theranos™
redefining healthcare

Anti-Thyroglobulin Antibody (IgG) Assay Development Report

Theranos, Inc.

March 9, 2012

Prepared by: Tina Noyes

TABLE OF CONTENTS

[TOC \o "1-3" \h \z \u]**LIST OF TABLES**

[TOC \h \z \c "Table"]

LIST OF FIGURES

[TOC \h \z \c "Figure"]

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

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

This assay is designed to quantitatively determine the presence of IgG antibodies to human thyroglobulin in human serum, plasma or whole blood (automatically processed into plasma by the Theranos System). This assay is calibrated to the IRP for Anti-Thyroglobulin Antibody (NIBSC code: 65/093) and has a reportable range of 10 – 3000 IU/mL.

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

- None of the tested benchtop ELISA kit methods produced acceptable results.
- The Siemens Immulite instrument should be used as a predicate method.

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

Highly purified human thyroglobulin serves as the capture surface for Auto-antibodies in the sample. After incubation of the appropriately-diluted sample on the capture surface, the surface is washed and then a solution of mouse anti-human IgG detection antibody is incubated on the surface. The surface is washed again. Finally an alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Human Thyroglobulin (highly purified Low IgG)	Genway	GWB-ED1A96
Analyte (Anti-Thyroglobulin Antibody)	NIBSC	65/093
Mouse Anti-Human IgG Antibody	Southern Biotech	9040-01

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

1.2 Antigen Screen

All available tests use purified native human thyroglobulin as a capture surface. This reagent was obtained from 4 sources to compare performance. The four antigens were all coated at 10 ug/mL and DAb#1 was used for the antigen screen on the Theranos 3.0 system. There were no significant differences between the four reagents. Antigen #1 was chosen based on price and reported purity, but the other sources would be equally acceptable as backup sources.

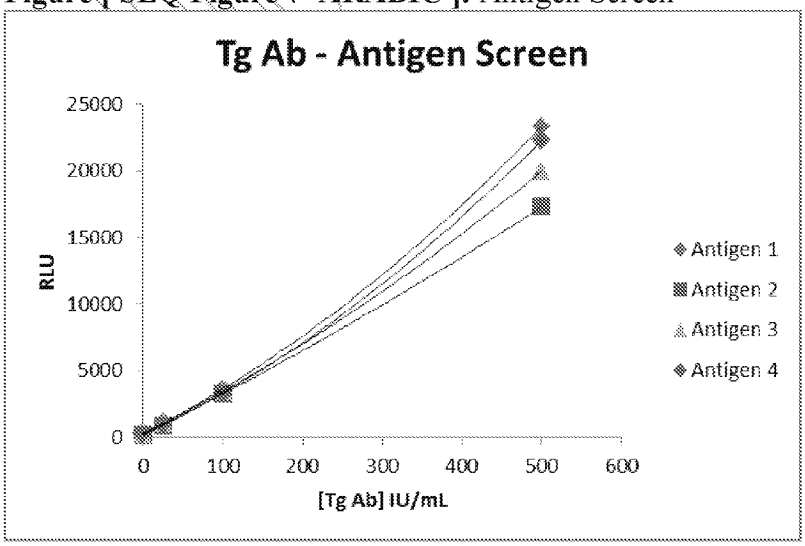
Table [SEQ Table * ARABIC]: Antigen Information

Antigen #	Manufacturer	Cat #
1	Genway	GWB-ED1A96
2	Genway	GWB-24E1F5
3	Fitzgerald	30R-AT006
4	Fitzgerald	30-AT01

Table [SEQ Table * ARABIC]: Antigen Screen (MTP)

[Tg Ab] IU/mL	Antigen 1			Antigen 2			Antigen 3			Antigen 4		
	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.
500	23323	3.2	94	17286	26.8	95	19972	23.7	85	22294	1.3	99
100	3608	17.5	15	3263	12.8	18	3434	13.8	15	3334	15.5	15
25	1134	11.4	5	888	12.3	5	1076	12.9	5	928	8.4	4
0	248	24.8		182	20.1		235	27.1		225	12.1	

Figure [SEQ Figure * ARABIC]: Antigen Screen



1.3 Detection Antibody Screen

Three anti-human IgG detection antibodies were screened – 2 mouse MAbs and one goat PAb. Detection antibody #2 showed the best modulation. DAb #3 also shows good modulation and could provide a backup reagent.

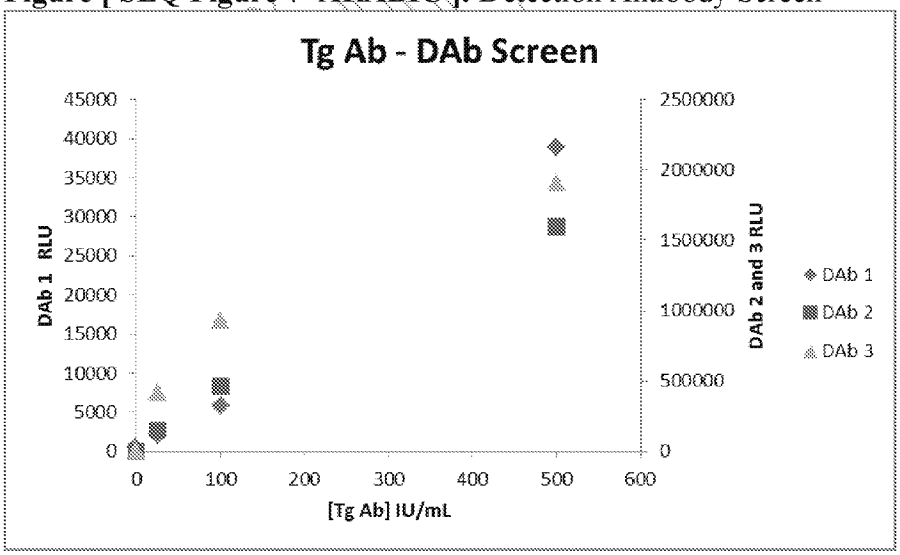
Table [SEQ Table * ARABIC]: Anti-Human IgG Detection Antibody Information

DAb#	Manufacturer	Cat #	Description
1	Novus	NB100-2046	Mouse Anti-Human IgG1 Antibody (Fc)
2	Southern Biotech	9040-01	Mouse Anti-Human IgG Fc (all subclasses)
3	Southern Biotech	2042-04	Goat Anti-Human IgG (HC)

Table [SEQ Table * ARABIC]: Detection Antibody Screen

[Tg Ab] IU/mL	DAb 1			DAb 2			DAb 3		
	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.
500	38909	6.1	85	1599610	6.9	1970	1907692	1.6	589
100	5822	23.6	13	465070	13.4	573	930790	4.4	287
25	2091	16.2	5	148705	11.1	183	417276	18.7	129
0	459	23.2		812	23.7		3239	24.4	

Figure [SEQ Figure * ARABIC]: Detection Antibody Screen



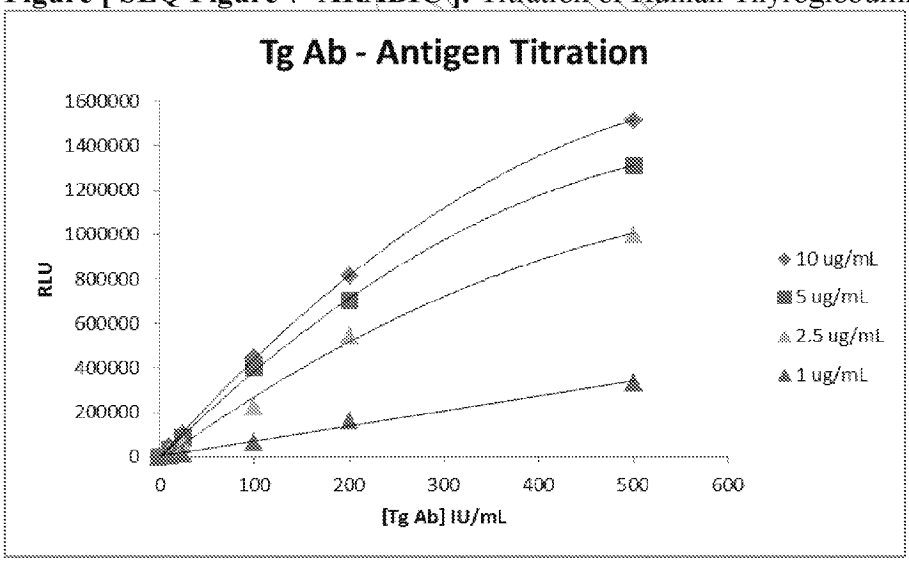
1.4 Surface Titration

The thyroglobulin surface was titrated to obtain the optimal coating conditions. The modulation was highest at 2.5 ug/mL however at 2.5 and 1 ug/mL the low end and background CVs started to increase. Coating at 5 ug/mL gave the best CVs and excellent modulation.

Table [SEQ Table * ARABIC]: Titration of Human Thyroglobulin Surface

[Tg Ab] IU/mL	10 ug/mL			5 ug/mL			2.5 ug/mL			1 ug/mL		
	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.	Mean RLU	CV %	Mod.
500	1513797	10.7	2125	1310004	7.8	3198	1001170	7.0	4324	334654	9.6	1391
200	815823	3.2	1145	704454	4.0	1720	546646	7.7	2361	159249	6.8	662
100	442868	14.1	622	399768	14.6	976	225802	8.1	975	64662	18.4	269
25	103300	5.7	145	88656	4.3	216	53520	24.3	231	14219	5.1	59
10	44439	19.3	62	32142	15.0	78	18084	16.4	78	5850	29.2	24
0	712	17.3		410	22.5		232	28.4		241	67.8	
Mean CV %		**11.7**			**11.4**			**15.3**			**22.8**	

Figure [SEQ Figure * ARABIC]: Titration of Human Thyroglobulin Surface



1.5 Alkaline Phosphatase Stabilizers

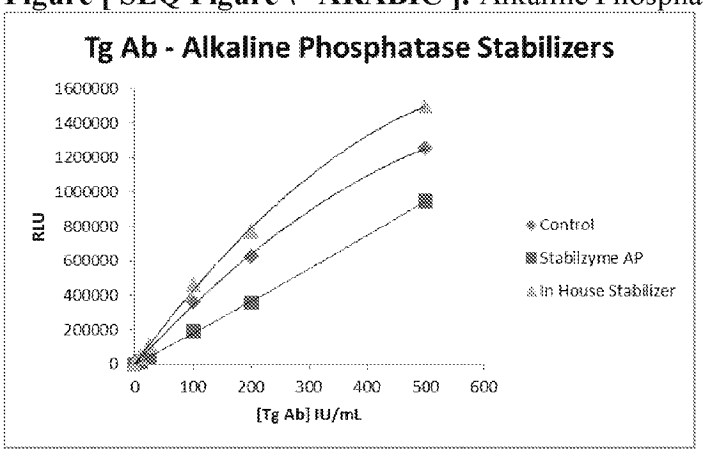
One commercial and one in-house alkaline phosphatase stabilizers were tested as detection antibody diluents, with the DAb at 100 ng/mL. Signal modulation as best with the In-House stabilizer consisting of 5 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 3% BSA in TBS.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizers

DAb Diluent	[Tg Ab] IU/mL	Signal, RLU			Back-Calculated Conc, IU/mL		
		Mean RLU	CV %	Mod.	Mean Conc	CV %	% Recovery
3% BSA (Control)	500	1253720	3.3	3186	508.8	5.2	102
	200	625394	10.8	1589	193.0	13.0	96
	100	362815	8.1	922	104.3	8.3	104
	25	73987	31.7	188	24.5	29.7	98
	10	29087	29.6	74	10.0	32.5	100
	0	393	29.0		0.0		
Stabilzyme AP	500	946937	10.1	3559	502.8	10.3	101
	200	356729	27.3	1341	190.4	26.3	95
	100	189328	2.6	712	104.6	2.4	105
	25	36704	5.0	138	24.5	4.2	98
	10	12431	23.2	47	10.0	18.8	100
	0	266	18.4		0.5		
In House Stabilizer*	500	1500816	4.6	3989	509.4	7.7	102
	200	769184	13.1	2044	193.9	17.5	97
	100	461067	1.6	1225	104.3	1.8	104
	25	104062	6.5	277	24.8	5.8	99
	10	39467	16.7	105	10.0	16.7	100
	0	376	27.1		0.0		

* 5 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 3% BSA in TBS

Figure [SEQ Figure * ARABIC]: Alkaline Phosphatase Stabilizers



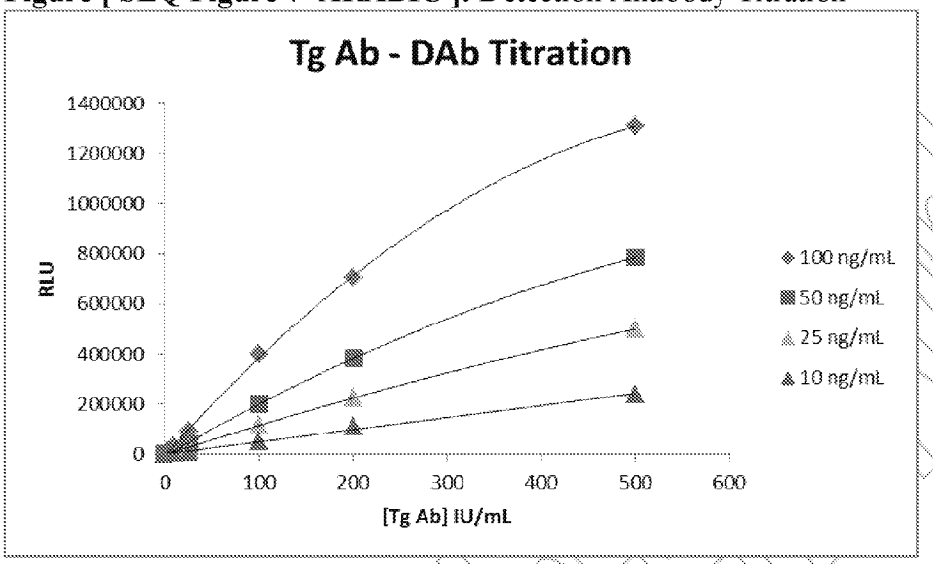
1.6 Detection Antibody Titration

The DAb was titrated in 5 mM Mg^{2+}, 0.1 mM Zn^{2+} and 3% BSA in TBS. The modulation was best with 100 ng/mL, and decreasing the DAb concentration further did not significantly reduce the background. Therefore 100 ng/mL was chosen as the final DAb concentration.

Table [SEQ Table * ARABIC]: Detection Antibody Titration

		Signal (RLU)				Back-Calculated Conc (IU/mL)		
[DAb] ng/mL	**[Tg Ab] IU/mL**	**Mean RLU**	**Log S**	**CV %**	**Mod.**	**Mean Conc**	**CV %**	**% Recovery**
100 ng/mL	500	1310004	6.12	7.8	3198	503.0	13.0	101
	200	704454	5.85	4.0	1720	198.9	5.3	99
	100	399768	5.60	14.6	976	100.9	15.9	101
	25	88656	4.95	4.3	216	25.0	3.6	100
	10	32142	4.51	15.0	78	10.0	15.0	100
	0	410		22.5		0.0		
50 ng/mL	500	783756	5.89	5.8	2532	492.8	8.1	99
	200	383608	5.58	2.0	1239	198.4	2.3	99
	100	198259	5.30	10.3	640	98.7	10.1	99
	25	39921	4.60	17.7	129	24.8	14.2	99
	10	13236	4.12	10.8	43	9.9	9.5	99
	0	310		22.7		0.0		
25 ng/mL	500	499793	5.70	16.3	1193	495.2	20.3	99
	200	224809	5.35	7.7	536	196.6	8.1	98
	100	113441	5.05	7.1	271	99.2	6.7	99
	25	23613	4.37	10.9	56	24.7	9.4	99
	10	8482	3.93	13.6	20	9.9	12.7	99
	0	419		82.2		0.3		
10 ng/mL	500	237566	5.38	9.7	1218	505.3	13.0	101
	200	109169	5.04	9.0	560	202.6	9.3	101
	100	52014	4.72	5.5	267	100.2	4.8	100
	25	9594	3.98	8.9	49	25.1	7.6	100
	10	3615	3.56	18.5	19	10.0	19.5	100
	0	195		10.4		0.1		

Figure [SEQ Figure * ARABIC]: Detection Antibody Titration



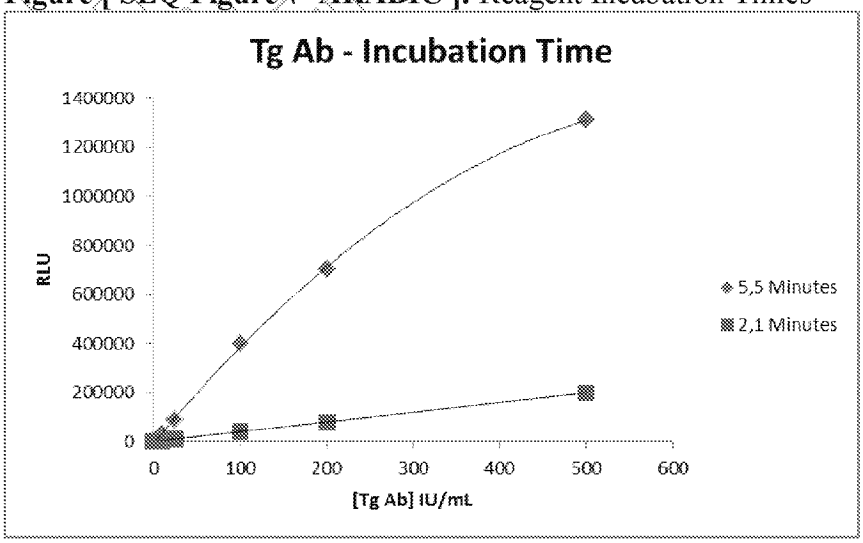
1.7 Reagent Incubation Time

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times respectively of 5, 5, 5 (original condition), and 2, 2, 1 minutes. With 2, 2, 1 minute incubations the modulation fell off and back-calculated concentration CVs were significantly higher. The 5,5,5 minute incubation protocol was chosen as the final condition.

Table [SEQ Table * ARABIC]: Reagent Incubation Time

Incubation Time	[Tg Ab] IU/mL	Signal (RLU)			Back-Calculated Conc (IU/mL)		
		Mean RLU	CV %	Mod.	Mean Conc	CV %	% Recovery
5,5 Minutes	500	1310004	7.8	3198	503.0	13.0	101
	200	704454	4.0	1720	198.9	5.3	99
	100	399768	14.6	976	100.9	15.9	101
	25	88656	4.3	216	25.0	3.6	100
	10	32142	15.0	78	10.0	15.0	100
	0	410	22.5		0.0		
2,1 Minutes	500	197713	24.6	920	465.4	22.8	93
	200	79928	16.0	372	203.1	14.7	102
	100	41719	13.0	194	111.9	11.9	112
	25	7608	18.0	35	23.5	16.5	94
	10	3000	22.4	14	10.0	20.6	100
	0	215	13.7		0.9		

Figure [SEQ Figure * ARABIC]: Reagent Incubation Times



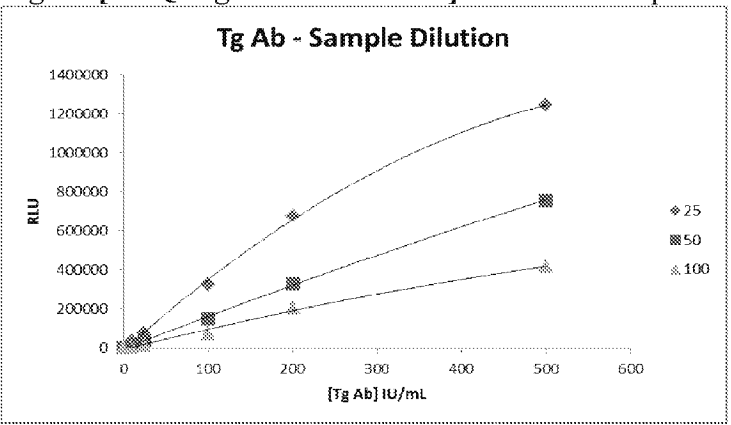
1.8 Sample Dilution

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:50 and 1:100 into 3% BSA in TBS blocking buffer. There was no detriment to precision or accuracy at a sample dilution up to 1:100, and the response was more linear across the projected range of the final assay (up to 3000 IU/mL), therefore 1:100 was chosen as the final sample dilution.

Table [SEQ Table * ARABIC]: Effect of Sample Dilution

Sample Dilution	[Tg Ab] IU/mL	Signal (RLU)			Back-Calculated Conc (IU/mL)		
		Mean RLU	CV %	Mod.	Mean Conc	CV %	% Recovery
25	500	1243623	4.9	2984	485.6	7.8	97
	200	675519	14.5	1621	208.5	18.1	104
	100	325632	14.7	781	94.9	14.0	95
	25	76000	13.3	182	25.2	13.2	101
	10	33329	15.7	80	9.9	21.0	99
	0	417	6.8				
50	500	758974	8.9	1648	483.0	8.7	97
	200	330965	7.7	719	213.3	7.6	107
	100	149060	9.9	324	97.2	9.8	97
	25	37465	15.7	81	25.0	15.5	100
	10	14783	5.4	32	10.0	5.4	100
	0	461	23.1		0.3		
100	500	416070	14.9	817	499.7	24.1	100
	200	206673	5.6	406	207.3	5.4	104
	100	74060	10.2	145	96.4	6.3	96
	25	14250	3.5	28	25.6	4.3	103
	10	7656	12.9	15	9.9	24.2	99
	0	509	17.6		0.0		

Figure [SEQ Figure * ARABIC]: Effect of Sample Dilution



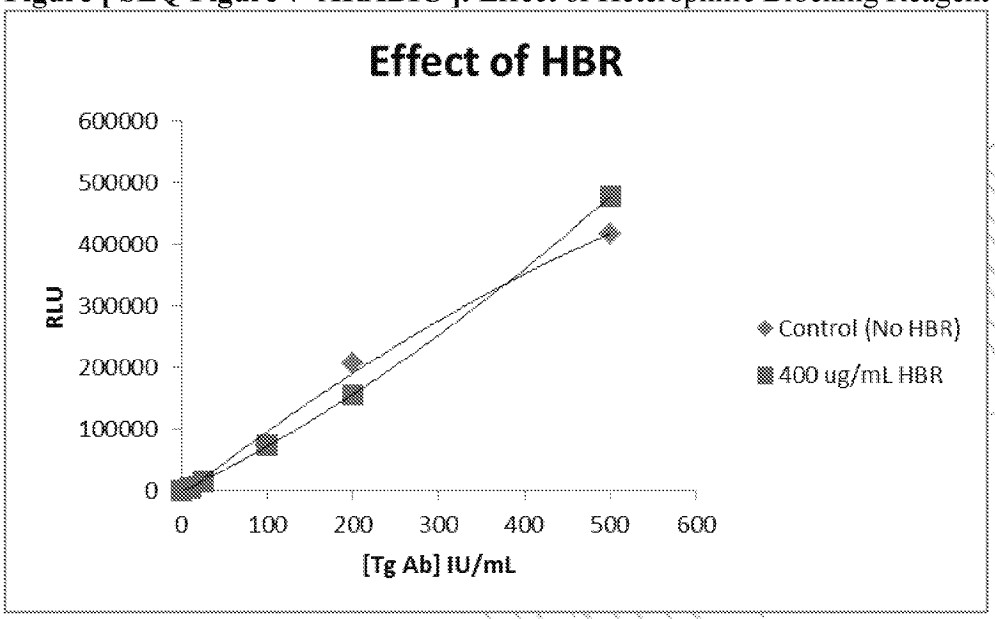
1.9 Heterophilic Blocking Reagent

The effect of Heterophilic Blocking Reagent (HBR) in the 3% BSA in TBS blocking buffer sample diluent was tested using 5 clinical samples with reported values < 20 IU/mL. The addition of HBR did not reduce the signal from any of the negative clinical samples or the mean signal of the negative samples. The modulation of the assay was not hindered by the addition of HBR, so HBR in a sample diluent for multiplex with other antibody assays would be acceptable.

Table [SEQ Table * ARABIC]: Effect of Heterophilic Blocking Reagent

			Signal (RLU)			Back-Calculated Conc (IU/mL)		
Diluent	**Sample Type**	**[Tg Ab] IU/mL**	**Mean RLU**	**CV %**	**Mod.**	**Mean Conc**	**CV %**	**% Recovery**
Control (No HBR)	Buffer Cal	500	416070	14.9	817	499.7	24.1	100
		200	206673	5.6	406	207.3	5.4	104
		100	74060	10.2	145	96.4	6.3	96
		25	14250	3.5	28	25.6	4.3	103
		10	7656	12.9	15	9.9	24.2	99
		0	509	17.6		0.0		
	Negative Clinical	< 20	16658	16.9		30.6		
		< 20	1231	7.7		0.0		
		< 20	883	10.3		0.0		
		< 20	854	11.3		0.0		
		< 20	2150	15.8		0.3		
400 ug/mL HBR	Buffer Cal	500	478519	3.1	1150	508.7	2.7	102
		200	156128	17.3	375	192.9	15.0	96
		100	74377	5.1	179	101.7	4.4	102
		25	14526	6.1	35	24.8	5.3	99
		10	5129	16.4	12	10.1	14.2	101
		0	416	8.7		0.9		
	Negative Clinical	< 20	16638	11.7		27.9		
		< 20	1653	26.5		3.8		
		< 20	1011	18.3		2.5		
		< 20	1467	12.1		3.4		
		< 20	2060	11.1		4.6		

Figure [SEQ Figure * ARABIC]: Effect of Heterophilic Blocking Reagent



1.10 Clinical Samples

A set of 29 clinical sera were obtained from Bioreclamation with reported values for Tg Ab measured on the Siemens Immulite system. A standard curve was used to calibrate the Theranos System from 10 – 3000 IU/mL. An anchor point at 5 IU/mL was included to confirm modulation at the low end.

These samples were also tested in the Genway Anti-Tg Antibody ELISA kit (Cat 40-101-325072). The same kit is marketed by several vendors. The Genway kit is calibrated on a single cutoff calibrator and generates qualitative results in "Antibody index" units. However according to the manufacturer, the Ab Index result can be converted to IU/mL by multiplying the result in Ab Index by 100. The genway kit results in either Antibody Index or IU/mL failed to correlate at all with the reported value or the theranos result, therefore the Genway ELISA was not considered a valid predicate method.

The lack of correlation of the Genway kit with the Siemens Immulite reported results and the Theranos results is not surprizing given that a recent publication concluded that "Despite the availability of an international reference preparation, current antithyroglobulin assays show unacceptable variance." [Taylor, KP et al, 2011].

The Theranos results showed excellent correlation with the Siemens Immulite reported results.

Table [SEQ Table * ARABIC]: Standard Curve

[Tg Ab] IU/mL	Signal, RLU		Back-Calculated Conc., IU/mL		
	Mean RLU	CV %	Mean Conc	CV %	% Recovery
3000	1359866	6.0	2951.0	5.9	98
2000	1014910	10.0	1894.8	16.2	95
1000	665003	5.7	1052.8	7.9	105
500	368223	11.6	515.6	10.5	103
200	135103	5.4	187.2	4.1	94
100	59763	19.4	91.7	17.8	92
50	32613	13.4	55.2	13.7	110
25	13176	13.8	24.8	13.0	99
10	5278	12.8	9.7	14.1	97
5	3055	20.9	5.0	29.8	101
0	449	13.6	0.2	30.4	

Conc = 10^(0.1133*(LOG(S))^3 - 1.5809*(LOG(S))^2 + 8.1908*(LOG(S)) - 13.439)

Figure [SEQ Figure * ARABIC]: Clinical Correlation Theranos to Reported Siemens Immulite Result

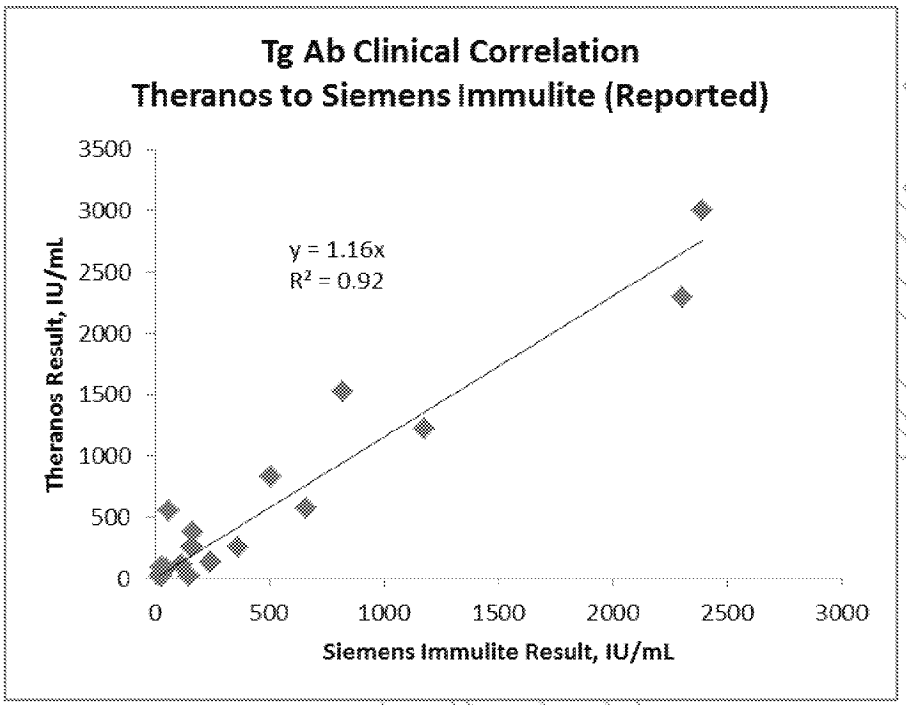


Table [SEQ Table * ARABIC]: Results for Clinical Sera

Tg Ab Results			
Sample ID	**Genway, IU/mL**	**Siemens Immulite, IU/mL**	**Theranos, IU/mL**
Cutoff	**150**	**20**	**20**
BRH527312	19	< 20	32
BRH527313	25	< 20	1
BRH527314	6	< 20	1
BRH527315	15	< 20	2
BRH527316	20	< 20	1
BRH527317	12	< 20	2
BRH527318	34	< 20	17
BRH527319	13	< 20	1
BRH527320	56	< 20	1
BRH527321	10	< 20	1
BRH527322	41	114	115
BRH527323	41	164	375
BRH527324	46	64	550
BRH527325	27	30	39
BRH527326	49	26	92
BRH527327	63	160	256
BRH527328	58	147	17
BRH527329	21	48	79
BRH527330	22	26	19
BRH527331	20	25	23
BRH527332	131	2390	3003
BRH527333	86	821	1526
BRH527334	159	> 3000	OORH
BRH527335	136	2305	2297
BRH527336	64	505	834
BRH527337	97	1178	1222
BRH527338	26	366	256
BRH527339	24	660	569
BRH527340	60	245	136

Legend

Negative
Borderline
Positive

1.11 Specificity

Samples that may potentially cause false positives in Thyroglobulin antibody assays were tested on the Theranos assay to test for specificity.

Three samples low in Anti-Tg antibody but high in Anti-TPO antibody were tested, there was no cross reactivity or false positives observed due to the presence of Anti-TPO antibodies.

Six rheumatoid factor (RF) positive samples were negative in the Theranos assay.

When HAMA positive samples that had been screened on the Siemens Immulite 2000 and found negative for anti-thyroglobulin antibody (< 40 IU/mL) were tested on the Theranos assay, over 50% false positives were observed. Individuals with HAMA can also have a variety of other auto-immune antibodies (in addition to anti-mouse antibodies) that can cross react with other autoimmune antibody detection assays. It was necessary to test blockers in order to address the false positives from HAMA samples.

Table [SEQ Table * ARABIC]: Specificity

Condition	Sample ID	Reported Condition Result	Unit	Siemens Immulite [Tg Ab] IU/mL	Theranos [Tg Ab] IU/mL
TPO Ab +	BRH527318	100	IU/mL	< 20	17.1
	BRH527330	159	IU/mL	26	18.5
	BRH527331	150	IU/mL	25	20.2
RF+	11672304	168	IU/mL	n/a	21.8
	11672321	162	IU/mL	n/a	15.4
	11672652	151	IU/mL	n/a	10.3
	11672657	108	IU/mL	n/a	6.9
	11672658	186	IU/mL	n/a	11.8
	11672680	114	IU/mL	n/a	4.7
HAMA+	10812791	13	ng/mL	< 20.0	70.3
	10697673	13	ng/mL	20.9	65.7
	10697707	29	ng/mL	22.3	153.7
	10669863	22	ng/mL	< 20.0	45.3
	10671270	19	ng/mL	< 20.0	76.0
	10671500	13	ng/mL	< 20.0	36.0
	10697651	19	ng/mL	< 20.0	74.4
	10580297	31	ng/mL	< 20.0	36.0
	10669775	20	ng/mL	< 20.0	19.7
	10669787	30	ng/mL	21.9	67.5
	10669839	19.5	ng/mL	< 20.0	56.2
	10669849	31.6	ng/mL	< 20.0	11.4

1.12 Remedy of HAMA Sample False Positives

1.12.1 Detection Antibody Testing

Alternate anti-human IgG detection antibodies were tested to ensure that the HAMA sample false positives were not a property of the selected MAb detection antibody. The goat anti-human IgG antibody and a different MAb clone that has been successfully used in other IgG assays were tested. HBR-1, rabbit and mouse IgG were tested spiked into the sample diluent. With these detection antibodies and blockers, the HAMA sample false positive rate remained unacceptable.

Table [SEQ Table * ARABIC]: Testing Alternative DAbs for HAMA False positives

Sample ID	Reported HAMA, ng/mL	Siemens Immulite [Tg Ab] IU/mL	Theranos [Tg Ab] IU/mL With Goat DAb	Theranos [Tg Ab] IU/mL With Alternate MAb DAb
10812791	13	< 20.0	66.6	110.5
10697673	13	20.9	67.0	218.9
10697707	29	22.3	130.4	255.7
10669863	22	< 20.0	27.5	161.3
10671270	19	< 20.0	63.5	n/a
10671500	13	< 20.0	20.7	n/a
10697651	19	< 20.0	88.5	238.8
10580297	31	< 20.0	30.5	86.5
10669775	20	< 20.0	17.3	32.0
10669787	30	21.9	45.2	94.1
10669839	19.5	< 20.0	53.6	78.8
10669849	31.6	< 20.0	12.4	8.6

Table [SEQ Table * ARABIC]: Testing Blockers Spiked in Diluent for HAMA False positives

Sample ID	400ug/mL HBR in diluent	2 mg/mL Mouse IgG in Diluent	5 mg/mL Rabbit IgG in Diluent
10812791	104.3	95.1	107.2
10697673	93.7	104.8	121.8
10697707	188.3	277.1	187.8
10669863	47.9	56.7	68.8
10671270	91.3		
10671500	35.0		
10697651	123.4	118.4	157.7
10580297	49.9	46.9	52.3
10669775	21.4	19.5	21.4
10669787	84.9	86.4	80.0
10669839	91.7	61.4	71.5
10669849	13.2	13.9	15.5

1.12.2 Coating Blocker Testing

On a microtitre plate, the thyroglobulin antigen surface was coated and different blockers were tested with 6 of the HAMA positive samples in addition to a standard curve. Some of the blockers used on the coated surface lowered the response from some of the HAMA samples, but none of the blockers resulted in an acceptable rate of false positives.

Table [SEQ Table * ARABIC]: Testing Coating Blockers for HAMA False positives, Results IU/mL (MTP)

Sample ID	Original Theranos System Result	3% BSA in TBS (Control)	Pierce Casein Blocker	Pierce Super Block	Pierce Starting Block	Pierce Sea Block	Abd Serotech Synblock
10812791	70.3	46.9	51.3	55.3	32.0	43.7	57.1
10697673	65.7	47.1	56.0	53.4	84.7	48.8	67.7
10697707	153.7	55.3	53.8	60.9	38.5	58.9	67.5
10669863	45.3	47.6	55.5	47.0	66.5	48.1	52.2
10697651	74.4	63.7	105.1	56.0	50.2	25.0	29.4
10580297	36.0	27.4	32.6	58.2	129.3	34.9	43.2

1.12.3 Diluent Testing

Various blockers were tested as sample diluents in combination with different coating blockers. Starting Block showed the most initial promise in the microtitre plate testing as a sample diluent.

However, in order to multiplex with other assays, it is desirable to dilute the sample first in standard 3% BSA blocking buffer. When Starting Block was used as a secondary sample diluent, its blocking effect was less potent.

After the initial testing, a new product was obtained from Surmodics – a protein free sample diluent specifically formulated to reduce HAMA and RF interference in antibody assays. It was tested with a 1:10 sample dilution in 3% BSA blocking buffer followed by another 1:10 sample dilution into the Surmodics Protein Free buffer to arrive at the final sample dilution of 1:100.

The coating surface was unchanged from the original assay conditions, using 3% BSA in TBS as a coating blocker. With this condition, all of the HAMA samples were reduced to negative levels, without a significant impact on the standard curve dose response. This condition represented the lowest cost and least change to the existing assay conditions and was chosen to test on the Theranos System for confirmation of specificity and clinical correlation.

Table [SEQ Table * ARABIC]: Testing Sample Diluents for HAMA False positives, Results IU/mL (MTP)

Diluent	Sample ID	Coating Blocker: Half-Half Sea/Starting Block	Coating Blocker: Sea Block	Coating Blocker: Starting Block
3% BSA in TBS	10812791	63.3	37.7	34.2
	10697673	91.6	31.3	98.3
	10697707	51.8	43.6	30.5
	10669863	52.0	39.4	56.2
	10697651	131.2	26.2	51.3
	10580297	45.5	26.9	116.3
3% BSA in TBS with 400 ug/mL HBR-1	10812791	29.3	37.7	26.9
	10697673	46.6	34.3	76.8
	10697707	30.2	46.8	29.8
	10669863	38.4	50.0	70.0
	10697651	64.3	27.2	46.6
	10580297	29.8	28.1	136.9
Starting Block	10812791	28.4	31.6	21.6
	10697673	34.4	37.7	n/a
	10697707	32.6	38.2	27.7
	10669863	34.4	51.4	34.9
	10697651	19.0	23.8	17.2
	10580297	21.2	24.2	27.5
Starting Block with 400ug/mL HBR-1	10812791	29.8	33.0	21.2
	10697673	35.1	37.7	29.5
	10697707	35.1	40.4	25.3
	10669863	35.0	42.3	29.2
	10697651	23.3	31.4	19.3
	10580297	24.1	24.6	29.8
Sea Block	10812791	45.6	43.8	25.6
	10697673	104.2	26.3	128.3
	10697707	54.7	59.3	26.7
	10669863	78.9	58.6	109.6
	10697651	83.7	15.8	33.7
	10580297	49.5	25.6	278.9
Sea Block with 400ug/mL HBR-1	10812791	39.1	41.5	24.0
	10697673	124.0	33.7	163.0
	10697707	47.0	56.2	26.9
	10669863	69.3	52.9	88.9
	10697651	116.3	19.4	59.9

	10580297	87.0	33.4	236.7

Table [SEQ Table * ARABIC]: Testing Sample Diluents for HAMA False positives, Results IU/mL (MTP)

	Coating Blocker 3% BSA in TBS Sample Diluent:	
Sample ID	**1:10 in 3% BSA, then 1:10 in Starting Block**	**1:10 in 3% BSA, then 1:10 in Surmodics Protein Free**
10812791	75.4	28.7
10697673	76.8	28.1
10697707	94.1	37.9
10669863	67.5	33.4
10697651	32.7	13.3
10580297	72.8	29.3

1.13 Specificity with Final Assay Conditions

To confirm that the problem seen with false positives in HAMA samples has been remedied satisfactorily, a new standard curve was generated using the final assay conditions on the Theranos 3.0 System and the specificity testing was repeated along with the clinical samples. With the new assay conditions using a 1:10 sample dilution into 3% BSA blocking buffer followed by a 1:10 dilution into Surmodics Protein Free sample diluent, there were no false positives in any RF positive or HAMA positive samples. The sensitivity of the assay and the dose response were unaffected by the use of this sample diluent.

Standard Curve

	Signal, RLU		Back-Calculated Concentration, IU/mL		
[Tg Ab] IU/mL	**Mean RLU**	**CV %**	**Mean Conc**	**CV %**	**% Recovery**
3000	923411	11.0	2922.3	18.5	97
2000	783396	16.4	1905.7	15.4	95
1000	487362	23.8	1114.2	18.8	111
500	217693	11.9	449.0	11.3	90
200	96717	29.0	206.9	14.0	103
100	50707	18.2	115.2	17.0	115
50	15355	29.1	39.7	28.6	79
25	9465	18.8	25.8	7.9	103
10	3587	19.5	10.5	13.2	105
5	1824	14.5	5.3	9.4	106
0	380	23.5	OORL		

Calibration Equation:

Conc = 2133.439 * (((1552408.712 - b1) / (S - 382.880)) - 1) ^ (1 / -1.164)
Signal Min = 1422
Signal Max =98,7963

Table [SEQ Table * ARABIC]: Specificity with Final Assay Conditions

Condition	Sample ID	Reported Condition Result	Unit	Siemens Immulite [Tg Ab] IU/mL	Theranos [Tg Ab] IU/mL
TPO Ab +	BRH527318	100	IU/mL	< 20	14.6
	BRH527330	159	IU/mL	26	9.9
	BRH527331	150	IU/mL	25	9.7
RF+	11672304	168	IU/mL	n/a	4.8
	11672321	162	IU/mL	n/a	5.0
	11672652	151	IU/mL	n/a	< 5.0
	11672657	108	IU/mL	n/a	8.8
	11672658	186	IU/mL	n/a	4.2
	11672680	114	IU/mL	n/a	< 5.0
HAMA+	10812791	13	ng/mL	< 20.0	9.8
	10697673	13	ng/mL	20.9	6.4
	10697707	29	ng/mL	22.3	13.0
	10669863	22	ng/mL	< 20.0	8.2
	10671270	19	ng/mL	< 20.0	7.7
	10671500	13	ng/mL	< 20.0	7.9
	10697651	19	ng/mL	< 20.0	5.8
	10580297	31	ng/mL	< 20.0	4.4
	10669775	20	ng/mL	< 20.0	7.0
	10669787	30	ng/mL	21.9	10.7
	10669839	19.5	ng/mL	< 20.0	10.4
	10669849	31.6	ng/mL	< 20.0	< 5.0

1.14 Clinical Results with Final Assay Conditions

A set of 29 clinical samples were tested in the Theranos System under the final assay conditions. These samples had reported values from Bioreclamation using the Siemens Immulite 2000 and were confirmed in house on the same instrument.

The Theranos System 3.0 results correlated very well with the reported and confirmed Siemens Immulite results. The correlation was plotted for all samples within range for both assays, and the slope was significant – however the Theranos Anti-Thyroglobulin Antibody assay is calibrated directly to the WHO International Reference Preparation of Anti-thyroglobulin serum.

Table [SEQ Table * ARABIC]: Clinical Results with Final Assay Conditions

			Original Conditions	Final Conditions
Sample ID	Reported (Siemens Immulite), IU/mL	In-House (Siemens Immulite), IU/mL	Theranos, IU/mL	Theranos, IU/mL
BRH527312	< 20	< 20.0	32.0	< 5.0
BRH527313	< 20	< 20.0	1.1	< 5.0
BRH527314	< 20	< 20.0	0.8	< 5.0
BRH527315	< 20	< 20.0	1.6	< 5.0
BRH527316	< 20	< 20.0	1.4	< 5.0
BRH527317	< 20	< 20.0	2.2	< 5.0
BRH527318	< 20	< 20.0	17.1	14.6
BRH527319	< 20	< 20.0	1.0	< 5.0
BRH527320	< 20	< 20.0	0.5	< 5.0
BRH527321	< 20	< 20.0	0.8	< 5.0
BRH527322	114	83.5	115.0	124.8
BRH527323	164	106	374.6	491.2
BRH527324	64	123	549.7	491.5
BRH527325	30	< 20.0	39.3	11.0
BRH527326	26	46.8	92.2	54.1
BRH527327	160	189	255.6	242.6
BRH527328	147	< 20.0	17.1	8.1
BRH527329	48	< 20.0	79.3	13.4
BRH527330	26	< 20.0	19.4	9.9
BRH527331	25	< 20.0	23.4	9.7
BRH527332	2390	1880	3003.0	3388.2
BRH527333	821	622	1526.5	1429.9
BRH527334	> 3000	2808	> 3000	> 3000
BRH527335	2305	1315	2297.0	3038.1
BRH527336	505	406	833.8	630.4
BRH527337	1178	722	1222.3	1433.7
BRH527338	366	218	255.9	289.4
BRH527339	660	322	568.6	595.0
BRH527340	245	< 20.0	135.9	47.5

Figure [SEQ Figure * ARABIC]: Clinical Correlation to Reported Siemens Immulite Results

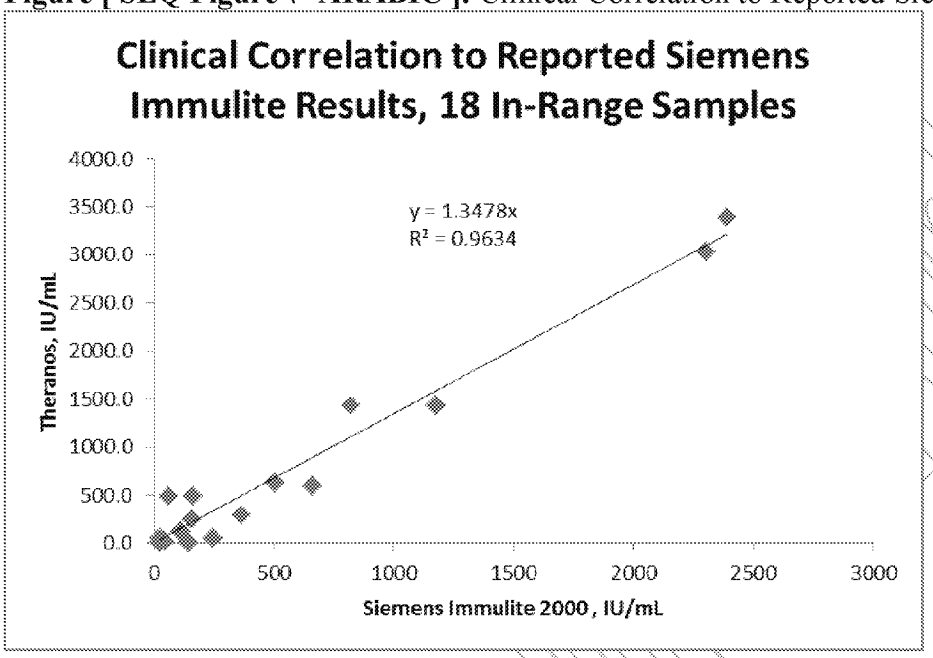
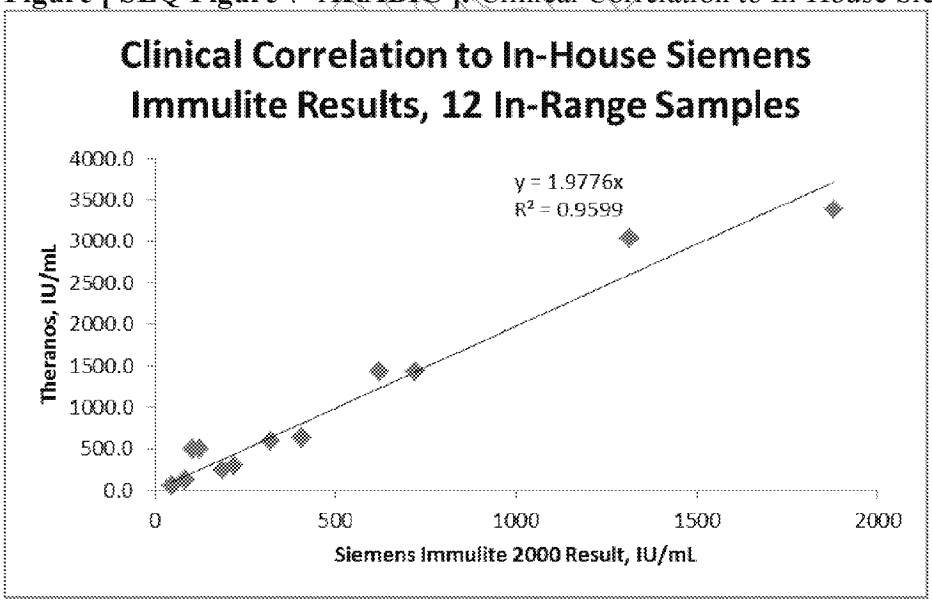


Figure [SEQ Figure * ARABIC]: Clinical Correlation to In-House Siemens Immulite Results



1.15 Stability

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light.

2 REFERENCES

- Latrofa et al, "Characterization of Thyroglobulin Epitopes in Patients with Autoimmune and Non-Autoimmune Thyroid Diseases Using Recombinant Human Monoclonal Thyroglobulin Autoantibodies." J Clin Endocrinol Metab, February 2008, 93(2):591–596.
- David Sinclair "Clinical and laboratory aspects of thyroid autoantibodies." Ann Clin Biochem 2006; 43: 173–183.
- Taylor, KP et al, "Concordance between thyroglobulin antibody assays." Ann Clin Biochem 2011; 48: 367–369.
- Ferrand, JRF et al, "Significance of thyroglobulin antibodies cross-reactive with thyroperoxidase (TGPO antibodies) in individual patients and immunized mice." Clin Exp Immunol 1993; 92:65-72.
- Nabipour, I et al,"Influence of levothyroxine treatment on serum levels of soluble Fas (CD95) and Fas Ligand (CD95L) in chronic autoimmune hypothyroidism." Endocr (2010) 38:406–411.
- Okosieme, OE, "Restricted thyroglobulin antibody epitope specificities in subjects with type 1 diabetes mellitus." European Journal of Endocrinology (2009) 161 489–493