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Total Thyroxine (TT4) 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"]

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

This assay is designed to detect total human Thyroxine (T4) in human whole blood (automatically processed into plasma by the Theranos system), plasma and serum. The assay has a reportable range of 0.7 to 25.7 ug/dL, and is calibrated to the European Commission Certified Reference Material IRMM-468. No NIBSC WHO material is available for thyroxine.

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

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

- Alpco TT4 ELISA (Cat# 25-TT4HU-E01)

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

This assay is a competitive ELISA.

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

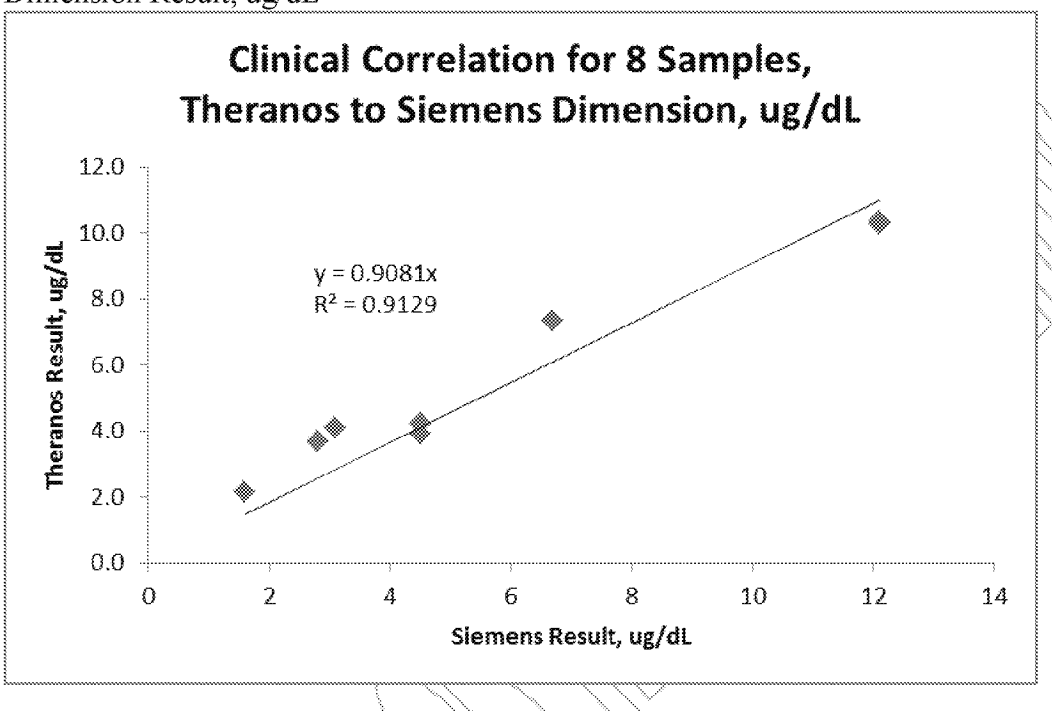
2.1 Training Set

In order to confirm full dissociation of T4 from binding proteins is achieved, a set of 8 clinical samples with pre-measured TT4 values were tested. Based on the serum calibrator standard curve, the calculated total T4 concentration correlated very well with the reported values for the 8 clinical samples.

Table [SEQ Table * ARABIC]: Clinical Samples - Training Set Results

Sample #	Siemens Dimension Reported Result, ug/dL Mean Conc.	Theranos Result, ug/dL Mean Conc	Theranos Result, ug/dL CV %
1	4.5	3.9	9.1
2	4.5	4.2	7.7
3	6.7	7.4	14.7
4	1.6	2.2	11.9
5	2.8	3.7	11.3
6	3.1	4.1	11.2
7	12.1	10.3	6.9
8	12.1	10.3	16.1

Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Reported Siemens Dimension Result, ug/dL



2.2 Plasma Screen

Plasma from 10 normal donors was screened in the Theranos System to confirm the normal range. The endogenous TT4 was calculated based on the serum standard curve. All samples fell into the reported normal range for TT4.

Normal Adult TT4 Range in Serum
Hypothyroid ≤ 4.5 ug/dL
Normal = 4.5 - 12 ug/dL
Hyperthyroid ≥ 12 ug/dL

Table [SEQ Table * ARABIC]: Normal Plasma Screen

Sample #	Mean Conc, ug/dL	CV %
1	8.8	1.2
2	11.7	11.5
3	10.6	21.7
4	7.5	3.3
5	10.8	0.9
6	7.6	22.0
7	8.4	4.3
8	8.6	3.7
9	9.6	8.7
10	6.6	9.8
Min	5.9	
Max	11.7	
Mean	8.6	

2.3 Interfering Matrixes and Spike Recovery

Spike recovery was tested in lipemic, icteric and hemolyzed serum to ascertain whether there may be interference in the assay results when measuring these types of samples. Lipemic, icteric and hemolyzed sera showed normal spike recovery as did normal plasma.

Table [SEQ Table * ARABIC]: Interfering Matrixes Spike Recovery

Sample	[Spiked] ug/dL	Mean Conc ug/dL	CV %	Minus Endogenous	% Recovery
Icteric Serum	20	24.1	20.9	16.0	80
	15	20.0	-	11.9	79
	10	17.2	12.1	9.1	91
	0	8.1	7.5	-	-
Hemolyzed Serum	20	22.3	7.7	16.9	85
	15	18.9	9.3	13.5	90
	10	14.3	24.7	8.9	89
	0	5.4	3.5	-	-
Lipemic Serum	20	24.0	6.1	15.7	79
	15	22.1	18.2	13.8	92
	10	18.0	21.4	9.7	97
	0	8.3	16.8	-	-

Table [SEQ Table * ARABIC]: Spike Recovery in Plasma

Sample	**[Spiked] ug/dL**	**Mean**	**CV %**	**Minus Endogenous**	**% Recovery**
#10	20	27.2	2.5	20.7	103
	15	19.4	0.9	12.9	86
	10	16.4	1.6	9.9	99
	0	6.5	5.2	-	-
#1	20	30.2	3.7	20.5	103
	15	25.3	22.1	15.7	104
	10	18.0	5.8	8.3	83
	0	9.7	2.2	-	-
#4	20	29.9	-	22.1	110
	15	23.6	1.2	15.8	105
	10	17.3	4.1	9.5	95
	0	7.8	3.5	-	-

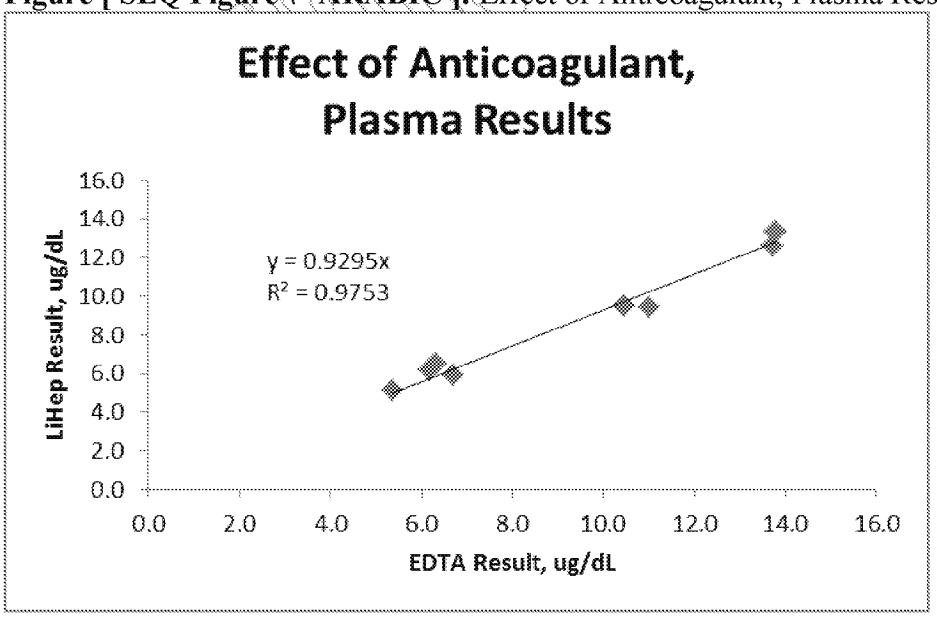
2.4 Effect of Anticoagulant on Plasma Results

The Theranos System will prepare plasma from both EDTA and lithium-heparin-treated blood. To determine if the TT4 assay is impacted by the choice of anticoagulant, plasma was prepared from EDTA tubes and from Li-Hep tubes for 4 normal donors, and the endogenous levels and a spiked level were tested. There was no difference between the EDTA and Li-Hep plasma results.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant, Plasma Results

Sample #		EDTA Result, ug/dL		Lithium-Heparin Result, ug/dL		
		Mean Conc.	CV %	Mean Conc.	CV %	% Difference from EDTA Result
1	unspiked	6.3	4.3	6.5	9.3	3
	spiked	11.0	19.0	9.4	1.2	-14
2	unspiked	5.4	6.0	5.2	5.4	-4
	spiked	10.5	8.7	9.5	5.2	-10
3	unspiked	6.2	5.2	6.2	6.6	0
	spiked	13.7	7.5	12.6	24.8	-8
4	unspiked	6.7	6.2	5.9	8.2	-12
	spiked	13.8	3.3	13.3	8.2	-3

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant, Plasma Results



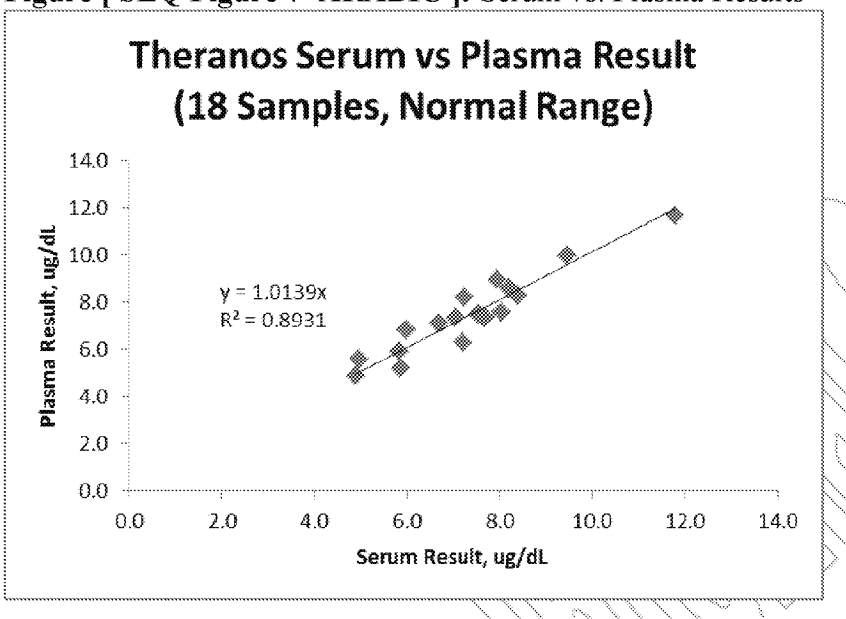
2.5 Serum vs. Plasma Results

Commercially available methods utilize serum only for measuring TT4 whereas the Theranos System will be capable of testing either serum prepared in the lab or plasma prepared on board from a whole blood sample. Blood was collected from 18 normal donors in a serum tube and in a lithium-heparin plasma tube. Serum and plasma were prepared and tested in the Theranos System. The results for serum and plasma were within 15% for each patient and the correlation between the serum and plasma results was excellent. All serum and plasma results were within the normal adult range of 4.5 – 12.0 ug/dL.

Table [SEQ Table * ARABIC]: Serum vs. Plasma Results

Sample #	Serum Result, ug/dL		Plasma Result, ug/dL		
	Mean Conc.	CV %	Mean Conc.	CV %	% Difference from Serum
1	6.7	4.6	7.1	4.2	6
2	8.0	3.9	7.6	5.4	-6
3	8.4	5.8	8.3	5.7	-1
4	7.0	9.5	7.3	7.9	4
5	7.2	11.3	6.3	2.5	-14
6	5.8	3.7	5.9	1.2	2
7	8.2	1.9	8.6	17.5	4
8	4.9	0.7	4.9	15.0	-1
9	7.5	7.1	7.5	6.4	-1
10	5.0	4.2	5.6	7.4	11
11	7.9	17.8	9.0	5.8	11
12	7.7	3.2	7.3	17.1	-5
13	6.0	5.0	6.8	9.0	12
14	5.9	7.6	5.2	2.3	-13
15	11.8	5.3	11.7	3.2	-1
16	9.4	3.5	10.0	22.5	5
17	7.6	5.2	7.4	17.4	-3
18	7.2	10.7	8.2	15.8	12

Figure [SEQ Figure * ARABIC]: Serum vs. Plasma Results



2.6 Cross Reactivity and Interference

To test for potential cross reactivity or interference, TT4 recovery was tested across the range in the presence of an excess of 5 substances with the potential to cross react or interfere with T4 assays. None of the test substances showed any interference or cross reactivity.

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

Test Substance	[Test Substance] ug/dL	[T4] ug/dL	Calculated Conc. ug/dL Mean	CV %	% Recovery
Control	-	25.7	25.6	5.4	100
		15.7	14.2	5.9	90
		10.7	12.5	14.5	116
		5.7	5.4	5.6	95
		2.7	2.8	3.6	102
		0.7	0.7	25.9	102
3,5-Diiodo-L-thyronine (T2)	3	25.7	27.9	6.1	109
		15.7	16.7	9.8	106
		10.7	10.6	7.5	99
		5.7	6.3	2.7	111
		2.7	2.7	20.6	101
		0.7	0.7	8.6	99
Diphenylhydantoin	30	25.7	30.2	-	118
		15.7	15.3	13.7	97
		10.7	10.3	2.1	96
		5.7	5.8	10.2	101
		2.7	2.7	7.1	100
		0.7	0.7	3.1	101
3-Iodo-L-tyrosine	3	25.7	28.6	15.6	111
		15.7	13.4	-	86
		10.7	12.0	5.0	112
		5.7	6.0	2.4	105
		2.7	3.2	14.0	117
		0.7	0.6	28.2	90
Triiodothyronine (T3)	3	25.7	28.3	19.7	110
		15.7	15.4	-	98
		10.7	10.9	3.7	102
		5.7	6.8	9.9	119
		2.7	2.8	3.7	103
		0.7	0.6	2.0	92
Sodium salicylate	40,000	25.7	29.4	11.4	114
		15.7	18.0	22.7	115
		10.7	11.5	6.7	108
		5.7	6.3	6.9	111
		2.7	3.4	-	124
		0.7	0.6	12.5	82

2.7 Calibration Verification

Calibration of the Theranos System was verified by obtaining the European Commission Certified Reference Material for Thyroxine (IRMM-468), reconstituting it according to the supplied directions, and making dilutions into the same depleted serum as the Theranos calibrators. In addition, 3-level serum controls from BioRad (Liquichek Immunoassay Plus) were measured in the Theranos System. Recovery of the reference material and the controls was excellent.

Standard Curve

[TT4] ug/dL	Signal, RLU		Conc. ug/dL		
	Mean	CV %	Mean	CV %	% Recovery
35.7†	2246	11.1	OORH	-	-
25.7	2153	8.8	27.0	8.3	105
15.7	3653	2.6	14.1	2.8	90
10.7	4692	8.3	11.1	7.0	104
5.7	10501	11.5	5.7	8.6	100
2.7	28541	4.6	2.6	2.6	96
1.7	41504	7.5	1.8	6.5	107
0.7	87339	7.9	0.7	13.2	98

† Anchor Point

Table [SEQ Table * ARABIC]: Recovery of IRMM-468 in T4-Depleted Serum

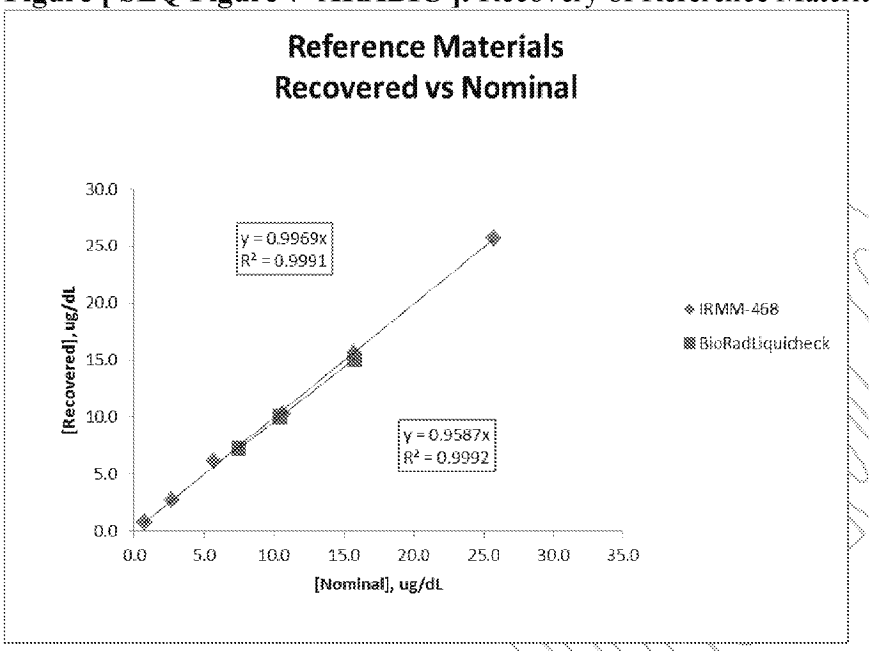
[Nominal], ug/dL	Signal, RLU		Conc. ug/dL		
	Mean	CV %	Mean	CV %	% Recovery
25.7	2223	11.3	25.7	15.9	100
15.7	3322	9.3	15.7	9.6	100
10.7	5041	5.6	10.3	4.3	96
5.7	9495	7.7	6.2	5.5	108
2.7	26811	3.3	2.7	2.6	100
0.7	90583	11.0	0.7	1.2	103

Table [SEQ Table * ARABIC]: Recovery of BioRad Liquichek Immunoassay Plus Controls

Level	Reported Mean Conc. †, ug/dL	Signal, RLU		Conc., ug/dL		
		Mean	CV %	Mean	CV %	% Recovery
1	7.44	7703	8.5	7.3	6.5	98
2	10.4	5281	9.2	10.0	9.3	96
3	15.8	3374	5.2	15.1	5.1	95

† Reported mean varies by instrument – this is the reported mean for Roche Elecsys E170/ COBAS

Figure [SEQ Figure * ARABIC]: Recovery of Reference Materials



2.8 Determination of LLOQ and ULOQ

LLOQ and ULOQ were determined using FDA guidelines for ELISA assay calibration. The ULOQ was 25.7 ug/dL and the LLOQ was 0.7 ug/dL. An anchor point was included at 35.7 ug/dL to assist in curve fit.

Table [SEQ Table * ARABIC]: Standard Curve

[TT4] ug/dL	Signal, RLU		Back-Calculated Conc ug/dL		
	Mean	CV %	Mean	CV %	% Recovery
35.7†	2246	11.1	OORH	-	-
25.7	2392	16.7	27.0	8.3	105
15.7	3653	2.6	14.1	2.8	90
10.7	4692	8.3	11.1	7.0	104
5.7	10501	11.5	5.7	8.6	100
2.7	28541	4.6	2.6	2.6	96
1.7	41504	7.5	1.8	6.5	107
0.7	87339	7.9	0.7	13.2	98

† anchor point

Measurement	Value	Units
LLOQ	0.70	ug/dL
ULOQ	25.70	ug/dL
LLOQ accuracy	97	%
LLOQ precision	17.9	%
Average Residuals	8	%
Error in prediction: Best case	8	%
Error in prediction: Expected	8	%

2.9 Clinical Correlation

Serum samples from hypothyroid, hyperthyroid, and normal patients were obtained with reported values from the Siemens Dimension system. The samples were tested in the Theranos System and in the Alpco ELISA kit.

Correlation of Theranos results to reported Siemens was excellent with the exception of 2 samples (# 12 and #15) that came out normal in the Theranos System but low in the Siemens system. However these 2 samples were reported as normal in the Alpco kit as well. No diagnosis information was available for these samples.

Correlation of the Theranos results to the Alpco kit results was excellent with the exception of 2 different outlier samples. Sample #3 showed normal results in the Theranos System and in the reported Siemens System, and the reported diagnosis was normal, but the Alpco result was exceptionally high – corresponding with a definite hyperthyroid range. In addition, sample #6 was reported as hypothyroid in the Theranos System and in the Siemens System as well as the reported diagnosis, but showed high borderline-hyperthyroid results in the Alpco kit. It appears possible that the Alpco kit – which utilizes a sheep polyclonal anti-T4 capture antibody – may show cross reactivity that results in abberant high results.

In conclusion, the Theranos System results correlate best with the reported results from the Siemens Dimension System.

Table [SEQ Table * ARABIC]: Clinical Sample Results

Sample #	Analyzer	Reported Conc, ug/dL	Alpco TT4 Kit, ug/dL	Theranos Result, ug/dL	Reported Diagnosis
1	Siemens Dimension	4.5	6.0	3.9	Hypothyroid
2	Siemens Dimension	4.5	7.0	4.2	Hypothyroid
3	Siemens Dimension	6.7	20.0	7.4	Normal
4	Siemens Dimension	1.6	4.4	2.2	Hypothyroid
5	Siemens Dimension	2.8	6.8	3.7	Hypothyroid
6	Siemens Dimension	3.1	11.9	4.1	Hypothyroid
7	Siemens Dimension	12.1	14.5	10.3	Hyperthyroid
8	Siemens Dimension	12.1	13.0	10.3	Hyperthyroid
9	Siemens Dimension	8.0	9.0	8.5	N/A
10	Siemens Dimension	7.6	10.6	6.9	N/A
11	Siemens Dimension	2.9	5.4	3.9	N/A
12	Siemens Dimension	4.5	9.3	9.0	N/A
13	Siemens Dimension	4.3	10.6	6.9	N/A
14	Siemens Dimension	12.0	12.9	10.6	N/A
15	Siemens Dimension	3.0	10.8	7.6	N/A
16	AVIDA Centaur	6.6	n/a	7.0	N/A

Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Reported Result

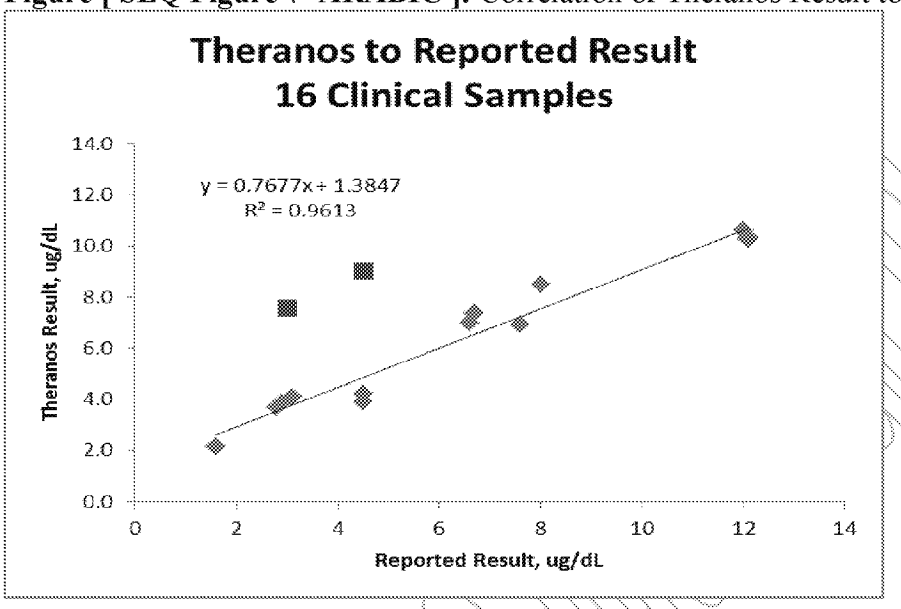


Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Alpco TT4 Kit Result

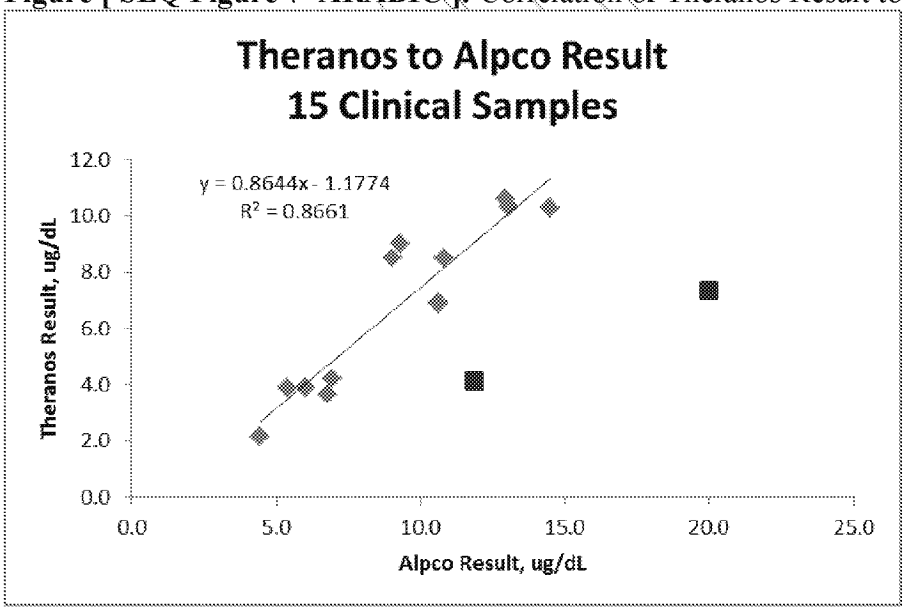
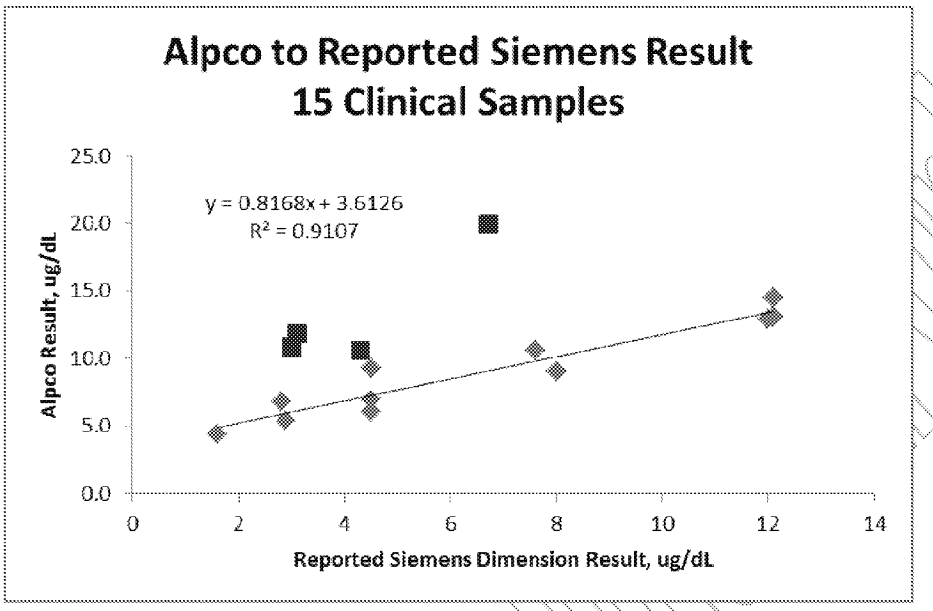


Figure [SEQ Figure * ARABIC]: Correlation of Alpco TT4 Kit Result to Reported Siemens Dimension Result



2.10 Stability

Stability of the reagents is being monitored.

3 CONCLUSION

We have successfully developed an immunoassay to detect Total Thyroxine (TT4) in human serum and plasma.