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Free Thyroxine Assay (fT4)
Assay Development Report

Theranos, Inc.

July 5, 2011

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

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

This assay is designed to detect free thyroxine (fT4) in human plasma, serum and whole blood. The assay has a reportable range of 0.4 to 6.8 ng/dL and is calibrated to the Certified Reference Material IRMM-468 from the Institute for Reference Materials and Measurements, Joint Research Centre, European Commission.

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:

- Alpco T4 (Thyroxine) (Free) ELISA (Cat# 25-FT4HU-E01)
- Alpco T4 (Thyroxine) (Free) LIA (Cat# 11-FT4HU-L01)
- Calbiotech Free Thyroxine (fT4) ELISA (Cat #F4107T)

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

A biotin-labeled goat anti-mouse antibody coated on an avidin surface serves as the capture surface for this competitive ELISA. The sample (whole blood, plasma or serum) is diluted with the diluent containing the anti-thyroxine antibody in solution and is followed by mixing with the thyroxine labeled with alkaline phosphatase (T4-AP). The mixture is then incubated on the capture surface for 10 minutes. Following this 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

Name	Supplier	Catalog #
Human Thyroxine	Sigma	T2376-5G
Goat Anti-Mouse IgG Biotin Conjugate	Pierce Biotechnology	P# 31805
Mouse Anti-Human T4 Antibody	US Biological	T5460-02A
Thyroxine labeled AP conjugate	Fitzgerald	65IT40
Phospho Glo Substrate	KPL	55-60-04
Superblock(TBS) Blocking Buffer	Pierce	37535
Blocking Buffer with low BSA conc. (0.03% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041

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

1.2 Antibody Screening (MTP)[TC "Detection Antibody Conjugate Verification" \f C \l "1"]

To determine the optimal pair for the fT4 competitive ELISA 22 T4 antibodies were tested on a microtitre plate against two commercially available T4-AP conjugates. The screening was performed with Validate FT4 Calibration Verification Test set from Maine Standards (lot# 91AA20910) diluted 1:5 into low BSA blocking buffer. The Anti-T4 Ab was set at 10ug/mL and the T4-AP conjugates were tested at 1:1000 and 1:100,000 fold dilutions. All dilutions were made in low BSA blocking buffer. The antibody screen summary is summarized in **Table 3**. Further optimizations were performed on the Theranos System and the data for the 2 best antibodies chosen is summarized in **Table 4**.

Table [SEQ Table * ARABIC]: Antibody Information

Number	Vendor	Cat #	Clone #	Type
1	Thermoscientific	MA1-22092	1H1	Mab
2	Thermoscientific	MA1-22093	XM212	Mab
3	Abcam	ab30833	Rabbit poly	Rabbit Pab
4	Abcam	ab31495	sheep poly	Sheep Pab
5	Genway	20-783-73698	BGN/0980/322	MAb
6	Genway	20-783-73697	BGN/0980/11	MAb
7	Genway	20-251-401270	2127455	MAb
8	Genway	20-251-401227	702431	MAb
9	Gene Tex	GTX41130	ME.125	MAb
10	Genway	20-511-240919	9101	MAb
11	Genway	20-511-240933	115-11011	MAb
12	Genway	20-511-240945	291-14641	MAb
13	Genway	20-511-240993	204-14525	MAb
14	Genway	20-511-241024	057-11007	Mab
15	US Biological	T5460	8.F.275	Mab
16	US Biological	T5460-04	1.B.169	MAb
17	US Biological	T5460-05A	1.B.171	MAb
18	US Biological	T5460-04B	3H278	MAb
19	US Biological	T5460-02A	9F75	Mab
20	US Biological	T5460-01D	9F227	MAb
21	US Biological	T5460-01G	9L720	MAb
22	Genway	18-783-78216	sheep poly	Sheep Pab

Table [SEQ Table * ARABIC]: Summary of Antibody Screening Results

Cab #	Type	Response
1	MAb	
2	MAb	
3	Rabbit Pab	
4	Sheep PAb	
5	MAb	
6	MAb	
7	MAb	
8	MAb	
9	MAb	
10	MAb	
11	MAb	
12	MAb	
13	MAb	
14	MAb	
15	MAb	
16	MAb	
17	MAb	
18	MAb	
19	MAb	
20	MAb	
21	MAb	
22	Sheep PAb	

Legend
- Good dose response
- Poor dose response
- No dose response

Table [SEQ Table * ARABIC]: Summary of Best Pairs (MTP)

	C19 as Cab					C3 as Cab				
[T4] ng/dL	**Values**	**Mean**	**Std.Dev.**	**CV%**	**Modulation**	**Values**	**Mean**	**Std.Dev.**	**CV%**	**Modulation**
0.03	2648	2814	234	8	1.7	10383	**10466**	117	1	1.0
	2979					10549				
0.3	1531	1630	140	9	1.0	10855	**10671**	260	2	1.1
	1730					10487				
0.4	1523	1570	67	4	1.2	8740	**9436**	983	10	1.8
	1618					10131				
1.7	1316	1308	12	1	1.5	5181	**5152**	41	1	2.1
	1299					5123				
4.64	923	883	56	6	1.3	2351	**2398**	67	3	2.6
	844					2446				
9.2	724	658	94	14		848	**935**	123	13	
	592					1022				
	S/B	**4.3**				**S/B**	**11.2**			

1.3 Cross Reactivity (Theranos System)

Thyroxine or 3,5,3',5'-tetraiodothyronine is the principal thyroid hormone in whole blood. The Theranos fT4 assay was tested for cross reactivity with the other major thyroid hormone T3 as well as other structurally similar tyrosine-based metabolites. Also included in the test were diphenylhydantoin and sodium salicylate that are commonly ingested drugs that bear close structural identity to T4. **Table 5** shows the structures of all the analytes tested. The assay conditions were with Anti-T4 antibody biotin conjugated at 1 µg/ml in solution diluted in low BSA blocking buffer, T4-AP conjugate diluted 1:50,000 fold in the above buffer and a 1:5 sample dilution. T4 calibrators were spiked depleted serum. The analytes tested were also spiked into the depleted serum. The test ranges of the analytes were sourced from literature. The highest level of the analytes tested, where applicable, corresponded to approximately 3-fold the highest concentration seen in normal serum. For each calibrator N=3 replicates were run. All the analytes tested showed RLU very similar to the background of the T4 control standard curve indicating no cross reactivity. D-Thyroxine showed close to 100% cross reactivity. The data are summarized in **Table 6**.

Table 5: Structures of T4 analytes tested for cross reactivity

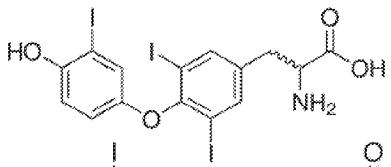
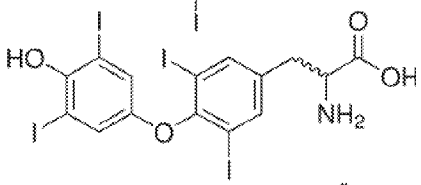
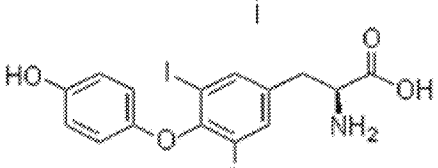
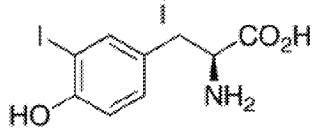
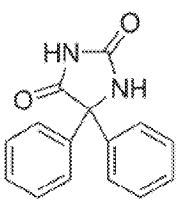
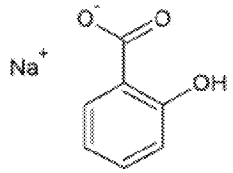
Analyte	Structure
Thyroxine(T4)	
3,3′,5-Triiodo-L-thyronine(T3)	
3,5-Diiodo-L-thyronine (T2)	
3-Iodo-L-tyrosine	
Diphenylhydantoin	
Sodium salicylate	

Table 6: Cross reactivity of Theranos fT4 ELISA with T4 analogs

CONTROL fT4 ng/dl	Mean	CV%	[T2] ng/mL Nominal	Mean	CV%	[Diphenylhydantoin] ng/mL Nominal	Mean	CV%
0.0	8313	12	0.0	6941	7	0.0	6941	7
0.50	6747	28	0.50	6249	12	10.00	6249	12
1	6010	11	1	8349	14	25	8349	14
2.5	2242	5	2.5	7378	7	50.0	7378	7
5.00	817	13	5.00	7944	11	75.00	7944	11
10	400	13	10	7351	12	150	7351	12
			30*	7885	16	300*	7885	16
CONTROL fT4 ng/dl	**Mean**	**CV%**	**[D-T4] ng/dL Nominal**	**Mean**	**CV%**	**[3-Iodo-L-tyrosine] ng/mL Nominal**	**Mean**	**CV%**
0.0	14121	17	0.0	9798	4	0.0	11845	1
0.50	9946	26	0.50	5097	16	0.75	10351	21
1	8378	20	1	5044	14	1	10906	16
2.5	3214	16	2.5	1549	10	2.5	12325	5
5.00	1212	7	5.00	585	9	5.00	10663	8
10	522	9	10	282	17	10	13734	11
			30*	184	9	30*	12288	6
CONTROL fT4 ng/dl	**Mean**	**CV%**	**[T3] ng/mL Nominal**	**Mean**	**CV%**	**[Sod. salicylate] ug/mL Nominal**	**Mean**	**CV%**
0.0	14001	6	0.0	10885	5	0.0	10903	4
0.50	10090	22	0.50	9406	16	10.00	9618	26
1	7716	11	1	11986	9	25	10872	17
2.5	3206	10	2.5	11629	7	50.0	11438	5
5.00	1174	1	5.00	12616	4	100.00	9688	5
10	491	7	10	11015	13	200	11581	7
			30*	11051	14	400*	10136	4

* These are 3 times the highest concentration found in serum

1.4 Training Set

8 clinical serum samples from Bioreclamation were tested on the Theranos System with the anti-T4 antibody at 1 µg/mL, T4-AP conjugate diluted at 50,000 fold in low BSA blocking buffer and a 1:5 sample dilution. Additionally, 6 calibration serum control samples from Maine Standards were also tested. All the above samples were tested in the Alpco fT4 ELISA, Alpco fT4 LIA and the Calbiotech fT4 ELISA. Clinical correlations were compared. Clinical correlation was later verified on a larger sample set with the final assay conditions. The chosen antibody produced excellent correlation with the commercial ELISA measured concentration for this sample set.

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

Sample	Alpco ELISA [fT4] ng/dL	Calbiotech [fT4] ng/dL	Alpco LIA [fT4] ng/dL	Theranos [fT4] ng/dL	Reported conc [fT4] ng/dL
Bio01	0.60	0.82	0.56	1.00	0.74
Bio02	0.57	0.75	0.44	1.10	0.74
Bio03	0.86	0.68	0.56	1.19	0.65
Bio04	0.32	0.46	0.22	0.44	0.4
Bio05	0.37	0.49	0.24	0.43	0.6
Bio06	0.37	0.52	0.59	0.73	1
Bio07	1.12	1.49	1.43	1.58	3.7
Bio08	0.86	1.02	1.10	1.19	2.9
MS Level 5	3.30	3.85	*nd*	5.31	
MS Level 4	2.33	2.84	*nd*	4.00	
MS Level 3	1.69	*nd*	*nd*	3.33	
MS Level 2	0.93	*nd*	*nd*	2.01	
MS Base serum	0.06	*nd*	*nd*	0.09	

Nd not determined

Table [SEQ Table * ARABIC]: Standard Curve in Depleted Serum

fT4 ng/dL Alpco Kit assigned	Signal Mean RLU	%CV	Back calculated Conc Mean Conc. ng/dL	%CV	% Recovery
0.08	9442	13	0.12		152
0.25	6792	8	0.31	21.9	123
0.41	6080	6	0.42	12.4	103
0.91	4137	5	0.72	6.7	80
1.73	1781	13	1.77	10.9	102
2.56	1092	15	2.81	13.2	110
3.93	647	14	4.44	13.2	113
5.86	471	7	5.89	6.8	101

Conc = 6.0581E-01 * (((9.2969E+03-1.3377E+02) / (RLU - 1.3377E+02)) - 1) ^ (1 1.4282E+00)

Figure [SEQ Figure * ARABIC]: Correlation of Alpco fT4 ELISA Result to Calbiotech fT4 ELISA Result

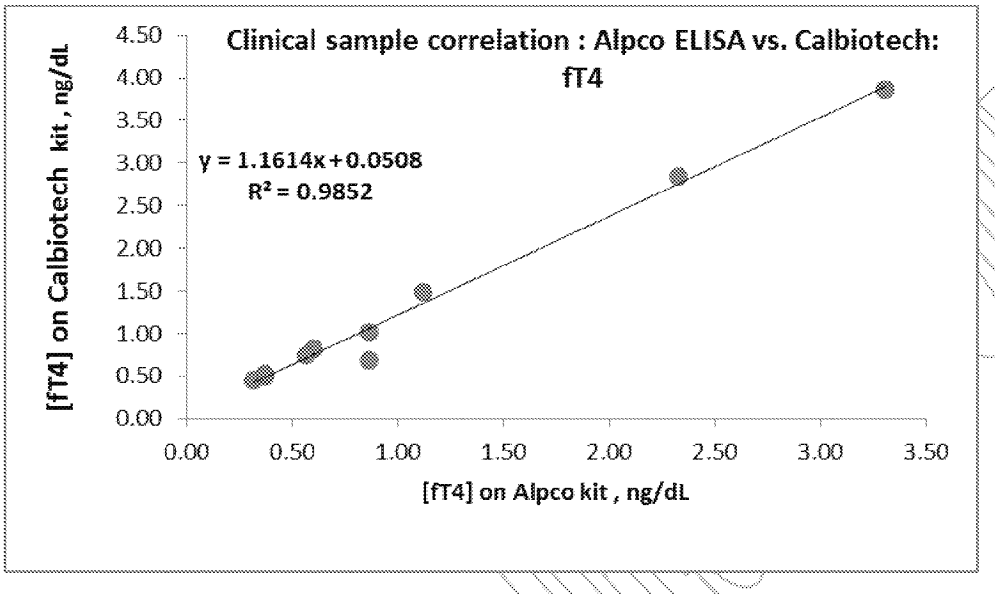


Figure [SEQ Figure * ARABIC]: Correlation of Alpco ELISA Result to Alpco LIA Result

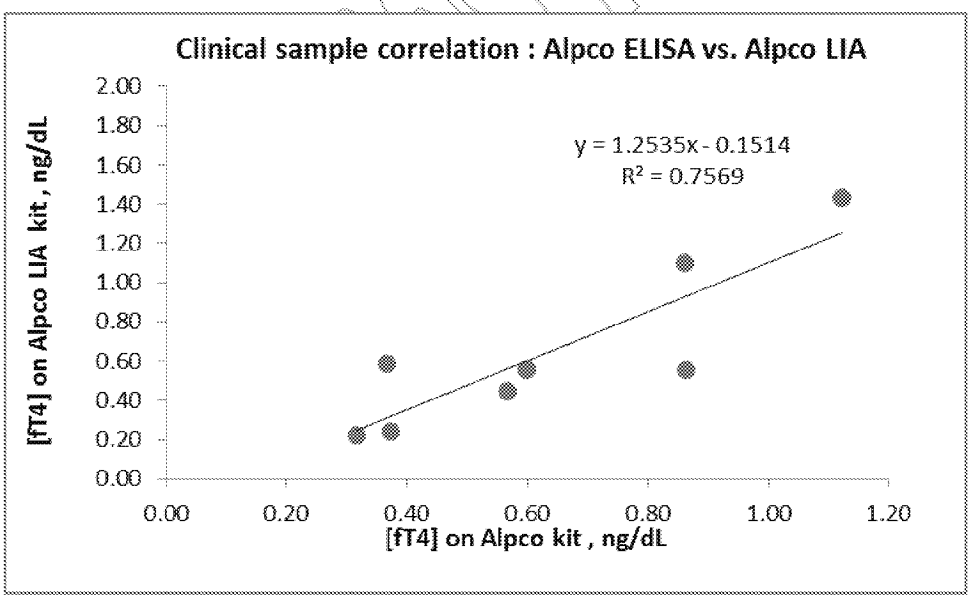


Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Alpco fT4 ELISA

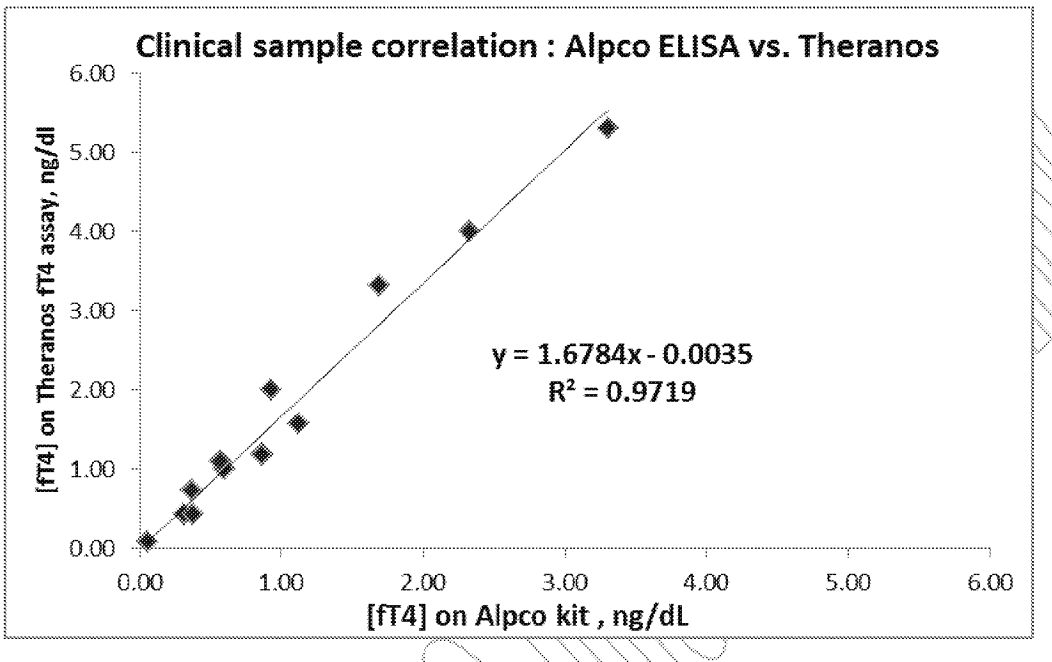


Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Calbiotech fT4 ELISA

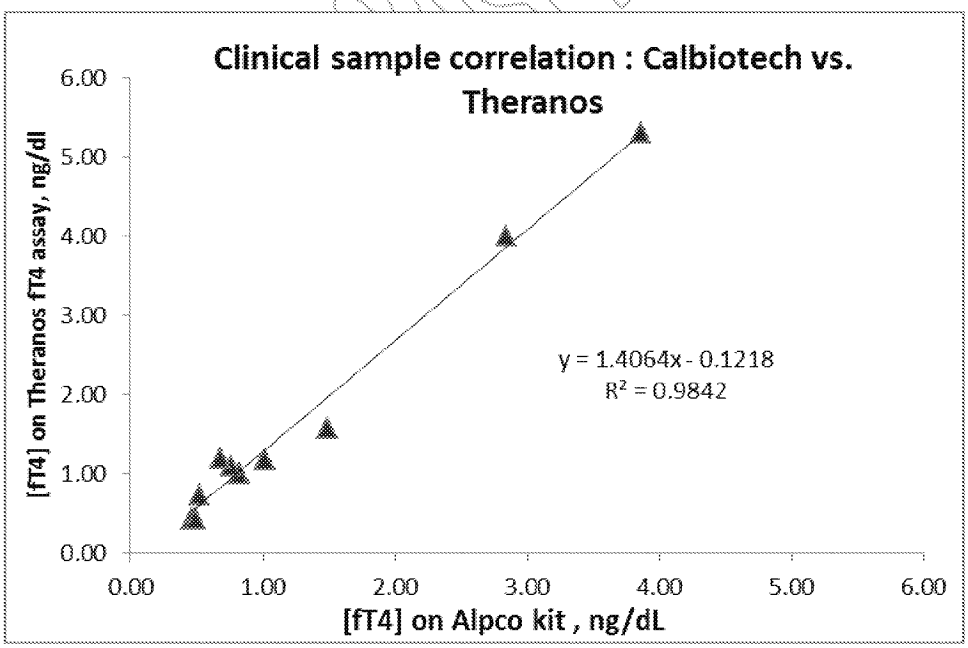


Figure [SEQ Figure * ARABIC]: Correlation of Theranos Result to Reported concentration

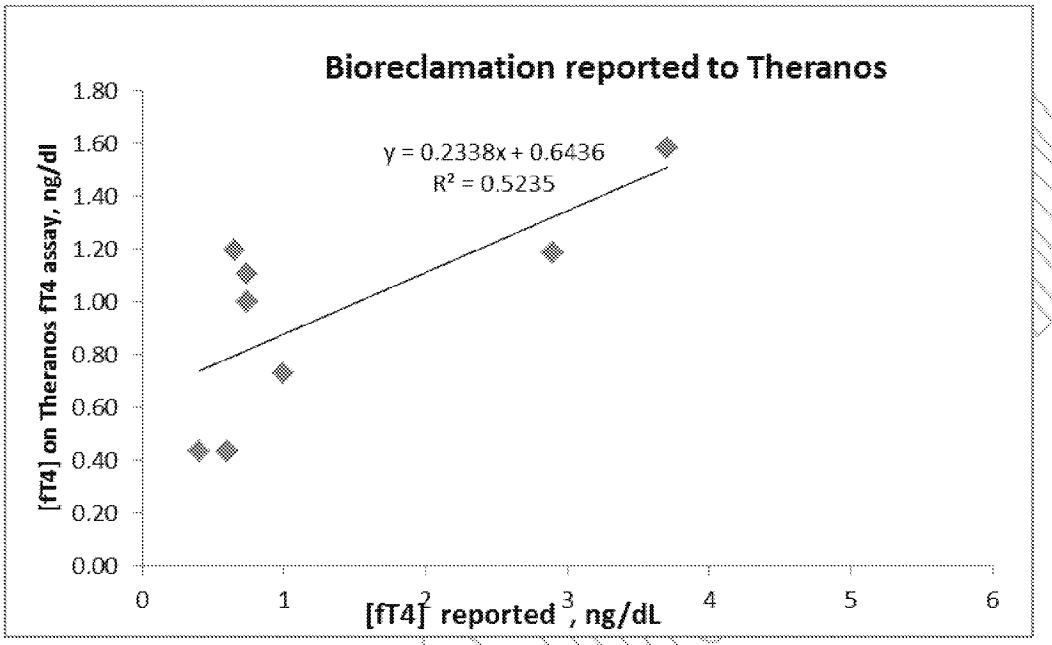


Figure [SEQ Figure * ARABIC]: Correlation of Alpco ELISA to Reported Concentration

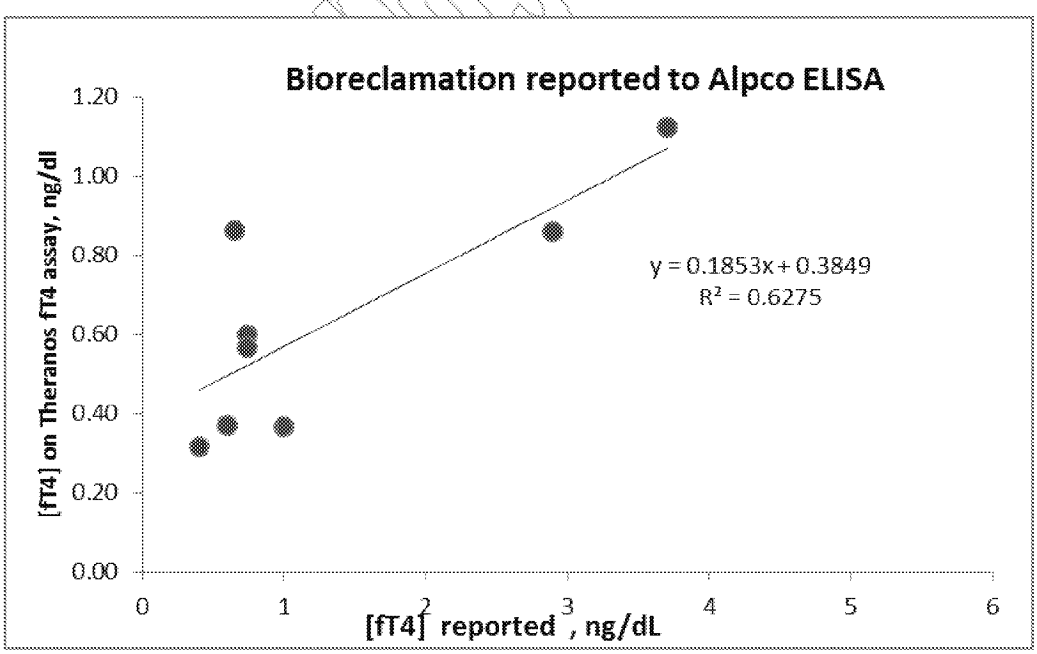


Figure [SEQ Figure * ARABIC]: Correlation of Calbiotech ELISA to Reported concentration

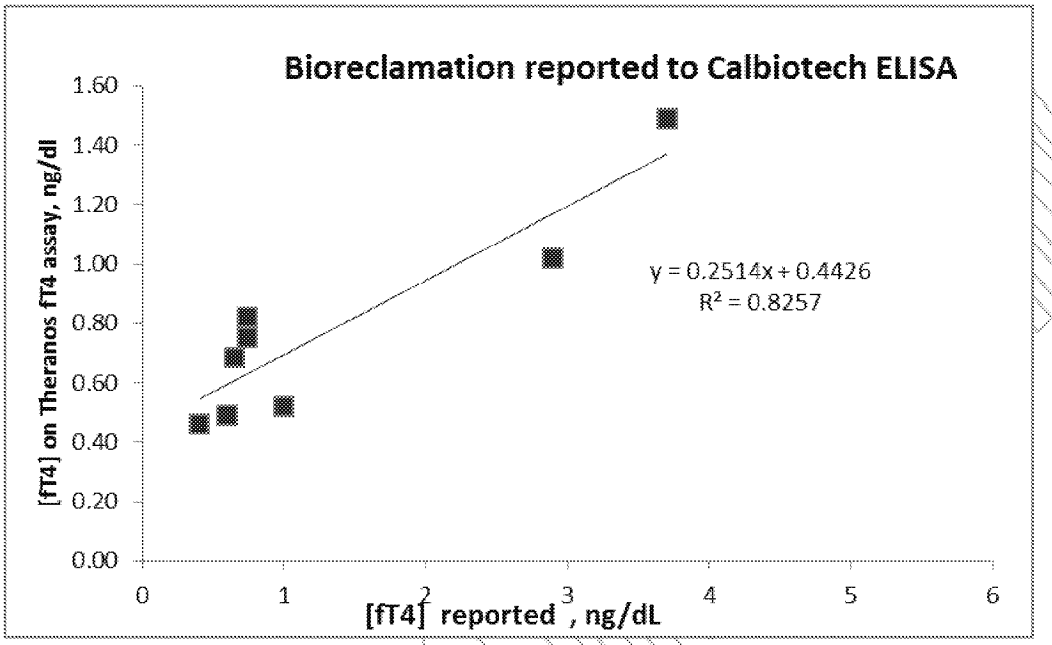
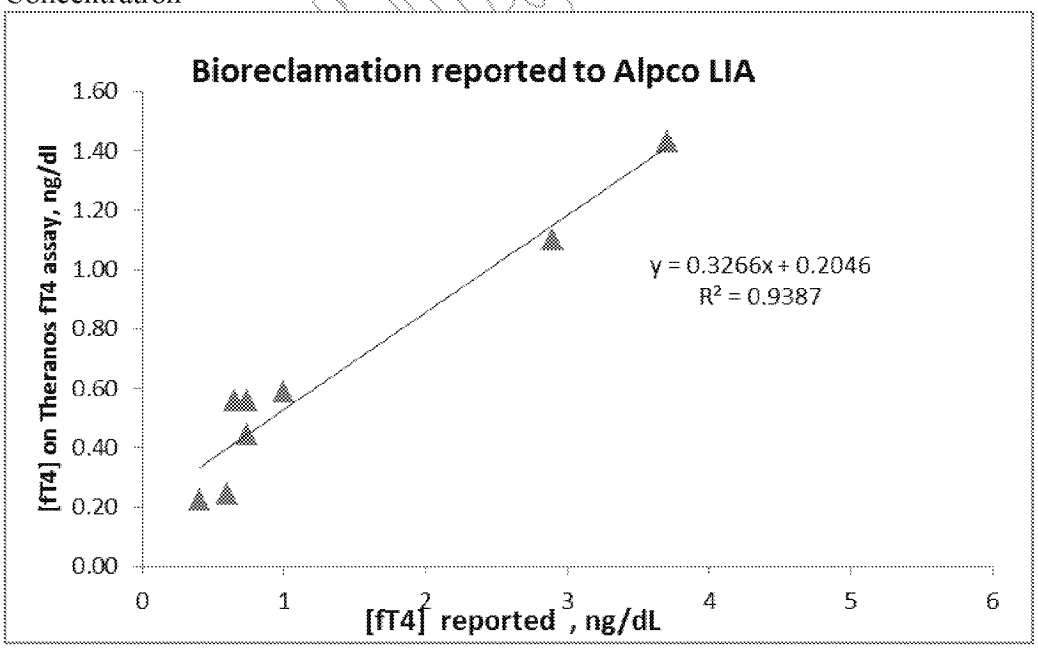


Figure [SEQ Figure * ARABIC]: Correlation of Alpco LIA Result to Reported Concentration



1.5 Matrix Screen and Spike Recovery

In order to investigate the effect of different matrices on the Theranos fT4 assay, a matrix screen was performed with spiked depleted serum, normal serum, normal plasma, whole blood and plasma from the same sample of whole blood with anti-T4 antibody biotin conjugated at 1 µg/ml in solution diluted in low BSA blocking buffer, T4-AP conjugate diluted 1:50,000 fold in the above buffer and a 1:5 sample dilution. Since T4 binds to human serum albumin with a high affinity calibrators made in assay buffer were not tested. For this experiment, concentrations were all calculated based on the depleted serum spiked standard curve. The T4 antibody performs equally in all matrices and can be finalized.

Table [SEQ Table * ARABIC]: Matrix Screen Results: Serum (normal & depleted) and Normal Plasma

Matrix	Alpco kit Assigned [fT4] ng/dL	Signal (RLU) Mean	Signal (RLU) CV %	Conc (ng/dL) Mean	Conc (ng/dL) CV %	Conc (ng/dL) % Recovery
Depleted serum	0.08	9442	13	0.12		152
	0.25	6792	8	0.31	21.9	123
	0.41	6080	6	0.42	12.4	103
	0.91	4137	5	0.72	6.7	80
	1.73	1781	13	1.77	10.9	102
	2.56	1092	15	2.81	13.2	110
	3.93	647	14	4.44	13.2	113
	5.86	471	7	5.89	6.8	101
Normal serum	0.78	5660	13	0.40	23.1	51.2
	0.79	3497	7	0.85	8.3	108.2
	1.4	2452	7	1.30	7.6	92.7
	2.04	1343	22	2.33	19.6	114.2
	2.94	955	11	3.28	11.2	111.7
	4.65	731	5	3.92	4.6	84.4
	6.41	484	15	5.94	13.7	92.7
Plasma	1.00	17145	14	0.98	10.6	98
(Directly Spiked)	1.08	12155	14	1.26	9.2	117
	1.77	6295	7	1.91	4.5	108
	2.48	3775	1	2.62	0.5	105
	3.57	2033	14	4.02	11.2	113
	5.7	1026	11	6.86	9.8	120
	8.74	774	6	9.57	0.8	109
	OORH	678	10	OORH		

T4 spiked depleted serum calibration curve

Conc = 6.0581E-01 * (((9.2969E+03-1.3377E+02) / (RLU - 1.3377E+02)) - 1) ^ (1 1.4282E+00)

Note: Most of the thyroxine in whole blood is bound to thyroxine binding proteins as well as human serum albumin. Only 0.03% of free thyroxine is available for measurement. Given this it is very difficult to ascertain the "nominal" concentration of thyroxine in a given matrix which complicates the spike recovery calculation. Throughout the assay development the spiked T4 matrix calibrators were made by using the "total" thyroxine (TT4) levels and each time these calibrators were run on the Alpco fT4 ELISa and the concentrations were assigned. The assigned concentrations were treated as "nominal" and this was used to calculate recovery. In the case of whole blood no recovery could be computed since these calibrators cannot be run on an ELISA. The back-calculated values from the spiked whole blood experiment (based on a depleted serum calibration curve) were used as the "nominal" in the plasma generated from spiked whole blood experiment and recoveries were computed on this basis.

Table 10: Matrix Screen Results: Whole Blood and Plasma from Spiked whole blood

	Nominal	Signal (RLU)		Conc (ng/dL)		
Matrix	**[TT4] ug/dL**	**Mean**	**CV %**	**Mean**	**CV %**	**% Recovery**
Whole Blood	0.00	24095	12	0.74	10.7	NA
	2.00	10216	12	1.41	7.9	NA
	5.00	3419	3	2.79	1.9	NA
	10.00	1774	5	4.40	3.6	NA
	25.00	861	3	8.92	4.3	NA
	35.0	731	11	9.70	2.9	
	From whole blood spike	**Signal (RLU)**		**Conc (ng/dL)**		
Matrix	**[fT4] ng/dL**	**Mean**	**CV %**	**Mean**	**CV %**	**% Recovery**
Plasma from spiked whole blood	0.74	16929	5	0.99	4.1	133
	1.41	7101	4	1.77	2.2	125
	2.79	2531	10	3.42	6.3	122
	4.40	1359	7	5.49	6.2	125
	8.92	658	12	10.84	5.6	122
	9.70	506	6	OORH		

T4 spiked depleted serum calibration curve

Conc = 6.0581E-01 * (((9.2969E+03-1.3377E+02) / (RLU - 1.3377E+02)) - 1) ^ (1 1.4282E+00)

Figure [SEQ Figure * ARABIC]: Matrix Screen

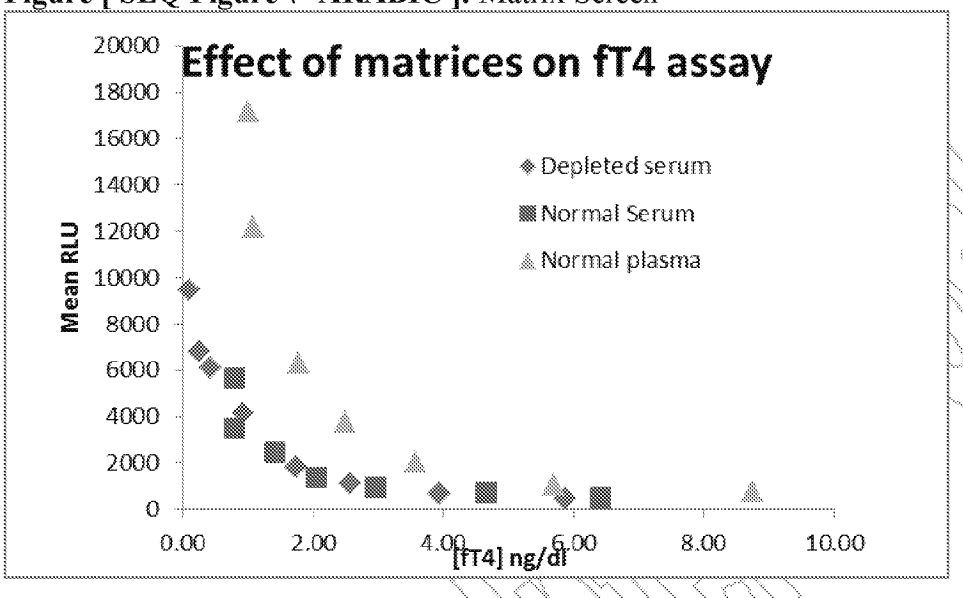


Figure [SEQ Figure * ARABIC]: Depleted serum spike recovery

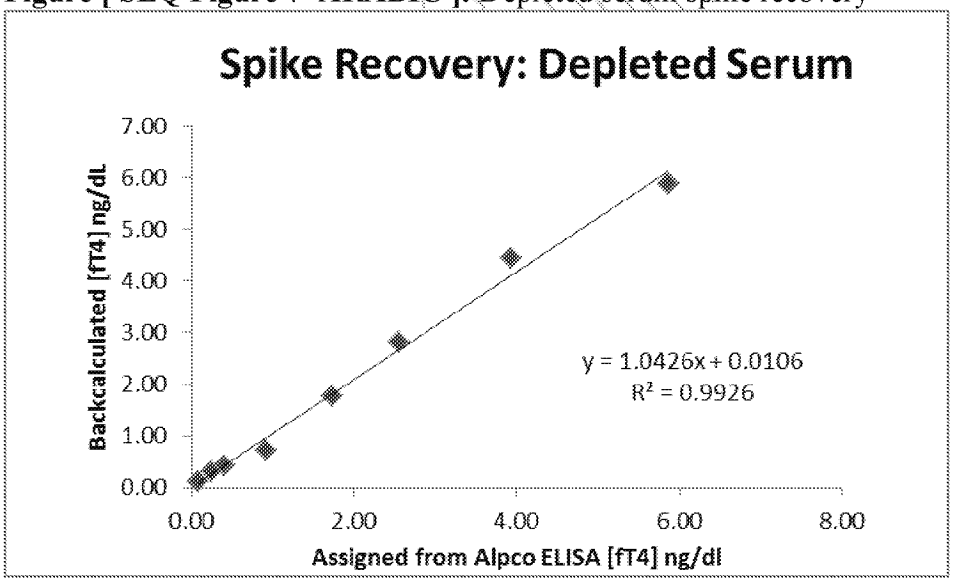


Figure [SEQ Figure * ARABIC]: Normal Serum Spike Recovery

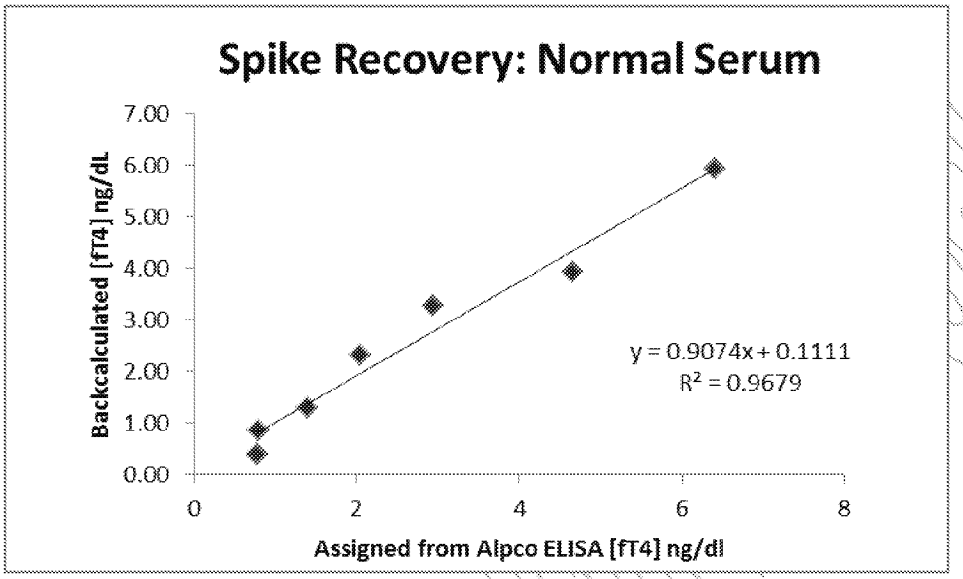
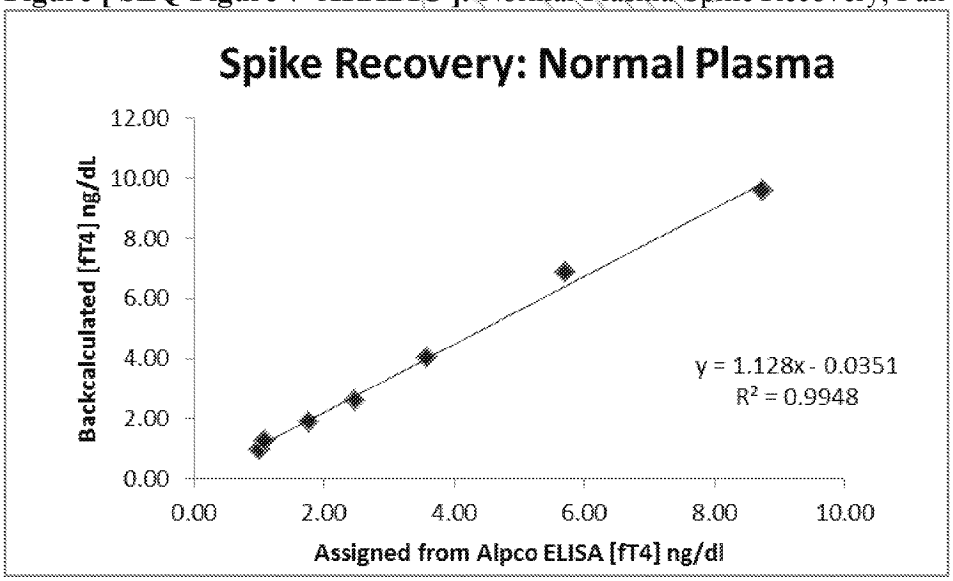


Figure [SEQ Figure * ARABIC]: Normal Plasma Spike Recovery, Pair 2



1.6 Finalization of Antibody Pair

Based on the results from the clinical sample training set, the matrix screen and the sensitivity of the calibrated assay the current conditions as well as the anti-T4 Antibody (Cab 19) were finalized.

1.7 Whole Blood/Plasma and Serum Screen

To verify the normal range in whole blood, 6 samples were screened. These results corresponded with the expected normal range in serum for adults of 0.8– 1.8 ng/dL.

Table 11: Whole Blood and Plasma Screen

	fT4 levels in whole blood				fT4 levels in plasma from whole blood			
Whole blood	**RLU**		**Conc. ng/dL**		**RLU**		**Conc. ng/dL**	
Sample #	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
6/9/11 1	3943	8	0.78	10.3	1448	17	2.16	16.7
6/9/11 2	3694	18	0.85	21.9	1352	13	2.29	10.5
6/9/11 3	4928	12	0.64	20.1	2324	33	1.55	27.7
6/10/11 1	3470	20	0.92	21.4	2032	8	1.56	7.8
6/7/2011	3098	14	1.03	15.4	1475	40	2.27	33.7
6/8/2011	3751	11	0.82	12.9	2010	5	1.56	4.5

T4 spiked depleted serum calibration curve

Conc = 6.0581E-01 * (((9.2969E+03-1.3377E+02) / (RLU - 1.3377E+02)) - 1) ^ (1 1.4282E+00)

Serum Screen

To verify the normal range in serum, 6 female and 6 male samples from normal subjects were screened. These results corresponded with the expected normal range in serum for adults of 0.8– 1.8 ng/dL for majority of the samples tested.

Table 12: Serum Screen

Serum sample serum ID#	RLU		Conc. ng/dL	
	Mean	CV%	Mean	CV%
M20	1542	7	2.01	6.7
M13	1993	18	1.61	18.7
M10	4105	17	0.74	21.7
M1	2672	26	1.24	25.4
M7	2895	7	1.10	7.5
M21	1253	20	2.37	18.8
F22	3557	6	0.87	6.6
F1	2978	16	0.98	15.8
F20	3505	8	0.89	9.0
F16	4611	13	0.71	19.8
F7	2110	8	1.50	7.5
F9	2358	5	1.35	4.6

T4 spiked depleted serum calibration curve

Conc = 6.0581E-01 * (((9.2969E+03-1.3377E+02) / (RLU - 1.3377E+02)) - 1) ^ (1 1.4282E+00)

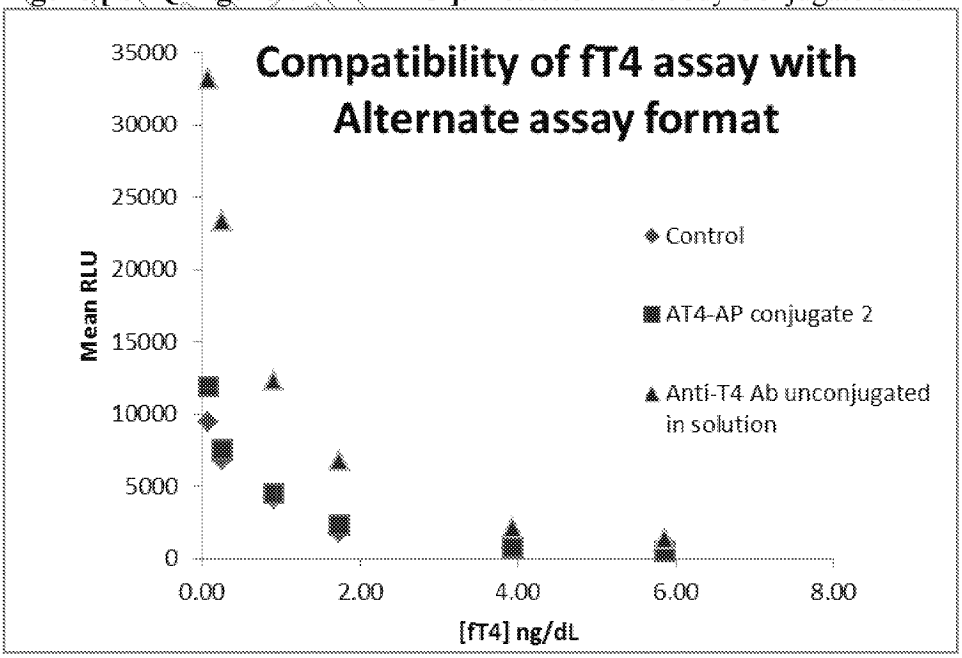
1.8Compatibility with alternate assay format

The control fT4 assay has the anti-T4 antibody conjugated to biotin in solution. Free and labeled (T4-AP conjugate) T4 compete in solution. The surface is an avidin surface. An alternate assay format was tested wherein the same T4 antibody is in solution in the unconjugated version and the surface is comprised of goat anti-mouse IgG. Another condition tested was an alternate T4-AP conjugate from different vendor. Both conditions matched very well and in one case the S/B was even better than control. The fT4 Assay is thus amenable to a change in format.

Table 13: Compatibility with alternate assay format

Control			T4-AP conjugate 2		Anti-T4 unconjugated	
fT4	**Signal**		**Signal**		**Signal**	
ng/dL	**Mean RLU**	**%CV**	**Mean RLU**	**%CV**	**Mean RLU**	**%CV**
Alpco Kit assigned						
0.08	9442	13	11891	13	33183	12
0.25	6792	8	7505	19	23358	8
0.91	4137	5	4529	14	12343	9
1.73	1781	13	2288	9	6778	3
3.93	647	14	714	10	2119	8
5.86	471	7	520	14	1359	18
Signal/Background	20		23		24	
Low end modulation	1.4		1.4		1.6	

Figure [SEQ Figure * ARABIC]: Detection Antibody Conjugate Stabilizers



1.9Capture Antibody Titration

The biotin conjugated anti-T4 antibody was titrated at 4 levels to determine the ideal concentration in solution. From the previous experiment T4-AP conjugate from vendor 2 was chosen to be used for the rest of the assay development, the concentration was the same 1:50,000 fold dilution. The calibrators were serum calibrators at a 1:5 sample dilution. The experiment was repeated with an alternate format: Anti-Mouse IgG surface and unconjugated anti-T4 antibody in solution. A concentration of 1 ug/mL was found to be optimal for modulation across the range and at the low end for both formats.

Table 14: Capture Antibody Titration

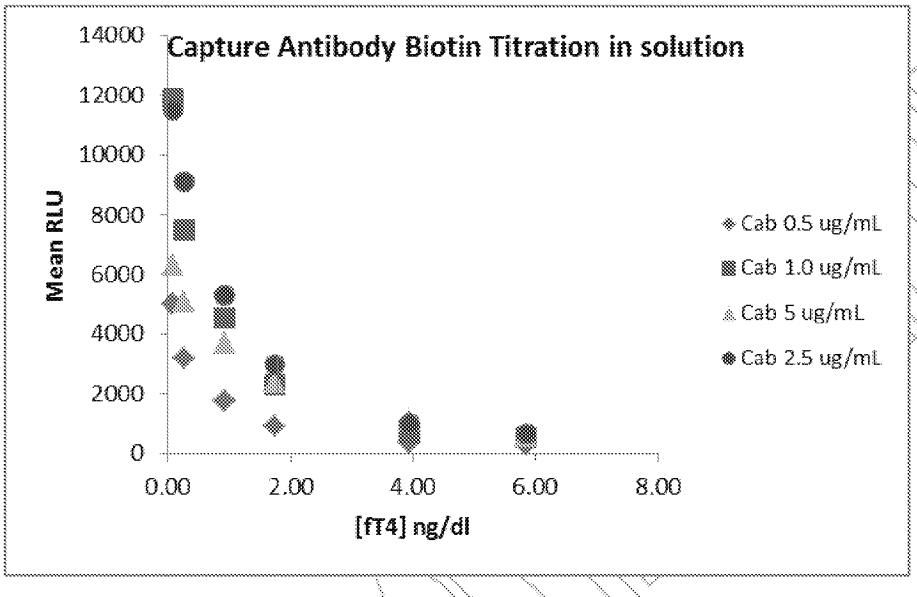
Anti-T4 Antibody Biotin conjugated

fT4 ng/dL Alpco Kit assigned	0.5 ug/mL Mean	0.5 ug/mL CV%	1 ug/mL Mean	1 ug/mL CV%	2.5 ug/mL Mean	2.5 ug/mL CV%	5 ug/mL Mean	5 ug/mL CV%
0.08	4990	9	11891	13	11500	12	6325	7
0.25	3209	5	7505	19	9112	11	5091	19
0.91	1748	9	4529	14	5318	9	3694	7
1.73	896	9	2288	9	2983	7	2384	10
3.93	367	4	714	10	995	9	1107	2
5.86	269	7	520	14	669	10	576	4
Signal/Background	19		23		17		11	
Low end modulation	1.6		1.6		1.3		1.2	

Anti-T4 Antibody unconjugated

fT4 ng/dL Alpco Kit assigned	0.5 ug/mL Mean	0.5 ug/mL CV%	1 ug/mL Mean	1 ug/mL CV%	2.5 ug/mL Mean	2.5 ug/mL CV%
0.08	12219	7	25949	4	38721	5
0.25	10264	15	19796	10	37299	18
0.91	5327	11	12579	11	28494	7
1.73	3265	4	5924	10	13711	10
3.93	1052	15	1474	9	3075	10
5.86	784	10	1145	5	2106	12
Signal/Background	16		23		18	
Low end modulation	1.2		1.3		1.0	

Figure [SEQ Figure * ARABIC]: Capture Antibody Titration



1.10 Detection Antibody Titration

The T4-AP labeled conjugate was tested using 3 conjugate stabilizers, 2 of them commercially available and one an in hosue formulation: Biostab (Fluka) , StabilZyme (Surmodics, Inc.) and low BSa (0.03%) blocking buffer at 1:50000 fold dilution. StabilZyme afforded the best S/B and low end modulation and was chosen as the stabilizer. The conjugate was titrated at 1:12,500, 25,000 and 50,000 in StabilZyme to determine the optimal concentration for the assay. A dilution of 1:25,000 fold dilution was chosen as it provided the best modulation and desired sensitivity.

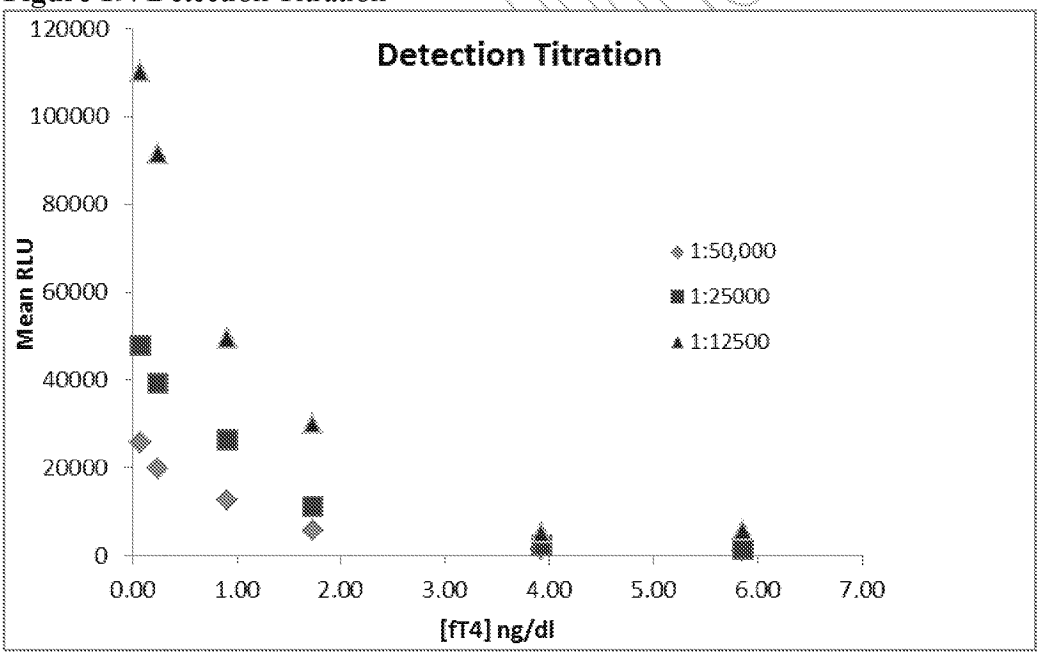
Table 15: Effect of different Stabilizers

fT4 ng/dL Alpco Kit assigned	Biostab Mean	Biostab CV%	StabilZyme Mean	StabilZyme CV%	(0.03%) BSA Blocking buffer Mean	(0.03%) BSA Blocking buffer CV%
0.08	9622	12	9906	10	17252	10
0.41	7315	16	6658	21	12892	13
2.56	1857	11	1373	11	2710	11
5.86	596	1	382	7	863	0
S/B	16		26		20	
Low end modulation	1.3		1.5		1.3	

Table 15. Detection conjugate titration

fT4 ng/dL Alpco Kit assigned	1:50,000 Mean	CV%	1:25,000 Mean	CV%	1:12,500 Mean	CV%
0.08	25949	4	47789	12	110313	10
0.25	19796	10	39176	18	91752	9
0.91	12579	11	26325	6	49618	7
1.73	5924	10	11378	12	29996	3
3.93	1474	9	2378	9	5285	14
5.86	1145	5	1646	10	5889	53
S/B	23		29		19	
Low end modulation	1.3		1.2		1.2	

Figure 19. Detection Titration



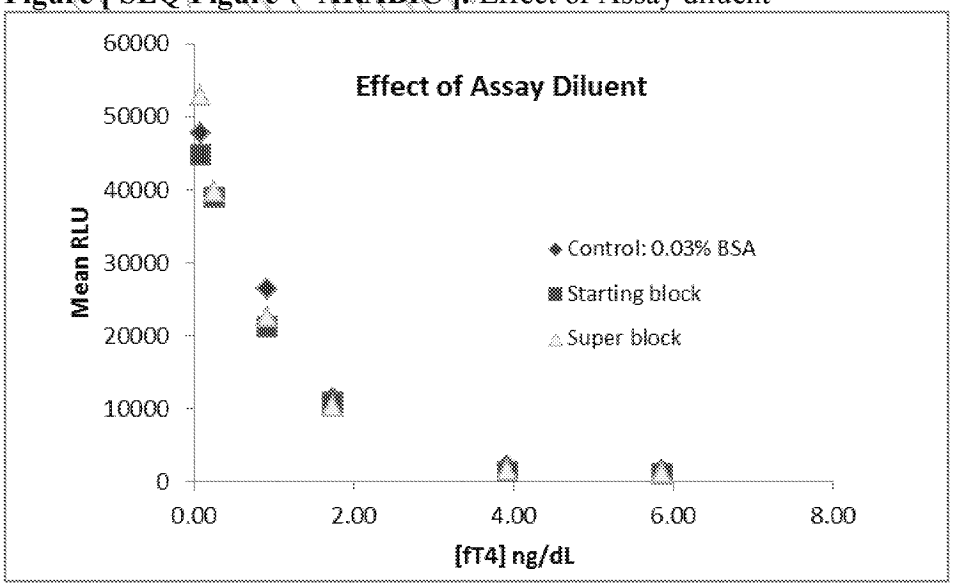
1.11 Effect of Assay diluent

The control assay diluent being used was 0.03% BSA blocking buffer. Two other commercially available blockers were tested and compared to the control. The commercial blocker SuperBlock blocking buffer afforded the best signal to background. The fT4 assay response is boosted by using a commercial blocker as the assay diluent.

Table 16: Effect of Assay diluent

fT4 ng/dL Alpco Kit assigned	CONTROL Mean	CV%	Starting Block Mean	CV%	Superblock Mean	CV%
0.08	47789	12	44807	12	52892	10
0.25	39176	18	38891	19	39731	10
0.91	26325	6	21304	10	22555	5
1.73	11378	12	10854	5	10451	8
3.93	2378	9	1423	4	1628	14
5.86	1646	10	1083	8	1241	18
S/B	29		41		43	
Low end modulation	1.2		1.2		1.3	

Figure [SEQ Figure * ARABIC]: Effect of Assay diluent



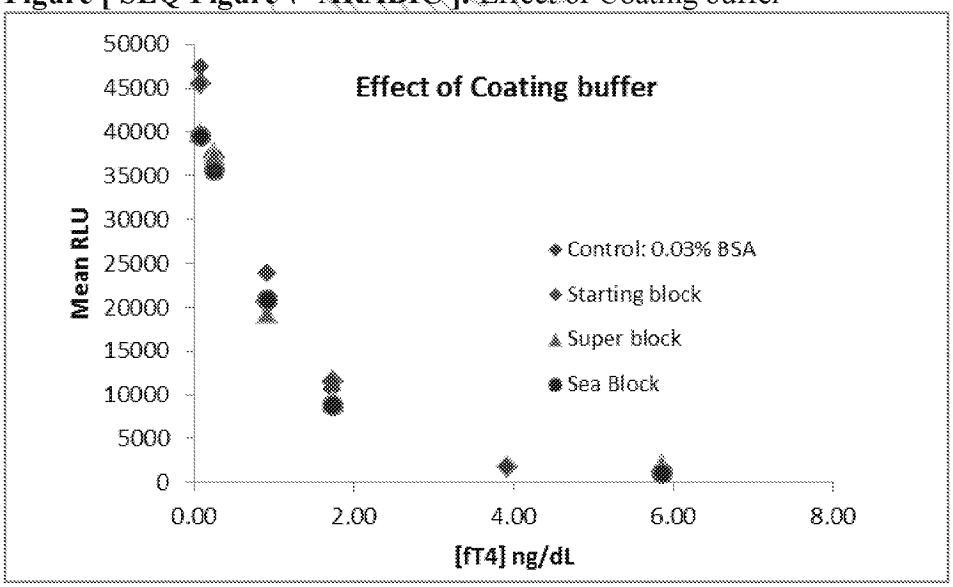
1.12 Effect of Coating Surface Buffer

The effect of changing the coating buffer was tested. The control coating buffer was again 0.03% BSA blocking buffer. Three commercially available blockers: Starting Block, Super Block and Sea Block were tested against the control buffer. Both low BSA as well as Starting Block as the coating surface buffer give godo modulation and dose response.

Table 17: Effect of Coating surface buffer

fT4	Control		Superblock		Starting Block		Sea Block	
ng/dL	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
Alpco Kit assigned								
0.08	45458	11	39914	11	47332	4	39493	4
0.25	37101	19	37506	6	37524	12	35657	14
0.91	20576	9	19272	18	23804	5	20855	1
1.73	11504	4	9273	4	10593	3	8754	18
5.86	1167	4	1255	11	1189	21	1094	15
S/B	39		32		40		36	
Low end modulation	1.2		1.1		1.3		1.1	

Figure [SEQ Figure * ARABIC]: Effect of Coating buffer



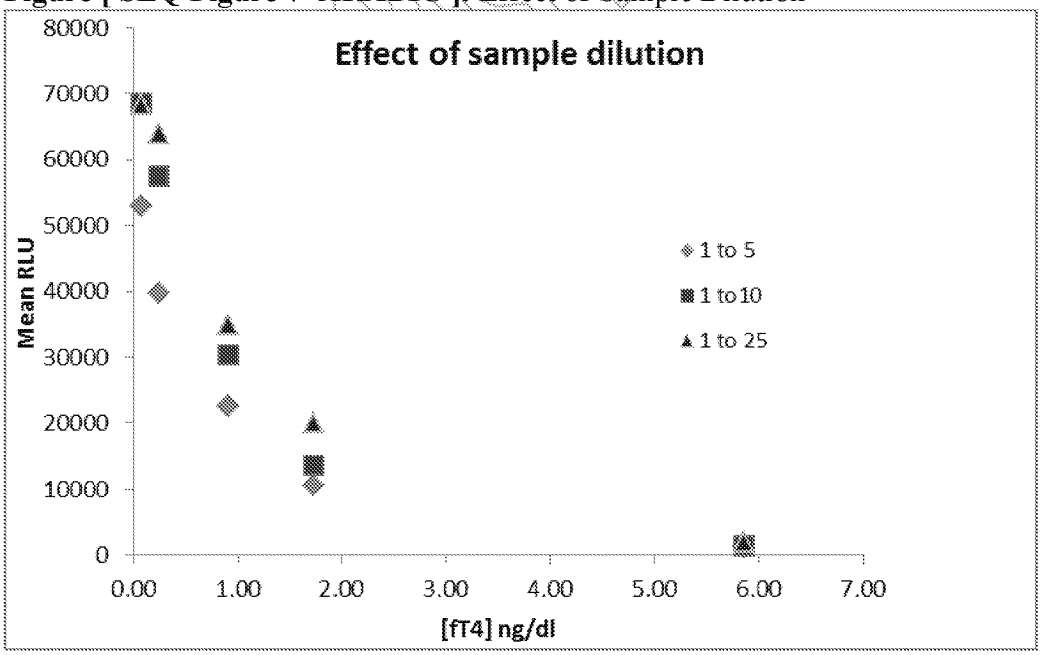
1.13 Effect of Sample Dilution

The effect of increasing sample dilution was tested at 1:5, 1:10 and 1:25 fold dilution. The S/B is high even at a sample dilution of 1:10. Given that all normal subjects have a fT4 level between 0.8 and 1.7 ng/dL both sample dilutions 1:5 and 1:10 afford excellent sensitivity. A 1:25 fold dilution is not desired since there is no dose modulation.

Table 18: Effect of Sample Dilution

fT4 ng/dL Alpco Kit assigned	1:5 Mean	1:5 CV%	1:10 Mean	1:10 CV%	1:25 Mean	1:25 CV%
0.08	52892	10	68601	11	68408	3
0.25	39731	10	57524	15	63995	7
0.91	22555	5	30415	7	34900	9
1.73	10451	8	13544	13	20026	2
5.86	1241	18	1444	17	1933	18
S/B	43		47		35	
Low end modulation	1.3		1.2		1.1	

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



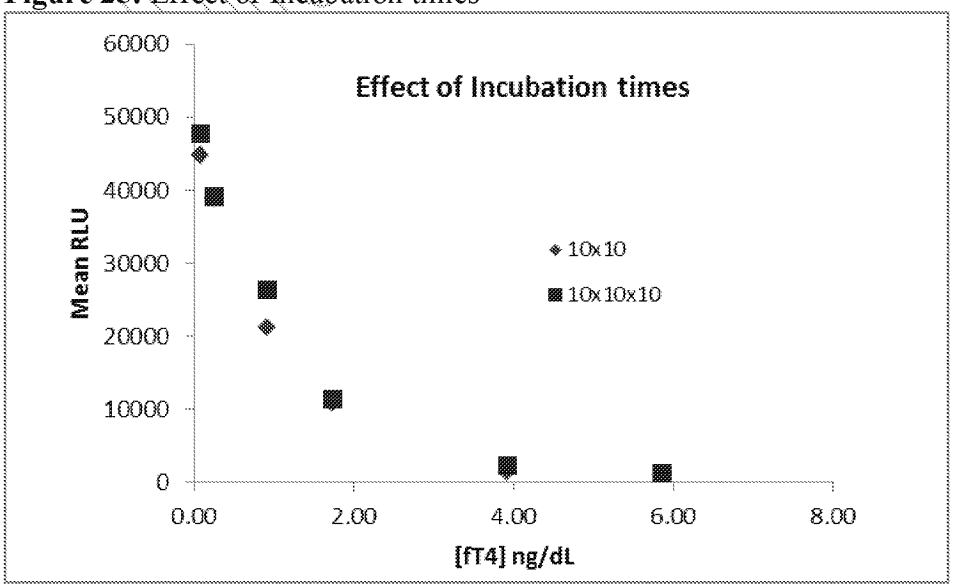
1.14 Effect of Incubation times

The effect of reagent incubation time on the fT4 assay was tested with 10x10 vs. 10x10x10 assay format. This being a competitive ELISa the antibody, sample and labeled T4 conjugate are mixed and then added to the surface where the compelx incubates for 10 min followed by wash and then a 10 minute incubation with the substrate. An additional "preincubation" of 10 minutes was added for the sample and antibody in solution before the conjugate was added and loaded onto surface. This was done to see if there was an added benefit of increasing incubation times. It was inferred that the additional 10 minute "preincubation" did not provide any dramatic increase in signal neither did it improve the sensitivity. The assay format of 10x10 was thus chosen as the final format.

Table 19: Effect of incubation times

fT4 ng/dL Alpco Kit assigned	10x10 Mean	10x10 CV%	10x10x10 Mean	10x10x10 CV%
0.08	44807	12	47789	12
0.25	38891	19	39176	18
0.91	21304	10	26325	6
1.73	10854	5	11378	12
3.93	1423	4	2378	9
5.86	1083	8	1246	10
S/B	41		38	
Low end modulation	1.2		1.2	

Figure 25: Effect of Incubation times



1.15 Calibration of fT4 assay

The fT4 assay was calibrated against the Certified Reference Material IRMM-468 from the Institute for Reference Materials and Measurements, Joint Research Centre, European Commission. A single lot of reagents were produced and the assay conditions used were as follows: anti-T4 antibody at 1 ug/mL in Superblock, 10 µg/ml Anti-mouse IgG surface coated using low BSA blocking buffer, T4-AP conjugate diluted 1:25,000 in StabilZyme and a sample dilution of 1:5, depleted serum spiked with IRMM-468.

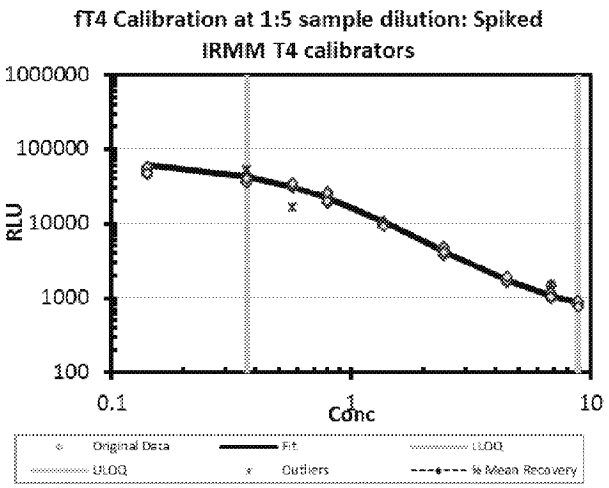
Note: The IRMM calibrators were first run on the Alpco ELISA kit to ascertain the "nominal" fT4 values. The dose response was compared to the in house T4 spiked depleted serum calibrators and the response and modulation were found to be equivalent.

Table 20: fT4 Calibration Curve

fT4 ng/dL Alpco Kit assigned	RLU		Concentration		% Recovery
	Mean	CV%	Mean	CV%	
0.14	49526	11	0.31	5.7	222
0.37	42752	18	0.38	29.9	102
0.57	33067	3	0.53	4.0	93
0.80	23195	16	0.77	15.0	97
1.36	9946	7	1.43	4.4	105
2.45	4308	10	2.42	6.2	99
4.46	1754	7	4.44	5.6	100
6.84	1215	21	6.28	19.1	92
8.84	838	7	8.74	2.5	99

Conc = 2.0560E+00 * (((4.8562E+00- 2.6348E+00) / (log 10(RLU) - 2.6348E+00)) - 1) ^ (1/1.2691E+00)

Figure 26: **Calibration curve**



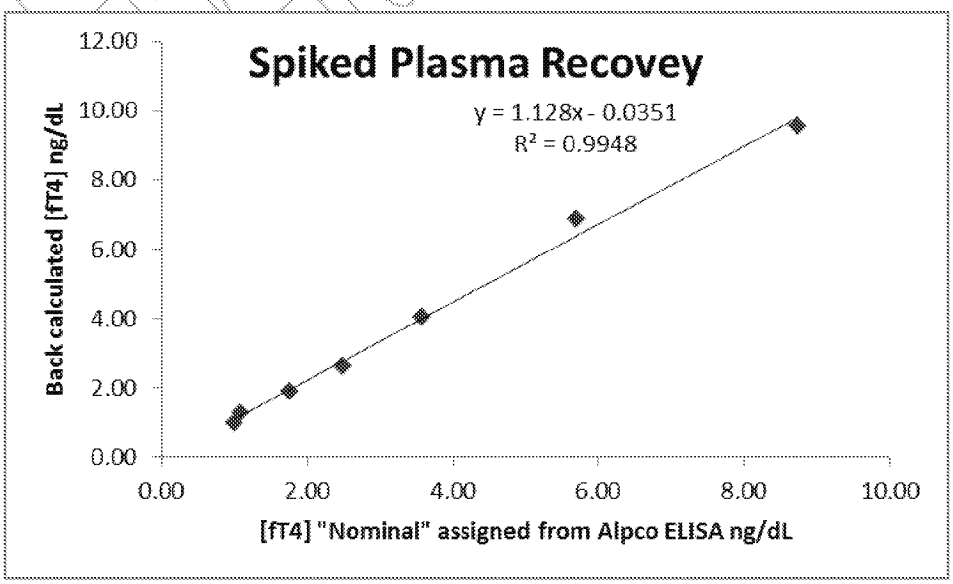
1.16 Spiked plasma recovery

Plasma from N=15 normal donors was pooled and T4 was spiked into it at levels spanning the range of the fT4 assay. The spiked plasma calibrators were run on an Alpco ELISA and assigned fT4 values. The above calibration was used to backcalculate the concentration of fT4 in the calibrators. The recovery was close to 100%.

Table [SEQ Table * ARABIC]: Spiked plasma recovery

fT4 ng/dL Alpco Kit assigned	RLU		Conc. ng/dL		% Recovery
	Mean	CV%	Mean	CV%	
1.00	17145	14	0.98	10.6	98
1.08	12155	14	1.26	9.2	117
1.77	6295	7	1.91	4.5	108
2.48	3775	1	2.62	0.5	105
3.57	2033	14	4.02	11.2	113
5.7	1026	11	6.86	9.8	120
8.74	774	6	9.57	0.8	109
OORH	678	10	OORH		

Figure [SEQ Figure * ARABIC]: Spiked Plasma Recovery



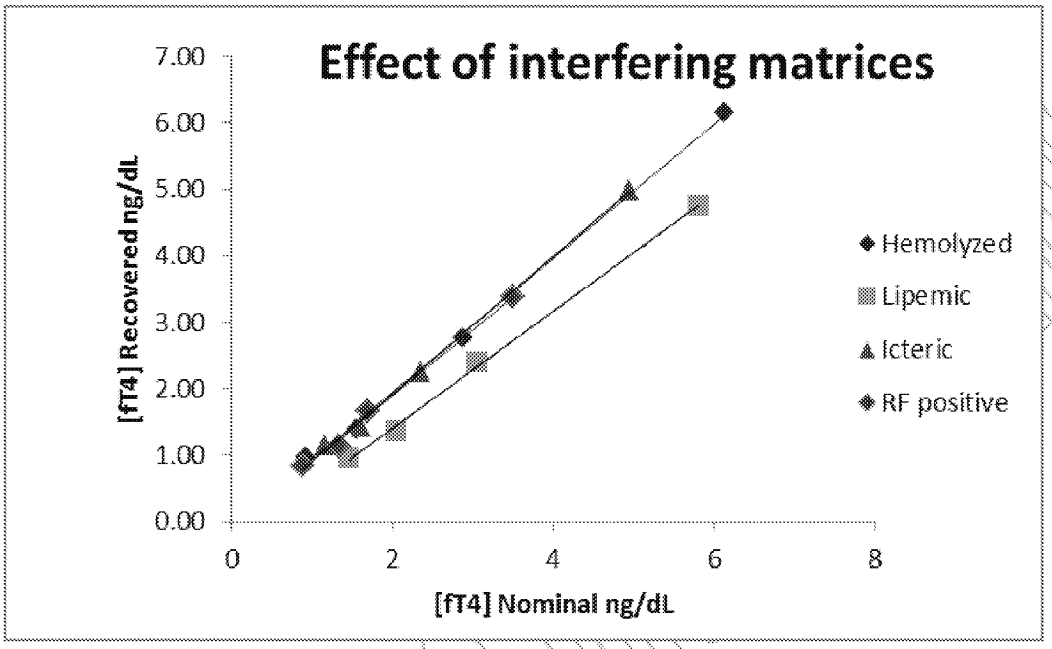
1.17Interfering Matrixes

Hemolyzed, lipemic, icteric and RF positive patient serum samples were obtained from a commercial source. The recovery of fT4 spiked into these potentially interfering matrixes was evaluated on the Theranos System. The serum calibration shown in section 1.15 was applied. The assay did not show any interference from hemolysed, icteric, or RF positive sample. The assay showed only about 70% recovery (<25% of nominal) for the lipemic sample tested.

Table 22: Interfering Matrixes

Interfering Matrix *(Sera)*	**fT4 ng/dL Alpco Kit assigned**	**RLU**		**Conc. ng/dL**		**% Recovery**
		Mean	**CV%**	**Mean**	**CV%**	
Hemolyzed	0.928	17292	8	0.97	6.1	105
	1.555	10342	12	1.40	8.2	90
	2.877	3484	9	2.76	5.9	96
	6.125	1216	14	6.16	13.2	100
Lipemic	1.454	17648	17	0.96	12.6	66
	2.042	10709	14	1.37	9.2	67
	3.053	4352	8	2.40	5.2	79
	5.806	1609	6	4.76	4.6	82
Icteric	1.153	13731	5	1.15	3.3	100
	1.612	10122	15	1.42	9.5	88
	2.356	4865	18	2.26	10.2	96
	4.939	1516	5	4.99	4.5	101
RF positive	0.886	20539	5	0.85	4.1	96
	1.338	13848	7	1.14	4.9	86
	1.701	7783	3	1.67	2.0	98
	3.494	2640	23	3.38	15.7	97

Figure 27. Effect of interfering matrices



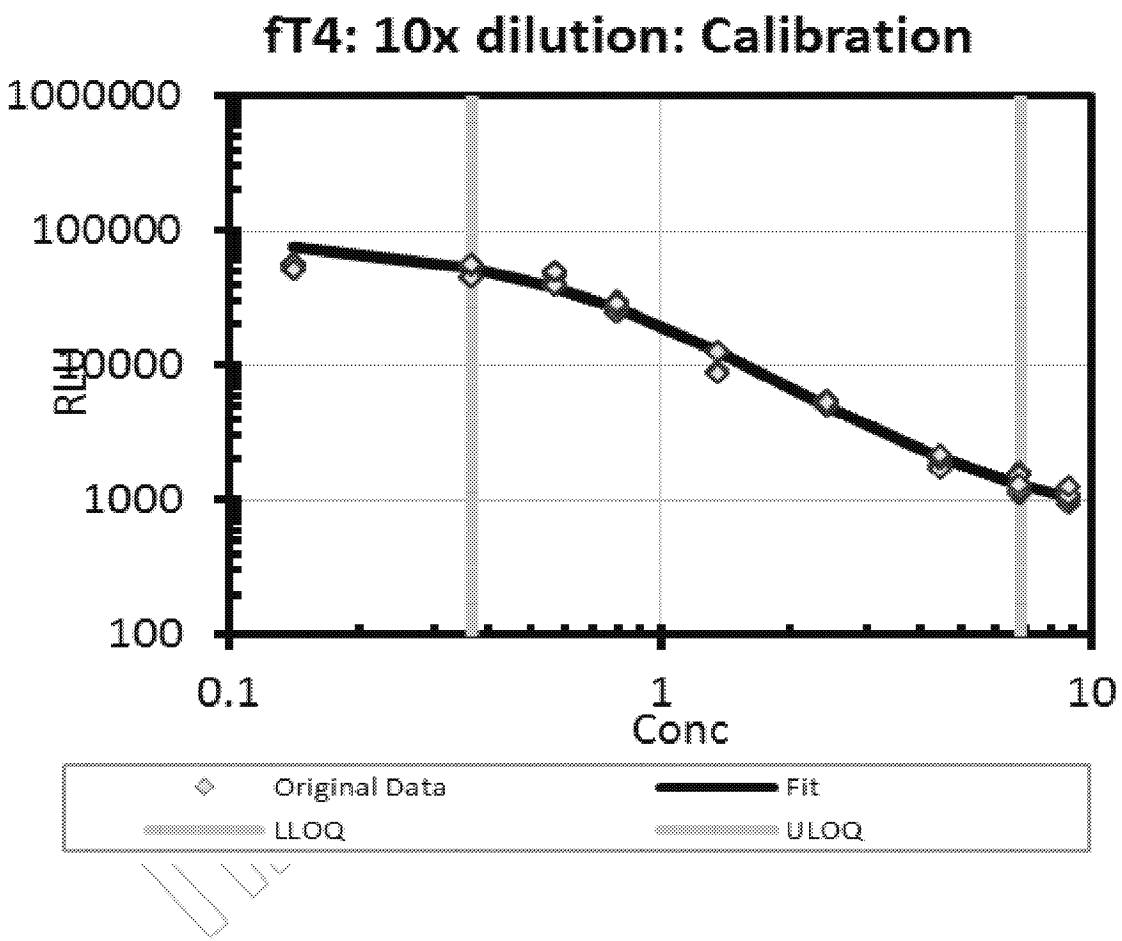
1.18Final Calibration: Comparison 5 and 10 fold sample dilution

In order to finalize the calibration for the FT4 assay the spiked IRMM T4 depleted serum calibrators were tested in a 1:10 sample dilution protocol against the 1:5 sample dilution. The table below compares the performance of the assay under the two conditions. A 10-fold dilution provides excellent precision and accuracy at the LLOQ (lower limit of quantitation) and ULOQ (upper limit of quantitation) which have been defined as 0.37 and 6.84 ng/dL. At the working range of the assay the recoveries are close to 20% of nominal. The precision for the calculated concentration is below 25% at ULOQ and LLOQ and <20% across the curve. The 10 fold sample dilution is picked as the final condition for thefT4 assay.

Table 23: Final Calibration

	10x Dilution					5x Dilution				
fT4 ng/dL Alpco Kit assigned	RLU Mean	RLU CV%	Concentration Mean	Concentration CV%	% Recovery	RLU Mean	RLU CV%	Concentration Mean	Concentration CV%	% Recovery
0.14	54284	5	0.35	9.5	247	49526	11	0.31	5.7	222
0.37	50796	11	0.39	17.5	107	42752	18	0.38	29.9	102
0.57	44965	11	0.46	14.7	81	33067	3	0.53	4.0	93
0.80	27235	10	0.78	8.9	98	23195	16	0.77	15.0	97
1.36	10700	25	1.53	15.7	113	9946	7	1.43	4.4	105
2.45	5227	1	2.35	0.9	96	4308	10	2.42	6.2	99
4.46	1952	11	4.68	9.2	105	1754	7	4.44	5.6	100
6.84	1323	16	6.88	17.2	101	1215	21	6.28	19.1	92
8.84	1070	9	9.32	12.8	105	838	7	8.74	2.5	99
S/B	51					59				
Low end modulation	1.1					1.2				
LLOQ	0.37 ng/dL					0.37 ng/dl				
ULOQ	6.84 ng/dl					8.84 ng/dL				
Curve fit	Log Lin4PL					LogLIn4PL				
Accuracy at LLOQ	107					120				
Precision at LLOQ	15.8					10				
Accuracy at ULOQ	107					105				
Precision at ULOQ	15.7					10.5				

Figure 28. Final calibration for Ft4 assay



1.19Final clinical sample correlation

The above final calibration curve was used to test a total of 20 different clinical samples chosen across the range of the assay. The samples were split into several categories: Calibration standards from Maine Standards, Bio Rad Immunoassay Controls, patient samples from Bioreclamation and 7 samples that were RF positive sera. All samples were run on the Alpco fT4 ELISA and the results were compared. The data show an excellent linear correlation for all the samples together as well as in separate categories in particular all 7 RF positive patient samples correlate well.

Table 24: Clinical sample correlation: Theranos vs. Alpco ELISA

Clinical Samples	Alpco fT4 ELISA ng/dL	Theranos ng/dL
MS Base	0.06	0.24
MS Level 2	0.93	0.92
MS Level 3	1.69	1.30
MS Level 4	2.33	2.54
MS Level 5	3.3	2.77
Biorec 2	0.57	0.87
Biorec 5	0.37	0.64
Biorec 9	1.26	1.14
Biorec 13	0.84	1.68
Biorec 14	1.13	1.09
Biorad 1	0.6	0.90
Biorad 2	2.19	1.89
Biorad 3	4.0	3.42
RF 1	1.095	0.82
RF2	1.254	0.93
RF 3	0.901	0.76
RF 4	0.932	0.85
RF 5	0.946	0.79
RF 6	1.439	1.02
Rf 8	1.358	0.95

Figure 29: All samples correlation

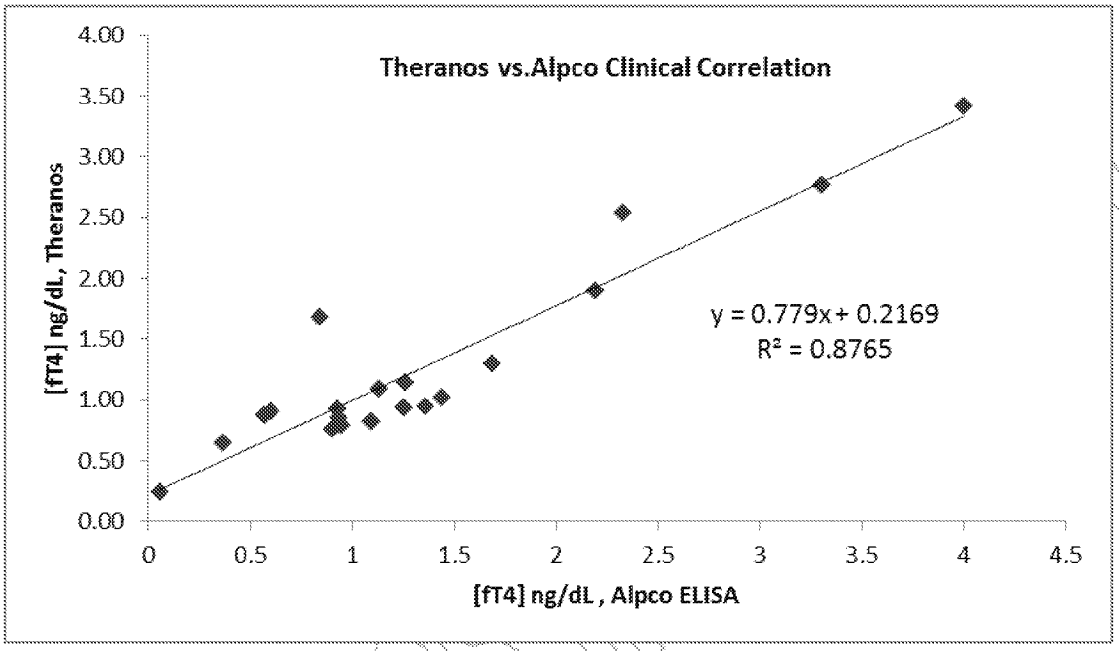
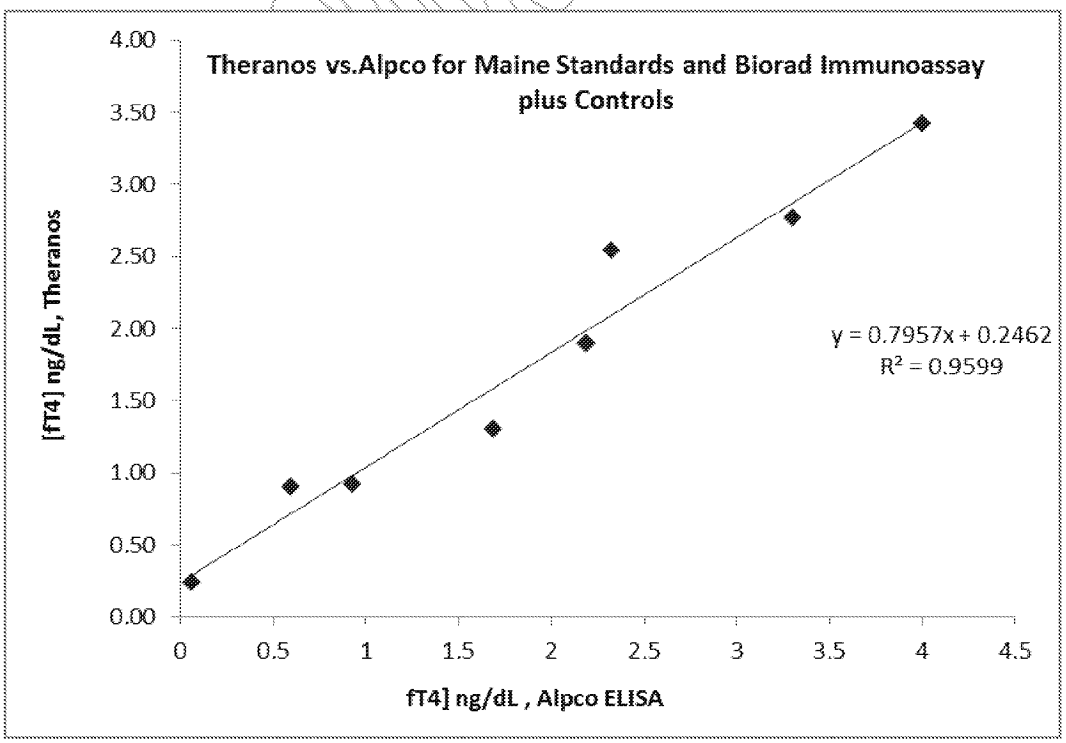


Figure 30: Correlation of Theranos fT4 assay with commercially available controls



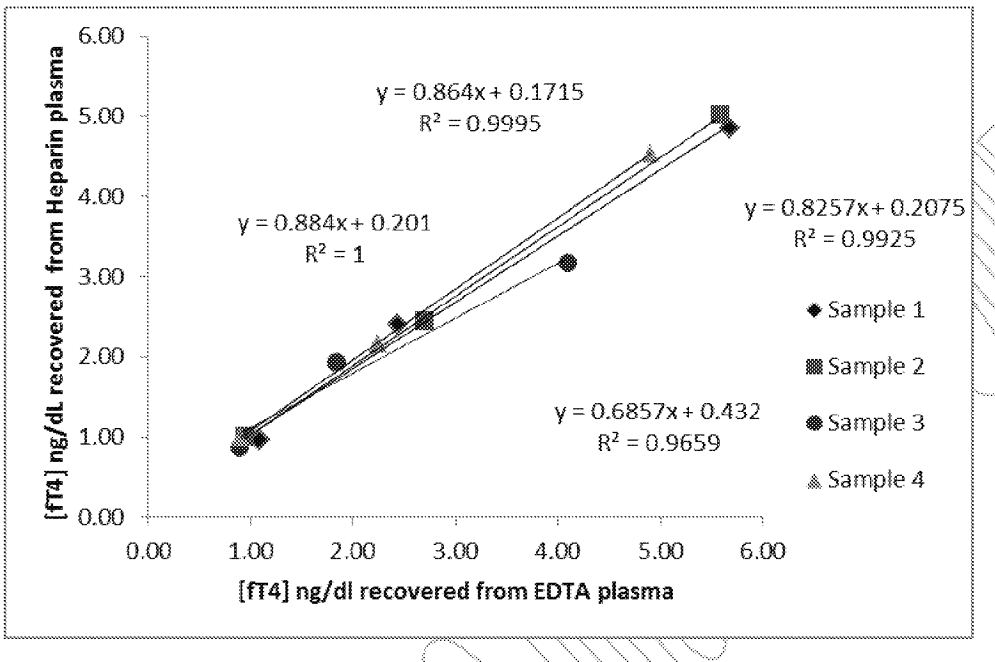
1.2 Effect of Anticoagulant: EDTA vs. Heparin plasma

Whole blood samples from 4 donors each collected in EDTA as well as Heparin tubes was obtained from the Stanford donor bank. Each sample was spun down and the respective plasma was generated. The plasma was screened for fT4 endogenous levels and was found to be 0.8-1.1 ng/dl which is the normal range. Each sample was then spiked at 2 levels above endogenous. Since all 3 commercial ELISAs for fT4 are specific for serum samples the above samples could not be run on them in order to obtain fT4 levels that could then be used to compute recoveries. Alternatively the heparin and EDTA sample data from each patient was correlated and the R2 values were found to be 0.99 for each sample indicating that there was no effect of the anticoagulant on the assay.

Table 25. Effect of anticoagulant

Whole Blood barcode	EDTA Plasma				Heparin Plasma			
	RLU		Conc. ng/dL		RLU		Conc. ng/dL	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
W07051110105700	17781	10	1.08	6.7	20675	3	0.97	2.4
	5002	15	2.44	10.1	5071	13	2.41	8.0
	1598	19	5.68	15.5	1868	8	4.84	7.0
	RLU		Conc. ng/dL		RLU		Conc. ng/dL	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
W07051110106000	21794	15	0.94	10.6	19713	16	1.01	12.1
	4289	17	2.70	11.0	4917	6	2.45	3.7
	1589	7	5.59	6.4	1865	22	5.02	20.5
	RLU		Conc. ng/dL		RLU		Conc. ng/dL	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
W07051110106100	23520	16	0.88	12.4	23922	13	0.87	10.7
	7876	11	1.83	6.6	7218	2	1.93	1.4
	2372	20	4.09	17.1	3291	4	3.17	2.9
	RLU		Conc. ng/dL		RLU		Conc. ng/dL	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
W07051110106200	22823	4	0.90	3.4	19744	4	1.00	2.9
	5777	19	2.24	13.2	5950	6	2.17	3.5
	1865	17	4.90	14.6	2018	7	4.54	6.3

Figure 31: Correlation of Recovered fT4 concentrations: Heparin vs EDTA plasma



1.3Stability

TBD