Urine Cotinine
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 is designed to detect cotinine in human urine. The assay has a reportable range of 5 to 2000 ng/mL. The Theranos urine 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 two reference assays are the FDA approved rapid test kits : (i) NicCheck I Test Strips(Clia waived.com) and (ii) Cot Cotinine one step (cassette) test. The Calbiotech ELISA also works for urine samples.

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	
Normal donor Urine samples	ProMedDx	

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

1.2 Assay Conditions[TC "Detection Antibody Conjugate Verification" \f C \l "1"]

The capture antibody, AP conjugate and assay conditions are the same as the serum cotinine assay that was already developed and tested on the Theranos 3.0. The conditions include: surface coated with anti-rabbit secondary antibody, anti-cotinine antibody at 1.4 µg/mL in Low BSA blocking buffer, Theranos Cotinine-4-AP conjugate at 1:50,000 loading concentration, a sample dilution of 25-fold and a reagent incubation time of 10'x10'. These conditions were adopted as the starting point for the urine cotinine assay and further optimizations and testing was carried out.

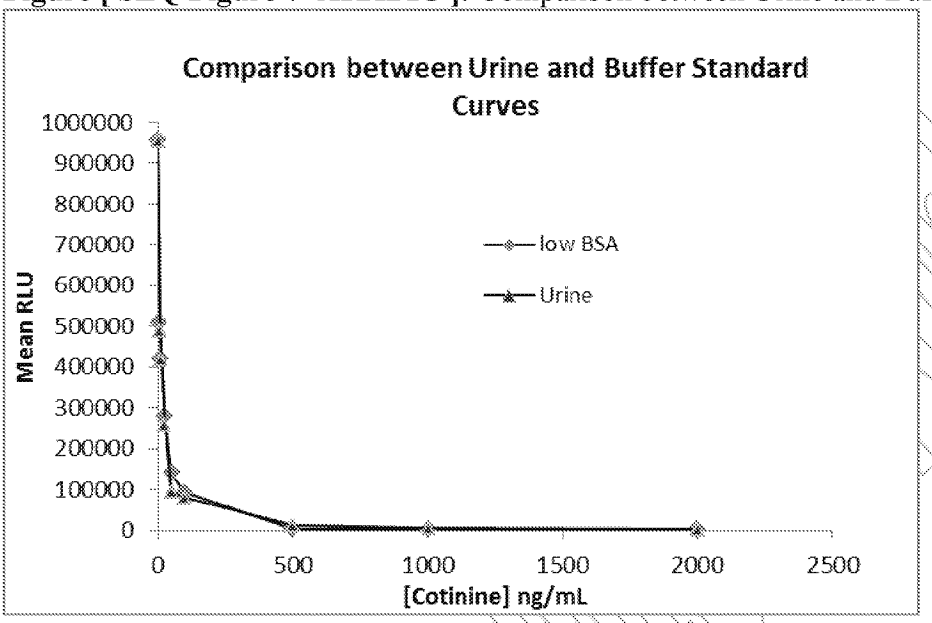
1.3 Matrix test for calibration

An 8 point standard curve using cotinine spiked into urine and into buffer was tested on the exisiting serum cotinien assay conditions. The purpose of this test was to evaluate any matrix effect between the buffer and urine matrices as well test the dose response in the target range for the urine assay. This would be important to finalize the matrix for the calibration of the urine cotinine assay. The data are summarized in Table 2 and Figure 1 depicts the dose response. It apeared that the two standard curves diverged at the top end of the curve. It was decided to finalize the urine matrix as the matrix for calibration of the urine cotinine assay. Further the overall signal to background and modulation at both the bottom and top end of the curve was excellent and met the target specificiations. Hence the same conditions (including reagent concentrations, sample dilutions as well as reagent incubation time) were retained.

Table [SEQ Table * ARABIC]: Comparison of the Standard Curve: Cotinine spiked into Urine vs. Buffer

[Cotinine] ng/ml	Urine			Buffer		
	Mean RLU	CV%	S/B	Mean RLU	CV%	S/B
2000.0	2793	10	342	2576	11	372
1000.0	5563	13	172	2975	9	322
500.0	12595	17	76	4968	16	193
250	29261	19	33	17707	37	54
100.0	79780	14	12	92129	4	10
50.0	93510	2	10	140100	8	6.8
25.0	259054	11	4	280161	6	3.4
10.0	419194	7	2.3	421825	7	2.3
5	488780	4	2	510054	7	1.9
0.0	954337	3	1	957106	14	1.0

Figure [SEQ Figure * ARABIC]: Comparison between Urine and Buffer Standard Curves



1.4 Calibration

A standard curve was run and Theranos calibration software was used to fit the data and determine the LLOQ and ULOQ in urine according to FDA guidelines for calibrating ELISA assays. The LLOQ was 5 ng/mL and the ULOQ was 2000 ng/mL. The data are summarized in Tables 3 and 4 and Figure 2.

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

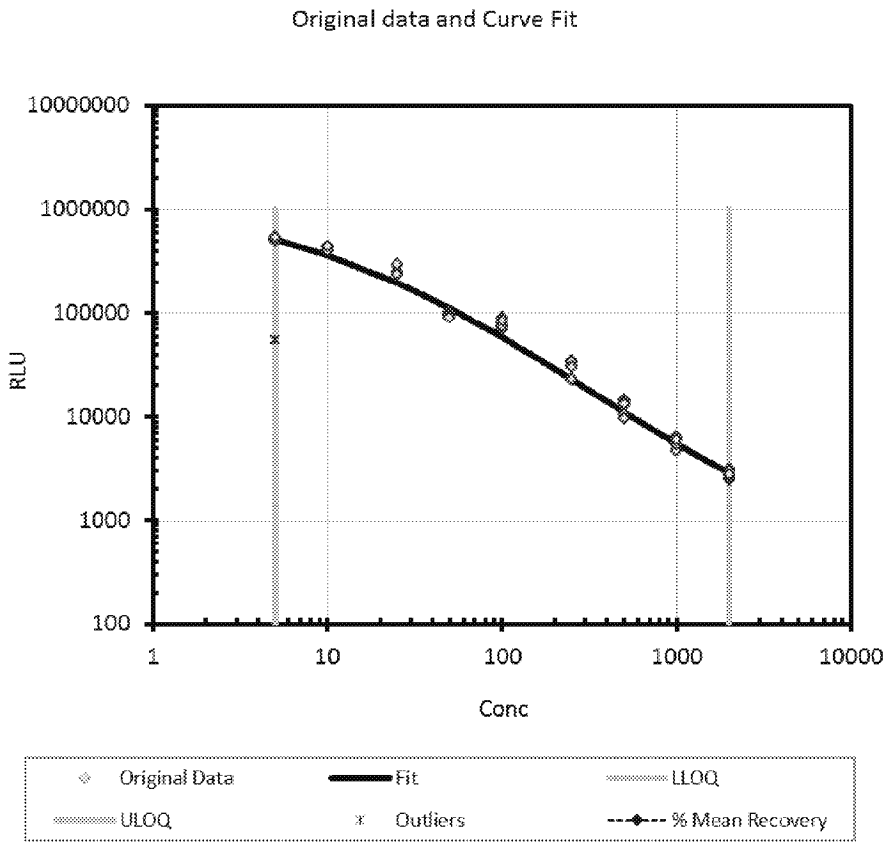
Cotinine	RLU			Conc. (ng/mL)		% Recovery
ng/ml	Mean	CV%	S/B	Mean	CV%	
2000.0	2793	10	342	2088	12	104
1000.0	5563	13	172	1002	14	100
500.0	12595	17	76	453	17	91
250	29261	19	33	204	20	82
100.0	78644	10	12	74	11	74
50.0	94983	2	10	60	2	121
25.0	259054	11	3.7	17	16	69
10.0	419194	7	2.3	8	12	77
5	513780	3	1.9	5	10	96
0.0	954337	3	1	1	12	

Conc = 319.917 * (((6.189 - 2.310) / (log10(RLU) - 2.310)) - 1) ^ (1 / 0.472)

Table [SEQ Table * ARABIC]: Calibration Parameters

LLOQ	5	ng/mL
ULOQ	2000	ng/mL
LLOQ accuracy	98	%
LLOQ precision	7.2	%
ULOQ accuracy	104	%
ULOQ precision	11.8	%

Figure [SEQ Figure * ARABIC]: Urine cotinine Calibration curve



1.5 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. Urine samples from 19 normal donors (smoking status unknown) were collected and tested on the Theranos urine cotinine assay as well as the FDA-approved strip and cassette rapid tests. The results are summarized in Table 5. All except two samples tested OORL (out of range low) on the Theranos urine cotinine assay. Sample #U4 and U16 were positive on the Theranos urine cotinine assay (> 5 ng/mL). The rapid tests both had a cut off of 200 ng/mL. Hence sample #U4 tested negative on both the rapid tests. # U16 gave a positive result of 221 ng/mL on Theranos assay and being greater than the cutoff value (200 ng/mL) on the rapid tests registered a positive result on both. The results demonstrate an excellent correlation of results between the Theranos and FDA-approved rapid tests for urine cotinine assay.

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

Sample ID	Theranos Reuslt ng/mL	COT one test 200 ng/ml cutoff	NicCheck I 200 ng/ml cutoff
U1	OORL	NEG	NEG
U2	OORL	NEG	NEG
U3	OORL	NEG	NEG
U4	114	NEG	NEG
U5	OORL	NEG	NEG
U6	OORL	NEG	NEG
U7	OORL	NEG	NEG
U8	OORL	NEG	NEG
U9	OORL	NEG	NEG
U10	OORL	NEG	NEG
U11	OORL	NEG	NEG
U12	OORL	NEG	NEG
U13	OORL	NEG	NEG
U14	OORL	NEG	NEG
U15	OORL	NEG	NEG
U16	221	POS	POS
U17	OORL	NEG	NEG
U18	OORL	NEG	NEG
U19	OORL	NEG	NEG

1.6 Interfering Matrices

In order to evaluate the effect of interfering substances, normal urine from 17 subjects was pooled and spiked with the following compounds to generate a sample urine matrix containing the specified substance at a clinically relevant high concentration: ascorbic acid at 50 mg/dL, protein at 250 mg/dL, hemoglobin at 0.3 mg/dL, glucose at 500 mg/dL, bilirubin 5 mg/dL and triglycerides at 20 mg/dl. Cotinine was spiked into each of the above urine samples at 1000, 500, 50 and 0 ng/ml. The samples were run on the Theranos assay. The data are reported in Table 6. The recoveries for all except the triglyceride sample were within the acceptance criteria of ± 25% of nominal. This indicated that the urine cotinine assay is not subject to interference from any of these compounds except triglycerides. It was concluded that urine samples with triglyceride levels > 20 mg/dl would interfere with the Theranos urine cotinine assay.

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

Interfering Substance	Nominal Spike Conc. ng/ml	RLU		Recovered Conc. ng/ml		% Recovery
		Mean	CV%	Mean	CV%	
Ascorbic Acid	1000.0	6329	21	894	22	89
	500.0	13631	2	414	1	83
	50.0	103888	10	55	12	110
	0	603894	0	3	0	
Bilirubin	1000.0	6432	14	873	12	87
	500.0	14050	11	403	10	81
	50.0	158068	3	33	4	66
	0	572779	22	6		
Glucose	1000.0	7159	1	772	0	77
	500.0	16030	9	354	9	71
	50.0	118384	9	48	11	96
	0	596425	2	4	4	
Hemoglobin	1000.0	7767	30	769	28	77
	500.0	12402	14	454	13	91
	50.0	151552	8	35	10	70
	0	462633	24	12	90	
Protein	1000.0	6868	17	716		72
	500.0	11992	13	470	12	94
	50.0	121380	6	46	7	92
	0	614896	16	3	39	
Triglycerides	1000.0	9059	2	634	12	63
	500.0	19870	37	308	36	62
	50.0	130511	10	42	11	84
	0	557269	16	4	40	

1.7 Clinical Correlation

20 urine samples from smoking subjects was collected from an outside commercial vendor and tested for cotinine results on the Theranos urine cotinine assay. The samples were also tested on the two FDA approved rapid tests. As seen in Table 7 there was excellent correlation between the results.

Table [SEQ Table * ARABIC]: Urine sample clinical correlation: Theranos vs. FDA-approved Cotinine Rapid Tests

	FDA approved	
Theranos Result ng/mL	**Cot 1 Cotinine Rapid test**	**NicChek I Rapid test**
OORH	Pos	LowPos
OORH	Pos	LowPos
OORL	Neg	Neg
OORH	Pos	LowPos
765	Pos	LowPos
OORH	Pos	LowPos
OORH	Pos	LowPos
OORL	Neg	Neg
894	Pos	LowPos
1793	Pos	LowPos
OORL	Neg	Neg
178	Pos	LowPos
347	Pos	LowPos
OORH	Pos	High pos
OORH	Pos	High pos
OORH	Pos	High pos
OORH	Pos	High pos
OORL	Neg	Neg
OORH	Pos	High pos
OORH	Pos	High pos