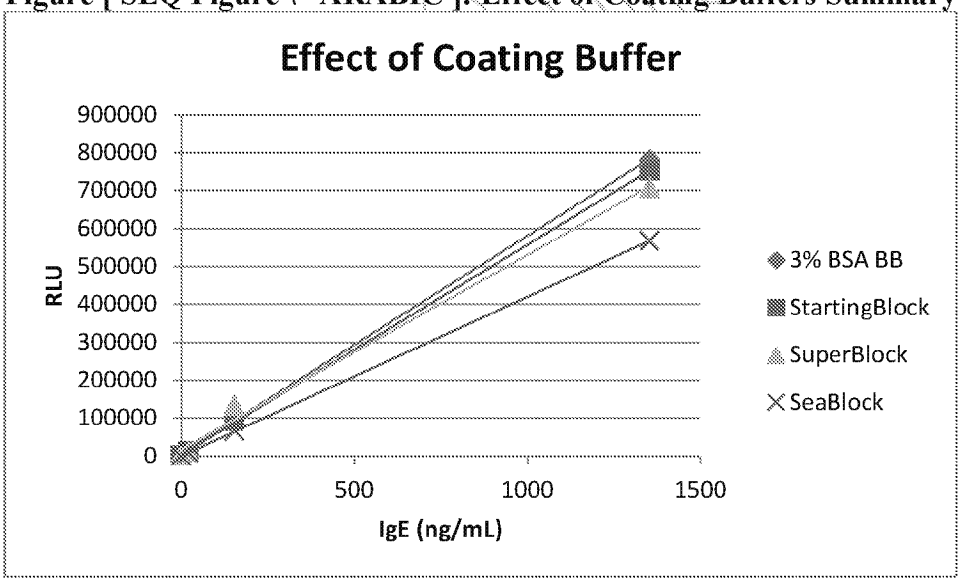
1.5 Effect of Coating Buffers

The in-house Fab A10 was coated on an avidin surface in 3% BSA blocking buffer (made in-house), SuperBlock, StartingBlock and SeaBlock (all commercially available). SeaBlock provided the best CVs in the assay, while maintaining acceptable modulation. This was selected as the coating buffer for the assay.

Table [SEQ Table * ARABIC]: Effect of Coating Buffers

	3% BSA BB			StartingBlock			SuperBlock			SeaBlock		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	782151	21	409	751858	10	339	707593	18	319	567569	16	256
153.6	105624	12	55	92027	42	41	133411	99	60	64554	11	29
20.6	11569	11	6	12335	22	6	9690	28	4	7100	4	3
0	1913	48	1	2220	31	1	1819	34	1	1472	26	1

Figure [SEQ Figure * ARABIC]: Effect of Coating Buffers Summary



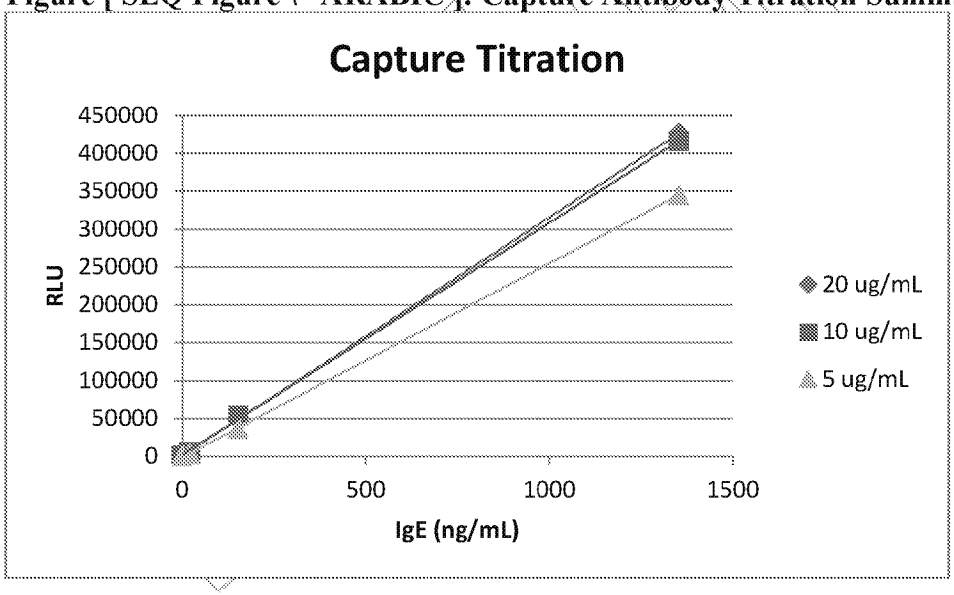
1.7 Capture Antibody Titration

In-house Fab A10 was coated on an avidin surface at 20, 10 and 5 µg/mL. The 5 µg/mL surface provided the most modulation, however CVs were unacceptably high. 10 µg/mL coating concentration was selected for the optimal combination of modulation and CVs.

Table [SEQ Table * ARABIC]: Capture Antibody Titration

	A10 20 µg/mL			A10 10 µg/mL			A10 5 µg/mL		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	426214	6	319	416102	18	456	345443	18	588
153.6	51826	9	39	54238	15	59	36004	17	61
20.6	5355	25	4	5882	11	6	3050	32	5
0	1336	48	1	913	16	1	587	27	1

Figure [SEQ Figure * ARABIC]: Capture Antibody Titration Summary



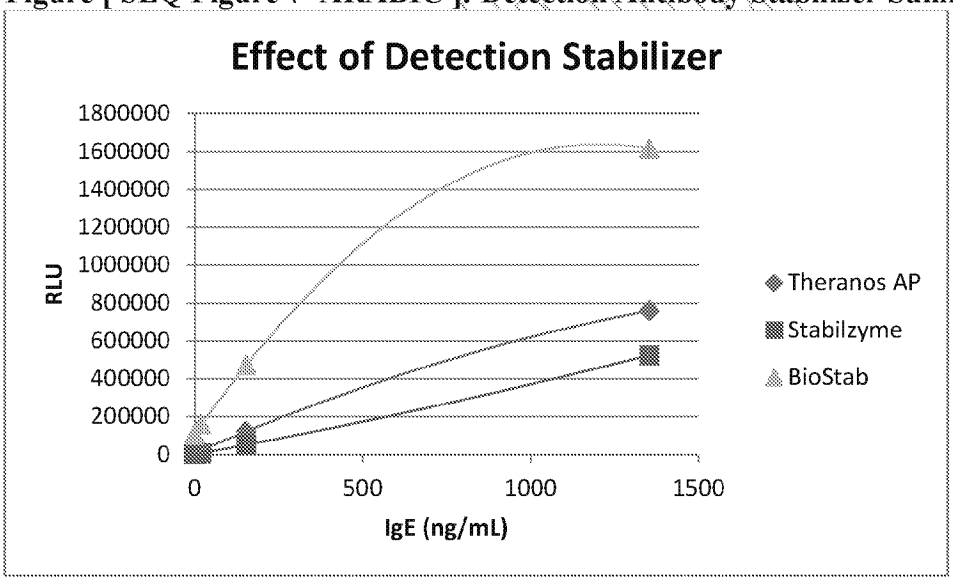
1.8 Effect of Detection Antibody Stabilizer

The detection antibody was diluted in three different AP stabilizer solutions: Theranos in-house, Stabilzyme, and BioStab. BioStab had very high background signal. Stabilzyme provided the best modulation and CVs, while Theranos in-house stabilizer showed potential for improvement with titration. Both Stabilzyme and Theranos in-house stabilizers were moved forward for further testing.

Table [SEQ Table * ARABIC]: Detection Antibody Stabilizer

	Theranos			Stabilzyme			BioStab		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	760148	16	92	525001	15	473	1615345	14	16
153.6	121873	26	15	50938	8	46	471406	7	5
20.6	21023	14	3	5997	20	5	160799	19	2
0	8265	39	1	1110	20	1	104106	19	1

Figure [SEQ Figure * ARABIC]: Detection Antibody Stabilizer Summary



1.9 Detection Antibody Titration

The detection antibody was diluted to 100, 50, and 25 ng/mL in both Stabilzyme and Theranos in-house AP stabilizers. Detection antibody at 50 ng/mL in Stabilzyme had the best modulation and CVs, without excess background or saturation at the top end of the curve. This was set as the detection formulation.

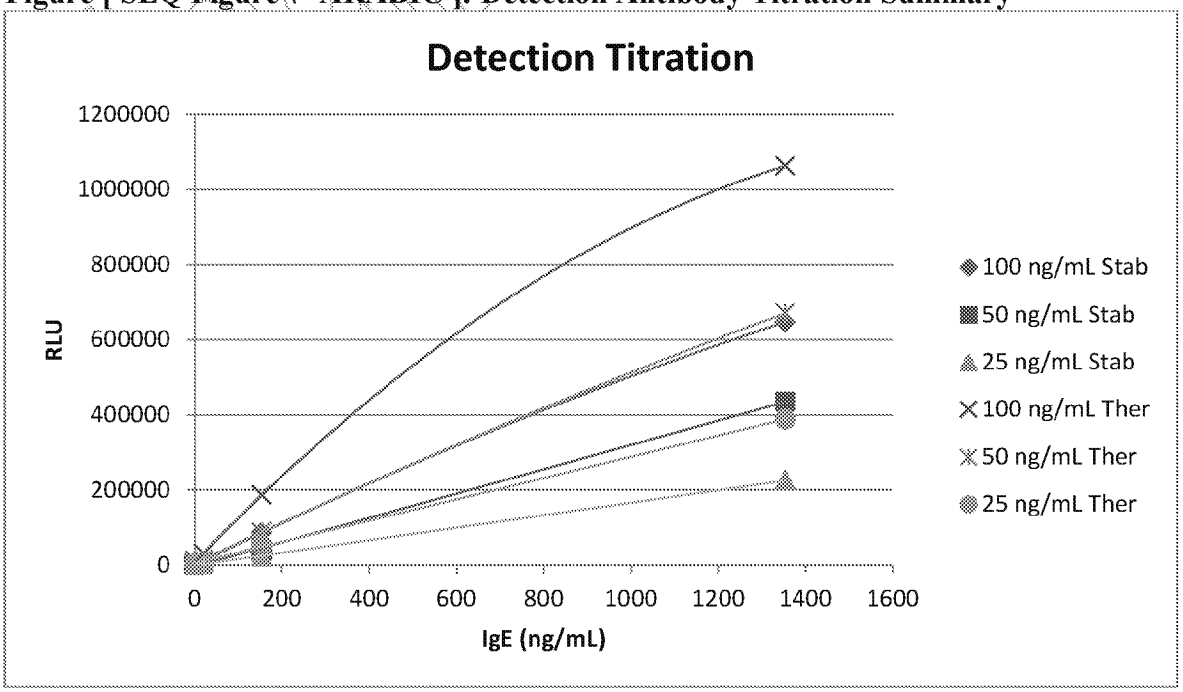
Table [SEQ Table * ARABIC]: Detection Antibody Titration, Stabilzyme

	100 ng/mL			50 ng/mL			25 ng/mL		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	647504	6	400	435050	7	450	225232	17	233
153.6	86322	15	53	39651	17	41	23822	12	25
20.6	9376	20	6	4857	8	5	2594	14	3
0	1619	21	1	967	25	1	584	14	1

Table [SEQ Table * ARABIC]: Detection Antibody Titration, Theranos in-house

	100 ng/mL			50 ng/mL			25 ng/mL		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	1062812	21	92	670757	12	125	388050	17	72
153.6	186382	10	16	88513	19	17	54447	18	10
20.6	30727	31	3	14252	37	3	9251	17	2
0	11573	10	1	5357	33	1	2875	4	1

Figure [SEQ Figure * ARABIC]: Detection Antibody Titration Summary



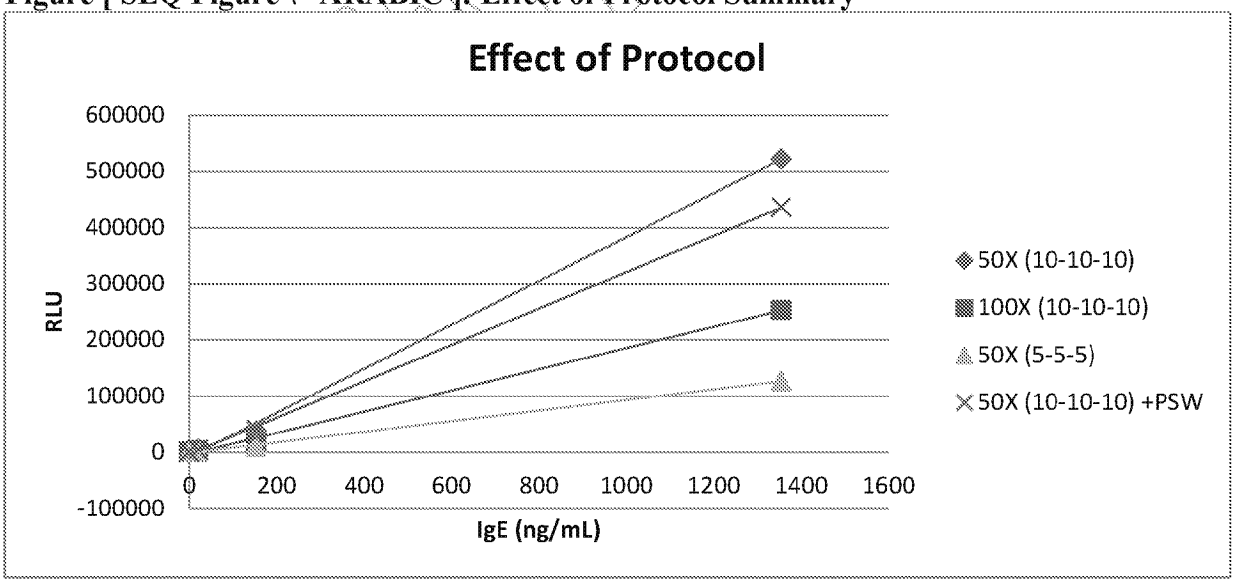
1.10 Effect of protocol (Sample dilution and incubation times)

The effect of adjusting the assay protocol was evaluated. First, incubation time was kept the same (10-10-10 minutes), but a sample dilution of 100X was tested for comparison to the original 50X dilution. There was no advantage to increasing the sample dilution. Next, the incubation times were tested at 5-5-5 minutes. This also showed no advantage, although results were still acceptable if it becomes necessary to reduce incubation time in the future. Finally, a post-sample wash (PSW) was added to the 50X, 10-10-10 protocol. There was no advantage to adding the additional wash step. The protocol was finalized as Generic2_50X.

Table [SEQ Table * ARABIC]: Effect of Protocol

	50X (10-10-10)			100X (10-10-10)			50X (5-5-5)			50X (10-10-10) +PSW		
IgE (ng/mL)	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod	Mean RLU	CV	Mod
1353.6	522888	4	635	252979	13	326	126388	9	241	435670	5	486
153.6	37029	23	45	17910	28	23	10340	18	20	39188	16	44
20.6	5109	15	6	3637	15	5	1400	4	3	4767	20	5
0	824	16	1	775	34	1	524	32	1	896	12	1

Figure [SEQ Figure * ARABIC]: Effect of Protocol Summary



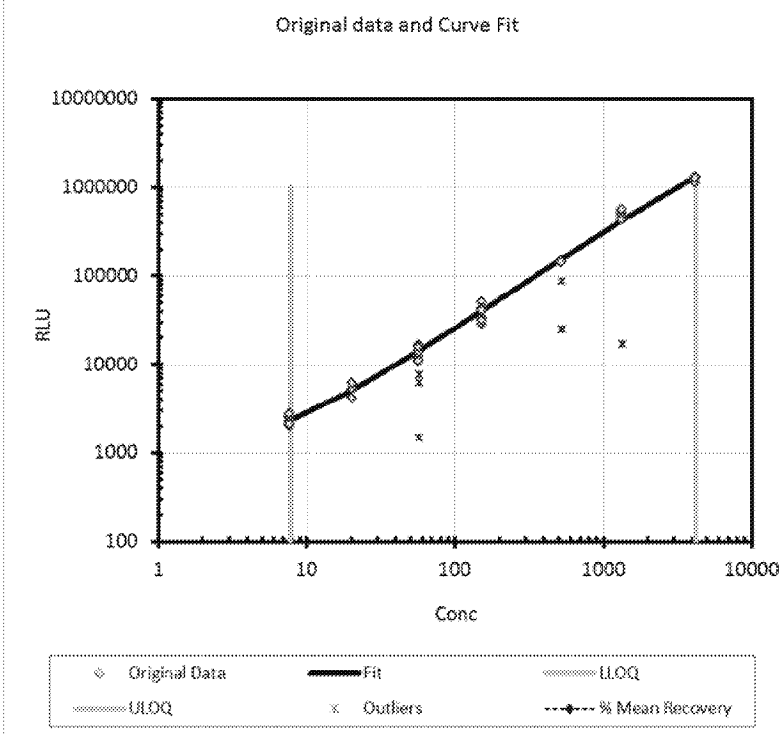
1.11 Determination of Expected LLOQ and ULOQ

The full 8-point calibration curve was run under finalized assay conditions to establish the LLOQ/ULOQ. Target was to meet or exceed the originally developed assay reportable range of 25-2025 ng/mL. The finalized assay with the A10 Fab resulted in a curve with LLOQ of 7.8 ng/mL and ULOQ of 4221.6 ng/mL.

Table [SEQ Table * ARABIC]: Final 8-point Calibration Curve

IgE (ng/mL)	Mean RLU	CV	Modulation
4221.6	1251237	7	1416
1353.6	499973	10	566
525.6	145735	1	165
153.6	38213	22	43
57.1	13888	17	16
20.6	5442	17	6
7.8	2399	12	3
0	884	5	1

Figure [SEQ Figure * ARABIC]: Calibration Curve (LLOQ/ULOQ)



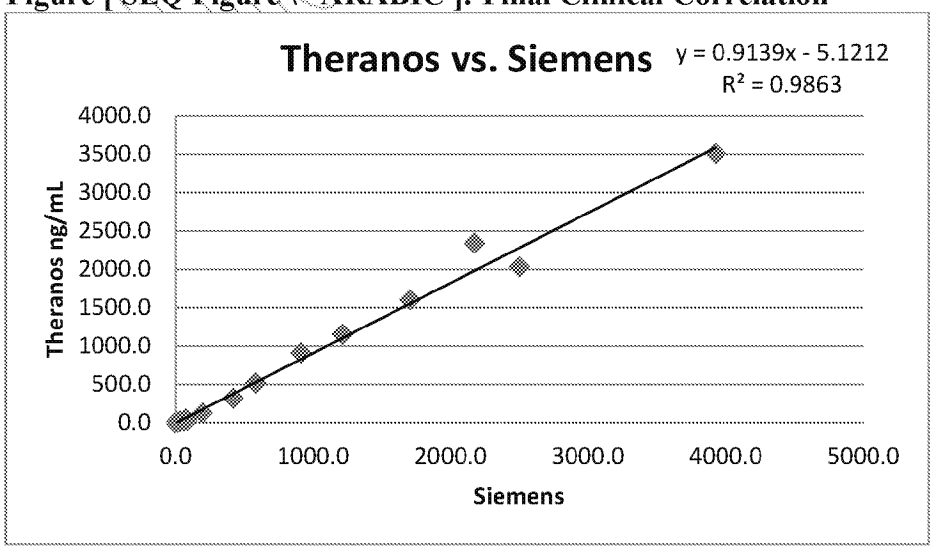
1.12 Final Clinical Correlation

Fifteen serum samples with varying levels of IgE across the range of the calibration curve were tested on the Siemens Immulite 2000 system and in the finalized Theranos assay with Fab A10 as capture. Correlation between the assays was very good, with an R^2 value of 0.9863 and a slope of 0.9139.

Table [SEQ Table * ARABIC]: Final Clinical Correlation

Sample	Mean RLU	CV	Theranos Result (ng/mL)	Siemens Result (ng/mL)
1	1117972	9	3502	3926
2	12124	24	49	71
3	7973	12	32	82
4	89855	20	316	422
5	1407	9	OORL	5
6	652074	12	2026	2501
7	3331	15	12	23
8	6533	18	26	61
9	4941	3	20	35
11	756115	12	2346	2174
12	153270	19	517	586
13	34369	7	131	195
14	360997	7	1147	1214
15	283151	12	913	914

Figure [SEQ Figure * ARABIC]: Final Clinical Correlation



1.12 Reproducibility

Three separate lots of in-house Fab A10 were produced and coated on tips. A full calibration curve and 5 clinical samples were run in the assay on each lot of coated tips. Results were very comparable across all three lots, indicating that the production process is reproducible.

Table [SEQ Table * ARABIC]: Reproducibility, Calibration Curves

	Lot 1		Lot 2		Lot 3	
IgE (ng/mL)	Inter Mean	Inter CV	Inter Mean	Inter CV	Inter Mean	Inter CV
4221.6	1244654	8	1182667	14	1161366	18
1353.6	535815	11	497939	6	536195	5
525.6	162651	27	143218	15	145054	19
153.6	37869	39	37413	18	38536	26
57.1	16254	27	17430	14	17069	14
20.6	4349	17	5261	17	5197	30
7.8	1991	27	2079	12	3092	25
0	1165	35	970	25	1026	25

Table [SEQ Table * ARABIC]: Reproducibility, Clinical Samples

	Measured IgE, ng/mL				
Sample #	Lot 1	Lot 2	Lot 3	Mean	Lot-to-lot CV
1	3006	3176	3560	3247	9
2	53	47	46	49	7
9	14	17	16	16	9
10	1560	1369	1369	1432	8
12	342	363	398	367	8

1.13 Stability Studies

Stability of reagents at was tested to 12 weeks. Four levels of IgE calibrators were run in the assay at each time-point. Results were comparable at all time-points tested, indicating the assay reagents are stable for at least 12 weeks when stored at 4°C.

Table [SEQ Table * ARABIC]: Stability

	Mean RLU				
IgE (ng/mL)	Day 1	Week 1	Week 4	Week 8	Week 12
1500	350765	294867	387092	302003	348345
150	34236	35825	32953	27553	28604
15	5957	6410	4703	5576	4929
0	2134	2262	2547	2382	1697

Figure [SEQ Figure * ARABIC]: Stability