theranos™
redefining healthcare

Serum Cotinine Assay Development Report

Theranos, Inc.

December 3, 2012

Prepared by: Sharada Sivaraman

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 is designed to detect cotinine in human serum and plasma. The assay has a reportable range of 10 to 1000 ng/mL. The Theranos serum cotinine assay is calibrated using the Certified Reference Material (-)-Cotinine (Cat#C-016) from Cerilliant.

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

The reference assay is the commercially available ELISA from OraSure Technologies, Inc. (OTI) Cotinine Micro-Plate EIA.

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

A biotin-labeled anti-rabbit antibody coated on avidin serves as the capture surface. The sample is diluted and combined with rabbit anti-cotinine antibody and an enzyme labeled cotinine conjugate. This mixture is incubated on the capture surface for 10 minutes. After the incubation, the surface is washed and substrate is incubated on the surface for 10 minutes, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]; Materials

Name	Supplier	Catalog #
(-)-Cotinine	Cerilliant	C-016
Alkaline Phosphatase Substrate	Theranos	T-ALKP-SB01
Low BSA Blocking Buffer (0.03% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041
Rabbit PAb Anti-Cotinine	Lifespan	LS-C128356
Cotinine-4-Alk Phos Conjugate	Theranos	
Charcoal absorbed Serum	ABT Technologies	

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

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

9 anti-Cotinine antibodies (Table 1) were commercially available and ordered. All 9 antibodies were coated on a 384 well microtitre plate (MTP) at 10, 5, 2.5 1, 0.1 and 0 ug/mL and tested for binding to the commercial Cotinine-HRP conjugate from 3 different vendors (Table 3) at a dilution of 1:1000 from the stock in Stabilzyme Noble (HRP small molecule conjugate stabilizer). The data are summarized in Table 4, 5 and 6. The antibodies showed varying binding affinities to each of the commercial cotinine HRP conjugates. Except Ab#8 all the remaining antibodies showed binding to at least 1 of the commercially available cotinine HRP conjugates and these were all chosen for further evaluation on the competitive format on the Theranos system.

Table [SEQ Table * ARABIC]: Antibody Information
[LINK Excel.Sheet.8 "\\\\theranos.local\\folders\\Projects\\Experiment Log\\E0800 - E0899\\E0866\\Reports\\Serum cotinine assay development .xls" "Ab info!R20C3:R29C7" \a \f 5 \h * MERGEFORMAT]

Table [SEQ Table * ARABIC]: Conjugate Information

Conjugate#	Vendor	Cat #	Type
1	Randox	HRP9478	Cotinine HRP conjugate
2	YJ Bio	HN3545-H	Cotinine HRP conjugate
3	Eastcoast Bio	P82-99-02A	Cotinine-4-HRP Enzyme Conj.
4	YJ Bio	HN3545A	Cotinine AP conjugate

Table [SEQ Table * ARABIC]: Antibody-Conjugate Binding Screen with Randox HRP Conjugate

Randox Cotinine HRP Conjugate									
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**	**Ab#**	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
1	10	2252	8	0.9	6	10	2705	2.6	0.6
	5	2263	15.4			5	2699	7.3	
	2.5	2290	4.3			2.5	2477	27.2	
	1	2378	21.9			1	2322	13.5	
	0.5	2768	9.6			0.5	3317	23.7	
	0	2378	10.7			0	4377	8.1	

2	10	3587	4.5	1.1	7	10	1625	7.1	1.2
	5	3455	14			5	1225	13.2	
	2.5	3566	18.7			2.5	1074	1.5	
	1	3210	2.3			1	1082	2.6	
	0.5	3257	1			0.5	1324	21.6	
	0	3172	6.3			0	1392	7.3	
4	10	3446	9.3	0.9	8	10	3314	23.9	0.9
	5	3231	7.6			5	2717	14.6	
	2.5	8514	99.1			2.5	3065	9.6	
	1	2500	0.7			1	3474	24.1	
	0.5	2602	26.9			0.5	3602	22.2	
	0	3938	18.9			0	3739	12.6	
5	10	117859	0.2	74	9	10	2576	10.2	1.4
	5	83495	0.9			5	2081	26	
	2.5	46345	12.8			2.5	1856	25.2	
	1	8620	9.9			1	1541	35.4	
	0.5	1561	0.1			0.5	1729	42.7	
	0	1594	27.8			0	1878	28.2	

Table [SEQ Table * ARABIC]: Antibody-Conjugate Binding Screen with YJ Bio HRP Conjugate

YJBio Cotinine HRP Conjugate									
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**	**Ab#**	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
1	10	26677	0.4	11	6	10	134251	5.2	47
	5	18458	1.2			5	108980	15.8	
	2.5	7857	3.4			2.5	31793	7.3	
	1	2292	8.5			1	3772	13.6	
	0.5	2430	11.6			0.5	2836	17.2	
	0	2392	10.9			0	2866	20.7	
2	10	160136	6.5	40	7	10	22009	2.4	10
	5	124764	0.9			5	16325	5.8	
	2.5	41286	15.6			2.5	6003	9.3	
	1	5300	5.2			1	1757	3.1	
	0.5	3544	30.5			0.5	1747	34.8	
	0	3968	0.1			0	2117	18	
4	10	73637	7.1	21	8	10	7820	3.9	2.4
	5	53463	4.1			5	3857	22.8	
	2.5	28376	3.1			2.5	7996	23.5	
	1	6200	5.3			1	3435	15.2	
	0.5	4412	29.1			0.5	2678	30.9	
	0	3555	9.5			0	3314	0.7	
5	10	2318	4.7	1.2	9	10	23137	6.6	12
	5	2250	13			5	17849	0.4	
	2.5	1737	6.1			2.5	5403	7	
	1	1640	4			1	1935	11.9	
	0.5	1761	13			0.5	1732	5.5	
	0	1878	8.8			0	2098	9.7	

Table [SEQ Table * ARABIC]: Antibody-Conjugate Binding Screen with Eastcoast Bio HRP Conjugate

Eastcoast Bio Cotinine HRP Conjugate									
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**	**Ab#**	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
1	10	1810	3.2	0.7	6	10	2303	13.2	0.6
	5	1718	13.5			5	2397	15.9	
	2.5	1738	1.2			2.5	2421	35.7	
	1	2153	5.1			1	2099	22.1	
	0.5	2086	2.5			0.5	2595	16.3	
	0	2647	15.7			0	3710	5.6	
2	10	3207	1.4	1.3	7	10	1417	21.3	1.1
	5	3437	10.2			5	1430	19	
	2.5	3221	13.1			2.5	960	4.2	
	1	2298	45.5			1	1199	18.9	
	0.5	3188	7.1			0.5	1270	32.3	
	0	2475	35.5			0	1326	27	
4	10	595125	0.3	180	8	10	4138	9.3	0.8
	5	510991	0.8			5	5421	32.7	
	2.5	247896	4.4			2.5	3793	14.7	
	1	34421	4.3			1	3469	40.8	
	0.5	12549	3.5			0.5	3377	37.1	
	0	3270	8.9			0	5775	56.9	
5	10	1280	6.6	0.8	9	10	2368	31.9	1.2
	5	1285	19			5	2049	21.9	
	2.5	1275	2.4			2.5	1744	30.5	
	1	1234	5.2			1	1547	18.5	
	0.5	1383	4.2			0.5	1391	37.8	
	0	1701	4			0	1914	1	

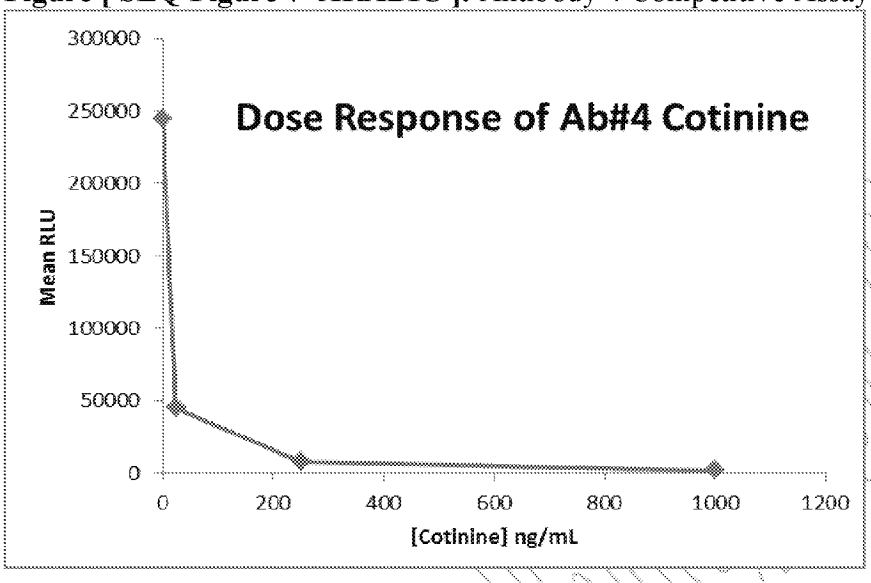
1.3 Competitive Assay Screen (Theranos 3.0 System)

The 7 antibodies were evaluated for their performance in the competitive format by coating the capture surface with the appropriate secondary antibody. Following this each of the unlabeled anti-cotinine antibodies were mixed into the sample along with the Cotinine -HRP conjugate at a 1:10 sample dilution and tested for response in a competitive assay with cotinine spiked depleted serum calibrators. Only antibody 3 showed a dose response in the competitive assay. Antibodies 1 and 2 showed high binding to the HRP conjugate but no response to the free analyte.

Table [SEQ Table * ARABIC]: Competitive Assay Screen (Theranos System)

Ab#	[Cotinine]	Inter-Cartridge		S/B
	ng/ml	Mean	CV%	
1	1000	5417	9	1
	250	5628	10	
	25	6577	3	
	0	6758	6	
2	1000	4110	10	1
	250	4451	4	
	25	3859	25	
	0	4791	8	
4	1000	1811	2	135
	250	8014	11	31
	25	44967	12	5
	0	244516	5	1
5	1000	28038	9	4
	250	43409	6	3
	25	70596	11	2
	0	113618	12	1
6	1000	3397	14	1
	250	4736	2	
	25	3452	41	
	0	4862	6	
7	1000	5717	8	1
	250	6144	3	
	25	6858	11	
	0	3980	8	
9	1000	5301	1	1
	250	5463	0	
	25	6304	13	
	0	4655	7	

Figure [SEQ Figure * ARABIC]: Antibody 4 Competitive Assay Dose Response



1.4 Standard Curve and Training Set

An 8 point standard curve using cotinine spiked depleted serum calibrators was used to calibrate the dose reponse of the Antibody#4. The modulation was excellent and recovery was within expected criteria(Table 8 and Figure 2). Since the dose reposnse and assay would need further optimization with the alkaline phosphatase labeled tracer the current conditions were used to evaluate the response of a small set of clinical samples(N=10). The same samples were also run on the Calbiotech Cotinine ELISa kit. The clinical sample correlation and data are summarized below in Table 9 and Figure 3. The correlation to the Calbiotech Cotinine ELISa kit was excellent at a R^2 value of 0.94 and slope of 0.88. The Ab#4 was finalized for further evaluation.

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

[Cotinine]	RLU		S/B	Conc. ng/mL		%Recovery
ng/ml	Mean	CV%		Mean	CV%	
1000.0	603	10	219.3	890.6	8.0	89.1
500	834	3	158.5	633.9	6.3	126.8
250	2317	11	57.1	235.7	7.8	94.3
100	6652	1	19.9	85.2	10.0	85.2
50	8629	6	15.3	64.9	5.5	129.8
25	19976	0	6.6	24.4	2.4	97.6
10	36564	5	3.6	10.0	22.3	99.9
0	132244	6	1.0	OORL		

Conc = 235.43* (((5.114-1.611) / (log10(RLU) - 1.611)) - 1) ^ (1 / 0.526)

Figure [SEQ Figure * ARABIC]: Cotinine Standard Curve in Depleted Serum

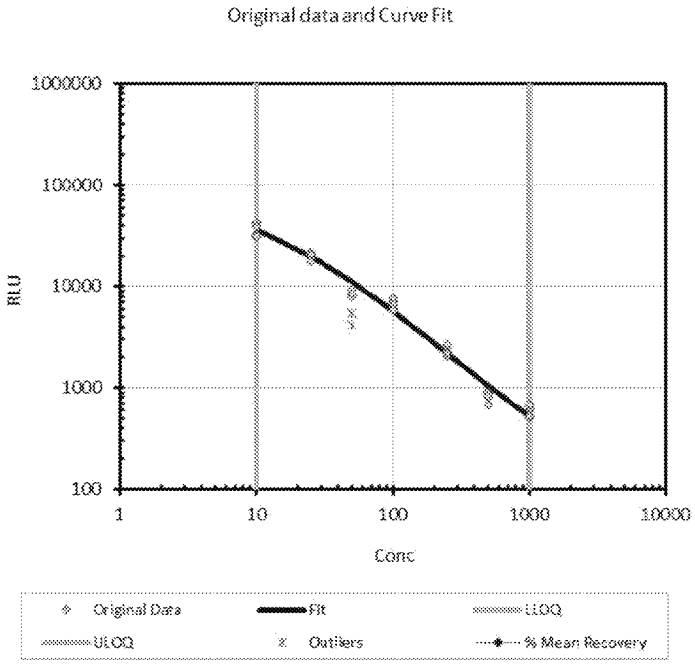
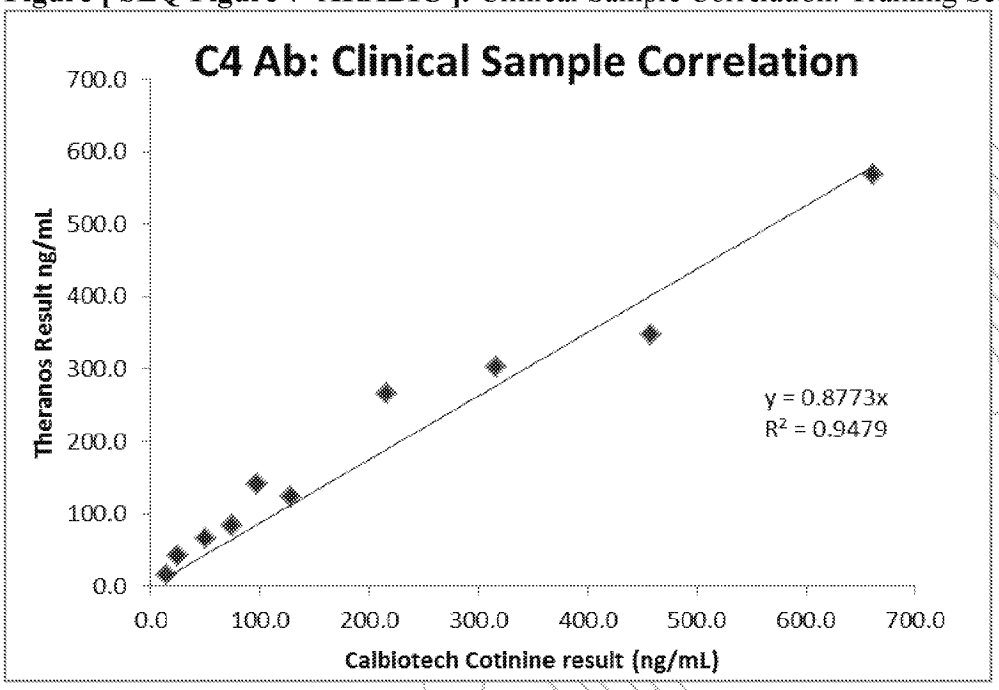


Table [SEQ Table * ARABIC]: Clinical Sample Correlation: Training Set

Sample ID	Calbiotech ELISA result ng/mL	Theranos Result Mean	CV%	%Recovery
M11	216.0	266.6	2.4	123
F4	129	124.0	8.7	96
M14	458	346.6	19.6	76
M15	97.9	140.4	17.5	143
BR 44	316	302.5	4.1	96
BR72	662	567.1	39.0	86
M9	25	41.4	2.5	166
Sm08	50	66.4	11.3	133
Sm16	75	83.1	12.6	111
BR67	15	16.5	13.2	110

Figure [SEQ Figure * ARABIC]: Clinical Sample Correlation: Training Set



1.5 Cross reactivity

The Theranos serum cotinine assay was tested for cross reactivity against nicotine and similar analogs: nicotinic acid, niacinamide, nicotine and nicotinic acid N-oxide. Each potential cross reactant was tested at 100,000 ng/ml and was spiked into 2 cotinine calibrators at 25 and 0 ng/ml. This experiment was able to evaluate cross reactivity and interference at the same time. The results are summarized in Table 10.
No cross reactivity was detected at the concentrations of the compounds tested. The compounds tested also did not interfere with the assay in the presence of 25 ng/ml of cotinine as shown from the recoveries which ranged from 96-122 %.

Table [SEQ Table * ARABIC]: Cross reactivity and Interference: Nicotine and analogs of Nicotine

Cross Reactant	Cross Reactant conc ng/ml	[Cotinine] ng/ml	RLU Mean	RLU CV%	Conc. Mean ng/ml	Conc. CV%	% Cross Reactivity	% From Control
Niacinamide	100,000	0	10041	4	55	4	0	119
	100,000	25	15974	5	32	6		
Nicotinic acid	100,000	0	7820	12	73	13	0	122
	100,000	25	15996	14	33	18		
Nicotine	100,000	0	15622	3	33	7	0	101
	100,000	25	19369	9	25	12		
Nicotinic acid N-oxide	100,000	0	14514	32	38	29	0	96
	100,000	25	20605	10	24	14		

1.6 Binding to Cotinine Alkaline Phosphatase (AP) Conjugates

Cotinine AP conjugates from two sources in house and commercial vendor (YJBio) were tested as candidates for the labeled tracer in the Theranos cotinine assay by testing their binding to Ab#4 and Ab#3. The experimental conditions were the same as described in section 1.2 Both antibodies demonstrated dose dependent binding to the cotinine AP conjugates as summarized in Table 11.

Table [SEQ Table * ARABIC]: Cotinine AP conjugates binding to Antibodies #3 and #4

YJ Bio Cotinine AP Conjugate				
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
3	10	2027151	5.3	1330
	5	1648077	0.6	1081
	2.5	1118514	8.9	734
	1	93985	49.5	62
	0.5	126305	25.5	83
	0	1524	9.5	1
4	10	2502129	4.9	1407
	5	2054719	0.2	1156
	2.5	1416624	8.5	797
	1	121991	48.4	69
	0.5	166290	26	94
	0	1778	11.2	1
Theranos Cotinine AP Conjugate				
3	10	4110207	4.2	755
	5	3937633	0.5	724
	2.5	3270405	2.2	601
	1	2461187	5.1	452
	0.5	677420	5	124
	0	5441	3	1
4	10	4911792	3.2	719
	5	4734018	0.6	693
	2.5	3976419	1.4	582
	1	3042843	3.9	445
	0.5	863556	4.4	126
	0	6833	2.4	1

1.7 Competitive assay screen with Cotinine AP conjugates

The Theranos Cotinine assay was evaluated for performance using the cotinine AP conjugates. Table 12 summarizes the data with Ab#4 and the commercial cotinine AP conjugate from YJBio. The data shows that this tracer –Ab combination provides excellent modulation. The availability of two versions of the in house cotinine AP conjugates differing in the position (-3 or -4) at which the AP is linked to the molecule prompted inclusion of Ab#3 into the testing since this is a rabbit polyclonal raised against cotinine -3-BSA as the immunogen. Ab#4 is another rabbit polyclonal that is raised against cotinine-4-BSA as the immunogen. It was expected that these antibodies would show selective binding to the respective in house cotinine conjugates. This was indeed observed as summarized in Table 13. The experimental conditions were as follows: Anti-rabbit IgG was coated on the surface. The sample was diluted with diluent that also contained the respective anti-cotinine antibodies and mixed with the cotinine AP cojugate such that the final sample dilution was 1:5.

Table [SEQ Table * ARABIC]: Competitive assay: YJBio Cotinine AP conjugate and Ab#4

YJBio Cotinine AP Conjugate						
Ab#	**Cotinine AP conjugate dilution**	**[Ab] µg/mL**	**[Cotinine] ng/ml**	**Inter-Cartridge Mean**	**CV%**	**S/B**
4	1:10,000	1	1000	4810	6	298
			25	228770	12	6
			0	1435404	8	1
		0.5	1000	2859	4	378
			25	102778	21	11
			0	1079756	12	1
		0.1	1000	1410	14	554
			25	69499	19	11
			0	781342	2	1
4	1:25,000	1	1000	3708	39	196
			25	89685	16	8
			0	728522	8	1
		0.5	1000	1649	18	597
			25	52435	13	19
			0	984365	36	1
		0.1	1000	566	12	779
			25	23631	11	19
			0	440945	9	1

Table [SEQ Table * ARABIC]: Competitive assay: Theranos Cotinine AP conjugates: Ab#4 and Ab#3

Theranos Cotinine AP conjugate						
Ab#	**AP conjugation site with respect to Cotinine**	**Cotinine AP conjugate dilution**	**[Cotinine] ng/ml**	**Inter-Cartridge Mean**	**CV%**	**S/B**
3	3-position	1:10,000	1000.0	43970	3	9
			250	162347	7	2
			25	349813	18	1
			0	396004	7	1
4	3-position	1:10,000	1000.0	850716	18	4
			250	1713015	4	2
			25	3367738	7	1
			0	3631983	9	1
3	4-position	1:10,000	1000.0	10672	11	11
			250	36512		3
			25	54987		2
			0	121345		1
4	4-position	1:10,000	1000.0	13496	11	166
			250	48827	19	46
			25	520696	12	4
			0	2236499	10	1

The Theranos Cotinine-4-AP conjugate was finalized as the labeled tracer in combination with the antibody#4. The remainder of the assay development was carried out with this combination. The YJBio cotinine AP conjugate was the backup labeled tracer.

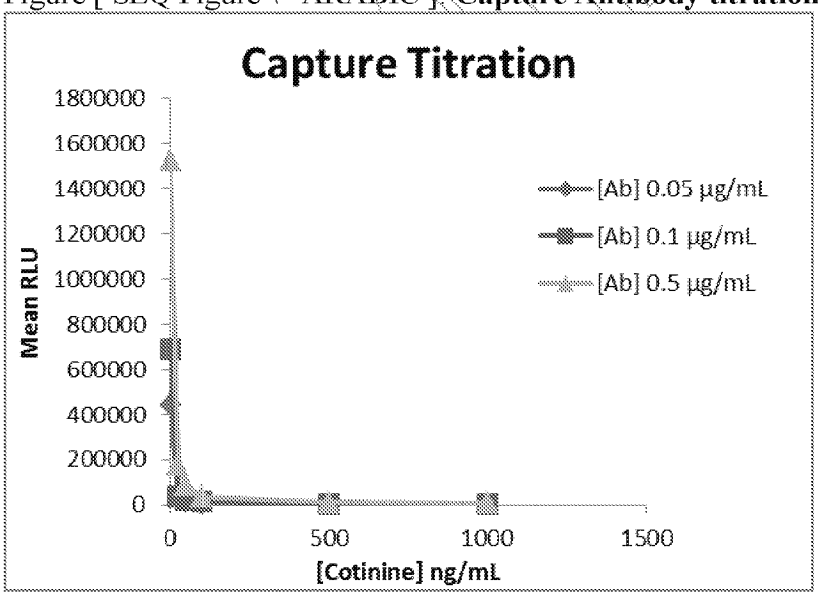
1.8 Capture Titration

The capture antibody was titrated to determine the optimal concentration. The antibody is diluted 1.4-fold into the final sample mixture, the loading concentration is shown. A concentration of 0.1 µg/mL produced the best response with the Theranos cotinine 4-AP conjugate at 1:50,000 loading concentration. A concentration of 0.1 µg/mL was finalized as the concentration of the primary antibody in solution.

Table [SEQ Table * ARABIC]: Capture Antibody Titration

[Cotinine]	[Ab] 0.05 µg/mL				[Ab] 0.1 µg/mL				[Ab] 0.5 µg/mL			
ng/ml	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.
1000	4447	26	100	1.1	4726	12	146	1.3	9065	4	168	1.1
500.0	4919	22	91	1.4	5935	13	116	2.1	10108	18	151	3.5
100	6654	29	67	2.2	12531	6	55	1.5	35710	10	43	2.4
50.0	14823	9	30	1.9	18783	3	37	2.0	85705	8	18	2.0
25	28560	19	16	15.6	38387	24	18	18.0	174783	2	9	8.7
0	446091	2	1		689626	7	1		1524114	4	0	

Figure [SEQ Figure * ARABIC]: **Capture Antibody titration**



1.9 Effect of AP conjugate stabilizer

The Cotinine AP conjugate was formulated in 3 different AP conjugate stabilizer and the dose resposne was evaluated as shown in Table 15. The Theranos Small Moleculae AP Conjugate Stabilizer showed the best modulation between the different calibrator levels and the best overall S/B. It was finalized as the AP conjugate stabilizer.

Table [SEQ Table * ARABIC]: Effect of AP conjugate stabilizer

[Cotinine]	Theranos Small Molecule AP Conjugate Stabilizer				Biostab				StabilZyme			
ng/ml	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.
1000	3360	10	150	1.5	5114	12	133	1.1	5586	9	96	0.9
500.0	5104	9	99	1.5	5372	10	127	1.9	5008	11	107	2.4
100	7761	3	65	2.0	10216	4	67	1.9	12149	3	44	1.8
50.0	15540	17	32	2.0	19196	8	36	1.9	21919	1	24	1.8
25	30992	14	16	16.3	37100	24	18	18.4	40194	15	13	13.3
0	503871	8	1		681787	14	1		536485	4	1	

1.10 Cotinine AP conjugate titration

The Theranos Cotinine AP conjugate was titrated at 3 different concentrations shown on Table 16. Increasing the conjugate concentration increased the sensitivity at the bottom of the curve and lowered the modulation between the top two calibrator levels. It was decided to finalize the conjugate concentration at 1:50,000.

Table [SEQ Table * ARABIC]: Effect of AP conjugate stabilizer

[Cotinine]	1:50,000				1:100,000				1:500,000			
ng/ml	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.
1000	3360	10	150	1.5	1800	1	157	1.1	420	23	172	1.1
500.0	5104	9	99	1.5	1966	17	144	2.0	462	2	157	2.1
100	7761	3	65	2.0	3941	17	72	1.6	971	10	74	1.6
50.0	15540	17	32	2.0	6478	6	44	1.8	1522	7	48	1.3
25	30992	14	16	16.3	11698	20	24	24.2	1982	2	36	36.5
0	503871	8	1		282890	12	2		72332	10	1	

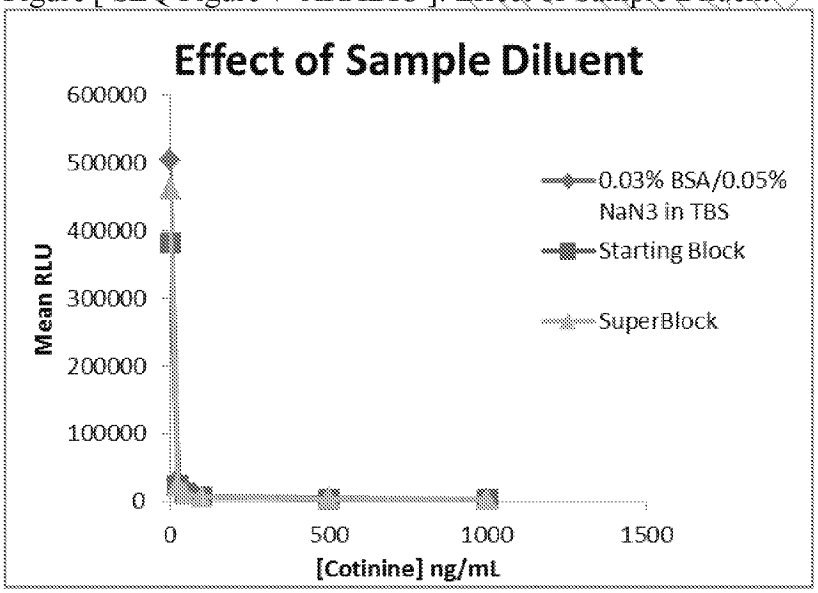
1.11 Effect of Sample Diluent

To determine if various blocking buffers might improve the assay when used as sample diluents, 2 commercial blockers were tested against the Low BSA buffer. The commercial blockers further lowered the modulation so it was decided to finalize the low BSA blocking buffer (0.03% BSA/0.05% NaN_3 in TBS) as the sample diluent. Data are summarized in Table 17 and Figure 5.

Table [SEQ Table * ARABIC]: Effect of Sample diluent

[Cotinine]	0.03% BSA/0.05% NaN3 in TBS				Starting Block				SuperBlock			
ng/ml	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.	Mean	CV%	S/B	Mod.
1000	3360	10	150	1.5	3672	8	104	0.9	3544	10	130	1.2
500.0	5104	9	99	1.5	3262	6	117	2.4	4218	6	109	1.8
100	7761	3	65	2.0	7687	12	50	1.6	7516	13	61	1.4
50.0	15540	17	32	2.0	12358	4	31	1.9	10687	56	43	2.1
25	30992	14	16	16.3	23350	4	16	16.3	22939	10	20	20.1
0	503871	8	1		381734	1	1		460756	8	1	

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



1.12 Effect of matrices

At this point in the cotinine assay development cotinine calibrators were prepared by spiking cotinine into the following matrices: (i) normal donor serum, (ii) normal donor EDTA plasma (iii) charcoal absorbed serum (depleted) serum and (iv) normal donor potassium oxalate/sodium fluoride plasma. The latter was tested to evaluate compatibility with the reference test. All the sera/plasma were screened on the Calbiotech cotinine ELISA and found to have undetectable levels of cotinine. The assay showed no significant matrix effect, the dose responses overlaid with each other. It was decided to calibrate the assay using the depleted serum matrix. The data are summarized in Table 18.

Table [SEQ Table * ARABIC]: Effect of matrices

[Cotinine]	Depleted Serum			Normal Serum			Normal EDTA Plasma			Normal Pot. oxalate/NaF Plasma		
ng/ml	Mean	CV%	S/B	Mean	CV%	S/B	Mean	CV%	S/B	Mean	CV%	S/B
1000	3603	10	123	3048	8	113	6565	10	73	3168	10	152
500.0	4789	9	92	4106	6	84	6811	6	70	3637	6	133
100	8216	3	54	10138	12	34	10480	13	45	9431	13	51
50.0	11948	17	37	15568	4	22	15499	56	31	12606	56	38
25	21823	14	20	24211	4	14	34464	10	14	17613	10	27
0	442510	8	1	345888	1	1	476422	8	1	483147	8	1

1.13 Effect of sample dilution

The cotinine assay dose response and modulation was tested by increasing the sample dilution from 5-fold to 10-fold and 25-fold. As can be seen from summary Table 19 all three sample dilutions show good modulation and S/B. 25 fold sample dilution is preferred to minimize the use of sample and aid multiplexing. The good modulation at the low end of the assay is retained at this sample dilution. The 25 fold sample dilution was finalized for the cotinine assay.

Table [SEQ Table * ARABIC]: Effect of sample dilution

[Cotinine]	5x			10x			25x		
ng/ml	Mean	CV%	S/B	Mean	CV%	S/B	Mean	CV%	S/B
1000	3603	10	123	6627	7	84	5140	6	91
500.0	4789	15	92	6695	16	83	7985	3	59
100	8216	1	54	19275	1	29	25168	6	19
50.0	11948	5	37	27502	2	20	52894	11	9
10	21823	20	20	91602	64	6	171882	14	3
0	442510	7	1	556162	1	1	470146	8	1

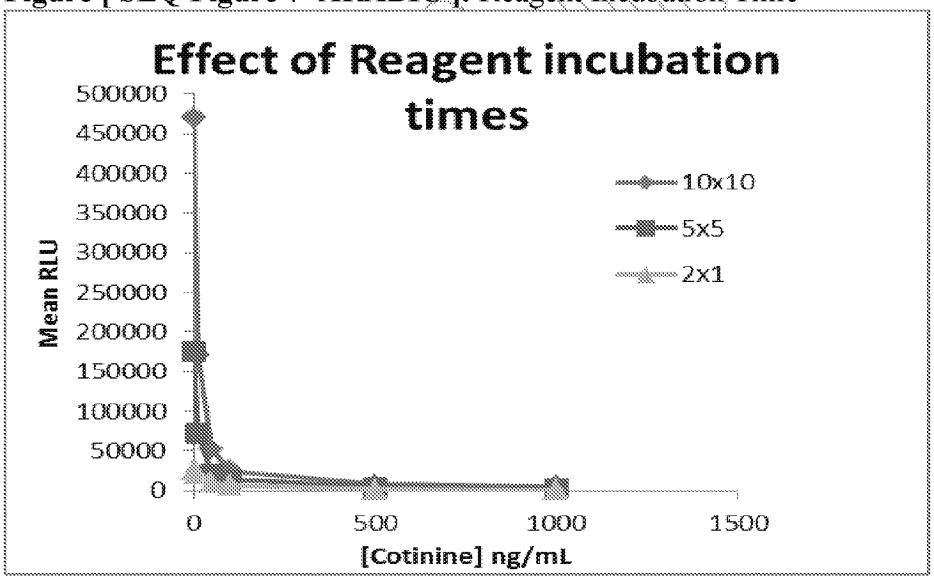
1.14 Effect of reagent incubation time

Shorter incubation times were tested compared to the original condition of 10 minute sample mixture and substrate incubations. Five minute and 2, 1 minute incubation times were tested. The overall dose reposnse was significantly lowered aqt shorter incubation times and the modulation at the bottom end of the assay weas lost. The control condition of 10x10 reagent incubation times was finalized.

Table [SEQ Table * ARABIC]: Reagent Incubation Time

[Cotinine]	10x10			5x5			2x1		
ng/ml	Mean	CV%	S/B	Mean	CV%	S/B	Mean	CV%	S/B
1000	5140	6	91	3123	7	56	576	6	44
500.0	7985	3	59	4695	16	38	1394	3	18
100	25168	6	19	14675	1	12	6653	6	4
50.0	52894	11	9	21502	2	8	9652	11	3
10	171882	14	3	71602	64	2	21235	14	1
0	470146	8	1	176362	1	1	25203	8	1

Figure [SEQ Figure * ARABIC]: Reagent Incubation Time



1.15 Calibration

A standard curve was run and Theranos calibration software was used to fit the data and determine the LLOQ and ULOQ in depleted serum according to FDA guidelines for calibrating ELISA assays. The LLOQ was 10 ng/mL and the ULOQ was 1000 ng/mL.

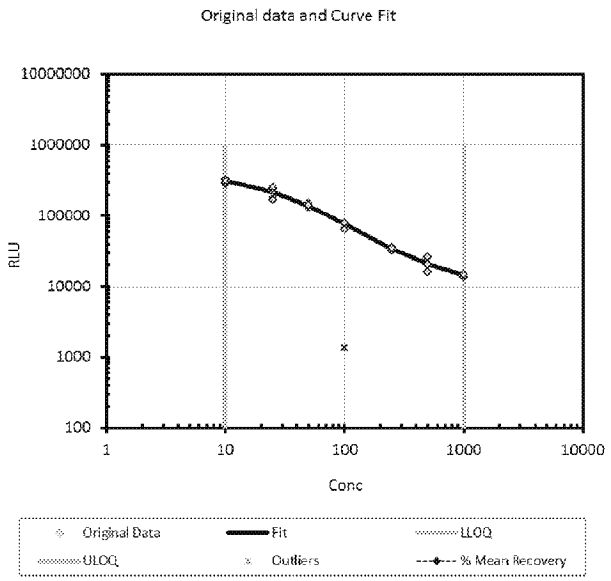
Table [SEQ Table * ARABIC]: Determination of LLOQ and ULOQ

Cotinine ng/ml	RLU		S/B	Conc. (ng/mL)		% Recovery
	Mean	CV%		Mean	CV%	
1000.0	14319	1	69	1052	3.8	105
500.0	21377	17	46	508	27.8	102
250.0	34206	4	29	251	5.1	101
100.0	72320	13	14	108	14.4	108
50.0	142682	4	7	48	5.4	96
25.0	219942	19	4.5	26	17.9	103
10.0	309193	5	3.2	10	20.2	105
0.0	991766	6	1.0	OORL		

Conc = 28.54* (((394776.9-10004.8) / (RLU -10004.8)) - 1) ^ (1 /1.242)

Table [SEQ Table * ARABIC]: Calibration Parameters

LLOQ	10	ng/mL
ULOQ	1000	ng/mL
LLOQ accuracy	105	%
LLOQ precision	20	%
ULOQ accuracy	105	%
ULOQ precision	3.8	%



1.16 Normal plasma screen

The same lot of reagents was used for the remainder of the assay development so the calibration equation from Table 21 was used for back calculating cotinine concentrations in samples in all the experiments in the next experiments. Whole blood samples from 10 male and 10 female normal donors (smoking status unknown) were collected using (i) K2 EDTA and (ii) potassium oxalate/NaF collection tubes for each sample. The plasma generated from these whole blood samples was screened on the Theranos assay and also tested on the predicate method. The results are summarized in Table 22. All samples except one OORL (out of range low) on both the Theranos cotinine assay as well as the (OTI) Cotinine Micro-Plate EIA. The results for the pot.oxalate/NaF data was within 20% of the EDTa plasma data for sample #F6. The predicate method results for this sample correlated well with the Theranos result. This test confirmed that there is no effect of the potassium oxalate /NaF anticoagulant on the Theranos cotinine assay.

Table [SEQ Table * ARABIC]: **Normal plasma screen**

	Theranos Result		**(OTI) Cotinine Micro-Plate EIA**	
Sample ID	**EDTA PLAsma Result (ng/mL)**	**Pot. oxalate plasma Result (ng/mL)**	**EDTA PLAsma Result (ng/mL)**	**Pot. oxalate plasma Result (ng/mL)**
ME1	OORL	OORL	OORL	OORL
ME2	OORL	OORL	OORL	OORL
ME3	OORL	OORL	OORL	OORL
ME4	OORL	OORL	OORL	OORL
ME5	OORL	OORL	OORL	OORL
ME6	OORL	OORL	OORL	OORL
ME7	OORL	OORL	OORL	OORL
ME8	OORL	OORL	OORL	OORL
ME9	OORL	OORL	OORL	OORL
ME10	OORL	OORL	OORL	OORL
F1	OORL	OORL	OORL	OORL
F2	OORL	OORL	OORL	OORL
F3	OORL	OORL	OORL	OORL
F4	OORL	OORL	OORL	OORL
F5	OORL	OORL	OORL	OORL
F6	45.0	36.0	39.0	31.0
F7	OORL	OORL	OORL	OORL
F8	OORL	OORL	OORL	OORL
F9	OORL	OORL	OORL	OORL
F10	OORL	OORL	OORL	OORL

1.17 Whole blood spike recovery and Hematocrit effect

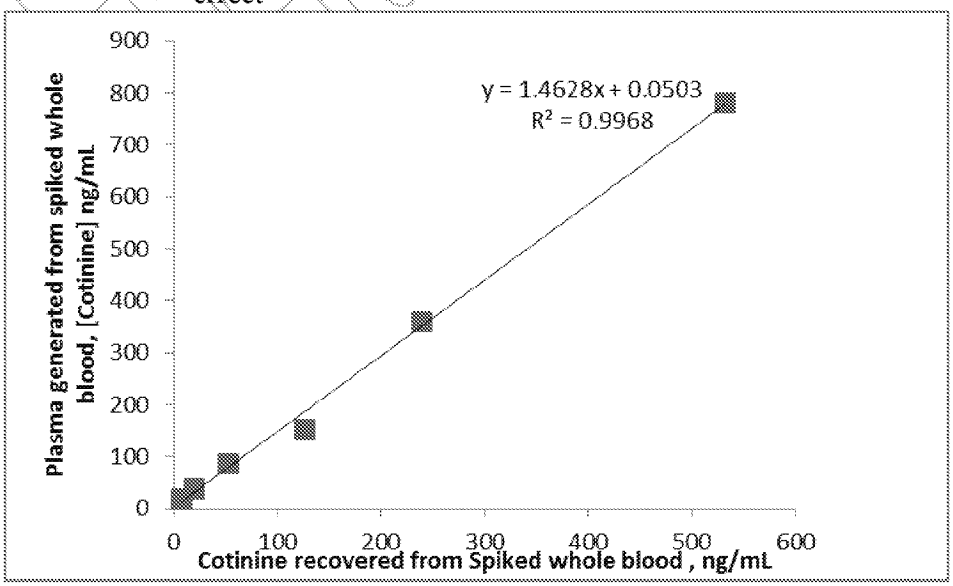
Spike recovery in EDTA whole blood and plasma was tested in the Theranos System. In order to determine the hematocrit effect, spiked whole blood was measured on the Theranos System, then plasma prepared from the spiked whole blood was measured and the results were compared. The results indicate that cotinine does concentrate into plasma. The result measured in the plasma is slightly lower than the expeceted 1.6-fold increase.

Table [SEQ Table * ARABIC]: Spike Recovery in Whole Blood Plasma generated from spiked whole blood

	Whole Blood, spike recovery					Plasma from spiked whole blood, recovery				
Nominal [Cotinine] ng/ml	RLU Mean	RLU CV%	Conc, ng/mL Mean	Conc, ng/mL CV%	% Recovery	RLU Mean	RLU CV%	Conc, ng/mL Mean	Conc, ng/mL CV%	% Recovery
1000.0	3951	2	825	16.7	83	3201	25	OORH		
500.0	4251	4	532	21.0	106	4213	5	782	27.1	156
250.0	5373	7	240	16.5	85	4997	7	359	3.2	144
100.0	6502	7	126	16.9	126	6087	9	151	25.0	151
50.0	10785	18	53	18.5	106	7885	8	85	16.1	170
25.0	22748	14	19	16.8	78	13636	22	38	28.4	151
10.0	48606	7	8	7.4	83	23500	7	18	5.2	181
0.0	504918	3	OORL			536445	3	OORL		

OORH= out of range high

Figure [SEQ Figure * ARABIC]: Spike Recovery in Whole Blood and Plasma: Hematocrit effect



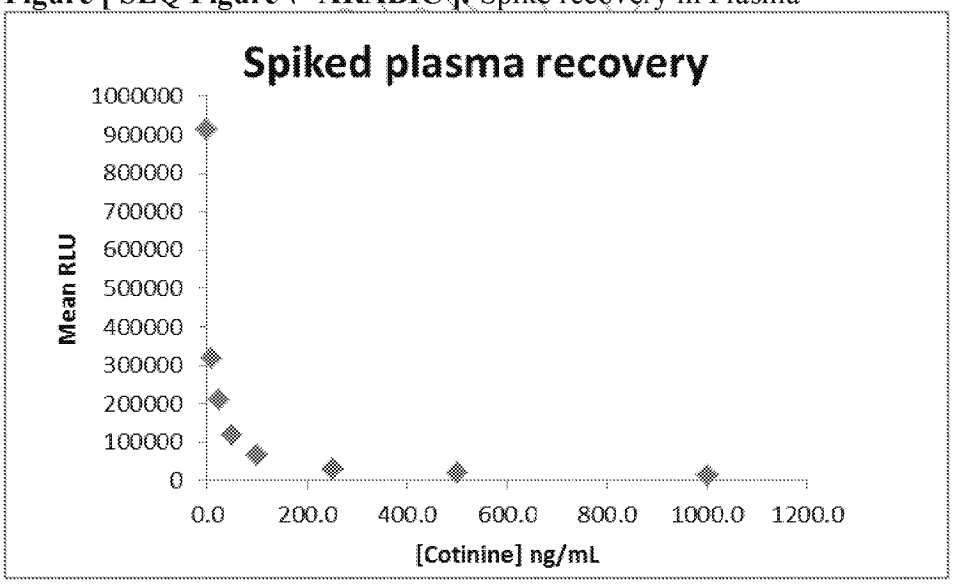
1.18 Spike recovery in Plasma

Recovery of cotinine spiked directly into EDTa plasma with low endogenous levels of cotinine was tested and the data are summarized in Table 25. The recovery was excellent and was within the accepted criterion of 20% of nominal.

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

Nominal [Cotinine] ng/ml	RLU		Conc, ng/mL		%
	Mean	CV%	Mean	CV%	Recovery
1000.0	14854	3	968	8.7	97
500.0	19275	7	573	11.0	115
250.0	29286	10	308	12.5	123
100.0	66086	1	119	1.1	119
50.0	119105	2	60	2.7	120
25.0	210979	16	27	20.5	110
10.0	319281	1	9	2.7	92
0.0	912247	6	OORL		

Figure [SEQ Figure * ARABIC]: Spike recovery in Plasma



1.19 Cross reactivity and Interference with *trans* hydroxycotinine

Trans 3' hydroxycotinine is an important metabolite of cotinine metabolism and is the main nicotine metabolite detected in smoker's urine. 3-OH cotinine is also a known cross reactant with cotinine in immunoassays. It was spiked into cotinine negative serum at the following concentrations: 1000, 750, 250, 150 and 50 ng/mL. *Trans* hydroxycotinine showed a cross reactivity of 9-20% in the Theranos cotinine assay (Table 26). In order to test the interference of this metabolite on the assay, *trans* hydroxycotinine was piked at a final concentration of 6000 ng/ml into serum spiked with cotinine at 1000,750, 250, 150 and 50 ng/mL. The recoveries were reported as a percentage from control. The data are summarized in Table 27 As both tables indicate *trans* hydroxycotinine is a cross reactant of the Theranos cotinine assay.

Table [SEQ Table * ARABIC]: **Cross reactivity with 3'-*Trans* –Hydroxycotinine**

Trans **hydroxycotinine ng/mL**	**RLU**		**Conc. ng/mL**		
	Mean	**CV%**	**Mean**	**CV%**	**% Cross reactivity**
1000.0	68894	7	114	7.7	11
750.0	108782	15	68	18.3	9
250.0	162582	6	40	7.9	16
150.0	287355	1	13	2.9	9
50.0	314277	0	10	0.2	20
0.0	1034855	20	OORL		

Table [SEQ Table * ARABIC]: **Interference with 3'-*Trans* –Hydroxycotinine**

[Cotinine]	**RLU**		**Conc. ng/mL**		
ng/mL	**Mean**	**CV%**	**Mean**	**CV%**	**% Control**
1000.0	15644	16	926	37.3	93
750.0	21032	5	497	5.8	66
250.0	65082	8	122	10.3	49
150.0	177355	2	35	4.1	24
50.0	234277	12	22	24.3	44
0.0	953030	13	OORL		

1.20 Interference Test for RF and HAMA positive samples

The Theranos Cotinine assay was tested for interference from RF positive and HAMA positive samples. 5 samples of each type were tested on the Theranos system as well as the CLIA lab cotinine assay. For the CLIA assay, the (OTI) Cotinine Micro-Plate EIA all results > 50 ng/mL are considered positive and results <10 ng/ml are considered negative. The results for all 10 samples matched the results from the reference method. The data are summarized in Table 27 and Table 28.

Table [SEQ Table * ARABIC]: **RF positive sample testing**

Sample ID	RLU Mean	RLU CV%	Theranos	CLIA Result
RF1	1013751	2	OORL	OORL
RF2	791903	4	OORL	OORL
RF3	907008	8	OORL	OORL
RF4	856522	6	OORL	OORL
RF5	843499	5	OORL	OORL
RF6	898265	8	OORL	OORL

Table [SEQ Table * ARABIC]: **HAMA positive sample testing**

Sample ID	RLU Mean	RLU CV%	Theranos Mean ng/mL	Theranos %CV	CLIA Result
H1	894687	8	OORL		OORL
H2	142728	1	48	2	35
H3	27284	7	336	9	OORH
H4	44419	11	186	13	OORH
H5	297806	7	12	20	12
H6	47004	10	176	12	OORH

1.21 Interfering Matrices

Hemolyzed, lipemic and icteric serum samples were obtained from a commercial source. The recovery of cotinine spiked into these potentially interfering matrices 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 and lipemic samples. The assay showed only about 70% recovery (<25% of nominal) for the lipemic sample tested.

Table [SEQ Table * ARABIC]: **Effect of interfering matrices**

	Hemolyzed					Lipemic					Icteric				
Spiked [Cotinine] ng/mL	RLU Mean	RLU CV%	Conc. ng/ml Mean	Conc. ng/ml CV%	% Recovery	RLU Mean	RLU CV%	Conc. ng/ml Mean	Conc. ng/ml CV%	% Recovery	RLU Mean	RLU CV%	Conc. ng/ml Mean	Conc. ng/ml CV%	% Recovery
1000.0	4447	7	OORH		NA	7247	6	OORH		NA	7288	11	OORH		NA
500.0	20015	7	531	12	86	14030	3	1116	9.3	223	18273	3	617	4.9	123
100.0	37761	7	224	8	105	51478	1	156	0.9	156	62296	1	126	0.9	126
50.0	50729	12	160	13	98	113628	3	64	3.8	255	216776	10	26	18.7	102
25.0	55186	5	145	5	104	299154	2	12	7.5	117	319529	0	9	1.6	91
0.0	68564	5	114	6		771815	7	OORL			816261	1	OORL		

The data are summarized in Table 30. The spike recoveries of cotinine in the hemolyzed serum matrix were within acceptable range. It appeared that recovery from the lipemic and icteric matrices was higher than the acceptable ±25%. This indicated that grossly lipemic and icteric sample types should be avoided as they interfere with the assay.

1.22 Cross reactivity with Common Drugs

The following drugs: chloramphenicol, ampicillin, aspirin, ibuprofen, caffeine and acetaminophen were tested on the Theranos serum cotinine assay at a concentration of 10,000 ng/ml. None of them displayed any cross reactivity with the assay as was seen with the result on the Theranos assay (Table 31).

Table [SEQ Table * ARABIC]: **Cross reactivity with common drugs**

Drug Name	Conc	Mean RLU		Theranos Result
	ng/mL	Mean	CV%	
Chloramphenicol	10,000	947747	12	OORL
Ampicillin	10,000	893031	2	OORL
Aspirin	10,000	919902	7	OORL
Ibuprofen	10,000	893110	23	OORL
Caffeine	10,000	872746	16	OORL
Acetaminophen	10,000	884695	2	OORL

1.23 Clinical Sample Correlation

65 serum samples from smoking subjects and 28 from non-smoking subjects were obtained from commercial sources. The vendors obtained these samples based on an interview with normal donors who provided information on their smoking status. All the samples (smokers and non-smokers) were tested on the OTI Cotinine ELISA. A majority of the smoking subjects' samples showed positive results on the CLIA assay which is a semi-quantitative assay with a cut off at 25 ng/mL. In order to compare the results to the Theranos assay which is a quantitative assay with a ULOQ of 1000 ng/mL and LLOQ of 10 ng/mL, a further dilution was performed to obtain the quantitative result. Additionally 40 of the 65 smoker subject samples were run on the Calbiotech cotinine ELISA. The results are summarized in Table 32 for smoking subjects and Table 33 for non-smoking subjects. In general all samples that were OORH on the Theranos assay and positive (>25 ng/mL) on the reference assays were highlighted in red. OORL samples on the Theranos assay and samples that were <25 ng/mL on the reference assays were highlighted in green. Out of the 65 samples only 2 samples (BR smoker 4 and PM smoker 10) were out of range high on the Theranos assay (> 1000 ng/ml). These samples were also OORH on both the reference methods (diluting the samples 20-fold). Sample # BR smoker 7 was OORL on all three assays and sample ID VL smoker 8's negative result correlated with the Calbiotech ELISA. The data for the non-smoking subjects correlated well for the Theranos and reference methods as shown in Table 33.

Table [SEQ Table * ARABIC]: Smoking subjects results (N=40): Comparison between Theranos, OTI and Calbiotech ELISAs

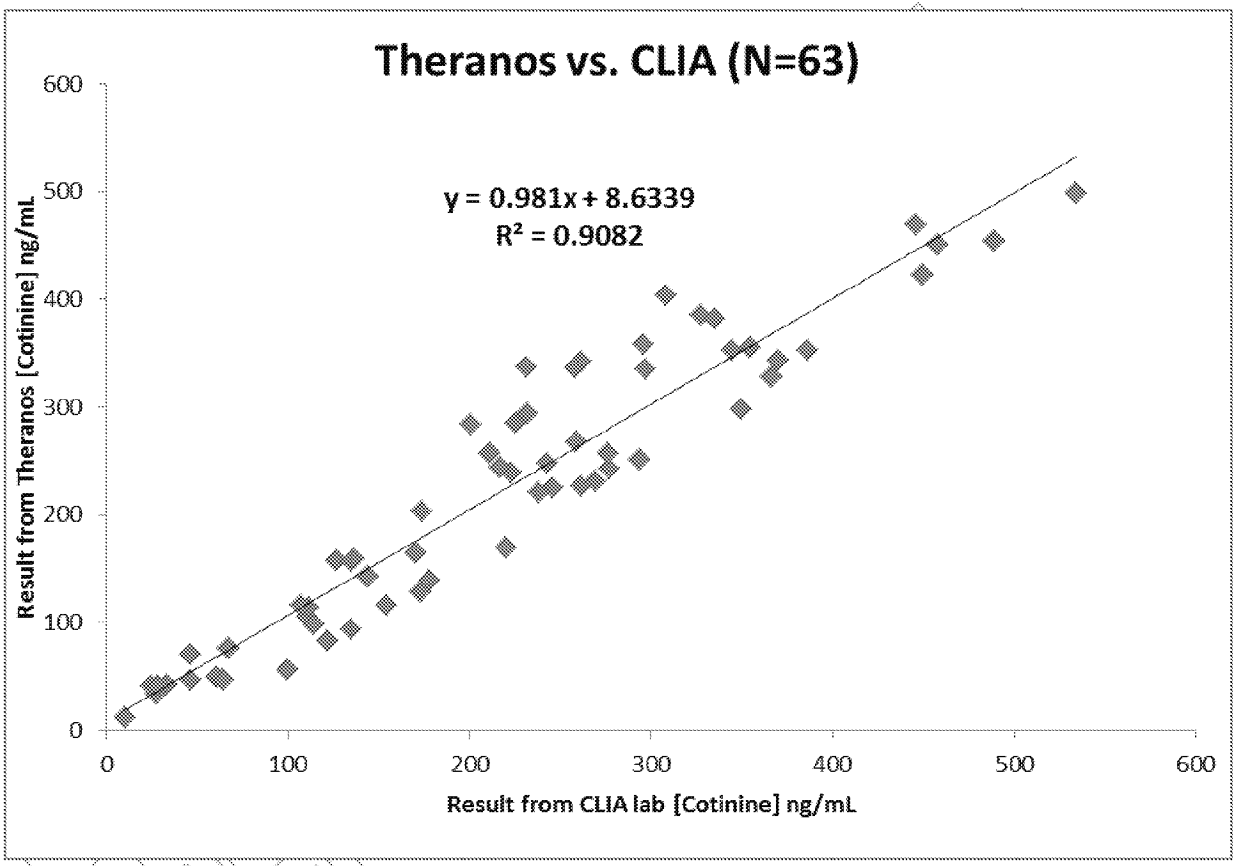
	Cotinine Results (ng/ml)				Cotinine Results (ng/ml)				Cotinine Results (ng/ml)	
Sample ID	Theranos	OTI Cotinine	Calbiotech ELISA	Sample ID	Theranos	OTI Cotinine	Calbiotech ELISA	Sample ID	Theranos	OTI Cotinine
BR Smoker 1	404	308	408	PM Smoker 1	342	262	298	VL Smoker 1	169	220
BR Smoker 2	454	289	386	PM Smoker 2	114	112	95	VL Smoker 2	116	107
BR Smoker 3	76	67	44	PM Smoker 3	336	258	252	VL Smoker 3	34	27
BR Smoker 4	OORH	OORH	OORH	PM Smoker 4	239	223	214	VL Smoker 4	352	387
BR Smoker 5	385	228	282	PM Smoker 5	203	174	184	VL Smoker 5	41	25
BR Smoker 6	337	231	243	PM Smoker 6	335	297	335	VL Smoker 6	231	270
BR Smoker 7	OORL	OORL	OORL	PM Smoker 7	49	61	52	VL Smoker 7	422	450
BR Smoker 8	312	558	667	PM Smoker 8	OORL	31	56	VL Smoker 8	OORL	OORL
BR Smoker 9	267	259	250	PM Smoker 9	142	144	173	VL Smoker 9	355	354
BR Smoker 10	285	226	197	PM Smoker 10	OORH	OORH	OORH	VL Smoker 10	327	367
BR Smoker 11	157	127	134	PM Smoker 11	225	246	292	VL Smoker 11	83	122
BR Smoker 12	469	446	317	PM Smoker 12	164	170	160	VL Smoker 12	297	350
BR Smoker 13	382	335	228	PM Smoker 13	139	178	241	VL Smoker 13	93	135
BR Smoker 14	210	345	286	PM Smoker 14	47	64	64	VL Smoker 14	221	238
BR Smoker 15	105	110	74	PM Smoker 15	358	296	287	VL Smoker 15	12	10
BR Smoker 16	501	399	312	PM Smoker 16	283	201	244	VL Smoker 16	116	154
BR Smoker 17	450	358	329	PM Smoker 17	156	135	175	VL Smoker 17	47	46
BR Smoker 18	159	136	158	PM Smoker 18	499	534	457	VL Smoker 18	251	294
BR Smoker 19	343	370	359	PM Smoker 19	243	277	267	VL Smoker 19	70	46
BR Smoker 20	227	262	207	PM Smoker 20	98	114	104	VL Smoker 20	247	243
								VL Smoker 21	129	173
								VL Smoker 22	257	277
								VL Smoker 23	56	99
								VL Smoker 24	256	211
								VL Smoker 25	244	216

Table [SEQ Table * ARABIC]: Non-Smoking subjects results (N=25): Comparison between Theranos, OTI and Calbiotech Cotinine assays.

	Cotinine Results (ng/ml)	
Sample ID	**Theranos**	**CLIA Cotinine ELISa**
BR Non Smoker 1	294	232
BR Non Smoker 2	OORL	OORL
BR Non Smoker 3	OORL	OORL
BR Non Smoker 4	OORL	OORL
BR Non Smoker 5	OORL	13
BR Non Smoker 6	OORL	OORL
BR Non Smoker 7	OORL	OORL
BRNon Smoker 8	OORL	15
BR Non Smoker 9	OORL	OORL
BR Non Smoker 10	OORL	21
BR Non Smoker 11	OORL	OORL
BR Non Smoker 12	OORL	OORL
BR Non Smoker 13	OORL	OORL
BR Non Smoker 14	OORL	OORL
BR Non Smoker 15	OORL	OORL
BR Non Smoker 16	OORL	18
BR Non Smoker 17	OORL	OORL
BR Non Smoker 18	OORL	OORL
BR Non Smoker 19	OORL	OORL
BR Non Smoker 20	42	33
PM Non Smoker 1	41	28
PM Non Smoker 2	OORL	OORL
PM Non Smoker 3	OORH	OORH
PM Non Smoker 4	OORL	OORL
PM Non Smoker 5	OORL	OORL
PM Non Smoker 6	OORL	OORL
PM Non Smoker 7	OORL	OORL
PM Non Smoker 8	OORH	OORH

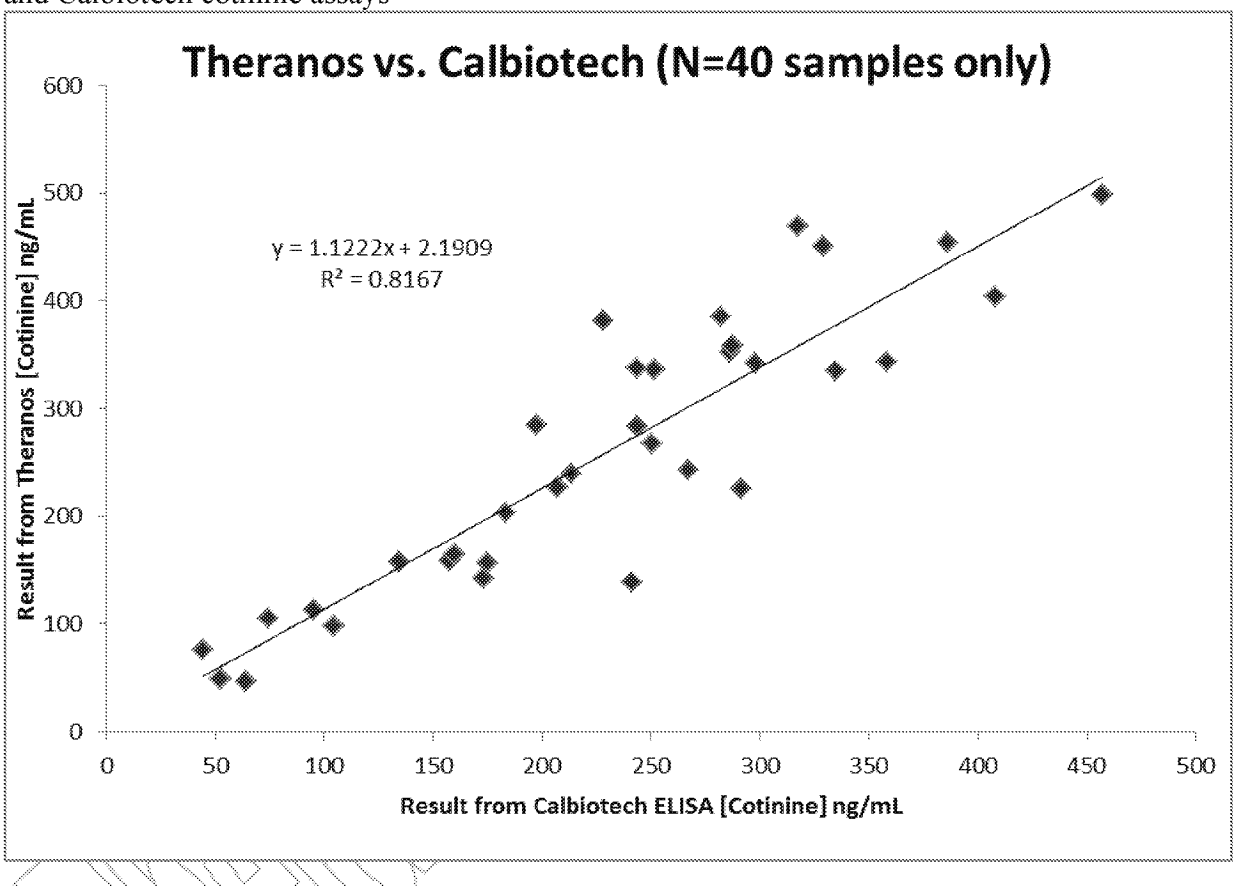
Since the Theranos serum cotinine assay is a quantitative assay the results of all samples that were within the range of the assay (1000-10 ng/mL) were depicted so as to derive a correlation between the results obtained from the two reference methods. Figure 9 depicts the clinical sample correlation results between the Theranos and OTI (CLIA) cotinine assays. Figure 10 shows the correlation for 40 of the 65 samples that were also run on the Calbiotech ELISA.

Figure [SEQ Figure * ARABIC]: Clinical sample correlation results between the Theranos and OTI (CLIA) cotinine assays.



The clinical correlation was excellent between the Theranos and OTI assays with an R^2 of 0.90 and slope of 0.98.

Figure [SEQ Figure * ARABIC]: Clinical sample correlation results between the Theranos and Calbiotech cotinine assays



The Theranos assay also correlated well with the Calbiotech ELISa as seen from the figure above. Figure 11 shows that the two reference methods correlate with one another well.

Figure [SEQ Figure * ARABIC]: OTI Cotinine and Calbiotech ELISa : method comparison.