



Free Testosterone (aka. fTST or fTEST) Assay Development Report

Theranos, Inc.

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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 total concentration of testosterone in human whole blood (automatically processed into plasma by the Theranos system), plasma or serum. The assay has been calibrated to the Thunderbolt ELISA platform. The assay has a current reportable range of 0.2 pg/mL to 22.55 pg/mL.

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

The reported ranges for available bench-top ELISA kits do not correspond with the reported ranges by ARUP as measured by LC-MS. There is a lack of agreement in the results from EIA bench-top kits and the RIA automated assays available [Wheeler et al, 2008]. LC-MS is considered the gold standard for Free Testosterone measurement. Due to lack of availability of LC-MS standardized calibrators and samples, the Theranos assay was instead calibrated to correlate with the Thunderbolt ELISA platform.

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

A biotin-labeled anti-mouse antibody coated on UltraAvidin 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 a mouse anti-TST antibody and an alkaline phosphatase-labeled testosterone conjugate (TST-AP). 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).

A greater amount of free TST in the sample results in lower binding of the TST-AP to the capture antibody. Thus the signal generated by the assay is inversely proportional to the concentration of TST in the sample.

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Testosterone	R&D Systems	From ELISA Kit Cat # KGE010, Part #893265
Mouse Anti-Testosterone Antibody (CAb)	Genway	20-322-392050
Goat Anti-Mouse IgG (Fc), Biotin Conjugated (Surface CAb)	Pierce/Thermo	31805
Testosterone Alkaline Phosphatase Conjugate (Custom product)	Fitzgerald	65-IT08
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
Steroid Depleted Serum (Calibrator Matrix)	Sunny Lab	SF236-2

2. ASSAY DEVELOPMENT

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

1.2 Conjugate Verification

During initial assay development, an alkaline phosphatase conjugate was not available. To verify that the commercial TST conjugates are recognized by the antibodies raised against TST-3-CMO-BSA, the biotin conjugate and the horseradish peroxidase (HRP) conjugate were tested with a set of antibodies. The HRP conjugate was used with Millipore Immobilon Chemiluminescent HRP Substrate. The biotin conjugate was detected by an UltraAvidin-alkaline phosphatase conjugate followed by KPL PhosphaGlo AP substrate.

A Nunc Maxisorb Microtiter plate was coated by passive absorption with 10.0, 1.0, and 0.1 ug/mL of the anti-TST antibodies and binding to a fixed concentration of the conjugates was tested. None of the antibodies responded to the biotin conjugate, but all of the antibodies responded well to the Fitzgerald HRP conjugate. Therefore initial assay development was done with the Fitzgerald HRP conjugate, while awaiting quotes for a custom TST-AP conjugate.

Table [SEQ Table * ARABIC]: Testosterone Conjugates Screened

Manufacturer	Cat #	Description
DCN	AGST-140	Testosterone-3-biotin
Fitzgerald	65-JT07	Testosterone-3-CMO-HRP
Leinco	A108	UltraAvidin- Alkaline Phosphatase

Table [SEQ Table * ARABIC]: Anti-Testosterone Antibodies Screened

Number	Manufacturer	Cat #	Type	Clone
1	Calbioreagents	M343	MAb	
2	Thermo	MA5-14686	MAB	14P1F9
3	Thermo	MA5-14687	MAB	14P2C8
4	Thermo	MA5-14715	MAB	P2G1
5	Genway	20-783-73686	MAB	4E1G2
6	Genway	20-322-392050	MAB	XM209
7	Genway	20-511-240888	MAB	7003
8	Genway	20-511-240937	MAB	7004
9	Genway	20-511-242359	MAB	B646M
10	Genway	20-511-242404	MAB	B641M
11	Genway	18-731-285334	Sheep PAb	
12	Genway	20-783-314372	MAB	
13	Fitzgerald	10-T07C	MAB	M211243
14	Fitzgerald	20C-CR2140R	Rabbit PAb	
15	Fitzgerald	20-TR03T	Rabbit PAb	
16	Fitzgerald	10-T07B	MAB	M211244
17	Fitzgerald	10-T07D	MAB	M211242
18	Fitzgerald	10-T07A	MAB	M021812
19	Fitzgerald	20R-TR018w	Rabbit PAb	
20	Fitzgerald	10C-CR2140M3	MAB	5127425
21	US Biological	T2950-19A	MAB	9L696
22	US Biological	T2950-24	MAB	5E801
24	US Biological	T2950-25A	Sheep PAb	
25	Diasource	5317006	Rabbit PAb	

Table [SEQ Table * ARABIC]: Antibody Response to Fitzgerald TST-3-CMO-HRP Conjugate (MTP)

Ab #	[Ab] ug/mL	Mean RLU	CV %	Modulation
1	10.0	496239	7.9	202
	1.0	46468	9.3	19
	0.1	2455	15.8	
2	10.0	292979	4.9	298
	1.0	3517	4.3	4
	0.1	984	5.7	
3	10.0	305970	3.4	299
	1.0	8506	4.2	8
	0.1	1022	11.3	
5	10.0	390825	1.0	281
	1.0	37005	5.2	27
	0.1	1393	3.1	
7	10.0	276514	4.6	291
	1.0	2529	3.9	3
	0.1	951	25.5	
8	10.0	282877	5.3	356
	1.0	3878	2.2	5
	0.1	795	18.9	
9	10.0	418956	2.7	128
	1.0	73615	12.5	22
	0.1	3282	16.3	
10	10.0	419319	1.1	101
	1.0	107326	1.2	26
	0.1	4158	4.1	
11	10.0	101273	0.4	140
	1.0	4721	11.0	7
	0.1	723	8.2	
12	10.0	521122	0.5	143
	1.0	74757		20
	0.1	3654	7.4	
13	10.0	432927	2.5	254
	1.0	53517		31
	0.1	1705	1.6	
14	1:1000	295415		107
	1:10,000	130726	1.3	47
	1:100,000	2771		

Table Continued: Antibody Response to Fitzgerald TST-3-CMO-HRP Conjugate (MTP)

Ab #	[Ab] ug/mL	Mean RLU	CV %	Modulation
15	1:1000	279882	2.6	90
	1:10,000	105956	3.4	34
	1:100,000	3098	11.6	
16	10.0	258257	0.9	224
	1.0	3309	2.8	3
	0.1	1155	19.3	
17	10.0	246206	3.3	237
	1.0	4226	1.7	4
	0.1	1041	8.0	
18	10.0	367679	3.0	75
	1.0	35215	8.1	7
	0.1	4923	13.1	
19	1:1000	113766	1.9	51
	1:10,000	60662	2.1	27
	1:100,000	2223	4.1	
20	10.0	481639	0.4	136
	1.0	71297	2.5	20
	0.1	3547	18.4	

1.3 Antibody Screen in Competitive Assay Format

Antibodies were screened for dose response to free TST in a serum-based matrix using a competitive assay format with 1:10 sample dilution and the Fitzgerald TST-HRP conjugate on the Theranos System. Antibodies # 1, 5, 14, 18, 19, 22 gave the best modulation and were chosen for further testing. These antibodies were then screened with the BioRad Liquicheck controls to verify response in a pure serum matrix, mean reported fTST values for the Siemens Coat-A-Count RIA are shown for these controls.

Table [SEQ Table * ARABIC]: Antibody Screen in Free TST Assay, Serum-Buffer Matrix (Theranos System)

Ab #	Total [TST] ng/mL	Mean RLU	CV %	Modulation
1	10.0	1608	19.5	36.2
	1.0	26680	12.0	2.2
	0.0	58264	1.5	
2	10.0	13848	4.7	1.8
	1.0	20870	7.1	1.2
	0.0	25142	13.1	

3	10.0	11553	17.5	2.0
	1.0	17975	10.4	1.3
	0.0	23061	3.6	
5	10.0	537	33.5	44.4
	1.0	12844	19.9	1.9
	0.0	23814	39.9	
6	10.0	1754	21.3	14.5
	1.0	16505	7.6	1.5
	0.0	25368	0.5	
7	10.0	11427	9.0	2.0
	1.0	19213	6.1	1.2
	0.0	23019	1.0	
8	10.0	11900	13.0	2.5
	1.0	22776	11.8	1.3
	0.0	29719	4.3	
9	10.0	5957	9.0	10.1
	1.0	39533	4.0	1.5
	0.0	60344	6.5	
10	10.0	4813	26.2	10.2
	1.0	40599	2.5	1.2
	0.0	48872	11.2	
11	10.0	16	18.5	0.8
	1.0	13	9.4	1.0
	0.0	13	44.6	
12	10.0	1358	23.3	13.6
	1.0	9213	30.1	2.0
	0.0	18471	6.3	
13	10.0	69	136.9	0.3
	1.0	43	59.9	0.5
	0.0	23	71.4	
14	10.0	3155	56.2	28.9
	1.0	46012	7.0	2.0
	0.0	91043	2.4	
15	10.0	5264	9.5	21.4
	1.0	50957	18.5	2.2
	0.0	112427	9.6	
16	10.0	14309	15.9	2.2
	1.0	19511	19.6	1.6
	0.0	31965	4.9	
17	10.0	11720	34.4	1.8

	1.0	16256	60.3	1.3
	0.0	20661	23.4	
18	10.0	290	69.6	62.4
	1.0	7324	18.8	2.5
	0.0	18081	36.3	
19	10.0	2852	48.5	13.4
	1.0	19389	19.7	2.0
	0.0	38279	11.9	
20	10.0	7969	3.8	3.3
	1.0	20769	22.1	1.3
	0.0	26647	7.7	
22	10.0	1665	23.6	20.5
	1.0	13179	11.6	2.6
	0.0	34111	3.7	
24	10.0	13	53.8	1.3
	1.0	10	58.0	1.7
	0.0	17	72.6	
25	10.0	12465	7.1	7.4
	1.0	40304	6.0	2.3
	0.0	92677	3.2	

Table [SEQ Table * ARABIC]: Antibody Screen in Free TST Assay, BioRad Serum Controls (Theranos System)

Ab #	[fTST] pg/mL	Mean RLU	CV %	Modulation
1	18.1	5350	22.1	5.2
	7.7	14402	6.3	1.9
	1.9	27995	7.1	
5	18.1	3188	24.1	10.4
	7.7	17437	9.3	1.9
	1.9	33130	2.1	
14	18.1	20622	6.8	3.3
	7.7	47642	3.2	1.4
	1.9	68857	2.8	
18	18.1	2756	18.5	7.8
	7.7	13621	32.8	1.6
	1.9	21587	14.9	
19	18.1	84462	4.4	2.5
	7.7	134488	9.1	1.6
	1.9	214737	3.8	
22	18.1	8202	15.7	4.5

	7.7	21228	14.4	1.7
	1.9	36940	13.7	

1.4 Capture Antibody and TST Conjugate Titration

Since the free and total testosterone assays will be multiplexed, the optimal levels of capture antibody and TST-AP conjugate determined in the total TST assay were used for the free TST assay, and excellent modulation was seen.

1.5 Determination of LLOQ and ULOQ

LLOQ and ULOQ were determined using FDA guidelines for ELISA assay calibration. The ULOQ was 22.55 pg/mL and the LLOQ was 0.20 pg/mL.

Table [SEQ Table * ARABIC]: Standard Curve

Nominal [fTST] pg/mL	Signal, RLU				Back- Calculated Conc, pg/mL		
	Rep 1 Mean RLU	CV%	Rep 2 Mean RLU	CV%	Mean Conc	CV%	% Recovery
22.55	1317	16.1	1505	16.8	25.74	29.3	114
15.05	2205	10.5	2604	11.8	12.80	9.7	85
7.55	4069	7.7	4879	3.9	7.87	4.0	104
3.80	6047	9.2	7154	13.8	4.88	24.4	129
1.55	11071	5.7	12396	8.6	1.13	29.0	73
0.42	13903	8.6	15807	1.7	0.42	7.7	100
0.20	15209	1.2	18110	4.3	0.23	20.5	120

Measurement	Value	Units
LLOQ	0.20	pg/mL
ULOQ	22.55	pg/mL
LLOQ accuracy	120	%
LLOQ precision	20.5	%
Average Residuals	8	%
Error in prediction: Best case	19	%
Error in prediction: Expected	20	%

1.6 Normal Donor Screen and Serum vs Plasma Matrix

To verify the normal range, 5 normal female and 5 normal male samples were collected in both serum and LiHep collection tubes and the prepared serum and plasma was tested in the Theranos system. The results from serum and plasma samples showed excellent correlation. In addition, the measured ranges for males and females fell within the expected reported ranges for normal males and females in free testosterone ELISA assays. The range for females was 0.31 – 1.15 pg/mL and for males 7.2 – 9.84 pg/mL.

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

Sample #	Gender	Serum		LiHep Plasma	
		Mean Conc	CV%	Mean Conc	CV%
3	M	7.50	10.0	7.64	3.2
8	M	9.84	7.7	9.05	9.5
10	M	7.20	3.4	8.01	7.1
12	M	8.91	10.7	8.47	15.4
14	M	7.64	4.8	7.43	17.8
7	F	0.89	1.9	1.62	35.3
11	F	1.15	17.7	1.12	10.5
15	F	0.31	4.6	0.44	23.6
17	F	1.15	19.6	1.06	11.0
18	F	1.04	33.4	0.62	15.2

Figure [SEQ Figure * ARABIC]: LiHep Plasma vs Serum Results
[SHAPE * MERGEFORMAT]

1.7 Hematocrit Effect and Effect of Anticoagulant

To test for the effect of anticoagulant and to test for hematocrit, 4 male and 4 female normal whole blood samples were obtained both in EDTA and in Lithium-Heparin tubes. Plasma was prepared from both types of tubes, and the whole blood from the EDTA tubes was also measured on the Therasos system.

There did not appear to be a significant difference between results obtained from Lithium-heparin plasma vs EDTA plasma. EDTA plasma may show a slightly higher result – and since serum and lithium heparin plasma correlation was excellent, the recommended anticoagulant will be lithium heparin.

In addition, free testosterone concentrated only slightly into plasma in comparison to whole blood – presumably as a result of its association with cells and proteins precipitated out in the preparation of the plasma. In conclusion, whole blood, serum, EDTA or Li-Hep plasma are all suitable matrixes for measuring free testosterone in the Therasos system all can be accurately calibrated with the serum standard curve.

Table [SEQ Table * ARABIC]: Whole Blood, Lithium Heparin Plasma and EDTA Plasma Results

Sample #	Gender	Whole Blood		Li-Hep Plasma		EDTA Plasma	
		Mean Conc	CV%	Mean Conc	CV%	Mean Conc	CV%
3	M	7.59	13.7	6.62	18.3	9.13	3.3
4	M	9.78	4.2	9.48	9.9	11.21	6.0
9	M	9.21	5.8	7.73	7.1	9.81	11.9
10	M	7.54	11.0	9.05	10.8	8.28	5.8
5	F	1.15	36.1	0.68	14.1	1.35	9.3
6	F	1.16	9.3	0.78	6.7	0.91	36.0
7	F	1.60	19.9	0.48	29.7	1.21	14.3
8	F	0.96	26.0	0.74	70.1	0.70	19.2

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant

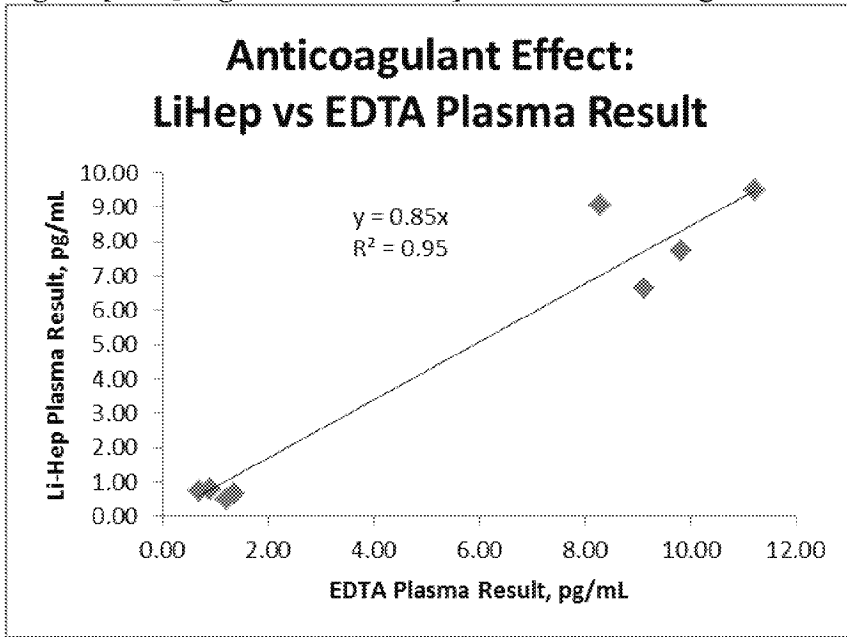
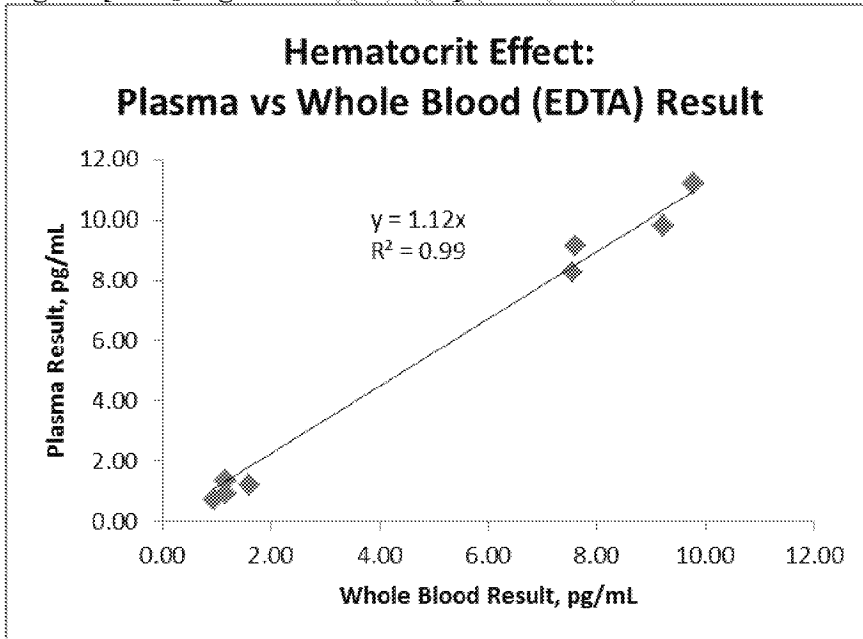


Figure [SEQ Figure * ARABIC]: Hematocrit Effect



1.8 Calibration Verification

The Theranos free testosterone assay has been calibrated to the Thunderbolt free testosterone assay. To verify the calibration compared to other ELISA and RIA methods, BioRad Liquicheck Immunoassay Plus Controls (Lot 40770) were tested on the Theranos System. The Theranos result fell within the reported range for the Siemens Coat-A-Count RIA and correlated well with the reported mean concentration for the Coat-A-Count RIA and the Thunderbolt ELISA result.

Table [SEQ Table * ARABIC]: BioRad Liquicheck Immunoassay Plus Controls Lot 40770

Level	Reported Values for Siemens Coat a Count RIA, pg/mL		CLIA Thunderbolt Result, pg/mL	Theranos Result, pg/mL	
	Mean Conc pg/mL	Range		Mean Conc.	CV%
1	1.9	1.48 -2.32	1.50	2.09	18.7
2	7.7	6.1 - 9.3	5.00	8.43	8.8
3	18.1	15.9 - 20.3	19.80	17.22	11.3

Figure [SEQ Figure * ARABIC]: BioRad Liquicheck Controls Theranos to Siemens Coat-A-Count RIA

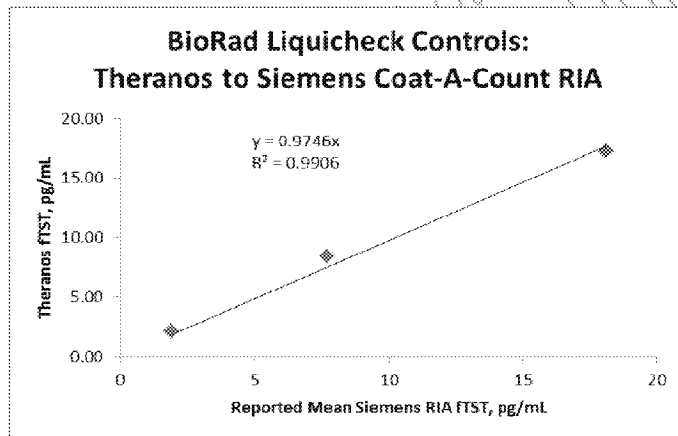
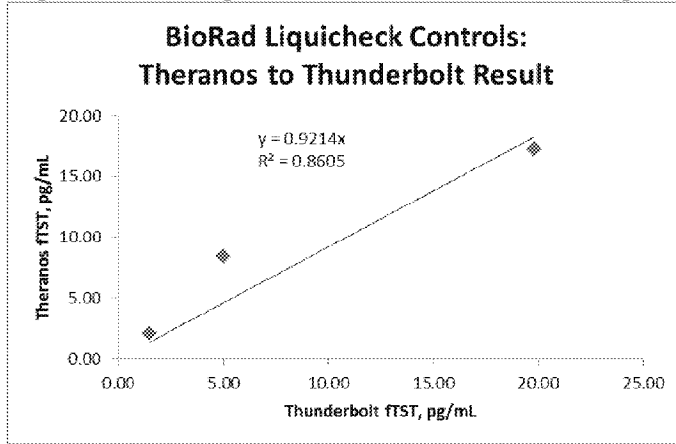


Figure [SEQ Figure * ARABIC]: BioRad Liquicheck Controls Theranos to Thunderbolt



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1.9 Clinical Samples

A set of 25 samples, 4 females and 21 males, were tested on the Theranos system and on the Thunderbolt System in the CLIA lab. Additionally, the free testosterone levels were compared with the total testosterone levels reported by the Siemens Advia system, since a good correlation between free and total testosterone in males is expected [Winters et al, 1998].

The correlation of the Theranos result to the Thunderbolt result was good, with the exception of one outlier – sample number 23. When the total testosterone correlations were examined, the Theranos free testosterone result correlated much more closely with the reported total testosterone levels for all samples including sample 23, whereas the Thunderbolt free testosterone result compared to the total testosterone result shows sample 23 as an outlier. Therefore it is likely that the Theranos result is more accurate for sample 23, however comparison to LC-MS results for the samples would be necessary to determine the true free testosterone level.

Figure [SEQ Figure * ARABIC]: Clinical Sample Correlation Free TST Results

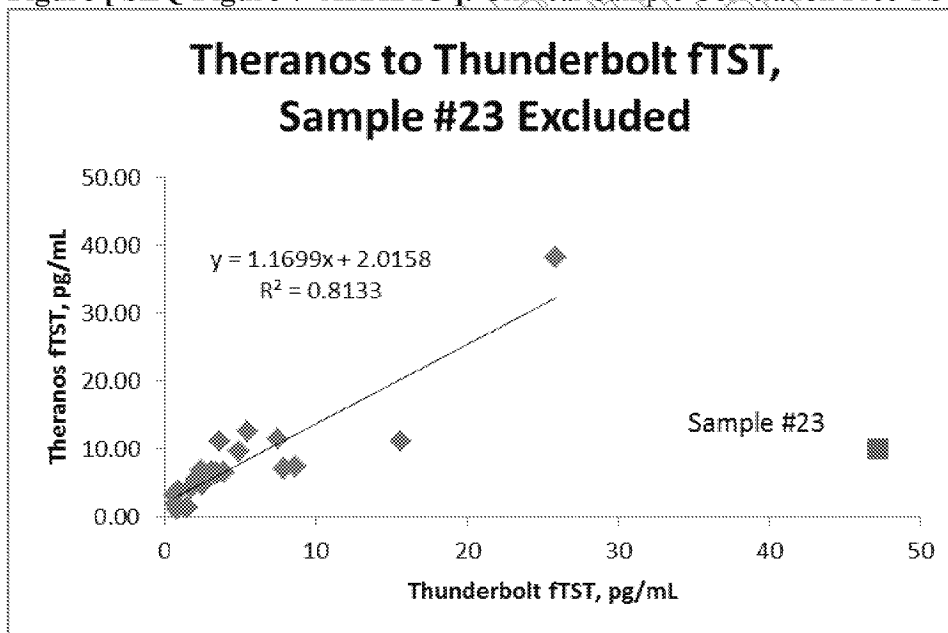


Table [SEQ Table * ARABIC]: Clinical Sample Results

Sample #	Gender	[Total TST] Reported (ADVIA), ng/mL	Thunderbolt [fTST], pg/mL	Theranos [fTST], pg/mL
1	F	0.16	0.82	1.07
2	F	0.13	1.53	1.34
3	F	0.29	< 0.17	0.68
4	F	0.05	< 0.17	0.74
5	M	1.61	3.50	6.41
6	M	1.16	2.51	4.57
7	M	2.09	0.63	3.19
8	M	1.57	0.88	3.64
9	M	2.02	2.04	5.61
10	M	1.43	0.71	1.81
11	M	5.54	15.60	11.20
12	M	4.31	1.72	4.28
13	M	3.55	2.43	6.96
14	M	3.53	4.93	9.69
15	M	4.04	3.31	6.34
16	M	6.50	3.88	6.50
18	M	6.50	3.68	11.18
19	M	4.04	3.13	6.76
20	M	7.50	5.51	12.54
21	M	4.24	7.87	6.98
22	M	6.57	8.66	7.40
23	M	4.57	47.14	10.01
24	M	12.30	25.86	38.20
25	M	8.10	7.51	11.54
26	M	10.45	113.30	OORH

Figure [SEQ Figure * ARABIC]: Theranos Free TST to Total TST Correlation

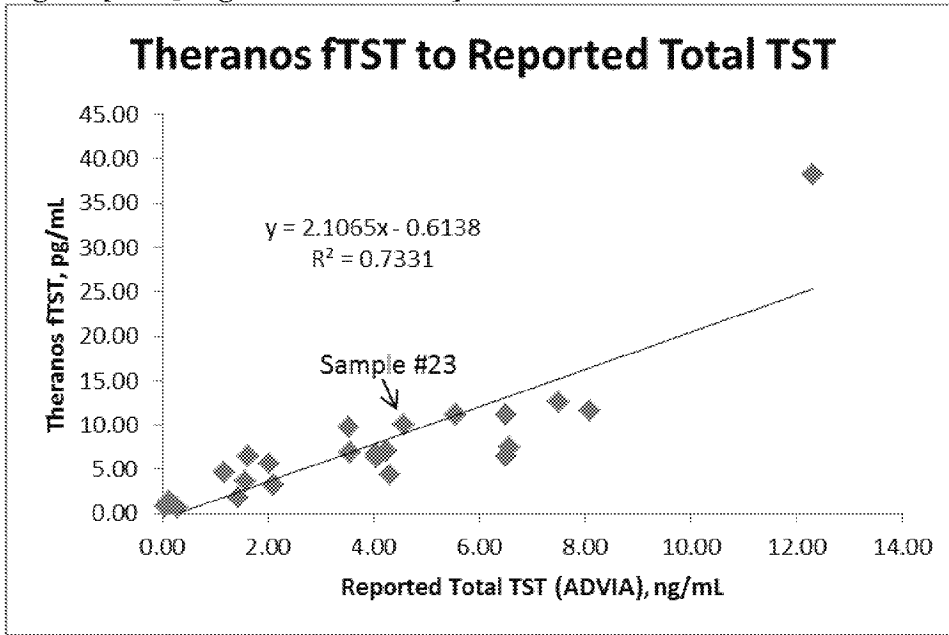
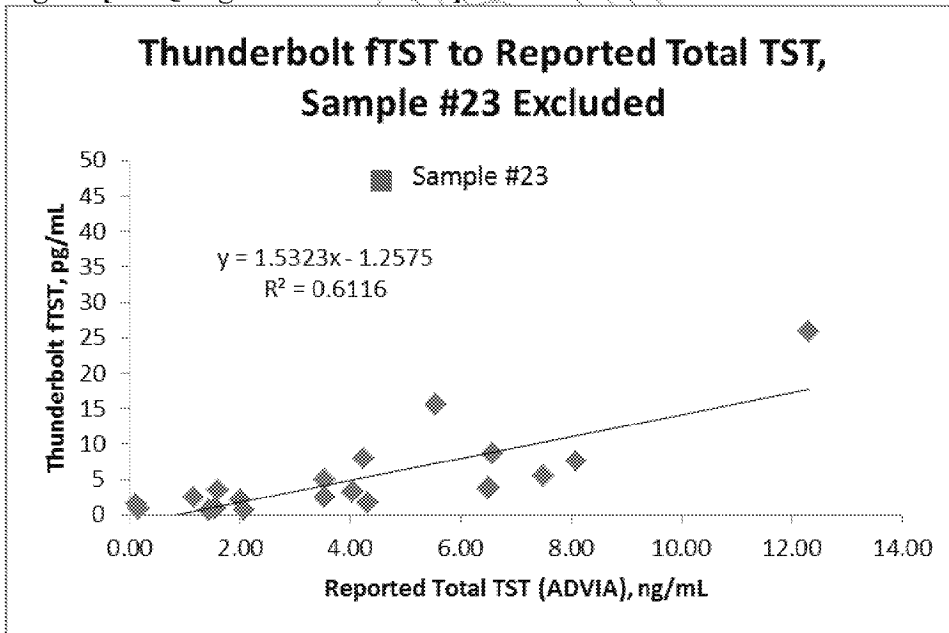


Figure [SEQ Figure * ARABIC]: Thunderbolt Free TST to Total TST Correlation



1.10 Stability

Stability of reagents is being monitored.

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