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Thyroglobulin (Tg)
Assay Development Report

Theranos, Inc.

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 detect human Thyroglobulin (Tg) in human whole blood, plasma and serum. The assay has a reportable range of 0.25 to 500 ng/mL, and is calibrated to the Thyroglobulin Reference Standard CRM 457.

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

The following commercial ELISA kits have been used in house as predicate methods:

- Orgentec ELISA Kit Cat# ORG5TG
- Siemens Immulite 2000 Thyroglobulin Test Cat #L2KTY2

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

A biotin-labeled anti-Tg antibody coated on an avidin surface serves as the capture surface for the sandwich ELISA. The sample (whole blood, plasma or serum) is diluted and then incubated on the capture surface, then an enzyme-labeled anti-Tg antibody is incubated on the surface for 10 minutes. After the detection antibody incubation, the surface is washed and the substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

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

2.1 Anti-Tg Antibody Interference

Since anti-Tg auto-antibodies (ATG) may be present in some samples to be tested for Tg, and these antibodies are known to interfere in Tg assays, the assay was tested for ATG interference at a level twice the cutoff for positive ATG. All Tg assays require screening of the same sample for ATG and reflex to LC-MS if the sample is positive for ATG. However due to the variability of avidity of these autoimmune antibodies [1], and the fact that some Tg assays show interference for ATG near the cutoff level [5], it is desirable to design the assay such that potential interference from ATG is minimal.

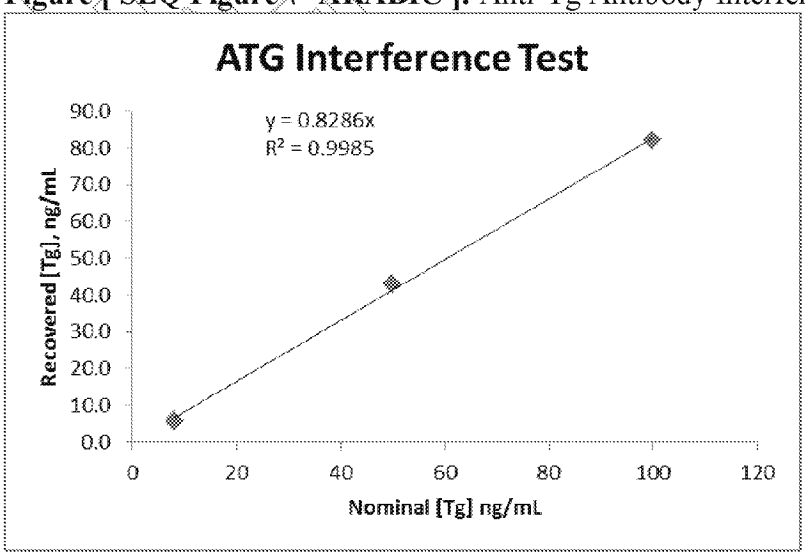
Test calibrators were made in assay buffer and with 4 levels of Tg spiked in ATG positive plasma. The WHO ATG IRP (NIBSC 65/093) which consists of a pool of ATG positive plasma, was used for the interference test at a level of 50 IU/mL.

The assay showed no significant interference from ATG at 50 IU/mL.

Table [SEQ Table * ARABIC]: Anti-Tg Antibody Interference

[Tg] ng/mL	[Anti-Tg Ab] IU/mL	Conc, ng/mL		
		Mean Conc	CV %	% Recovery
100	50	82.1	35.8	82
50	50	43.2	14.7	86
8	50	5.7	3.9	71

Figure [SEQ Figure * ARABIC]: Anti-Tg Antibody Interference



2.2 Cross Reactivity and Interference

The assay was tested for cross reactivity and interference from very high levels of thyroxine (T4), triiodothyronine (T3), follicle stimulating hormone (FSH) and alpha fetoprotein (AFP). No cross reactivity or interference was seen.

Table [SEQ Table * ARABIC]: Standard Curve

[Tg] ug/mL	Signal, RLU		Conc, ng/mL		
	Mean RLU	CV %	Mean Conc	CV %	% Recovery
500	449619	16.6	513	20.4	103
100	108537	8.2	102	8.4	102
50	50543	24.4	47	24.4	95
10	12087	22.9	10	26.9	103
5	7304	17.0	6	22.2	111
1	2154	20.9	1	35.8	86
0.25	1201	10.1	0	20.2	110
0	741	31.5	0	64.3	

Table [SEQ Table * ARABIC]: Cross Reactivity and Interference

Test Substance	[Tg] ng/mL	[Test Substance] ng/mL	Signal, RLU		Conc, ng/mL			
			Mean RLU	CV %	Mean Conc	CV %	% Recovery	% Cross Reactivity
AFP	100	1000	110529	8.0	103.96	8.2	104	
	10	1000	12637	5.5	10.89	6.4	109	
	0	1000	551	20.3	OORL			ND
FSH	100	1000	93979	5.5	88.15	5.6	88	
	10	1000	11158	14.3	9.40	17.1	94	
	0	1000	561	24.6	OORL			ND
T3	100	50	85449	16.4	80.13	16.6	80	
	10	50	13360	29.6	10.00	28.5	100	
	0	50	612	53.9	OORL			ND
T4	100	1000	123184	9.5	116.25	9.8	116	
	10	1000	11136	21.2	9.38	25.4	94	
	0	1000	901	26.4	OORL			ND

2.3 Determination of LLOQ and ULOQ

A standard curve was run and Theranos calibration software was used to fit the data and determine the LLOQ and ULOQ according to FDA guidelines for calibrating ELISA assays. The LLOQ was 0.25 ng/mL and the ULOQ was 500 ng/mL.

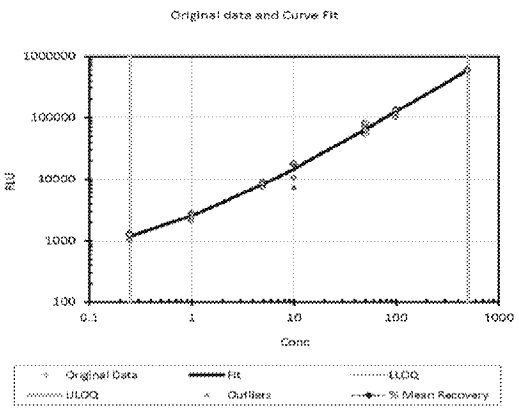
Table [SEQ Table * ARABIC]: Standard Curve

	Signal, RLU		Conc, ng/mL		
[Tg] ug/mL	Mean RLU	CV %	Mean RLU	CV %	% Recovery
500	600280	2.6	508.74	2.9	102
100	121317	14.7	94.71	15.1	95
50	68754	17.6	52.72	18.3	105
10	17848	3.0	12.43	3.4	124
5	8110	11.7	4.91	14.5	98
1	2514	13.2	0.97	20.6	97
0.25	1201	10.1	0.28	5.8	113
0	529	7.8	OORL		

Conc = 92.813 * (((7.904 - 2.247) / (log10(S) - 2.247)) - 1) ^ (1 / -0.298)
SMin = 1066, SMax = 670840

Table [SEQ Table * ARABIC]: Determination of LLOQ and ULOQ

Parameter	Value	Units
LLOQ	0.25	ng/mL
ULOQ	500.00	ng/mL
LLOQ accuracy	103	%
LLOQ precision	9.1	%
ULOQ accuracy	102	%
ULOQ precision	1.9	%
Average Residuals	11	%



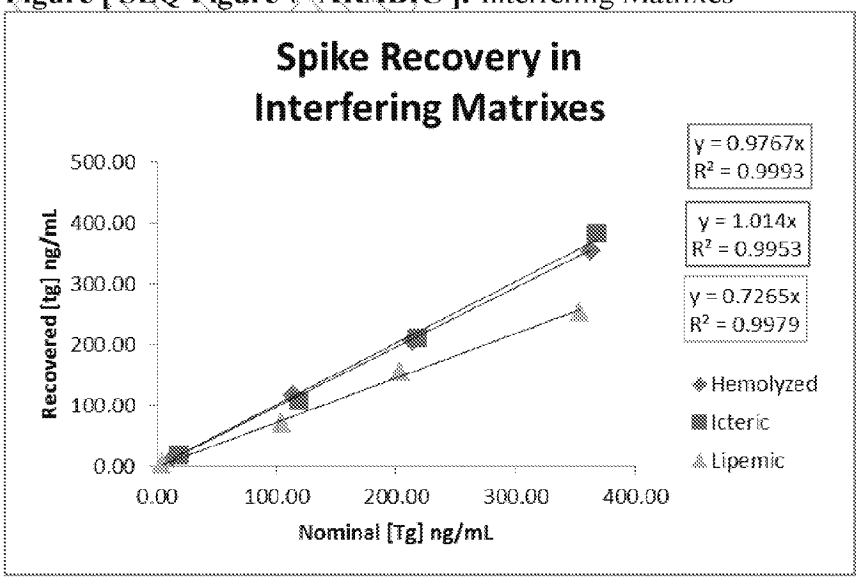
2.4 Interfering Matrixes

To test for interference in the assay, lipemic, icteric and hemolyzed serum was obtained and spiked with thyroglobulin. Recovery was excellent in hemolyzed and icteric serum but recovery was lower in lipemic serum (triglycerides = 212 mg/dL). Samples with triglycerides above 200 mg/dL may cause erroneously low results in this assay.

Table [SEQ Table * ARABIC]: Interfering Matrixes

Sample Type	Spike [Tg] ng/mL	Conc, ng/mL			
		Mean	CV %	Minus Endogenous	% Recovery
Hemolyzed	350	354.45	5.0	340.97	101
	200	206.17	34.4	192.70	103
	100	117.05	18.5	103.58	117
	0	13.47	19.8		
Icteric	350	383.06	15.0	365.07	109
	200	210.79	14.7	192.81	105
	100	107.61	34.5	89.63	108
	0	17.98	24.8		
Lipemic	350	254.39	11.5	250.47	73
	200	155.05	11.7	151.12	78
	100	71.23	16.4	67.30	71
	0	3.93	21.0		

Figure [SEQ Figure * ARABIC]: Interfering Matrixes



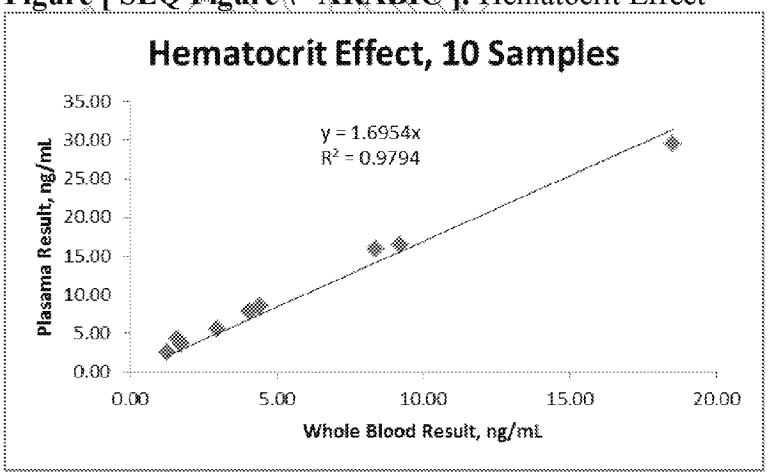
2.5 Hematocrit Effect

To determine whether thyroglobulin concentrates fully into plasma, 10 EDTA whole blood samples were collected and the whole blood and the plasma derived by centrifuging the whole blood were each measured on the Theranos System. As expected, thyroglobulin concentrated into the plasma resulting in a slope of approximately 1.7.

Table [SEQ Table * ARABIC]: Hematocrit Effect

	Thyroglobulin, ng/mL	
Sample ID	**Whole Blood**	**EDTA Plasma**
01	1.24	2.59
02	4.06	7.76
03	1.61	4.32
04	4.43	8.48
05	OORL	OORL
06	9.23	16.45
07	2.98	5.56
08	1.71	3.61
09	8.35	15.89
10	18.51	29.47

Figure [SEQ Figure * ARABIC]: Hematocrit Effect



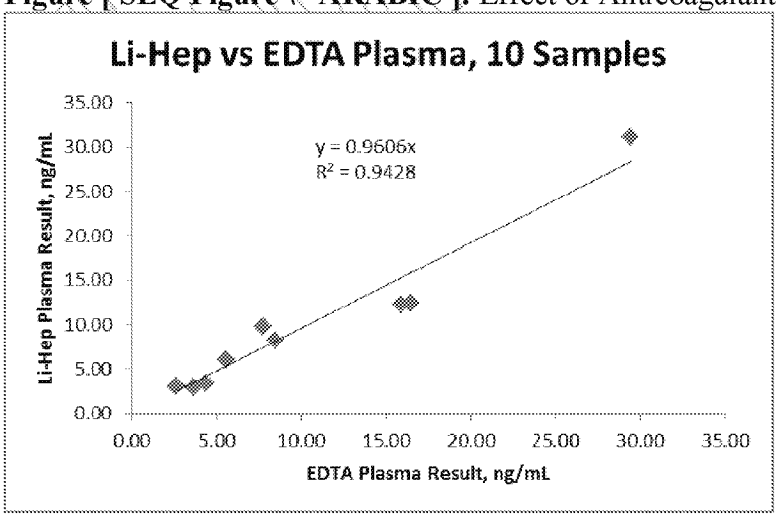
2.6 Effect of Anticoagulant

To determine whether the choice of anticoagulant affects the assay, matched samples in EDTA and Lithium Heparin tubes were collected from 10 normal donors. The samples were tested in the Theranos System and the results compared. There was no signifianct difference between results in EDTA and Li-Hep plasma.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant

	Thyroglobulin, ng/mL	
Sample ID	**EDTA Plasma**	**LiHep Plasma**
01	2.59	3.06
02	7.76	9.78
03	4.32	3.47
04	8.48	8.27
05	OORL	OORL
06	16.45	12.37
07	5.56	6.06
08	3.61	2.95
09	15.89	12.34
10	29.47	31.12

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant



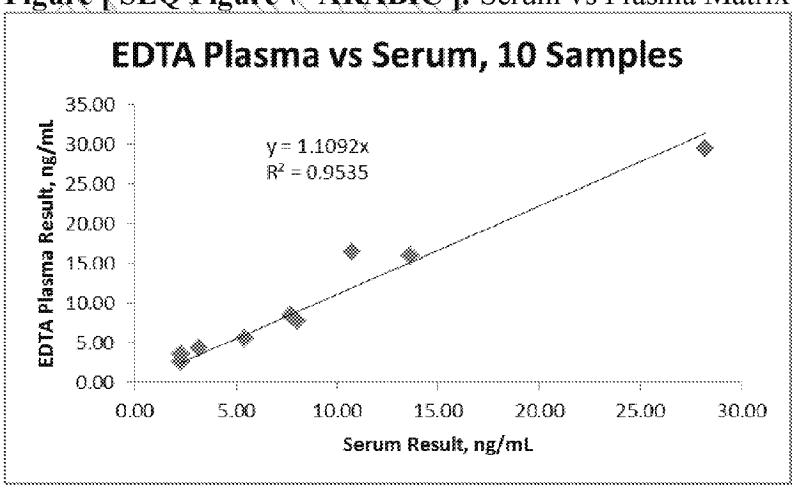
2.7 Serum vs Plasma Matrix

To determine whether a serum or plasma matrix affects the assay, matched samples in EDTA plasma and serum tubes were collected from 10 normal donors. The samples were tested in the Theranos System and the results compared. There was no signifianct difference between results in EDTA plasma and serum.

Table [SEQ Table * ARABIC]: Serum vs Plasma Matrix

	Thyroglobulin, ng/mL	
Sample ID	**EDTA Plasma**	**Serum**
01	2.59	2.26
02	7.76	8.06
03	4.32	3.15
04	8.48	7.74
05	OORL	OORL
06	16.45	10.71
07	5.56	5.43
08	3.61	2.29
09	15.89	13.64
10	29.47	28.22

Figure [SEQ Figure * ARABIC]: Serum vs Plasma Matrix



2.8 Clinical Correlation

A set of samples from patients with Hashimoto's Disease was obtained along with a set of normal samples. The samples were screened for ATG on the Siemens Immulite 2000 and only samples negative for ATG (< 22 IU/mL) were included in the clinical comparison, in accordance with the accepted guidelines for Tg testing and the manufacturer's directions for Orgentec and Immulite assays.

Correlation was excellent between the Theranos result and both the Orgentec kit and the Siemens Immulite results. The Orgentec ELISA is 510k cleared and calibrated to the CRM 457 thyroglobulin reference standard.

Table [SEQ Table * ARABIC]: Clinical Correlation

Sample ID	Sample Type	Matrix	Siemens ATA Result	Orgentec [Tg], ng/mL	Siemens [Tg], ng/mL	Theranos [Tg], ng/mL
3	Hashimoto's	Serum	Neg	7.8	14.0	7.6
6	Hashimoto's	Serum	Neg	3.5	5.8	2.8
7	Hashimoto's	Serum	Neg	7.0	7.8	2.9
8	Hashimoto's	Serum	Neg	6.8	12.6	9.0
12	Hashimoto's	Plasma	Neg	7.0	13.3	7.4
13	Normal	Serum	Neg	93.4	142.0	111.5
14	Normal	Serum	Neg	15.3	17.2	16.1
15	Normal	Serum	Neg	17.0	18.4	13.2
16	Normal	Serum	Neg	29.4	35.2	25.1
L1	Normal	Plasma	Neg	4.4	6.6	3.1
L2	Normal	Plasma	Neg	12.3	15.5	9.8
L3	Normal	Plasma	Neg	3.2	7.0	3.5
L4	Normal	Plasma	Neg	8.9	17.0	8.3
L6	Normal	Plasma	Neg	12.9	23.9	12.4
L7	Normal	Plasma	Neg	8.2	9.0	6.1
L8	Normal	Plasma	Neg	1.9	7.5	3.0
L9	Normal	Plasma	Neg	12.0	27.4	12.3

Figure [SEQ Figure * ARABIC]: Clinical Correlation Theranos to Orgentec 510k-Cleared Kit

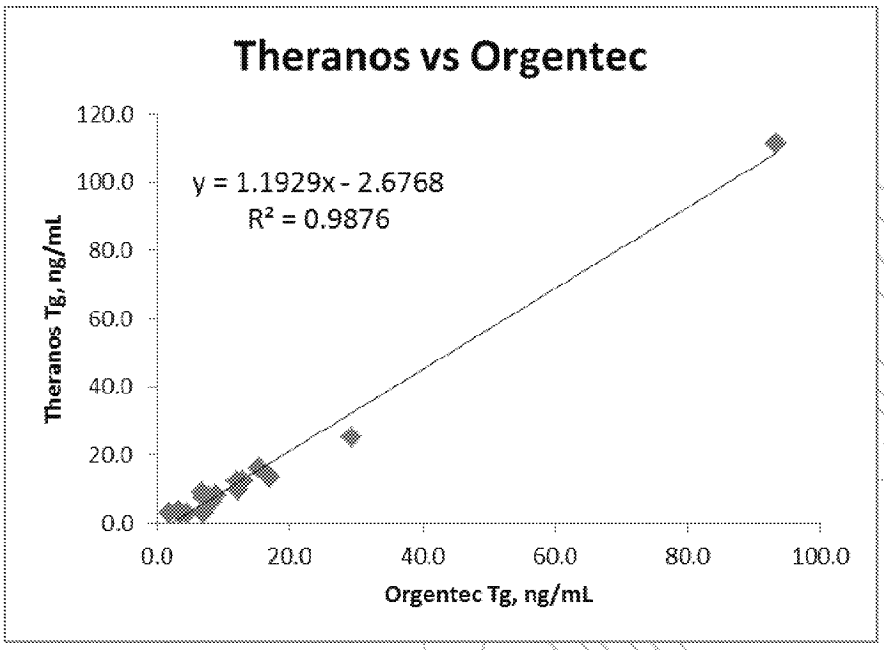
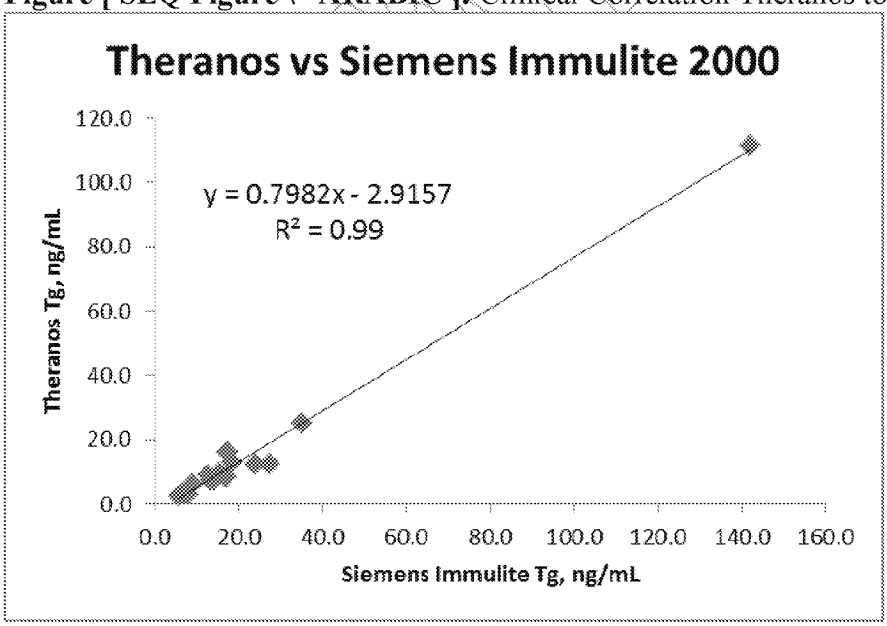


Figure [SEQ Figure * ARABIC]: Clinical Correlation Theranos to Siemens Immulite 2000



3 CONCLUSION

We have successfully developed an immunoassay to detect thyroglobulin in human serum and plasma.

4 REFERENCES

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