theranos™
redefining healthcare

Total Testosterone (aka. TST or TEST) Assay Development Report

Theranos, Inc.

Nov 10, 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 total concentration of testosterone in human whole blood (automatically processed into plasma by the Theranos system), plasma or serum. The assay has a reportable range of 0.013 ng/mL to 15.03 ng/mL and is calibrated to the Siemens Immulite 2000 via BioRad LiquiCheck ImmunoAssay 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:

R&D Systems Testosterone Assay Kit Cat #KGE010
Alpco Testosterone (Serum) ELISA Cat #11-TESHU-E01
Genway Testosterone ELISA Kit (Plasma and Serum) Cat #40-521-475002

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 an excess of unlabeled estradiol (E2), a mouse anti-TST antibody, and an alkaline phosphatase-labeled testosterone conjugate (TST-AP). The function of the E2 is to displace the TST from Steroid Hormone Binding Globulin (SHBG) by competition. 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 total 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
Estradiol	Fitzgerald	30-AE30
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 phosphastase 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-IT07	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 Free TST Assay Format

Antibodies were screened for dose response to free TST (no reagent to displace TST from SHBG) in a serum-buffer matrix using a competitive assay format with 1:10 sample dilution and the Fitzgerald TST-HRP conjugate on the Theranos Systems. 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 values for the Siemens Iummulite 2000 are shown.

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

Ab #	[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 #	[TST] ng/mL	Mean RLU	CV %	Modulation
1	13.05	5350	22.1	5.2
	4.94	14402	6.3	1.9
	1.29	27995	7.1	
5	13.05	3188	24.1	10.4
	4.94	17437	9.3	1.9
	1.29	33130	2.1	
14	13.05	20622	6.8	3.3
	4.94	47642	3.2	1.4
	1.29	68857	2.8	
18	13.05	2756	18.5	7.8
	4.94	13621	32.8	1.6
	1.29	21587	14.9	
19	13.05	84462	4.4	2.5
	4.94	134488	9.1	1.6
	1.29	214737	3.8	
22	13.05	8202	15.7	4.5

	4.94	21228	14.4	1.7
	1.29	36940	13.7	

1.4 Antibody Screen with Displacer

The finalist antibodies were tested in a competitive assay with and without 20 ng/mL of sample estradiol as a displacer to test for response to Total TST in clinical samples. This experiment was performed using serum calibrators made in steroid-depleted pooled serum and with 3 clinical samples – calibrators and samples were measured in the R&D Kit to determine the Nominal Total TST. The lowest clinical sample is female and the other 2 are male.

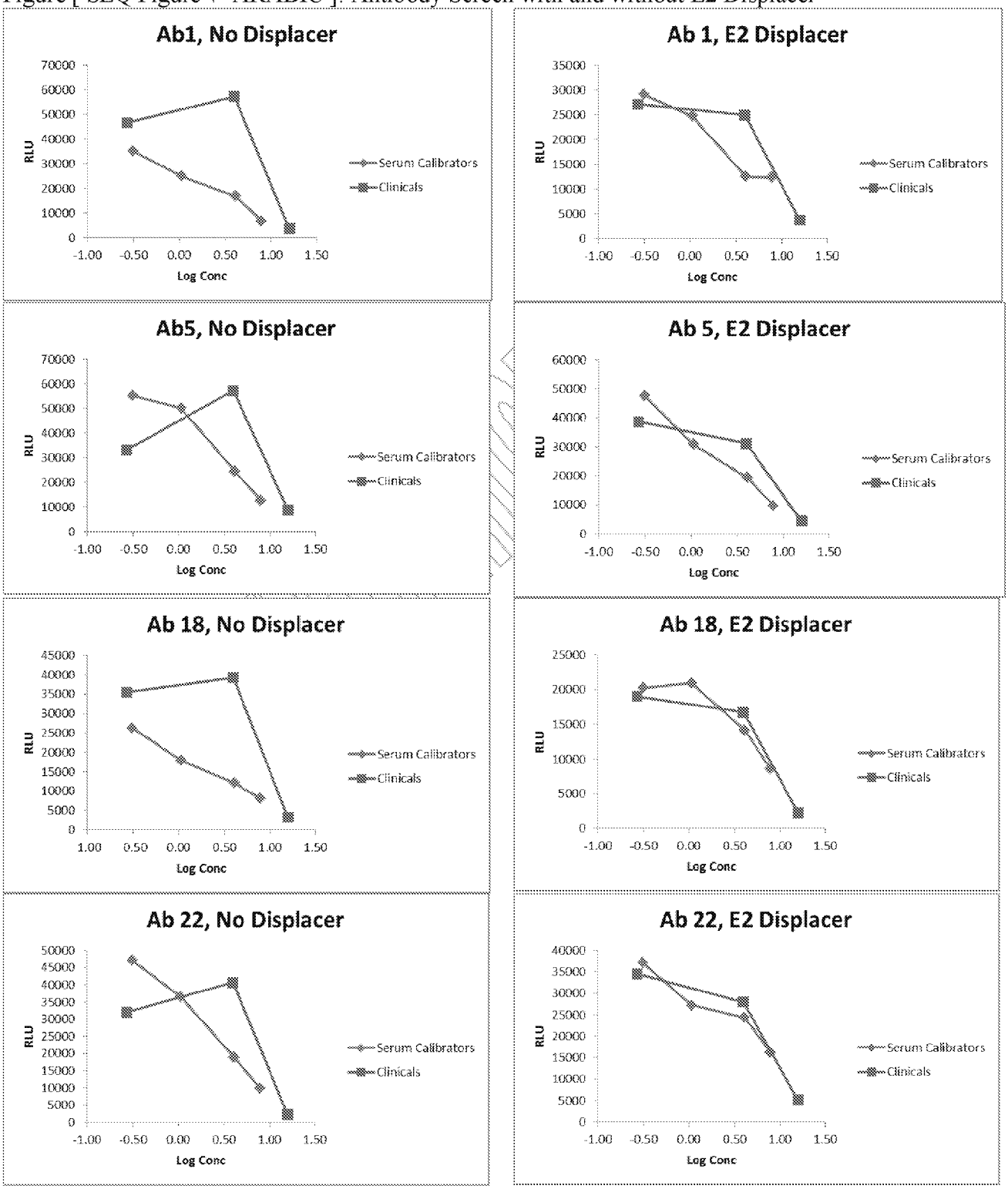
Antibody #5 showed the best modulation in the Total TST assay in response to the serum calibrators and the clinical samples. The data for all 4 antibodies confirmed that an excess of E2 successfully displaces TST from SHBG as evidenced by the tracking of the signal from serum calibrators and clinical samples and especially the 2 lowest samples. The level of E2 will be titrated to optimize the concentration.

Table [SEQ Table * ARABIC]: Antibody Screen with and without E2 Displacer

				No Displacer		2 ng/mL E2 Displacer	
Ab #	**Sample Type**	**[TST] ng/mL**	**Log Conc**	**Mean RLU**	**CV %**	**Mean RLU**	**CV %**
1	Serum Calibrators	7.83	0.89	6632	11.0	12371	9.0
		4.07	0.61	16842	5.4	12488	15.6
		1.06	0.03	25018	9.3	24716	0.9
		0.31	-0.51	35280	8.7	29194	18.2
	Clinical Samples	15.75	1.20	3849	8.9	3716	20.3
		3.95	0.60	57313	5.0	24907	6.8
		0.27	-0.57	46699	7.6	27164	13.2
5	Serum Calibrators	7.83	0.89	12683	6.8	9755	9.3
		4.07	0.61	24565	11.4	19429	8.0
		1.06	0.03	50259	3.8	30886	13.8
		0.31	-0.51	55219	13.4	47740	4.4
	Clinical Samples	15.75	1.20	8844	2.5	4453	12.7
		3.95	0.60	57226	1.8	31196	14.3
		0.27	-0.57	33211	9.1	38627	3.0
18	Serum Calibrators	7.83	0.89	8050	6.0	8592	14.1
		4.07	0.61	12110	25.3	14144	2.3
		1.06	0.03	17852	34.5	20918	7.1
		0.31	-0.51	26277	18.5	20233	3.9
	Clinical Samples	15.75	1.20	3222	19.7	2277	28.9
		3.95	0.60	39291	10.8	16754	3.3
		0.27	-0.57	35462	10.4	19041	5.4
22	Serum Calibrators	7.83	0.89	9909	10.8	16154	17.9
		4.07	0.61	18860	24.8	24318	4.3
		1.06	0.03	36558	24.6	27260	2.9
		0.31	-0.51	47171	12.6	37158	6.0
	Clinical Samples	15.75	1.20	2361	19.8	5070	6.2
		3.95	0.60	40633	2.8	27986	16.9
		0.27	-0.57	32067	3.7	34474	10.0

Note: Nominal [TST] determined by R&D ELISA.

Figure [SEQ Figure * ARABIC]: Antibody Screen with and without E2 Displacer



1.5 Titration of E2 Displacer

The optimal concentration of E2 needed to competitively displace TST from SHBG was determined by titration with serum calibrators and clinical samples. The loaded concentration of E2 was spiked 1:10 into the final sample mixture, with a sample dilution of 1:10. Normal E2 levels in females are 25-100 pg/mL in pregnancy may rise as high as 2 ng/mL. All levels tested represent a great excess of the highest physiological level expected.

Modulation across the range in serum calibrators and clinical samples was highest with 250 ng/mL E2 and the dose response to clinical samples and calibrators tracked well with E2 concentrations 20 ng/mL and greater. The assay condition chosen was 250 ng/mL.

Table [SEQ Table * ARABIC]: Tested Concentrations of E2 Displacer

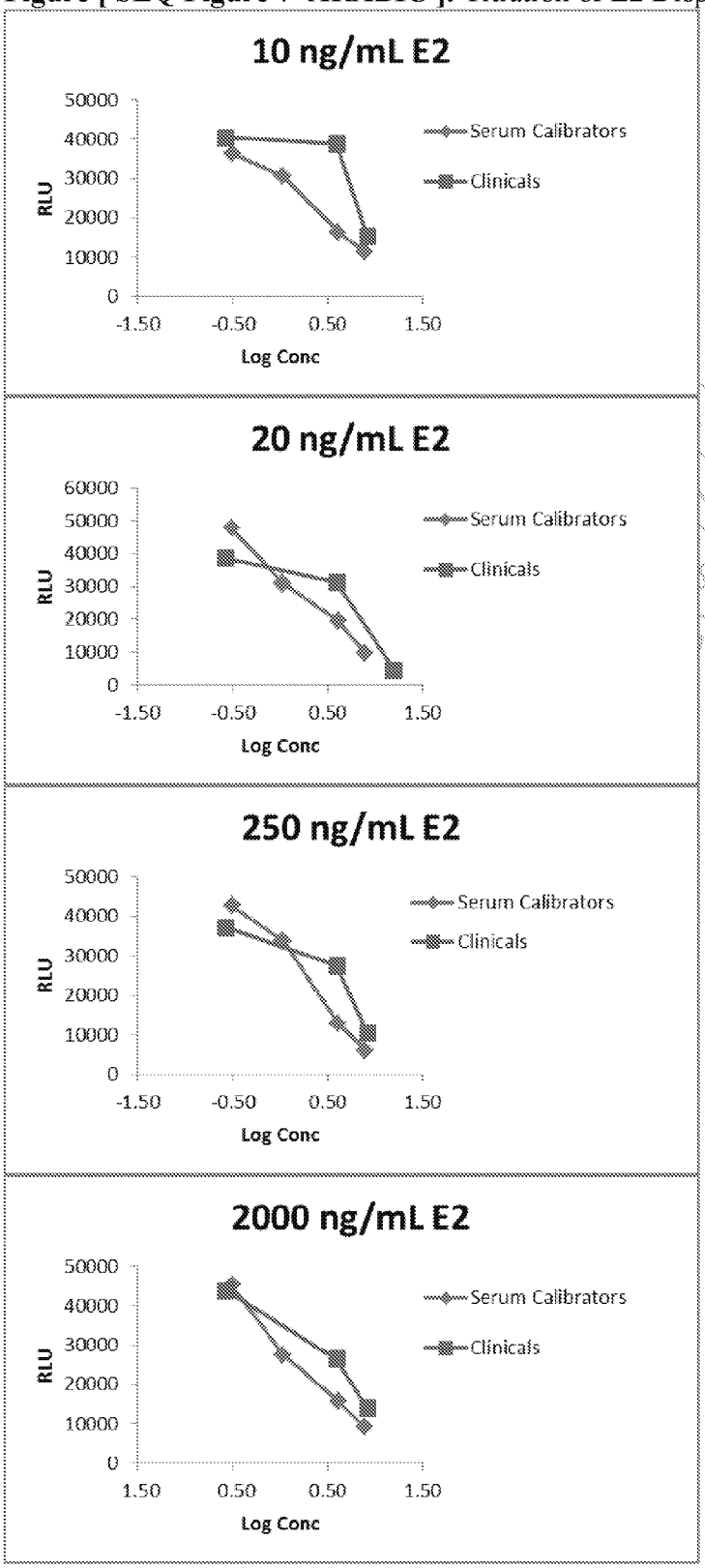
Loading [E2] ng/mL	Final [E2] in Sample Mix (ng/mL	E2, ng per mL of Sample
10	1	10
20	2	20
250	25	250
2000	200	2000

Table [SEQ Table * ARABIC]: Titration of E2 Displacer

[E2] ng/mL	Sample	[TST] ng/mL	Log Conc	Mean RLU	CV %	Modulation
10	Serum Calibrators	7.83	0.89	11191	20.5	3.3
		4.07	0.61	16354	3.7	2.2
		1.06	0.03	30537	23.0	1.2
		0.31	-0.51	36504	12.1	
	Clinical Sample #4	0.27	-0.57	40459	3.9	
	Clinical Sample #6	3.95	0.60	38887	5.6	
	Clinical Sample #14	8.27	0.92	15211	12.1	
20	Serum Calibrators	7.83	0.89	9755	9.3	4.9
		4.07	0.61	19429	8.0	2.5
		1.06	0.03	30886	13.8	1.5
		0.31	-0.51	47740	4.4	
	Clinical Sample #4	0.27	-0.57	38627	3.0	
	Clinical Sample #6	3.95	0.60	31196	14.3	
	Clinical Sample #17	15.75	1.20	4453	12.7	
250	Serum Calibrators	7.83	0.89	6197	15.5	6.9
		4.07	0.61	12860	13.0	3.3
		1.06	0.03	33776	4.1	1.3
		0.31	-0.51	42665	10.6	
	Clinical Sample #4	0.27	-0.57	37076	3.1	
	Clinical Sample #6	3.95	0.60	27516	17.1	
	Clinical Sample #14	8.27	0.92	10469	14.2	
2000	Serum Calibrators	7.83	0.89	9072	24.9	5.4
		4.07	0.61	15704	2.3	2.9
		1.06	0.03	27555	7.2	1.6
		0.31	-0.51	45303	2.9	
	Clinical Sample #4	0.27	-0.57	43761	2.9	
	Clinical Sample #6	3.95	0.60	26458	10.1	
	Clinical Sample #14	8.27	0.92	13789	1.4	

Note: Nominal [TST] determined by R&D ELISA.

Figure [SEQ Figure * ARABIC]: Titration of E2 Displacer

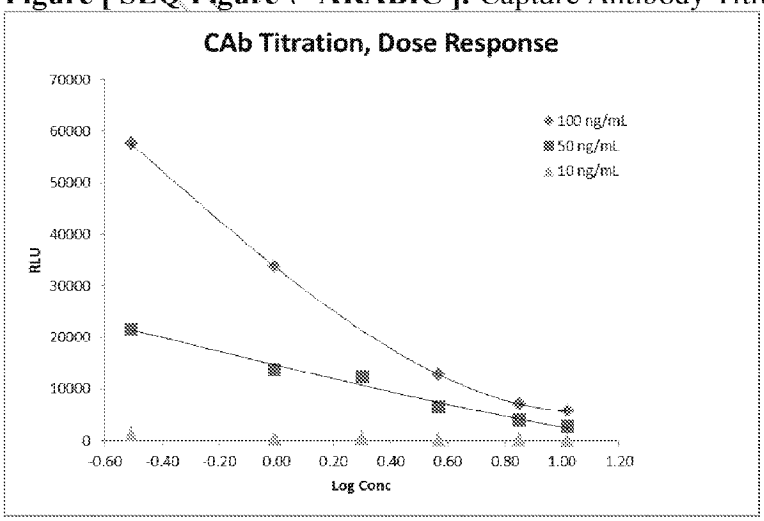


1.6 Capture Antibody Titration

The optimal concentration of capture antibody was determined by titration to be 100 ng/mL loading (final with sample mixture 10 ng/mL). The R&D TST analyte stock was re-assigned to correspond with the BioRad Liquicheck Controls reported values for Siemens Immulite 2000.

[CAb] ng/mL	[TST] ng/mL	Log Conc	Signal, RLU				Back-Calculated Conc, ng/mL		
			Mean RLU	CV %	Log S	Modulation	Mean Conc	CV %	% Recovery
100	10.45	1.02	5687	6.7	3.75	10.1	10.17	11.0	97
	7.07	0.85	7024	-	3.85	8.2	9.24	28.6	131
	3.69	0.57	12860	13.0	4.11	4.5	3.62	15.0	98
	2.00	0.30	-	-	-	-	-	-	-
	0.99	-0.01	33776	4.9	4.53	1.7	0.99	8.7	101
	0.31	-0.51	57590	12.9	4.76		0.32	34.5	102
50	10.45	1.02	2651		3.42	8.1	11.51		110
	7.07	0.85	3860	23.2	3.59	5.6	7.09	25.0	100
	3.69	0.57	6496	41.4	3.81	3.3	4.51	37.7	122
	2.00	0.30	12400	3.4	4.09	1.7	1.63	2.1	82
	0.99	-0.01	13715	31.4	4.14	1.6	1.12	47.8	114
	0.31	-0.51	21589	19.5	4.33		0.36	69.1	115
10	10.45	1.02	52	7.9	1.72				
	7.07	0.85	90	80.1	1.96				
	3.69	0.57	114	102.5	2.06				
	2.00	0.30	322	37.3	2.72				
	0.99	-0.01	332	20.0	2.52				
	0.31	-0.51	1277	65.8	3.11				

Figure [SEQ Figure * ARABIC]: Capture Antibody Titration



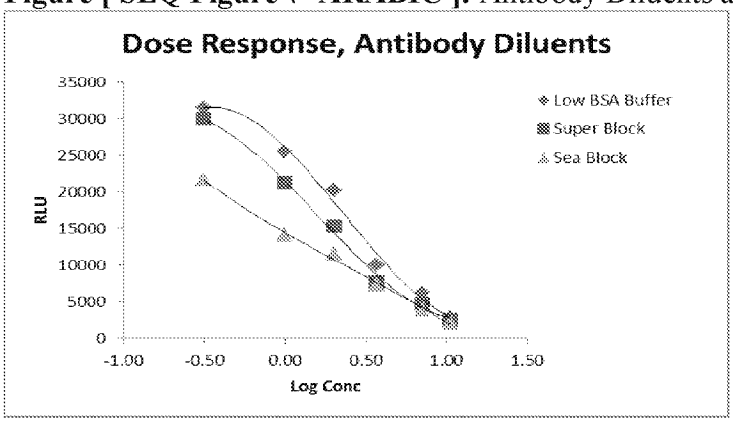
1.7 Antibody Diluents and Blockers

The effect of 3 different blocking buffers as antibody diluent was tested. Overall modulation was similar with all 3 blockers, but Sea Block appeared to improve the sensitivity of the assay and made the dose response more linear across the range. Therefore, Sea Block was chosen for the antibody diluent.

Table [SEQ Table * ARABIC]: Antibody Diluents and Blockers

			Signal, RLU				Back-Calculated Conc, ng/mL		
Antibody Diluent	**[TST] ng/mL**	**Log Conc**	**Mean RLU**	**CV %**	**Log S**	**Modulation**	**Mean Conc**	**CV %**	**% Recovery**
Low BSA Buffer	10.45	1.02	2742	25.4	3.44	11.5	12.50	22.4	120
	7.07	0.85	6254	6.0	3.80	5.0	5.47	0.7	77
	3.69	0.57	9971	6.8	4.00	3.2	4.71	4.5	128
	2.00	0.30	20263	6.2	4.31	1.6	1.70	17.1	85
	0.99	-0.01	25620	3.2	4.41	1.2	0.81	12.1	82
	0.31	-0.51	31577	12.8	4.50		0.38	69.2	122
Super Block	10.45	1.02	2589	19.3	3.41	11.6	11.75	21.8	112
	7.07	0.85	4830	3.8	3.68	6.2	6.14	2.9	87
	3.69	0.57	7711	12.5	3.89	3.9	4.42	9.4	120
	2.00	0.30	15385	9.5	4.19	1.9	1.92	20.3	96
	0.99	-0.01	21224	14.9	4.33	1.4	0.99	44.6	101
	0.31	-0.51	29969	8.6	4.48		0.33	36.3	
Sea Block	10.45	1.02	2073	4.7	3.32	10.5	10.44	2.7	100
	7.07	0.85	3933	3.9	3.59	5.5	7.09	0.3	100
	3.69	0.57	7308	13.1	3.86	3.0	3.84	14.1	104
	2.00	0.30	11515	12.4	4.06	1.9	1.88	18.6	94
	0.99	-0.01	14079	15.6	4.15	1.5	1.18	20.8	119
	0.31	-0.51	21800	10.9	4.34		0.32	43.5	104

Figure [SEQ Figure * ARABIC]: Antibody Diluents and Blockers



1.8 Alkaline Phosphatase Conjugate

A quote was obtained for a custom TST-3-CMO-AP conjugate from Fitzgerald. In preparation for conversion to an alkaline phosphatase tracer, the AP conjugate from an Enzo Life Sciences kit was tested with the KPL PhosphaGlo substrate and used for further assay development. In order to conserve this reagent it was tested diluted in AP stabilizing buffers. Modulation was similar to that observed with the Fitzgerald TST-3-CMO-HRP conjugate and Millipore HRP substrate. A 1:4 dilution into a House Stabilizer consisting of 0.03% BSA in TBS with 0.1 mM Zn^{2+} and 5mM Mg^{2+} was chosen for further development.

Table [SEQ Table * ARABIC]: Testing Enzo TST AP Conjugate

AP Conjugate	[TST] ng/mL	Mean RLU	CV %	Modulation
Neat	10.45	10160	11.0	9.4
	0.31	95292	2.7	
1:5 in House Stabilizer	10.45	2267	8.7	8.4
	0.31	18984	3.1	
1:5 in Biostab	10.45	2311	6.7	7.9
	0.31	18370	7.3	

1.9 Calibration Verification

There is no WHO or alternative international calibration standard for testosterone. Therefore, calibration of the Theranos TST assay was verified using the BioRad Liquicheck ImmunoAssay Plus traceable controls, Lot 40770. These controls were also tested in the 3 bench-top ELISA kits – R&D Systems, Genway and Alpco. It is evident that calibration of testosterone assays varies widely, even between instruments by the same manufacturer.

Based on the comparative recovery of these controls, the Theranos System is expected to correlate closely with the Siemens Immulite 2000 and the Roche Elecsys COBAS. Theranos results are expected to be approximately 1.4 to 1.5 fold higher than the Siemens ADVIA system.

Table [SEQ Table * ARABIC]: Standard Curve: Serum Calibrators Batch 1

		Signal, RLU				Conc, ng/mL		
[TST] ng/mL	Log Conc	Mean RLU	CV %	Log S	Modulation	Mean Conc	CV %	% Recovery
10.45	1.02	2527	4.6	3.4	8.7	10.59	6.5	101
7.07	0.85	3595	12.4	3.6	6.1	6.86	13.2	97
3.69	0.57	6816	12.8	3.8	3.2	3.59	13.8	97
2.00	0.30	9904	8.1	4.0	2.2	2.23	12.7	111
0.99	-0.01	15893	7.0	4.2	1.4	0.87	17.7	88
0.31	-0.51	21951	2.9	4.3		0.32	10.6	104

Conc = -2.1119*(log(S))^3 + 23.426*(log(S))^2 - 87.574*(log(S)) + 110.98

Table [SEQ Table * ARABIC]: Recovery of BioRad LiquiCheck ImmunoAssay Plus Controls

	Level 1	Level 2	Level 3	Units
Siemens ADVIA Centaur XP*	0.92	3.68	9.10	ng/mL
Roche Elecsys COBAS E*	0.808	4.68	12.40	ng/mL
Siemens Immulite 2000/2500*	1.29	4.94	13.05	ng/mL
R&D Systems Cat #KGE010	1.03	4.45	13.11	ng/mL
Genway Cat #40-521-475002	0.93	3.51	11.49	ng/mL
Alpco Cat #11-TESHU-E01	1.38	n/a	8.92	ng/mL
Theranos System	1.44	5.11	13.51	ng/mL

* Mean values reported by BioRad.

Figure [SEQ Figure * ARABIC]: Recovery of BioRad LiquiCheck ImmunoAssay Plus Controls

[SHAPE * MERGEFORMAT]

1.10 Training Set

A set of 16 clinical samples with reported values determined on the Siemens ADVIA system at the time of collection were obtained from Bioreclamation and measured on the predicate ELISA kits and in the Theranos assay.

Correlation of Theranos results with the reported Siemens ADVIA results were excellent across the range, with the slope approximately 1.4 as expected based on calibration differences observed in the calibration verification. The Theranos System is calibrated to the Siemens Immulite 2000 which will be used for in-house validation.

Correlation of Theranos results with the R&D ELISA kit results were excellent, though the R&D kit appears to over-recover low samples in comparison to all the other tested methods. Correlation with the Genway ELISA kit result was adequate while correlation with the Alpco ELISA kit was adequate with the removal of one outlier sample. This sample #11 was an outlier in the Alpco results compared to both the reported result and the Theranos result.

Table [SEQ Table * ARABIC]: Standard Curve: Serum Calibrators Batch 1

		Signal, RLU				Conc, ng/mL		
[TST] ng/mL	Log Conc	Mean RLU	CV %	Log S	Modulation	Mean Conc	CV %	% Recovery
10.45	1.02	2527	4.6	3.4	8.7	10.59	6.5	101
7.07	0.85	3595	12.4	3.6	6.1	6.86	13.2	97
3.69	0.57	6816	12.8	3.8	3.2	3.59	13.8	97
2.00	0.30	9904	8.1	4.0	2.2	2.23	12.7	111
0.99	-0.01	15893	7.0	4.2	1.4	0.87	17.7	88
0.31	-0.51	21951	2.9	4.3		0.32	10.6	104

Conc = -2.1119*(log(S))^3 + 23.426*(log(S))^2 - 87.574*(log(S)) + 110.98

Table [SEQ Table * ARABIC]: Clinical Sample Training Set Results

		[TST] ng/mL				
Sample#	**Gender**	**Reported***	**R&D**	**Genway**	**Alpco**	**Theranos**
1	F	0.16	2.13	0.55	0.25	0.19
2	F	0.13	0.76	0.20	0.63	0.14
3	F	0.29	0.73	0.17	0.10	0.28
4	F	0.05	0.27	0.06	OORL	0.28
5	M	1.61	4.64	2.23	1.79	2.17
6	M	1.16	3.95	1.49	1.17	2.21
7	M	2.09	3.88	0.90	1.06	2.66
8	M	1.57	3.77	0.49	0.60	2.00
9	M	2.02	4.04	1.70	1.94	3.93
10	M	1.43	2.86	0.95	0.87	2.16
11	M	5.54	11.25	OORH	9.18	9.20
12	M	4.31	6.85	3.33	2.74	5.43
13	M	3.55	6.53	2.91	2.03	4.35
14	M	3.53	8.27	4.91	3.00	5.23
15	M	4.04	7.76	3.67	2.67	6.11
16	M	6.50	11.33	4.83	3.09	7.89
17	M	9.44	15.75	NES	9.0	9.61

* Reported values were determined on the Siemens ADVIA instrument at the time of sample collection.

Figure [SEQ Figure * ARABIC]: Correlation of Theranos Results to Reported Siemens ADVIA Results

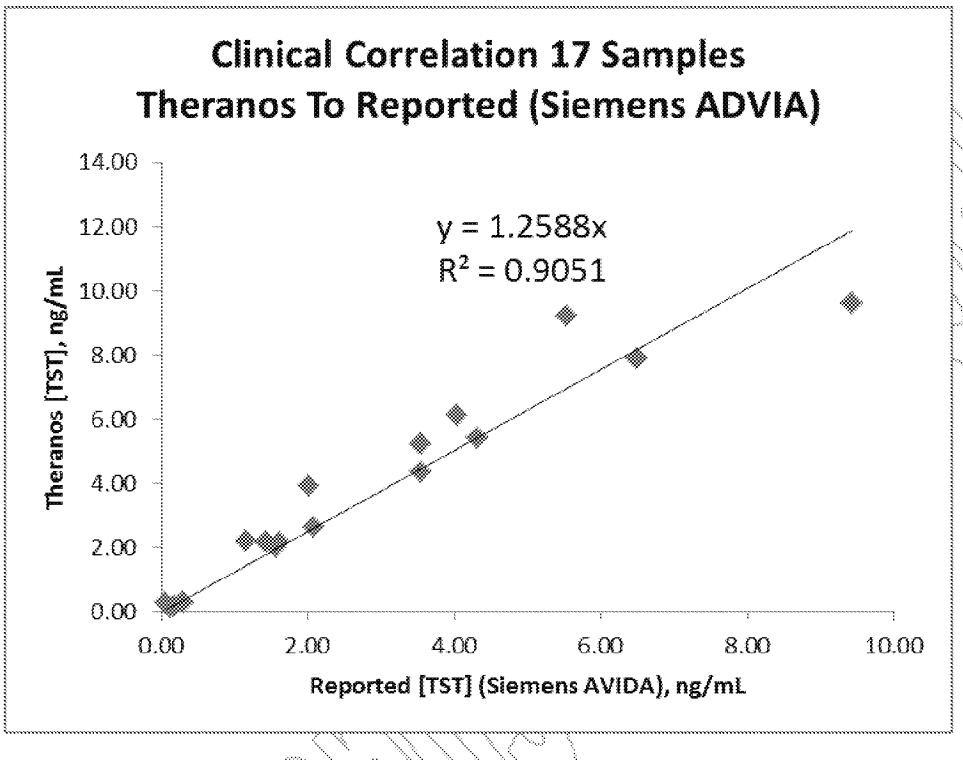
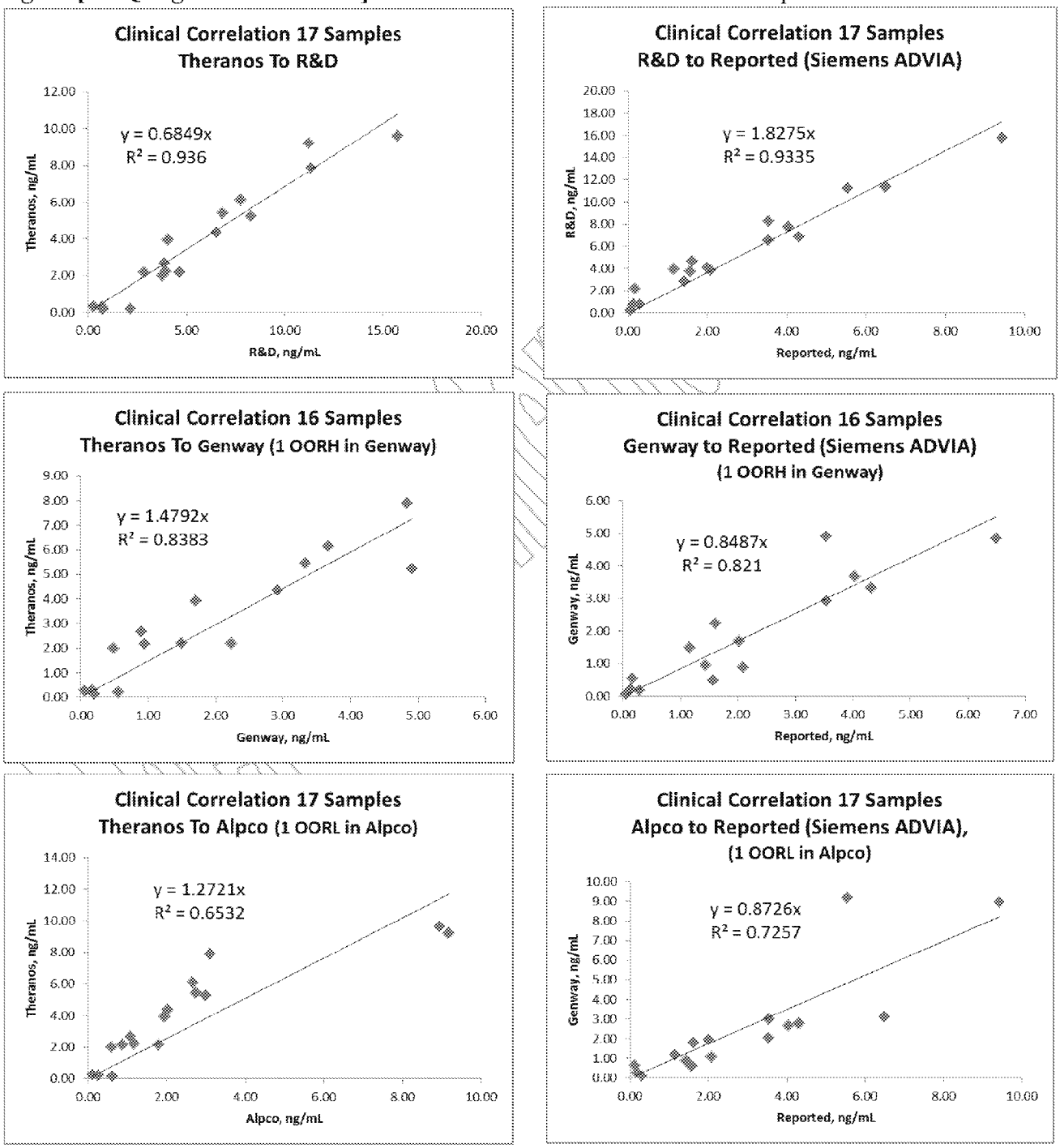


Figure [SEQ Figure * ARABIC]: Correlation of Theranos and Bench-Top ELISA Kit Results



1.11 Reagent Incubation Time

The effect of shorter reagent incubation times was tested. It was found that a 2 minute coincubation incubation followed by a 1 minute substrate incubation was ideal – modulation was equivalent or potentially better than the 5 minute incubation times. Due to the positional read time effect and the rapid kinetics in this short incubation time, the duplicate tips must be calibrated by tip position.

Table [SEQ Table * ARABIC]: Effect of Reagent Incubation Time

Incubation Time	[TST] ng/mL	Signal, RLU					Back-Calculated Conc, ng/mL		
		Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Modulation	Mean Conc	CV %	% Recovery
5, 5	10.45	3243	11.8	3254	10.7	7.3	11.11	22.6	106
	7.07	4460	1.5	4497	7.8	5.3	6.11	4.7	86
	3.69	6100	20.7	5643	0.3	4.2	4.10	16.2	111
	2.00	8920	25.2	10122	17.0	2.4	2.15	32.1	107
	0.99	14306	11.8	16094	3.8	1.5	0.93	15.1	94
	0.31	24074	20.9	23848	17.0		0.34	51.0	108
2, 1	10.45	552	15.2	628	6.2	8.9	11.20	19.8	107
	7.07	755	15.0	908	11.0	6.1	6.37	20.2	90
	3.69	1069	0.4	1247	3.1	4.5	3.83	2.6	104
	2.00	1613	15.8	1840	12.0	3.0	2.21	19.8	111
	0.99	2826	15.7	3220	18.9	1.7	0.93	30.1	94
	0.31	4610	7.9	5581	9.9		0.32	20.6	103

1.12 Sample Dilution

The effect of a higher sample dilution was tested with a final sample dilution of 1:25 versus the current 1:10. There was a small negative impact on assay modulation at the higher sample dilution. The assay may not be able to withstand a higher sample dilution so the current 1:10 sample dilution will continue to be used.

Final Sample Dilution	[TST] ng/mL	Signal, RLU					Back-Calculated Conc, ng/mL		
		Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Modulation	Mean Conc	CV %	% Recovery
1:10	10.45	552	15.2	628	6.2	8.6	11.20	19.8	107
	7.07	755	15.0	908	11.0	6.1	6.37	20.2	90
	3.69	1069	0.4	1247	3.1	4.4	3.83	2.6	104
	2.00	1613	15.8	1840	12.0	3.0	2.21	19.8	111
	0.99	2826	15.7	3220	18.9	1.7	0.93	30.1	94
	0.31	4610	7.9	5581	9.9		0.32	20.6	103
1:25	10.45	702	4.5	733	11.0	7.0	11.09	22.1	106
	7.07	824	11.7	897	4.2	5.9	7.15	16.3	101
	3.69	1381	16.2	1562	9.4	3.4	3.54	11.7	96
	2.00	2222	8.8	2428	8.6	2.2	2.30	1.3	115
	0.99	3337	19.5	4489	6.6	1.3	0.90	37.7	91
	0.31	4511	9.2	5600	1.3		0.34	22.1	110

1.13 Normal Sample Screen and Serum vs. Plasma

The normal range was verified and the effect of plasma or serum matrix was tested by measuring samples gathered from 5 male and 5 female donors. There was no significant difference between the results from the serum and the lithium-heparin plasma. In addition, the samples all fell into the expected normal range for adult males of 1.5 – 12 ng/mL and for females < 0.9 ng/mL.

Table [SEQ Table * ARABIC]: Standard Curve: Serum Calibrators Batch 2

	Signal, RLU				Conc, ng/mL		
[TST] ng/mL	Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Mean Conc	CV %	% Recovery
15.03	443	20.2	491	14.5	15.79	22.3	105
10.30	630	7.2	734	10.5	9.66	11.5	94
5.03	961	14.4	1071	12.4	5.55	19.0	110
2.53	1526	5.9	1821	9.9	2.49	3.2	98
1.03	2515	10.3	2917	4.8	1.04	14.4	101
0.28	4610	9.6	5749	2.9	0.28	12.7	102

Tip 1 Conc = 10^(-0.1313*(LOG(S))^3 + 0.7332*(LOG(S))^2 - 2.3783*(LOG(S)) + 4.7799)
Tip 2 Conc = 10^(0.1124*(LOG(S))^3 - 1.5143*(LOG(S))^2 + 4.6002*(LOG(S)) - 2.4166)

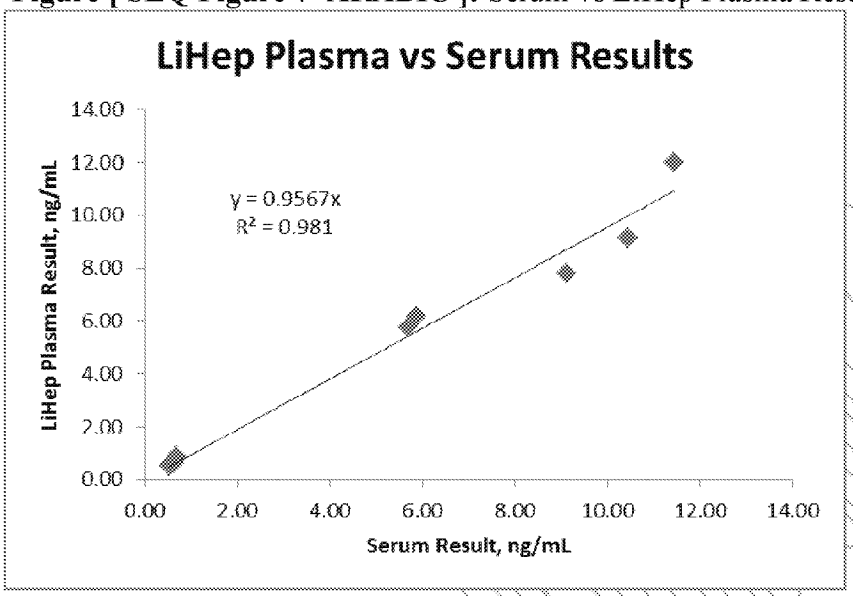
Table [SEQ Table * ARABIC]: Normal Donors - Serum vs LiHep Plasma Result

		Plasma Result, ng/mL		Serum Result, ng/mL	
Sample #	Gender	Mean Conc.	CV %	Mean Conc.	CV %
3	M	7.79	16.8	9.11	24.0
7	F	0.57	4.8	0.61	15.3
8	M	11.98	6.0	11.43	8.2
10	M	9.12	5.4	10.43	15.3
11	F	0.62	5.2	0.64	23.2
12	M	6.19	25.3	5.87	3.6
14	M	5.76	17.5	5.68	24.4
15	F	0.59	18.1	0.54	15.4
17	F	0.89	26.8	0.67	21.4
18	F	0.49	23.3	0.55	17.5

Table [SEQ Table * ARABIC]: Normal Ranges for Males and Females in Plasma and Serum

		Plasma, ng/mL	Serum, ng/mL
Male	Mean	8.17	8.50
	Min	5.76	5.68
	Max	11.98	11.43
Female	Mean	0.63	0.60
	Min	0.49	0.54
	Max	0.89	0.67

Figure [SEQ Figure * ARABIC]: Serum vs LiHep Plasma Result



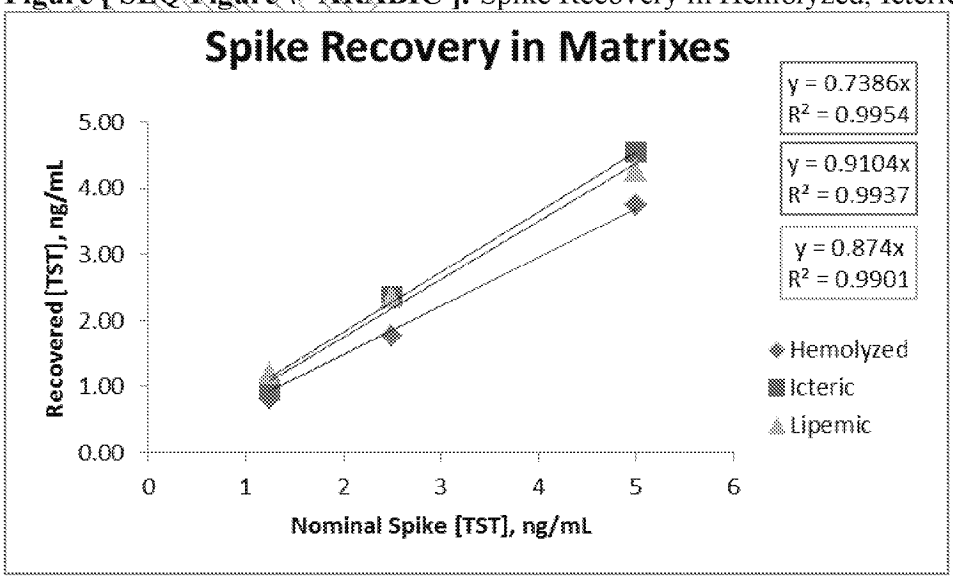
1.14 Interfering Matrixes

Spike recovery in severely hemolyzed, icteric and lipemic female serum obtained from ProMedDx and Vital was tested to determine if these sample types may cause aberrant effects in the TST assay. Spike recovery was excellent in icteric and lipemic serum but low in the severely hemolyzed serum. It should be noted that severe hemolysis could cause slightly low reported values in this assay.

Table [SEQ Table * ARABIC]: Spike Recovery in Hemolyzed, Icteric, and Lipemic Serum

Sample Type	Nominal Spike [TST] ng/mL	Conc, ng/mL			
		Mean Conc	CV %	Minus Endogenous	% Recovery
Hemolyzed	5	4.12	14.5	3.76	75
	2.5	2.14	6.7	1.78	71
	1.25	1.18	16.7	0.81	65
	0	0.37	18.9	-	-
Icteric	5	5.11	4.2	4.55	91
	2.5	2.92	16.9	2.36	95
	1.25	1.51	1.5	0.95	76
	0	0.55	16.8	-	-
Lipemic	5	4.60	20.1	4.26	85
	2.5	2.68	3.1	2.35	94
	1.25	1.52	3.9	1.19	95
	0	0.33	15.4	-	-

Figure [SEQ Figure * ARABIC]: Spike Recovery in Hemolyzed, Icteric, and Lipemic Serum



1.15 Effect of Anticoagulant

The Theranos System will prepare plasma from both EDTA and lithium-heparin-treated blood. To determine if the TST assay is impacted by the choice of anticoagulant, plasma was prepared from EDTA tubes and from Li-Hep tubes for 3 normal female donors, and spike recovery was tested. There was no significant difference between results in Li-Hep and EDTA samples.

Table [SEQ Table * ARABIC]: Effect of Anticoagulant on Plasma Results, Signal (RLU)

Sample #	Spiked [TST] ng/mL	EDTA Plasma				LiHep Plasma			
		Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %
1	5.00	1135	4.3	1292	5.8	1100	9.3	1174	9.4
	2.50	1676	3.0	2023	10.4	1755	-	1926	-
	1.25	2564	6.9	3022	14.0	2377	21.6	2916	20.5
	0.00	5230	18.4	6305	17.5	5665	3.8	6450	16.2
2	5.00	981	5.9	1152	3.0	919	20.7	977	19.7
	2.50	1329	12.1	1472	19.9	1337	9.9	1541	8.3
	1.25	1854	9.5	2134	2.4	1763	5.4	2130	6.8
	0.00	2815	4.8	3193	0.4	3015	23.0	3535	17.7
3	5.00	921	6.5	1101	4.7	1171	2.6	1343	2.3
	2.50	1592	21.3	1810	17.7	1676	0.2	2094	2.9
	1.25	2044	5.8	2344	2.2	2175	13.9	2442	11.9
	0.00	3939	6.7	4289	0.2	4504	6.0	4927	3.0

Table [SEQ Table * ARABIC]: Effect of Anticoagulant on Plasma Results, Conc. (ng/mL)

Sample #	Spiked [TST] ng/mL	EDTA Plasma				LiHep Plasma			
		Mean Conc, ng/mL	CV %	Minus Endogenous	% Recovery	Mean Conc, ng/mL	CV %	Minus Endogenous	% Recovery
1	5.00	4.13	6.0	4.13	83	4.54	12.5	4.54	91
	2.50	2.19	10.7	2.19	88	2.19	6.2	2.19	87
	1.25	1.03	18.3	1.03	82	1.20	34.2	1.20	96
	0.00	OORL	-	-	-	OORL	-	-	-
2	5.00	4.94	5.4	4.08	82	5.97	23.1	5.20	104
	2.50	3.42	20.6	2.55	102	3.24	11.1	2.48	99
	1.25	1.92	9.7	1.05	84	2.01	9.3	1.24	99
	0.00	0.87	7.5	-	-	0.77	37.3	-	-
3	5.00	5.33	6.9	4.92	98	3.92	2.9	3.64	73
	2.50	2.57	25.2	2.16	86	2.12	7.7	1.84	74
	1.25	1.62	6.6	1.21	97	1.50	19.8	1.21	97
	0.00	0.41	17.3	-	-	0.28	20.0	-	-

Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant on Plasma Results, Signal (RLU)

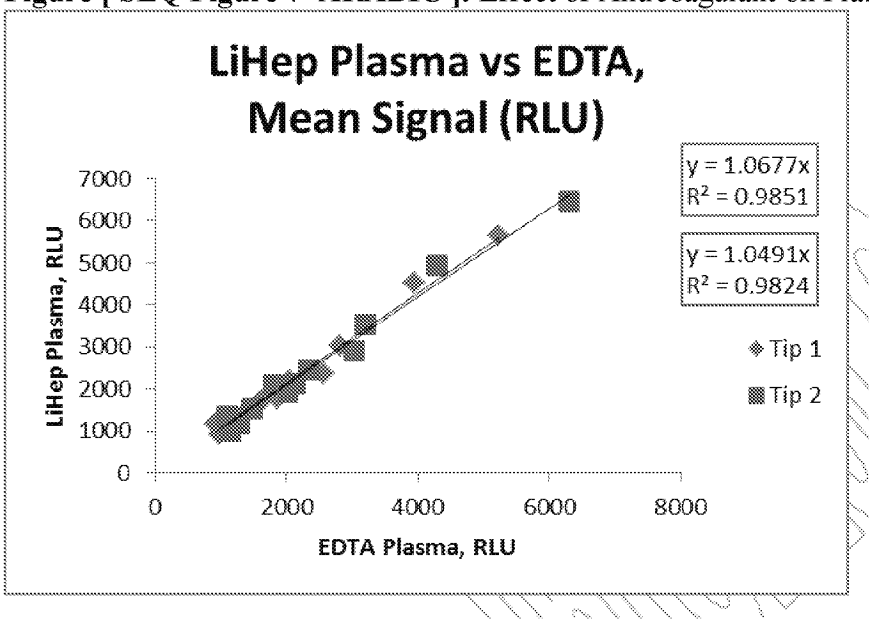
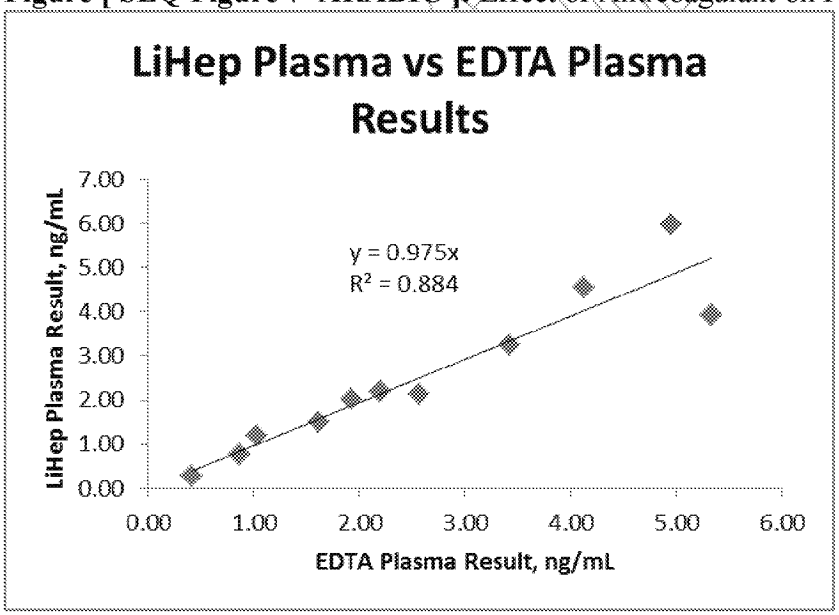


Figure [SEQ Figure * ARABIC]: Effect of Anticoagulant on Plasma Results, Conc. (ng/mL)



1.16 Cross Reactivity

The Theranos TST assay was tested for cross reactivity to dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), progesterone, and estradiol. A control standard curve was run in the same buffer calibrator matrix used for the test substances. No cross reactivity was observed, signal was above SMax for all the levels of all test substances.

Test Substance	[TST], ng/mL	[Test Substance], ng/mL	Signal, RLU				Conc, ng/mL		
			Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Mean Conc	CV %	% Recovery
Control	15.0	0.0	325	10.1	348	16.3	14.0	17.2	93
	10.0	0.0	396	14.9	432	13.7	10.7	20.7	107
	5.0	0.0	642	11.6	749	10.5	4.8	14.4	96
	2.5	0.0	1066	6.8	1164	2.3	2.6	3.2	104
	1.0	0.0	1950	2.7	2205	2.0	1.0	3.3	99
	0.0	0.0	5105	24.2	6265	17.9	OORL	-	-
DHT	0.0	2.5	4447	7.1	5216	6.9	OORL	-	0
	0.0	1.0	5415	4.4	6736	0.0	OORL	-	0
	0.0	0.5	4736		6187	8.8	OORL	-	0
	0.0	0.0	5775	3.2	6760	7.0	OORL	-	0
DHEA	0.0	30.0	6118	14.8	7086	0.6	OORL	-	0
	0.0	10.0	5766	5.1	6592	9.2	OORL	-	0
	0.0	3.3	6656	3.0	7917	2.9	OORL	-	0
	0.0	0.0	6013	6.4	7368	3.5	OORL	-	0
Progesterone	0.0	100.0	5699	12.4	6709	17.3	OORL	-	0
	0.0	50.0	5028	1.2	6005	6.3	OORL	-	0
	0.0	25.0	5587	28.7	6656	18.6	OORL	-	0
	0.0	0.0	5667	16.2	6783	11.8	OORL	-	0
Estradiol	0.0	2.0	6118	4.6	7652	5.1	OORL	-	0
	0.0	1.0	6434	0.5	7590	2.6	OORL	-	0
	0.0	0.5	5495	10.1	6563	16.3	OORL	-	0
	0.0	0.0	5683	9.5	6617	15.2	OORL	-	0

Tip 1 Conc = 0.579 * (((5112.115 - 144.959) / (S - 144.959)) - 1) ^ (1 / 1.018)
SMin = 297; SMax = 2223

Tip 2 Conc = 0.491 * (((6283.617 - 118.159) / (S - 118.159)) - 1) ^ (1 / 0.956)
SMin = 317; SMax = 2494

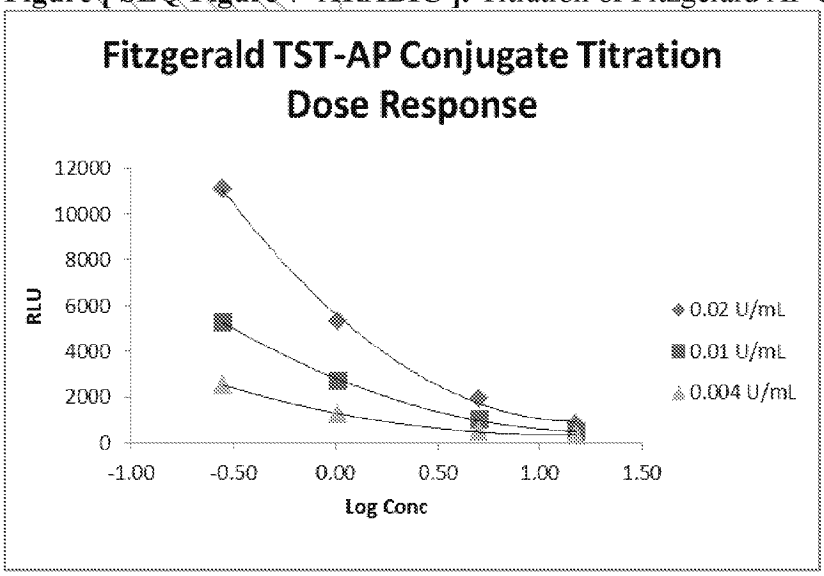
1.17 Titration of Fitzgerald Alkaline Phosphatase Conjugate

The final alkaline phosphatase conjugate produced by Fitzgerald was received and titrated in a buffer consisting of 0.1 mM Zn2+, 5mM Mg2+ in 0.03% BSA in TBS. The loading concentration of the conjugate is shown, the protocol dilutes the conjugate 1:10 into the final sample mixture. A loading concentration of 0.02 U/mL of the conjugate produced the best modulation across the range.

Table [SEQ Table * ARABIC]: Titration of Fitzgerald AP Conjugate

Conjugate, U/mL	[TST] ng/mL	Signal, RLU				Total CVs		
		Tip 1 Mean RLU	CV %	Tip 2 Mean RLU	CV %	Mean RLU	CV %	Modulation
0.02	15.03	780	5.2	882	3.3	831	7.9	13.4
	5.03	1844	-	2059	-	1952	7.8	5.7
	1.03	5296	19.2	5355	25.4	5326	18.4	2.1
	0.28	10298	5.4	11986	2.5	11142	9.3	-
0.01	15.03	437	0.5	477	4.0	457	5.6	11.6
	5.03	972	19.8	1054	14.1	1013	14.6	5.2
	1.03	2664	14.0	2778	16.1	2721	12.6	1.9
	0.28	4935	12.2	5628	14.5	5282	13.4	-
0.004	15.03	301	11.0	325	0.2	313	7.5	8.1
	5.03	507	8.8	481	18.0	494	11.8	5.1
	1.03	1191	1.5	1275	7.7	1233	6.1	2.0
	0.28	2330	5.4	2712	12.5	2521	12.0	-

Figure [SEQ Figure * ARABIC]: Titration of Fitzgerald AP Conjugate



1.18 Clinical Correlation under Final Assay Conditions

A new protocol was produced to allow the Total TST and Free TST assays to be multiplexed. To validate this protocol and the final AP conjugate from Fitzgerald, a calibration and a larger set of 26 clinical samples was run using the final assay conditions. The calibration was fit to a 4PL curve using the Theranos in-house calibration software.

Final Assay Conditions:

- 1:10 final sample dilution
- 2,1 minute reagent incubations
- 250 ng/mL Estradiol in 10% ethanol as displacer
- 100 ng/mL CAb in Sea Block
- 0.02 U/mL Fitzgerald TST-AP in 0.1 mM Zn^{2+}, 5mM Mg^{2+} in 0.03% BSA in TBS
- Multiplex protocol: TST_Total_Free

Table [SEQ Table * ARABIC]: Standard Curve

	Signal, RLU				Back-Calculated Conc, ng/mL		
[TST] ng/mL	Tip 3	CV%	Tip 4	CV%	Mean Conc	CV%	% Recovery
15.03	863	13.4	934	13.5	14.17	11.8	94
10.30	1170	7.5	1309	13.1	9.84	15.2	96
5.03	2182	10.4	2191	7.9	5.36	9.3	107
2.53	3172	14.4	3476	13.1	3.04	16.0	120
1.03	6253	4.4	7528	1.9	0.90	3.8	87
0.28	10135	5.8	11197	2.8	0.32	9.9	116
0.13	12222	5.1	13618	3.7	0.14	21.4	105
0.03*	11961	18.1	12864	15.3	OORL	-	-

* anchor point

Tip 3 Conc = 0.680 * (((14740.353 - 95.750) / (S - 95.750)) - 1) ^ (1 / 0.932)
Tip 4 Conc = 0.824* (((15679.559 - 19.741) / (S - 19.741)) - 1) ^ (1 / 0.978)

LLOQ = 0.13 ng/mL
ULOQ = 15.03 ng/mL

Table [SEQ Table * ARABIC]: Clinical Sample Total Testosterone Results, ng/mL

Sample #	Reported (Siemens ADVIA)	R&D Systems Kit	Theranos
1	0.16	2.13	0.40
2	0.13	0.76	0.41
3	0.29	0.73	0.46
4	0.05	0.27	0.28
5	1.61	4.64	2.13
6	1.16	3.95	1.74
7	2.09	3.88	2.64
8	1.57	3.77	1.82
9	2.02	4.04	2.39
10	1.43	2.86	1.38
11	5.54	11.25	9.12
12	4.31	6.85	5.13
13	3.55	6.53	5.72
14	3.53	8.27	4.74
15	4.04	7.76	5.55
16	6.50	11.33	6.66
17	9.44	15.75	9.61
18	6.50	5.93	7.47
19	4.04	3.58	5.40
20	7.50	7.53	8.71
21	4.24	13.98	5.34
22	6.57	9.54	6.91
23	4.57	16.68	5.73
24	12.30	17.49	13.32
25	8.10	8.92	8.00
26	10.45	31.30	14.37

Figure [SEQ Figure * ARABIC]: Clinical Correlation, Theranos to Reported (Siemens ADVIA)

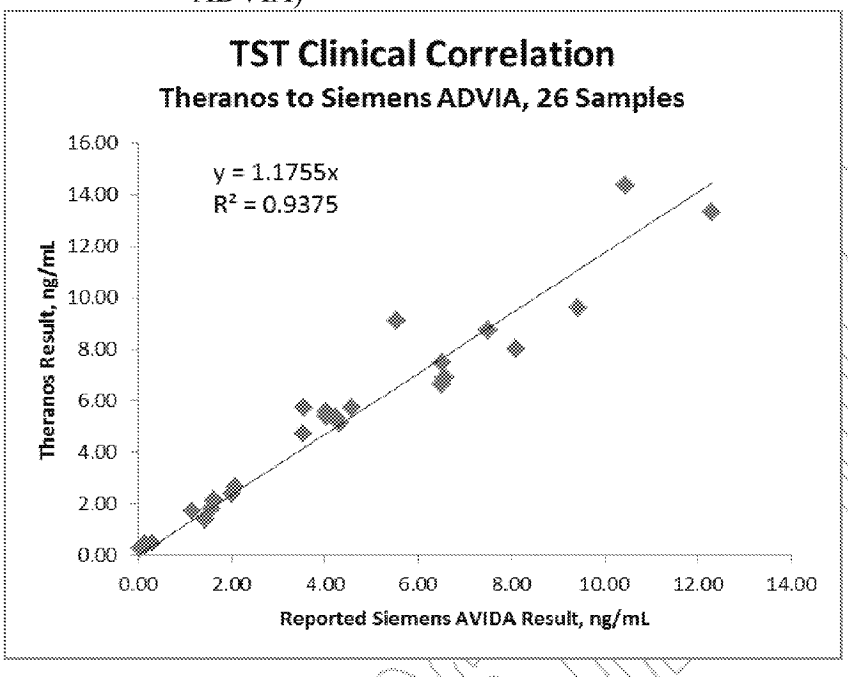


Figure [SEQ Figure * ARABIC]: Clinical Correlation, Theranos to R&D Systems Kit

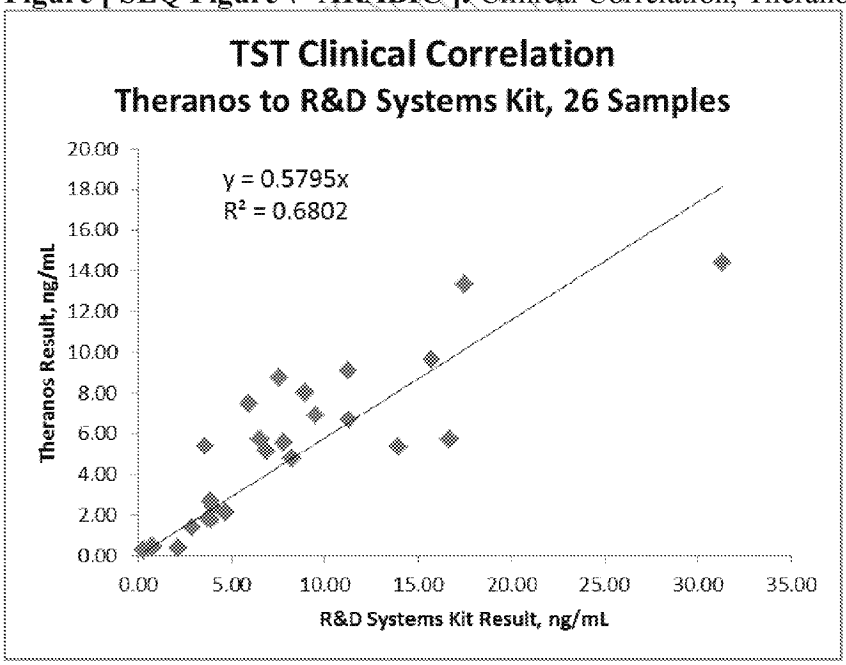
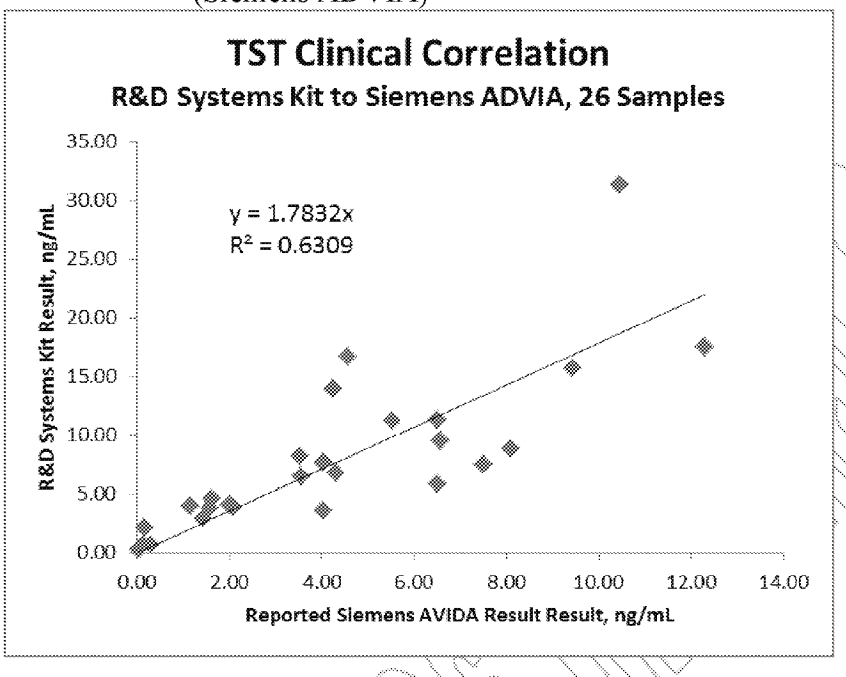


Figure [SEQ Figure * ARABIC]: Clinical Correlation, R&D Systems Kit to Reported (Siemens ADVIA)



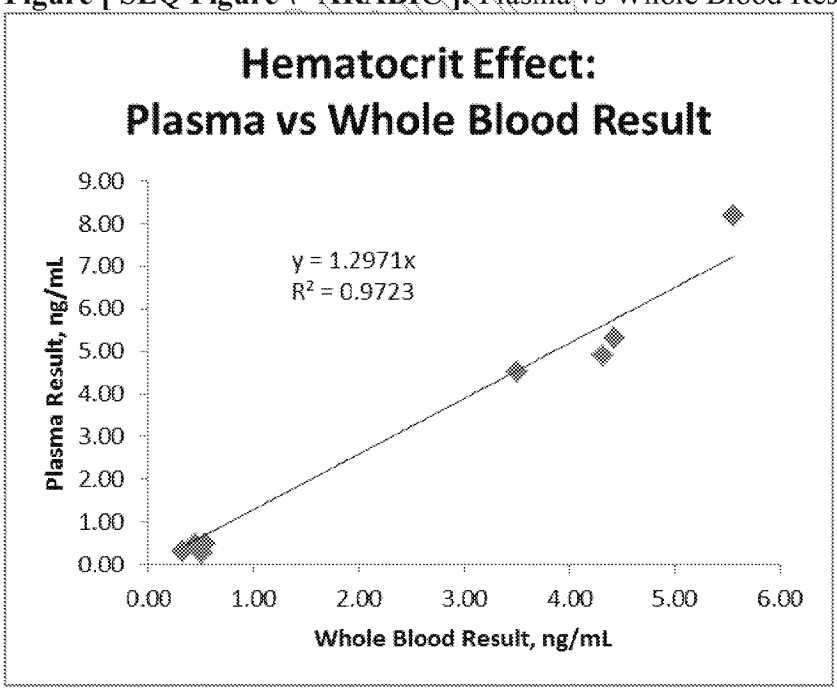
1.19 Hematocrit Effect

The hematocrit effect was tested by measuring 8 whole blood samples (4 male and 4 female) collected in EDTA tubes, and then measuring the plasma obtained from those samples. Total testosterone concentrated slightly into the plasma. Whole blood results could be calibrated on the serum standard curve with a hematocrit correction factor.

Table [SEQ Table * ARABIC]: Plasma vs Whole Blood Results

		Whole Blood		Plasma	
Sample #	Gender	Mean Conc	CV%	Mean Conc	CV%
3	M	3.51	21.3	4.53	1.5
4	M	5.56	4.3	8.18	1.7
5	F	0.51	30.0	0.26	15.1
6	F	0.45	9.2	0.48	9.6
7	F	0.55	16.2	0.46	11.6
8	F	0.33	18.2	0.32	9.9
9	M	4.31	18.4	4.90	14.7
10	M	4.42	14.9	5.32	5.6

Figure [SEQ Figure * ARABIC]: Plasma vs Whole Blood Results



1.20 Stability

Stability of reagents stored at 4°C is being monitored.

2 REFERENCES

1. Shrivastav TG., (2002) "Matrix interference in direct total testosterone enzyme immunoassay and its elimination with the use of non-cross reactive steroids in serum based standards." Health and Population: Perspective and Issues, 25 (2). pp. 55-64.
2. Gungor O, et al. "Endogenous testosterone and mortality in male hemodialysis patients: is it the result of aging?" Clin J Am Soc Nephrol. 2010 Nov;5(11):2018-23. Epub 2010 Jul 22.
3. Martínez AC, et al. "Testosterone and cortisol changes in professional basketball players through a season competition." J Strength Cond Res. 2010 Apr;24(4):1102-8.
4. Sanyal A, et al. "Regulation of bone turnover by sex steroids in men." J Bone Miner Res. 2008 May;23(5):705-14.