theranos™
redefining healthcare

T Uptake (aka T3 Uptake) Assay Development Report

Theranos, Inc.

Sept 6, 2011

Prepared by: Tina Noyes

theranos™
redefining healthcare

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 determines the percentage of thyroid hormone uptake in human whole blood (automatically processed into plasma by the Theranos system), plasma or serum. The assay has a reportable range of 25.6% to 57.0%, and is calibrated to BioRad Liquicheck Immunoassay Plus Controls and to Randox Immunoassay Premium Plus Controls.

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:

- US Biological T3 Uptake Cat #T8425-04

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

A biotin-labeled anti-sheep antibody coated on an avidin surface serves as the capture surface for the competitive ELISA. The sample (serum, plasma or whole automatically processed into plasma by the Theranos system) is diluted and mixed with an antibody, an alkaline phosphatase-labeled triiodothyronine conjugate (T3-AP), and an excess of unlabeled thyroxine (T4). The antibody is an anti-thyroxine antibody that also recognizes the T3-AP conjugate with similar affinity. The reaction mixture is incubated on the capture surface, then the surface is washed and the alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

The added unlabeled T4 competes with endogenous T4 and T3 for the available binding sites of Thyroxine Binding Globulin (TBG). The T3-AP conjugate does not compete for binding to TBG. The unlabeled thyroid hormones (both added and endogenous) that are not bound to TBG compete with the T3-AP conjugate for the capture antibody. The resulting multi-directional competition produces a sensitive assay to determine the percentage of unoccupied TBG binding sites in the sample.

A greater amount of unoccupied binding protein sites in the sample results in greater uptake of the unlabeled T4 by the sample, which results in lower residual amounts of free T4 and therefore more binding of the T3-AP to the capture antibody. Thus the signal generated by the assay is inversely proportional to the percentage of occupied binding sites, or % Uptake.

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Thyroxine	Sigma	T2376-5G
Sheep Anti-Thyroxine Antibody (CAb)	Calbioreagents	P078
Rabbit Anti-Sheep IgG (Fc), Biotin Conjugated (Surface CAb)	Fitzgerald	43C-CB1326
Triiodothyronine Alkaline Phosphatase Conjugate	Fitzgerald	65-IT25
Phospho Glo Substrate	KPL	55-60-04
Low BSA Blocking Buffer (0.03% BSA (Fraction V, 99% Pure) in TBS, 0.05% Sodium Azide)	Sigma	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041
Thyroid Hormone Depleted Serum (Calibrator Matrix)	Sunny Lab	SF509-2

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

1.2 Antibody Screening

1.2.1 Antibody Response to T3-AP and T4-AP[TC "Detection Antibody Conjugate Verification" \f C \l "1"]

To determine the optimal capture antibody for the T Uptake ELISA, antibodies were screened for their response to the T4-AP and T3-AP conjugates, with the goal of selecting antibodies that recognize both T4-AP and T3-AP. The monoclonal antibody selected for the Total T4 assay, already shown to have no cross reactivity to free T3, was included as a control. The concentration of the T4-AP and T3-AP are dilutions relative to the concentrated stock. It is possible for an antibody to cross react to T3-AP and not to free T3. The ideal antibody for this application would be such, since the assay would then not be affected by differences in fT3 concentration, while any potential differences in the fT4 concentration will be overwhelmed by the addition of an excess of T4.

Antibodies 1 and 6 showed the most equivalent response to T3-AP and T4-AP in response to both varied concentrations of T3-AP, T4-AP and to varied concentrations of the antibody in the presence of a steady concentration of the AP conjugates, and were therefore chosen for further testing.

Table [SEQ Table * ARABIC]: Antibody Information

Number	Vendor	Cat #	Type
1	Calbioreagents	P078	Sheep polyclonal
4	Fitzgerald	20C-CR1053R	Rabbit polyclonal
5	Fitzgerald	20-TR40	Rabbit polyclonal
6	Fitzgerald	20-TS40	Sheep polyclonal
8	Novus	NB100-66010	Sheep polyclonal
9	US Biological	T5460-02	Sheep polyclonal
12	US Biological	T5460-12	Rabbit polyclonal
13	US Biological	T5460-03	Rabbit polyclonal
14	US Biological	T5460-14	Sheep polyclonal
15	Genway	18-783-78216	Sheep polyclonal
16	Raybiotech	MD-14-0559	Rabbit polyclonal
27	East Coast Bio	P01-97-41R-IF	Rabbit polyclonal

Table [SEQ Table * ARABIC]: Antibody Screening – Response to Varied Concentration of T3-AP and T4-AP.

Ab #	[T3-AP]	Mean RLU	CV %	[T4-AP]	Mean RLU	CV %	Slope T3/T4 Response
Control (Ab #20)	0.001	22522	13.7	0.001	1272425	8.5	0.02
	0.0001	4571	26.5	0.0001	173148	15.2	
	0.00001	604	8.1	0.00001	19363	8.9	
1	0.001	1678210	8.4	0.001	1483288	5.5	1.09
	0.0001	427276	8.4	0.0001	284370	13.2	
	0.00001	52743	9.2	0.00001	29239	11.1	
4	0.001	45666	1.6	0.001	529757	9.9	0.08
	0.0001	17184	9.7	0.0001	81158	8.8	
	0.00001	2450	15.3	0.00001	9523	8.5	
5	0.001	56498	10.2	0.001	612091	4.3	0.08
	0.0001	21134	8.0	0.0001	91596	3.9	
	0.00001	2904	6.6	0.00001	11538	3.6	
6	0.001	1000863	3.1	0.001	1238750	13.6	0.77
	0.0001	247706	16.3	0.0001	189279	13.4	
	0.00001	28453	13.5	0.00001	23252	10.5	
8	0.001	217665	4.4	0.001	47782	10.0	4.58
	0.0001	24499	4.9	0.0001	5467	5.0	
	0.00001	2619	3.4	0.00001	1005	5.6	
9	0.001	13130	3.9	0.001	15404	8.5	0.84
	0.0001	1959	7.3	0.0001	1734	8.4	
	0.00001	310	6.6	0.00001	289	5.5	
12	0.001	42748	8.8	0.001	494024	3.2	0.08
	0.0001	14944	11.7	0.0001	79557	4.1	
	0.00001	2197	7.1	0.00001	7707	6.5	
13	0.001	65462	17.6	0.001	141026	12.2	0.42
	0.0001	20619	15.9	0.0001	18222	12.0	
	0.00001	2868	15.7	0.00001	2323	16.5	
14	0.001	177630	14.3	0.001	557155	10.3	0.29
	0.0001	57632	17.1	0.0001	73243	15.7	
	0.00001	7215	14.3	0.00001	8618	9.6	
15	0.001	192374	2.2	0.001	48050	6.5	3.99
	0.0001	23577	4.9	0.0001	5162	8.7	
	0.00001	2792	4.8	0.00001	1033	3.0	

Table Cont'd: Antibody Screening – Response to Varied Concentration of T3-AP and T4-AP.

Ab #	[T3-AP]	Mean RLU	CV %	[T4-AP]	Mean RLU	CV %	Slope T3/T4 Response
16	0.001	68095	5.7	0.001	138472	5.6	0.45
	0.0001	20213	8.4	0.0001	16999	8.7	
	0.00001	2818	7.9	0.00001	2071	7.4	
27	0.001	674940	5.0	0.001	1380790	4.2	0.43
	0.0001	248608	1.6	0.0001	205228	3.9	
	0.00001	33415	1.9	0.00001	23925	4.1	

Table [SEQ Table * ARABIC]: Antibody Screening – Response with Varied Antibody Concentration

Ab #	[Ab]	T3-AP @ 1:10,000 Mean RLU	T3-AP @ 1:10,000 CV %	T4-AP @ 1:10,000 Mean RLU	T4-AP @ 1:10,000 CV %	Slope T3/T4 Response
Control (Ab 20)	1 ug/mL	14544	20.5	817595	13.0	0.01
	0.1 ug/mL	6881	42.8	88275	14.3	
	0.01 ug/mL	4655	13.4	16237	8.0	
1	1 ug/mL	1051575	0.8	1182487	0.6	0.94
	0.1 ug/mL	116555	0.3	287274	9.0	
	0.01 ug/mL	19851	1.6	42817	1.7	
4	1:50,000	22604	11.5	171941	17.5	0.10
	1:500,000	7417	6.6	21855	1.2	
	1:5,000,000	5230	23.1	5162	17.2	
5	1:50,000	30952	8.6	253226	5.0	0.10
	1:500,000	9409	3.0	29613	2.9	
	1:5,000,000	6253	0.7	8499	14.6	
6	1:50,000	590128	2.7	831011	0.3	0.73
	1:500,000	57868	3.1	134525	0.5	
	1:5,000,000	15512	4.5	18884	0.7	
8	1:50	2355991	12.8	1264576		-1.20
	1:500	2317476	14.1	1402156	5.2	
	1:5000	2043710	12.7	1518015	9.4	
9	1:50,000	15678	2.6	14386	7.0	0.51
	1:500,000	11715	2.5	6128	1.5	
	1:5,000,000	10855	1.2	5414	1.2	
12	1:50,000	23219	7.0	194461	9.1	0.08
	1:500,000	8955	20.7	26729	9.2	
	1:5,000,000	8077	1.5	6409	10.1	
13	1:200,000	26714	32.2	59990	12.5	0.34
	1:2,000,000	10227	19.4	11516	3.8	

1:20,000,000	8586	28.2	6339	1.1	

Table Cont'd: Antibody Screening – Response with Varied Antibody Concentration

		T3-AP @ 1:10,000		T4-AP @ 1:10,000		
Ab #	**[Ab]**	**Mean RLU**	**CV %**	**Mean RLU**	**CV %**	**Slope T3/T4 Response**
14	1:50,000	89055	0.1	247715	3.2	0.32
	1:500,000	17424	6.6	27997	4.8	
	1:5,000,000	11550	21.6	7138	2.0	
15	1:50	2499271	1.7	1454608	1.2	-4.88
	1:500	2365085	5.3	1487157	1.3	
	1:5000	1906120	1.8	1472044	3.5	
16	1:200,000	40325		56197	9.9	0.64
	1:2,000,000	11630	13.8	10728	1.4	
	1:20,000,000	8425	5.3	6366	2.4	
27	1 ug/mL	33918	10.2	247483	11.3	0.10
	0.1 ug/mL	12966	10.4	27691	8.7	
	0.01 ug/mL	8324	24.9	8459	11.9	

1.2.2 Antibody Screening in T3 Uptake Assay Format

After determining the candidate capture antibodies based on response to T3-AP and T4-AP, the top choice antibodies were screened with BioRad Immunoassay Plus serum controls in the competitive assay format. The initial test was done at a 1:10 sample dilution and an excess of 50ug/dL added unlabeled thyroxine in the T3-AP conjugate mixture (both conjugate and sample are diluted 10x in this protocol).

Antibody 1 and 6 showed the best assay modulation as expected based on the response to T3-AP and T4-AP.

Table [SEQ Table * ARABIC]: Antibody Screen Competitive Format

Ab #	% Uptake	Mean RLU	CV %	Modulation
1	28.5	3006	11.4	
	39.6	1325	1.5	
	45.4	712	6.6	4.2
6	28.5	1827	2.1	
	39.6	825	5.7	
	45.4	480	7.0	3.8
8	28.5	10954	1.3	
	39.6	9779	4.8	
	45.4	10454	5.2	1.0
9	28.5	308	0.8	
	39.6	264	5.0	
	45.4	288	13.5	1.1
15	28.5	12566	12.9	
	39.6	11729	13.1	
	45.4	12733	2.4	1.0
16	28.5	2764	3.9	
	39.6	1518	12.9	
	45.4	1318	3.9	2.1

1.3 Titration of Reagents

Assay reagents were titrated to achieve the optimal assay response using the BioRad Immunoassay Plus serum controls as calibrators. Since antibody 1 and 6 both showed similar modulation in the screen, both antibodies were optimized with titration for better comparison. The best modulation with a 1:10 sample dilution was achieved with antibody#1 at 1 ug/mL (final) and T3-AP 1:10,000 with T4 at 20ug/dL (the conjugate/T4 mixture is diluted 1:10 into the final sample mixture). Back calculated CVs and recovery were excellent.

Table [SEQ Table * ARABIC]: Titration of Reagents for Antibody #1

				Signal, RLU			Back-Calculated % Uptake		
[T4] ug/dL	[Ab] ug/mL	[T3-AP]	% Uptake	Mean RLU	CV %	Modulation	Mean % Uptake	CV %	% Recovery
50	1	1:50,000	28.5	3006	11.4		29.1	3.8	102
			39.6	1325	1.5		38.0	0.5	96
			45.4	712	6.6	4.2	46.6	2.1	103
50	1	1:5000	28.5	24237	4.8		28.8	1.6	101
			39.6	10052	5.0		38.5	1.7	97
			45.4	5805	5.7	4.2	46.2	1.9	102
50	10	1:50,000	28.5	10618	15.4		29.0	4.6	102
			39.6	4180	4.5		38.3	1.4	97
			45.4	2242	2.8	4.7	46.3	0.9	102
50	10	1:10,000	28.5	143280	4.8		28.5	1.3	100
			39.6	43589	10.6		39.6	2.9	100
			45.4	26327	9.0	5.4	45.5	2.4	100
			29.9*	100126	7.5		31.5	2.1	105
20	1	1:10,000	28.5	97216	3.0		28.8	1.5	101
			39.6	22172	6.1		38.7	0.8	98
			45.4	9249	3.5	10.5	46.1	0.4	102
			29.9*	57174	2.4		32.0	1.2	107

* A clinical sample with a reported % Uptake (measured on Siemens Centaur) was included as an unknown.

Table [SEQ Table * ARABIC]: Titration of Reagents for Antibody #6

[T4] ug/dL	[Ab]	[T3-AP]	% Uptake	Signal, RLU Mean RLU	Signal, RLU CV %	Signal, RLU Modulation	Back-Calculated % Uptake Mean % Uptake	Back-Calculated % Uptake CV %	Back-Calculated % Uptake % Recovery
50	1:50,000	1:50,000	28.5	1827	2.1		28.9	0.7	101
			39.6	825	5.7		38.3	2.0	97
			45.4	480	7.0	3.8	46.4	2.5	102
50	1:50,000	1:5000	28.5	14277	7.1		28.4	2.5	100
			39.6	4959	8.0		40.7	2.7	103
			45.4	3817	7.5	3.7	44.5	2.6	98
50	1:5000	1:50,000	28.5	6756	1.8		29.0	0.6	102
			39.6	3022	6.1		38.0	2.0	96
			45.4	1650	15.4	4.1	46.7	5.1	103
50	1:5000	1:10,000	28.5	123095	6.5		29.0	1.5	102
			39.6	36748	9.3		38.2	2.0	96
			45.4	15525	10.8	7.9	46.5	2.6	102
			29.9	71388	3.7		32.8	0.8	110
20	1:50,000	1:10,000	28.5	45908	20.0		28.4	1.0	100
			39.6	9304	4.0		40.0	2.9	101
			45.4	5195	5.9	8.8	45.1	0.9	99
			29.9	24879	13.0		32.4	1.9	108

* A clinical sample with a reported % Uptake (measured on Siemens Centaur) was included as an unknown.

1.4 Establishment of In-House Calibrators

To create a 6 point calibrator set, serum depleted in T4 and T3 (but not in TBG) was spiked with T4 and serially diluted to produce a range of % Uptake. These calibrators were measured in the US Biological T3 Uptake Kit and compared to the BioRad Immunoassay Plus Control set to assign the T3 Uptake values. Then these calibrators were run in the Theranos assay to verify the assay response, and used for subsequent optimization.

Table [SEQ Table * ARABIC]: Standard Curve with Serum Calibrators Assigned to US Biological Kit

% Uptake	Signal, RLU Mean RLU	Signal, RLU CV %	Back-Calculated % Uptake Mean	Back-Calculated % Uptake CV %	Back-Calculated % Uptake % Recovery
57.0	738	7.8	57.1	0.6	100
51.1	1204	2.2	51.0	0.8	100
41.6	2071	5.8	40.2	2.9	97
34.2	2597	2.5	36.0	1.2	105
28.6	4694	13.8	28.2	4.4	98
25.6	8336	0.6	25.7	0.0	100

% Uptake =10^(0.5655*(LOG(S))^3 - 5.6935*(LOG(S))^2 + 18.618*(LOG(S)) - 18.148)

1.5 Cross Reactivity and Interference

Cross reactivity and interference with relevant substances was tested for antibody #1 in a Total T4 assay format, since it is impossible to do so in the dual-competitive format as the affinity of test substances for TBG is variable or non-existent. Test substances were spiked into T3, T4-depleted serum (endogenous T4 = 0.7 ug/dL) with or without added T4. Antibody#1 showed no cross reactivity or interference from any of the test substances.

		Signal, RLU			Calculated Conc. ug/dL		
Test Substance	**[Test Substance] ug/dL**	**[TT4] ug/dL**	**Mean RLU**	**CV %**	**Mean**	**CV %**	**% Recovery**
Control	0.00	27.7	2538	4.2	28.2	4.9	102
	0.00	15.7	4955	5.1	15.5	3.6	99
	0.00	10.7	7555	2.7	11.2	2.4	104
	0.00	5.7	13123	5.8	5.8	9.3	102
	0.00	2.7	19262	4.2	2.7	10.5	100
	0.00	0.7	29662	10.5			
Triiodothyronine (T3)	25.00	0.7	25857	8.5	OORL	-	-
	10.00	0.7	26235	14.6	OORL	-	-
	1.00	0.7	28317	3.7	OORL	-	-
	0.25	0.7	30765	2.3	OORL	-	-
	0.05	0.7	25659	13.8	OORL	-	-
	0.00	0.7	31044	2.9	OORL	-	-
Triiodothyronine (T3)	3.00	15.7	4662	11.1	16.3	8.6	104
	3.00	5.7	11221	16.3	6.1	6.5	106
	3.00	0.7	28233	2.4	OORL		
3,5-Diiodo-L-thyronine (T2)	3.00	15.7	5129	7.3	15.1	5.2	96
	3.00	5.7	13745	7.3	5.4	12.1	94
	3.00	0.7	27873	6.5	OORL		
3-Iodo-L-tyrosine	3.00	15.7	4343	11.2	17.2	8.5	109
	3.00	5.7	13859	2.9	5.3	5.0	93
	3.00	0.7	35008	4.1	OORL		
3,3',5' Triiodothyronine (Reverse T3)	0.15	15.7	5885	8.5	13.7	6.6	87
	0.15	5.7	13500	13.3	5.6	21.8	98
	0.15	0.7	27662	8.3	OORL		

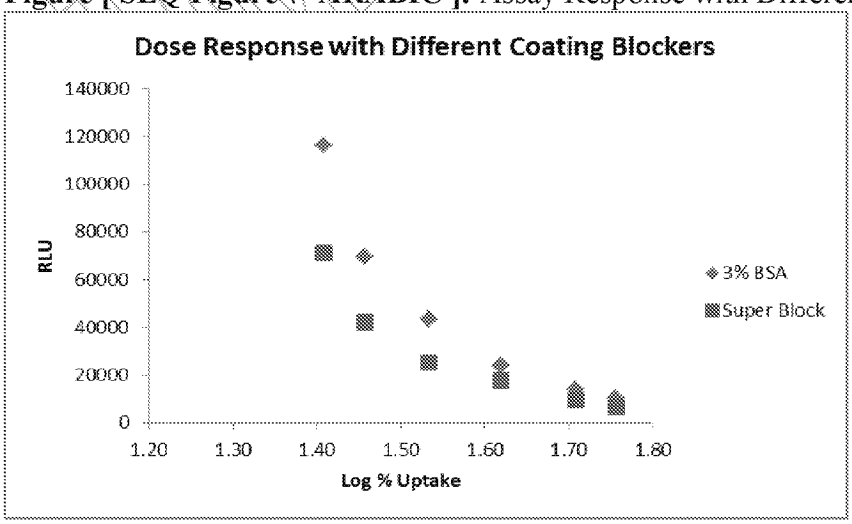
1.6 Effect of Coating Blockers

To optimize the anti-sheep capture surface, 3% BSA Blocking buffer was tested versus BSA-free Super Block used for the initial assay testing. Standard 3% BSA blocking buffer produced higher signal and modulation was similar for both buffers however modulation in the mid-range was better with 3% BSA coating. Thus 3% BSA blocking buffer was chosen as the coating buffer for further optimization.

Table [SEQ Table * ARABIC]: Assay Response with Different Coating Buffers

		Signal, RLU			Back-Calculated % Uptake		
Coating Buffer	**% Uptake**	**Mean RLU**	**CV %**	**Modulation**	**Mean**	**CV %**	**% Recovery**
3% BSA in TBS	57.0	10420	3.0	11.2	57.1	1.0	100
	51.1	14075	12.0	8.3	51.5	4.3	101
	41.6	24048	6.8	4.8	42	2.6	101
	34.2	43281	2.0	2.7	33.9	0.7	99
	28.6	69617	7.8	1.7	29.2	2.2	102
	25.6	116260	4.0		25.7	0.8	100
Super Block	57.0	6383	3.3	11.1	56.7	0.5	99
	51.1	9275	4.5	7.7	52.1	1.3	102
	41.6	17821	15.6	4.0	40.8	6.7	98
	34.2	25328	3.4	2.8	34.9	1.5	102
	28.6	42035	1.7	1.7	28.8	0.5	100
	25.6	71128	12.8		25.8	1.3	101

Figure [SEQ Figure * ARABIC]: Assay Response with Different Coating Buffers



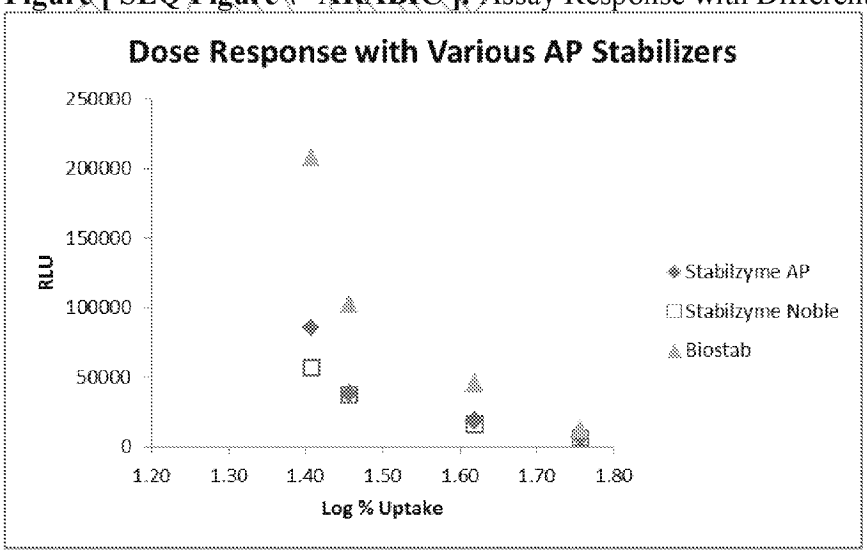
1.7 Effect of Alkaline Phosphatase Stabilizers

Different commercial alkaline phosphatase stabilizing buffers were tested. Biostab produced the best assay modulation overall, though Stabilzyme AP showed very similar modulation and could also be used.

Table [SEQ Table * ARABIC]: Assay Response with Different AP Stabilizers

		Signal, RLU			Back-Calculated % Uptake		
Stabilizer	**% Uptake**	**Mean RLU**	**CV %**	**Modulation**	**Mean**	**CV %**	**% Recovery**
StabilZyme AP	57.0	6616	7.6	12.9	56.5	2.5	99
	41.6	18752	3.6	4.6	40	1.2	96
	28.6	38078	7.1	2.2	31.7	2.3	111
	25.6	85419	2.0		24.3	0.6	95
StabilZyme Noble	57.0	6745	6.7	8.4	57.5	2.6	101
	41.6	16458	7.9	3.4	40.7	3.1	98
	28.6	37447	4.9	1.5	29.6	1.8	103
	25.6	56736	2.1		25.2	0.8	98
Biostab	57.0	12637	4.0	16.4	57.8	1.2	101
	41.6	45642	5.0	4.6	39.3	1.5	94
	28.6	102662	5.6	2.0	30.8	1.7	108
	25.6	207776	7.3		24.9	2.2	97

Figure [SEQ Figure * ARABIC]: Assay Response with Different AP Stabilizers



1.8 Sample Dilution

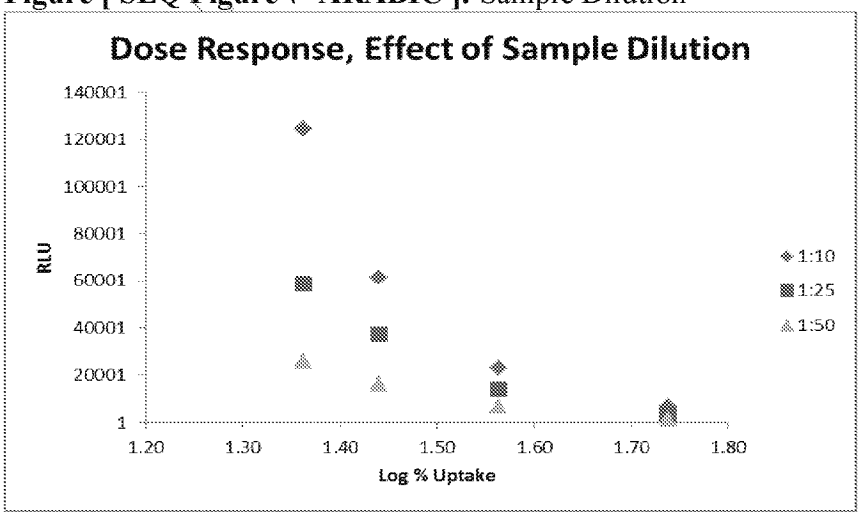
The effect of final sample dilutions of 1:10, 1:25 and 1:50 was tested. Since the amount of unlabeled added T4 has already been optimized at 20ug/dL of sample, this level of T4 and T3-AP will be maintained in proportion to the amount of final diluted sample mixture. A final sample dilution of 1:25 was chosen for further optimization, however a 1:50 sample dilution may also be possible. Back-calculated CVs and recovery were excellent at all dilution levels.

Table [SEQ Table * ARABIC]: Sample Dilution

Sample Dilution	% Uptake	Signal, RLU			Back-Calculated % Uptake		
		Mean RLU	CV %	Modulation	Mean	CV %	% Recovery
1:10	57.0	6526	9.1	19.1	57.1	0.0	100
	41.6	22882	19.7	5.4	42.1	8.0	101
	28.6	61284	6.4	2.0	28.9	1.9	101
	25.6	124394	5.1		25.8	0.1	101
	29.9*	63102	1.7		28.6	0.5	96
1:25	57.0	3511	3.2	16.7	57.2	0.1	100
	41.6	13879	4.6	4.2	41.8	1.8	101
	28.6	37233	5.4	1.6	28.9	1.7	101
	25.6	58594	3.8		25.8	0.7	101
	29.9*	34784	4.4		29.5	1.4	99
1:50	57.0	1688	7.1	15.3	58.9	2.1	103
	41.6	6847	5.2	3.8	38.6	1.5	93
	28.6	16331	3.2	1.6	29.7	1.0	104
	25.6	25870	4.8		25.9	1.5	101
	29.9*	15466	12.3		30.3	3.7	101

* A clinical sample with a reported % Uptake (measured on Siemens Centaur) was included as an unknown.

Figure [SEQ Figure * ARABIC]: Sample Dilution



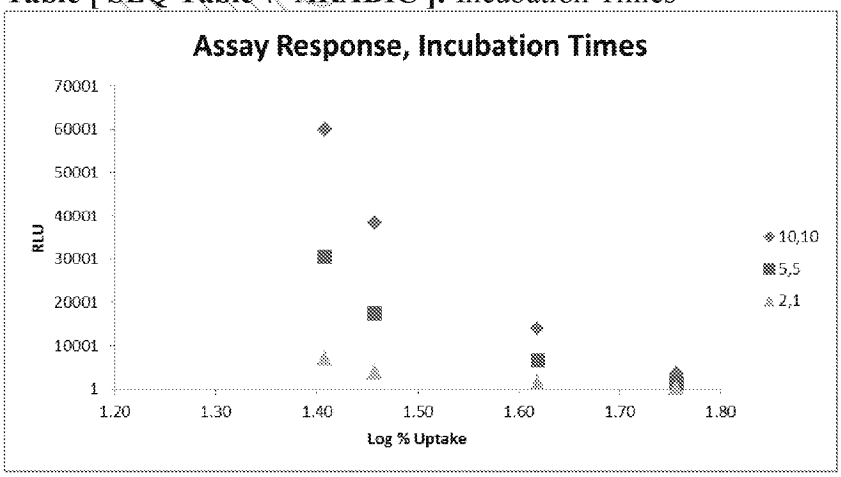
1.9 Incubation Time

The effect of shortened incubation was tested with sample mixture-substrate incubation times of 10-10 minutes, 5-5 minutes, and 2-1 minutes. With shortened substrate incubation the read time difference between the duplicate tips becomes significant so the response was calibrated by tip position. The competitive assay was sufficiently sensitive with a short 2-1 minute incubation time (total reagent incubation time 3 minutes) so these conditions were used for further assay development.

Table [SEQ Table * ARABIC]: Incubation Time

Incubation Time	% Uptake	Signal, RLU			Back-Calculated % Uptake		
		Tip 1 Mean RLU	Tip 2 Mean RLU	Modulation	Mean	CV %	% Recovery
10, 10 Minutes	57.0	3480	3542	17.0	56.8	0.2	100
	41.6	13726	14032	4.3	41.4	1.8	100
	28.6	36072	38394	1.6	28.5	1.3	100
	25.6	57143	60045		25.5	0.4	99
5, 5 Minutes	57.0	1528	1340	22.8	56.8	1.2	100
	41.6	5881	6614	4.6	41.5	3.1	100
	28.6	17935	17552	1.7	28.6	2.3	100
	25.6	28651	30491		25.5	0.8	100
2, 1 Minutes	57.0	297	323	21.6	56.7	2.1	99
	41.6	1316	1556	4.5	41.5	1.1	100
	28.6	3454	3955	1.8	28.6	1.6	100
	25.6	5559	6968		25.6	1.3	100

Table [SEQ Table * ARABIC]: Incubation Times



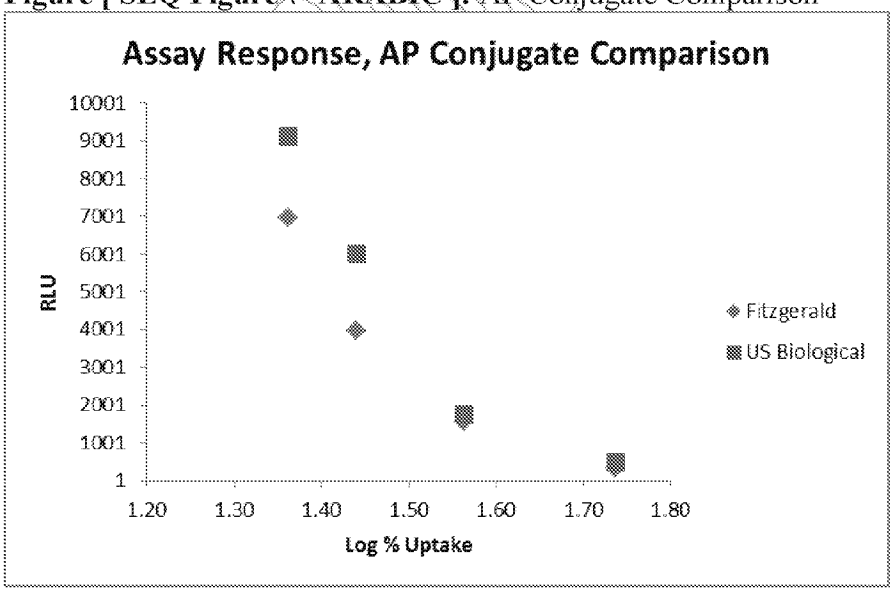
1.10 Alkaline Phosphatase Conjugate Comparison

Commercial T3-AP conjugates are available from Fitzgerald and from US Biological, so both sources were evaluated in the assay. Both conjugates performed similarly. Since the Fitzgerald conjugate had been used for development thus far, it was retained as the first choice however the US Biological conjugate can be used as a backup.

Table [SEQ Table * ARABIC]: AP Conjugate Comparison

T3-AP Conjugate	% Uptake	Signal, RLU			Back-Calculated % Uptake		
		Tip 1 Mean RLU	Tip 2 Mean RLU	Modulation	Mean	CV %	% Recovery
Fitzgerald	57.0	297	323	21.6	55.4	3.3	97
	41.6	1316	1556	4.5	35.7	0.9	86
	28.6	3454	3955	1.8	27.3	1.7	95
	25.6	5559	6968		23.5	3.4	92
US Biological	57.0	410	480	19.0	54.4	2.2	95
	41.6	1607	1753	5.2	37.2	2.1	90
	28.6	5147	6004	1.5	26.5	0.7	92
	25.6	7687	9101		23.6	2.0	92

Figure [SEQ Figure * ARABIC]: AP Conjugate Comparison



1.11 Plasma and Serum Screen

Matching Lithium-Heparin plasma and serum from 10 normal donors was screened in the Theranos System to confirm the normal range and test for matrix effects. The normal serum samples ranged from 27.3% to 31.7% with the mean 29.1% uptake. Plasma samples ranged from 27.2% to 32.6% uptake with the mean 29.0%. There was no significant difference between serum and plasma results and all results corresponded with an expected normal range for T3 Uptake of 25.0% to 35.0%[1].

Standard Curve

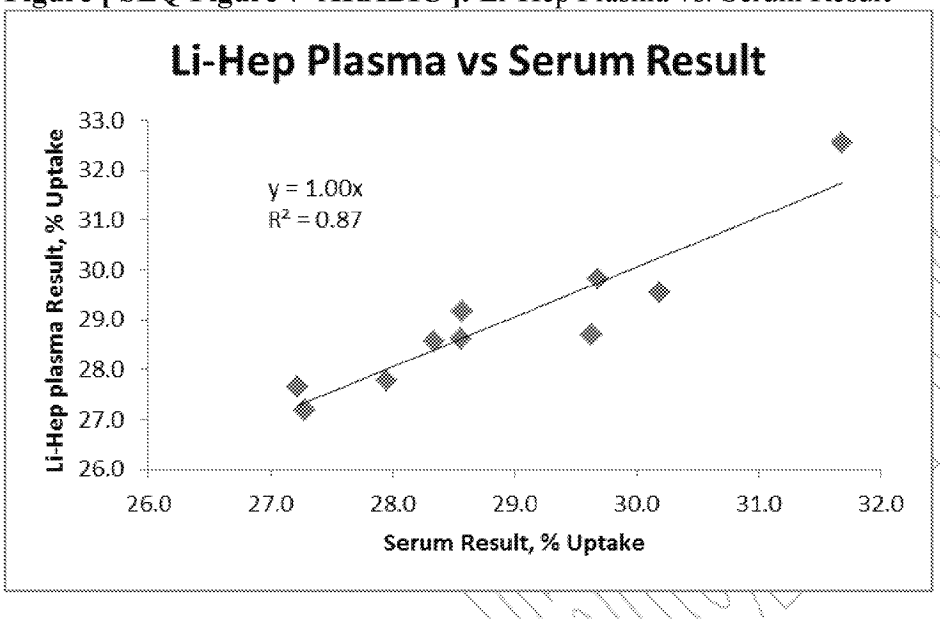
% Uptake	Signal, RLU		Back-Calculated % Uptake		
	Tip 1 Mean RLU	Tip 2 Mean RLU	Mean	CV %	% Recovery
57.0	324	388	56.8	2.0	100
51.1	509	600	51.7	1.3	101
41.6	1450	1612	41.1	3.6	99
34.2	2593	3194	34.4	1.4	101
28.6	4639	5163	28.8	1.7	101
25.6	6155	7278	25.7	8.9	100

Tip 1 % Uptake =10^(-0.0443*(LOG(S))^3 + 0.3382*(LOG(S))^2 - 1.0636*(LOG(S)) + 2.9936)
Tip 2 % Uptake =10^(-0.0513*(LOG(S))^3 + 0.4196*(LOG(S))^2 - 1.3594*(LOG(S))+ 3.3505)

Table [SEQ Table * ARABIC]: Normal Plasma and Serum Screen

Sample #	Serum		Li-Hep Plasma		% Difference from Serum Result
	Mean % Uptake	CV %	Mean % Uptake	CV %	
2	30.2	2.0	29.5	1.9	-2.1
3	28.6	1.6	29.2	1.3	2.1
6	27.3	1.7	27.2	3.4	-0.4
8	29.6	2.1	28.7	0.9	-3.1
10	27.2	4.7	27.7	3.8	1.6
12	28.0	1.4	27.8	2.9	-0.6
13	29.7	1.9	29.8	1.3	0.5
14	28.3	2.0	28.6	3.0	0.8
15	28.6	4.9	28.6	2.2	0.2
16	31.7	7.1	32.6	4.0	2.7
Min	27.3		27.2		
Max	31.7		32.6		
Mean	28.9		29.0		

Figure [SEQ Figure * ARABIC]: Li-Hep Plasma vs. Serum Result



1.12 Effect of Anticoagulant on Plasma Results

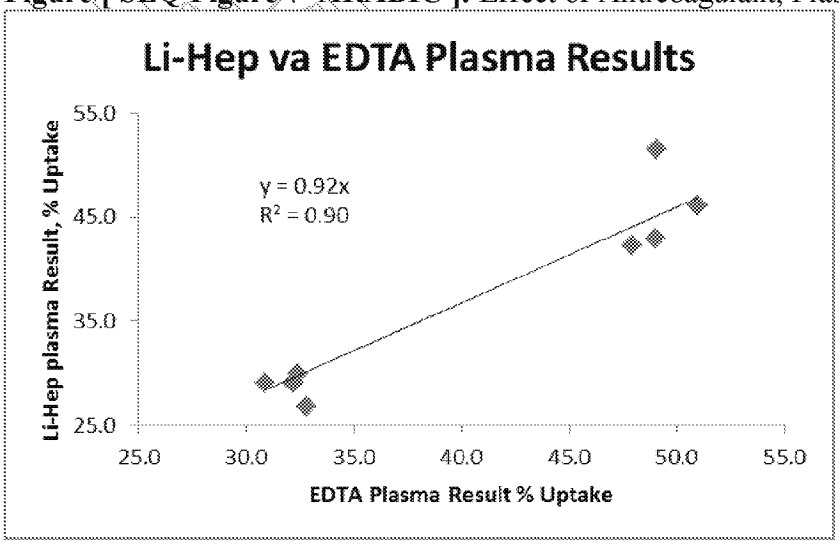
The Theranos System will prepare plasma from both EDTA and lithium-heparin-treated blood. To determine if the % Uptake assay is impacted by the choice of anticoagulant, plasma was prepared from EDTA tubes and from Li-Hep tubes for 4 normal donors, and the endogenous levels and a T4-spiked level were tested.

There was a significant different between results from Lithium Heparin vs. EDTA plasma; results from EDTA plasma were about 8% higher than Li-Hep plasma results. Since Li-Hep plasma results correlate very well with serum results and serum is the standard matrix measured in reference methods, Lithium Heparin plasma is recommended for this assay.

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

		EDTA Plasma		Lithium Heparin Plasma		
Sample #	**Spike**	**Mean % Uptake**	**CV %**	**Mean % Uptake**	**CV %**	**% Difference in LiHep vs EDTA**
1	Unspiked	30.9	6.2	29.0	1.8	-6.0
2	Unspiked	32.4	3.1	30.0	4.8	-7.3
3	Unspiked	32.8	4.0	26.8	1.9	-18.1
4	Unspiked	32.2	2.2	29.1	2.0	-9.5
1	Spiked 25 ug/dL T4	51.0	1.8	46.1	7.2	-9.6
2	Spiked 25 ug/dL T4	49.0	0.9	42.9	1.4	-12.4
3	Spiked 25 ug/dL T4	47.9	3.9	42.3	1.7	-11.6
4	Spiked 25 ug/dL T4	49.0	2.8	51.6	2.2	5.1

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



1.13 Clinical Correlation

The T Uptake % is used to compute the Free Thyroxine Index (FTI) from the Total T4. The formula is FTI = (T4 conc.) x (% T-Uptake)/100. Clinical samples and control sera from BioRad and Randox were tested in the US Biological T3 Uptake ELISA kit and in the Theranos System, and the FTI calculated for each method using the Theranos TT4 result. The correlation was excellent, with no aberrant results.

Figure [SEQ Figure * ARABIC]: Clinical Sample Results, Free Thyroxine Index (FTI)

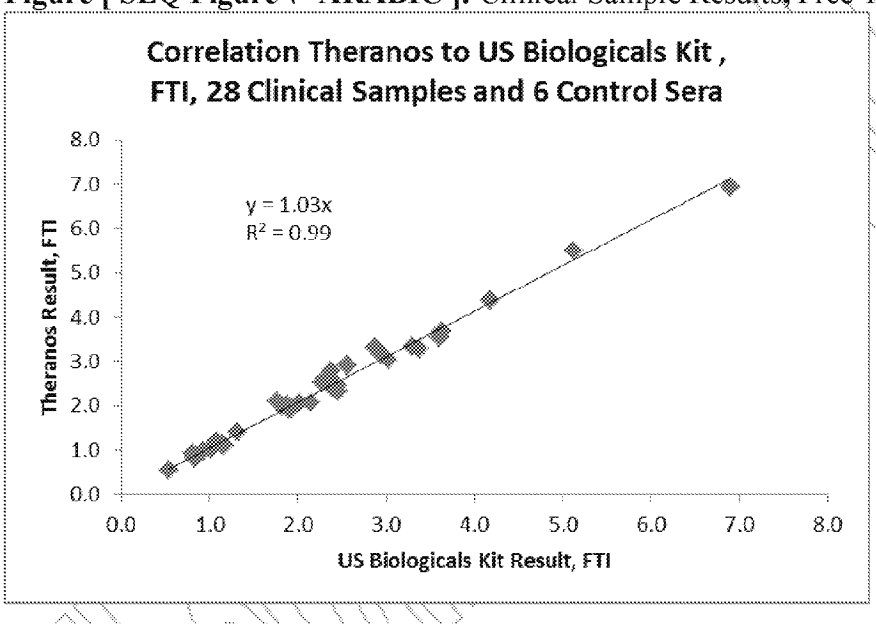


Figure [SEQ Figure * ARABIC]: Clinical Sample Results, % Uptake

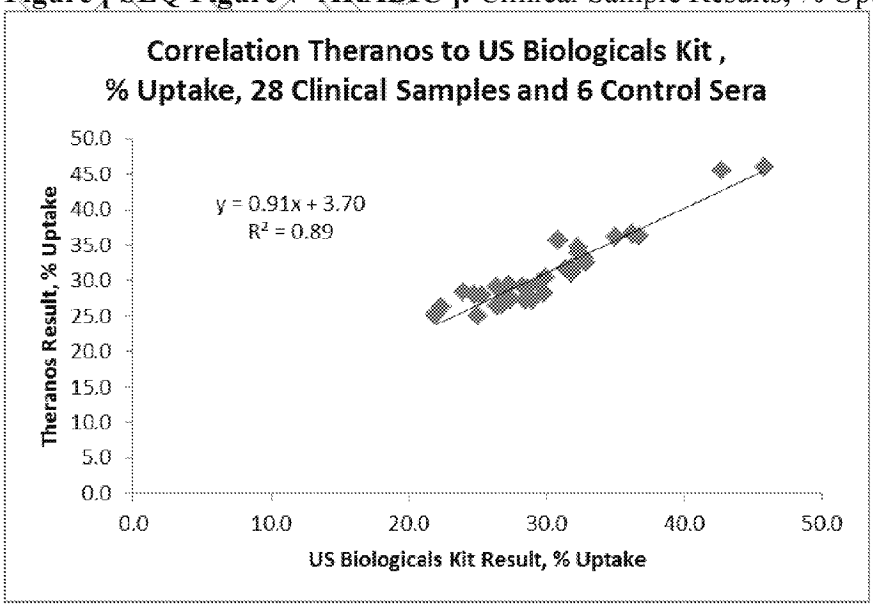


Table [SEQ Table * ARABIC]: Clinical Sample Results

Sample Type	Sample #	Theranos [TT4], ug/dL	% Uptake		FTI	
			US Biological	Theranos	US Biological	Theranos
BioRad Control	1	7.3	26.6	26.7	1.9	1.9
BioRad Control	2	10.0	36.2	36.5	3.6	3.7
BioRad Control	3	15.1	45.8	46.0	6.9	6.9
Randox Control	1	2.7	35.0	36.2	0.9	1.0
Randox Control	2	2.3	36.7	36.3	0.8	0.8
Randox Control	3	3.1	42.7	45.4	1.3	1.4
Clinical Sera Set 1	1	3.9	26.4	26.5	1.0	1.0
Clinical Sera Set 1	2	4.2	26.8	26.6	1.1	1.1
Clinical Sera Set 1	3	7.4	24.0	28.3	1.8	2.1
Clinical Sera Set 1	4	2.2	25.0	25.0	0.5	0.5
Clinical Sera Set 1	5	3.7	21.9	25.2	0.8	0.9
Clinical Sera Set 1	6	4.1	26.4	29.1	1.1	1.2
Clinical Sera Set 1	7	10.3	29.5	29.6	3.0	3.0
Clinical Sera Set 1	8	10.3	24.8	28.0	2.6	2.9
Clinical Sera Set 1	9	8.5	28.9	27.2	2.5	2.3
Clinical Sera Set 1	10	6.9	26.5	28.8	1.8	2.0
Clinical Sera Set 1	11	3.9	29.9	28.1	1.2	1.1
Clinical Sera Set 1	12	9.0	25.2	27.9	2.3	2.5
Clinical Sera Set 1	13	6.9	27.3	29.4	1.9	2.0
Clinical Sera Set 1	14	10.6	22.4	26.3	2.4	2.8
Clinical Sera Set 1	15	7.6	28.5	27.3	2.2	2.1
Clinical Sera Set 1	16	7.0	27.4	27.3	1.9	1.9
Clinical Sera Set 2	1	11.0	26.7	28.7	2.9	3.2
Clinical Sera Set 2	2	7.1	28.3	29.2	2.0	2.1
Clinical Sera Set 2	3	15.8	32.3	34.8	5.1	5.5
Clinical Sera Set 2	4	10.6	31.8	31.0	3.4	3.3
Clinical Sera Set 2	5	2.9	28.6	28.8	0.8	0.8
Clinical Sera Set 2	6	12.9	32.3	33.9	4.2	4.4
Clinical Sera Set 2	7	10.1	32.7	33.1	3.3	3.3
Clinical Sera Set 2	8	8.0	30.0	30.3	2.4	2.4
Clinical Sera Set 2	9	7.8	31.4	31.7	2.4	2.5
Clinical Sera Set 2	10	10.9	32.9	32.4	3.6	3.5
Clinical Sera Set 2	11	7.6	31.9	31.7	2.4	2.4
Clinical Sera Set 2	12	9.3	30.9	35.6	2.9	3.3

1.14 Calibration Verification

There is no NIBSC WHO or international standard material for the calibration of T3 uptake assays, however immunoassay controls were obtained from BioRad and Randox and tested in the Theranos System. Some example reported values for these controls on different systems are shown. Recovery of the control sera in the Theranos System compared to an arbitrary reported value and to the US Biological kit result was excellent.

Randox Immunoassay Premium Plus Controls (Cat 1A3112) Reported Mean Values

System	Level 1	Level 2	Level 3	Unit
Pishtaz Teb- ELISA	35.0	32.0	38.0	%U
Siemens Immulite 1000	37.7	30.9	44.5	%U

BioRad Liquicheck ImmunoAssay Plus Controls (Cat 360X) Reported Mean Values

System	Level 1	Level 2	Level 3	Unit
Siemens Dimension VISTA	26.7	40.2	46.7	%U
Siemens Dimension	28.5	39.6	45.5	%U

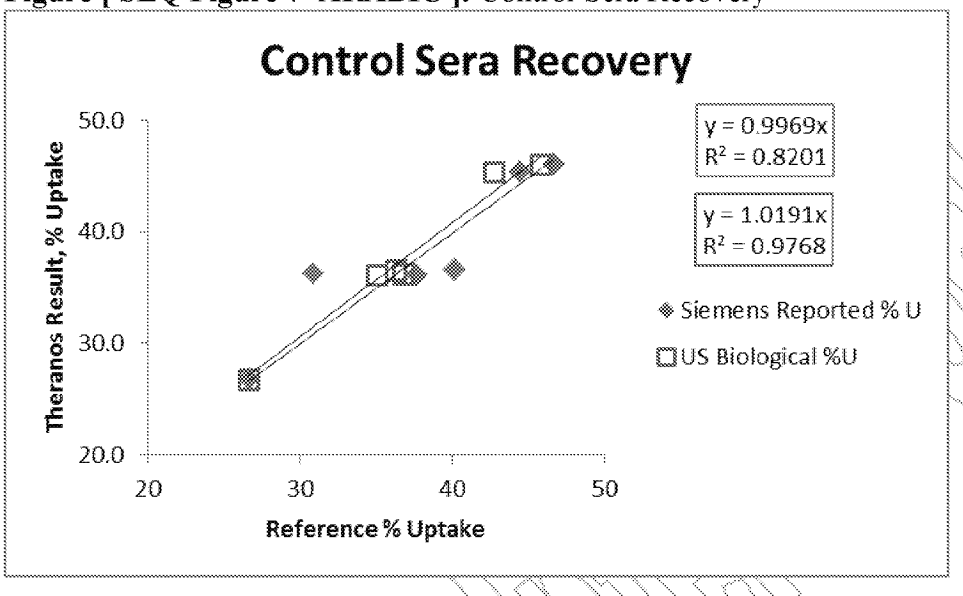
Standard Curve

	Signal, RLU		Back-Calculated % Uptake		
% Uptake	Tip 1 Mean RLU	Tip 2 Mean RLU	Mean	CV %	% Recovery
57.0	366	436	57.1	1.2	100
51.1	606	708	51.2	1.9	100
41.6	1373	1552	41.8	3.8	100
34.2	2602	3121	34.3	5.0	101
28.6	4432	5230	28.8	1.4	100
25.6	6334	6995	25.7	3.5	100

Table [SEQ Table * ARABIC]: Control Sera Recovery

Set	Level	Reported (Siemens), % Uptake	US Biological, % Uptake	Theranos, % Uptake	CV %	% Recovery	
						To Reported	To US Biological
BioRad	1	26.7	26.6	26.7	3.8	100	101
BioRad	2	40.2	36.2	36.5	3.3	91	101
BioRad	3	46.7	45.8	46.0	1.0	99	100
Randox	1	37.7	35.0	36.2	5.6	96	103
Randox	2	30.9	36.7	36.3	1.8	117	99
Randox	3	44.5	42.7	45.4	2.2	102	106

Figure [SEQ Figure * ARABIC]: Control Sera Recovery



1.15 Stability

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

Standard Curve

	Signal, RLU		Back-Calculated % Uptake		
% Uptake	Tip 1 Mean RLU	Tip 2 Mean RLU	Mean	CV %	% Recovery
57.0	366	436	57.1	1.2	100
51.1	606	708	51.2	1.9	100
41.6	1373	1552	41.8	3.8	100
34.2	2602	3121	34.3	5.0	101
28.6	4432	5230	28.8	1.4	100
25.6	6334	6995	25.7	3.5	100

Table [SEQ Table * ARABIC]: Stability of Reagents

			Signal, RLU		% Uptake		
Date	Day	% Uptake	Tip 1 Mean RLU	Tip 2 Mean RLU	Mean	CV %	% Recovery
08/29/11	0	26.6	5359	6622	26.7	3.8	101
		36.2	2131	2513	36.5	3.3	101
		45.8	942	1074	46.0	1.0	100
09/28/11	30	26.6	5191	5804	27.6	4.5	104
		36.2	2163	2456	36.6	4.5	101
		45.8	952	1068	46.0	0.8	100

Figure [SEQ Figure * ARABIC]: Stability, Signal (RLU)

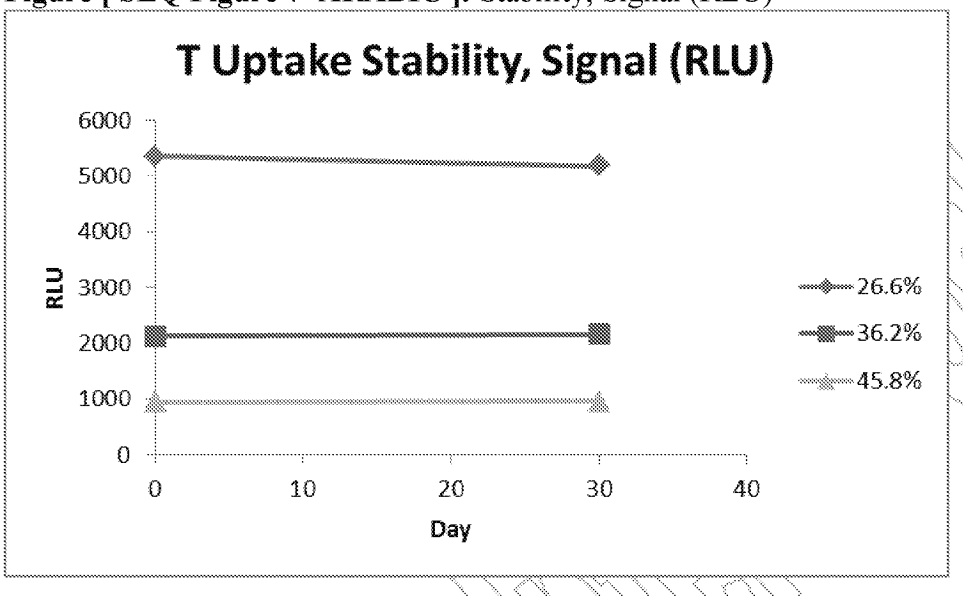
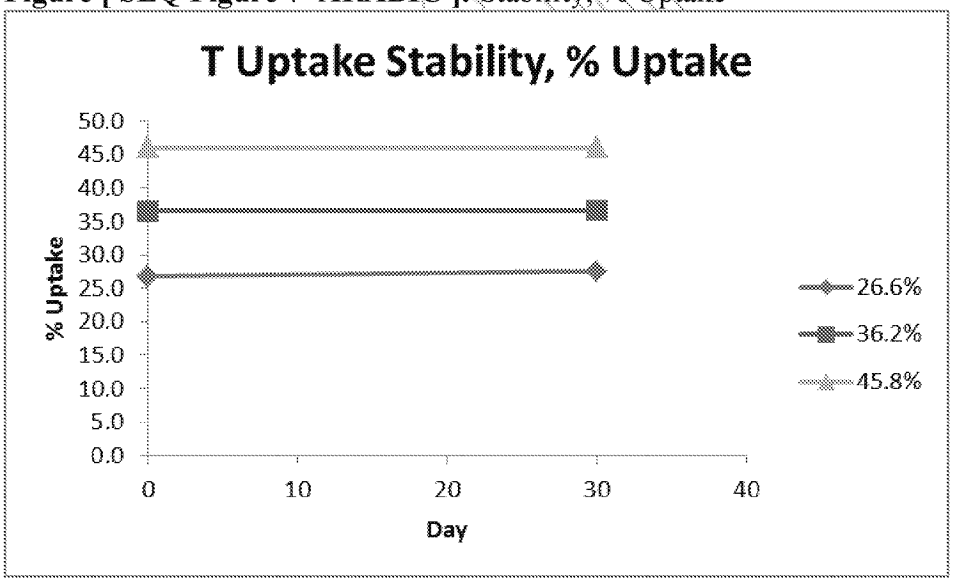


Figure [SEQ Figure * ARABIC]: Stability, % Uptake



2 REFERENCES

1. Szpunar et al, J Nucl Med 22: 793-795,1981.