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Opiate Assay Development Report

Theranos, Inc.

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1. ASSAY INFORMATION

1.1 Assay Specifications

Opiates belong to the large biosynthetic group of benzylisoquinoline alkaloids, and can be found naturally in opium poppy. The major psychoactive opiates are morphine, codeine, and thebaine. Papaverine, noscapine, and approximately 24 other alkaloids are also appeared in opium. Also there are others considered as semi-synthetic opioids such as hydrocodone, hydromorphone, oxycodone, and oxymorphone, while derived from opiates, are not opiates themselves.

For example, Heroin (diacetylmorphine) is semi-synthetic opioids derived from the opiate morphine, meaning that it is metabolized by the body into morphine after administration. One of the major metabolites of heroin, 6-monoacetylmorphine (6-MAM), is also a morphine prodrug. Nicomorphine (morphine dinicotinate), dipropanoylmorphine (morphine dipropionate), desomorphine (di-hydro-desoxy-morphine), methyldesorphine, acetylpropionylmorphine, dibenzoylmorphine, diacetyldihydromorphine, and several others are also derived from morphine.

This assay is designed to quantitatively detect only 3 semi-opiates (Heroin, 6-AM, and Codeine) in urine, serum, plasma and whole blood. The assay has a reportable range of 10 ng/ml to 800ng/ml. This assay is used as a qualitative screening with positive cutoff for urine at 300ng/ml. For serum/plasma positive cutoff is at 25ng/mL. The reported value above 300ng/mL in Urine is considered positive and below 300ng/mL is negative. On the other hand the cutoff for serum/plasma is at 25ng/ml. The reported value above 25ng/mL in plasma, serum or whole blood is positive and below 25ng/mL is negative.

1.2 Reference Assays

The following commercial methods were used as comparison methods:

- Neogen Cat #130415 Opiate Group ELISA Kit (urine, whole blood, and Oral fluid)
- Abnova Cat#KA0937 Opiates ELISA KIT (Urine, plasma/serum, and whole blood, but directions are specified only for urine)
- Advia Chemistry Cat#2011-02 Opiate_2 (urine only)

1.3 Materials and Methods

The Theranos opiate assay format is designed as a competitive immunoassay. An anti-sheep secondary antibody was coated first on the reaction tips and followed by a primary sheep anti-heroin antibody. The sample is diluted 100X and combined with an enzyme labeled Heroin-AP 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).

Figure 1: Opiate assay principle

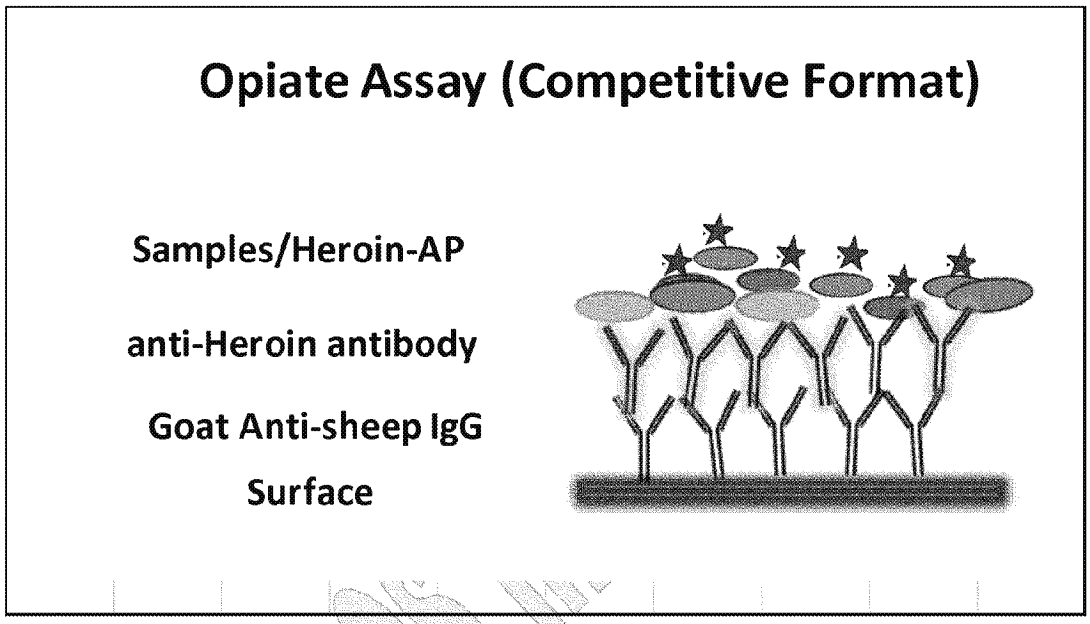


Table 1: Materials

Name	Supplier	Catalog #
Heroin	Cerilliant	H-038
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
Sheep polyclonal to Heroin antibody	Abcam	ab123991
Heroin-ALP Conjugate	YJ Bioscience	KO1709
Rabbit anti-sheep IgG (Fc)	Fitzgerald	41-RS45

2 ASSAY DEVELOPMENT

2.1 Assay Formats Screen

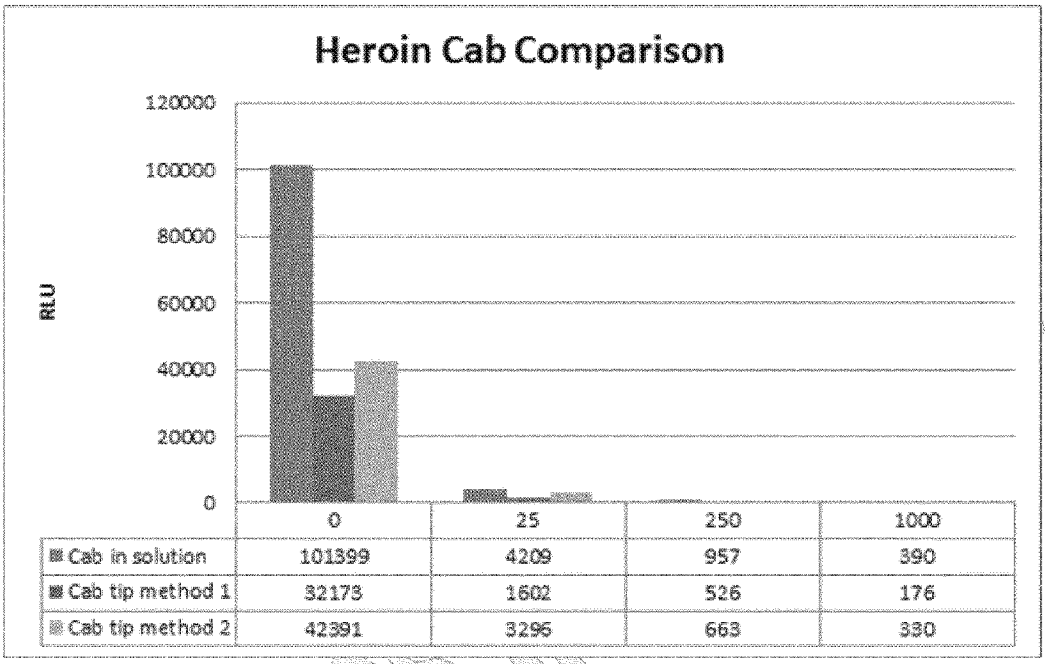
In order to simplify the assay format for easier multiplexing in the drugs screening panels, three methods (see below) were tested for coating the anti-sheep antibody on the surface rather than mixing it in solution in a homogenous competitive assay. Both methods showed very comparable results to the capture antibody in solution. Method 1 was chosen as the final assay condition.

- CAb in solution: 20 ng/mL Sheep Anti-heroin PAb in solution with sample mixture with 20 ug/mL UltraAvidin, 10 ug/mL Biotinylated Anti-Sheep Ab coated on the surface
- Method 1: 20 ug/mL Anti-Sheep Ab, 20 ng/mL Sheep Anti-heroin PAb
- Method 2: 20 ug/mL UltraAvidin, 10 ug/mL Biotinylated Anti-Sheep Ab, 20 ng/mL Sheep Anti-heroin PAb

Table 2: Assay format screen

Condition	Heroin [ng/mL]	Average	%CV	Modulation
Cab in solution	1000	390	18.5	260
	250	957	4.3	106
	25	4209	4.5	24
	0	101399	9.7	1
Cab tip method 1	1000	176	27.1	183
	250	526	17.7	61
	25	1602	4.2	20
	0	32173	11.6	1
Cab tip method 2	1000	330	25.8	128
	250	663	23.3	64
	25	3296	16.4	13
	0	42391	26	1

Figure 2: Assay format screen



2.2 Antibodies Screen on Theranos System 3.0

The two antibodies were chosen to screen on Theranos system 3.0. The protocol Generic2_5X_Coincubation_10_10 was used. In this screen, the secondary antibody, Goat anti-sheep as the capture surface. Then the unlabeled anti-Heroin antibody at 20ng/mL in souluton mixed into the sample along with the YJ Heroin-AP conjugate at the dilution of 1:125,000. The response on the Theranos System was showing some modulation for both anitbody 1 and 2. Since the signal of antibody 2 was a litle higher than antibody 1. Antibody 2 was chosen to move forward for further opimatization.

Table 3: Antibodies screen

Antibody	Sample [ng/mL]	Average	%CV	Modulation
Cab 1	1000	70543	6	3.22
	250	90243	8	2.52
	25	136194	15	1.67
	0	227248	16	1.00
Cab 2	1000	30006	0	4.83
	250	45216	10	3.21
	25	70054	18	2.07
	0	145046	10	1.00

2.3 Capture Antibody Titration

To improve the surface capture of the assay, the capture antibody concentration was further titrated. The assay was performed using a Generic2_50x_Competitive_10-10 protocol on the Theranos system. Heroin-AP conjugate final concentration dilution at 1,000,000. The best response was shown with a capture concentration of 100ng/mL.

Table 4a: Capture antibody titration #1

Final: Heroin-AP 1:1,000,000 dilution		Final: Ab 500ng/mL			Final: Ab 200ng/mL		
Capture Ab	[Heroin] ng/mL	Mean RLU	Modulation	Btw pt	Mean RLU	Modulation	Btw pt
Low BSA	1000	871	218.6	4.7	491	272.7	1.9
	250	4107	46.4	8.8	913	146.8	13.6
	25	36319	5.2	5.2	12447	10.8	10.8
	0	190489	1.0		133986	1.0	
Urine	1000	1885	97.4	1.9	795	112.6	2.2
	250	3602	51.0	8.4	1733	51.6	8.5
	25	30192	6.1	6.1	14767	6.1	6.1
	0	183634	1.0		89514	1.0	
Serum	1000	1261	217.3	3.8	582	299.7	2.8
	250	4854	56.4	6.2	1606	108.6	11.1
	25	30289	9.0	9.0	17881	9.8	9.8
	0	273995	1.0		174441	1.0	

Table 4b: Capture antibody titration #2

Final: Ab 100ng/mL			Final: Ab 50ng/mL			Final: Ab 20ng/mL		
Mean RLU	Modulation	Btw pt	Mean RLU	Modulation	Btw pt	Mean RLU	Modulation	Btw pt
302	297.7	2.0	411	119.2	1.4	158	149.5	2.1
611	147.3	17.7	592	82.7	8.7	325	72.8	5.0
10831	8.3	8.3	5156	9.5	9.5	1635	14.5	14.5
89991	1.0		48948	1.0		23636	1.0	
367	221.6	2.5	214	129.8	3.7	196	79.6	1.2
919	88.4	11.1	786	35.4	6.6	244	64.1	5.8
10243	7.9	7.9	5210	5.3	5.3	1413	11.1	11.1
81289	1.0		27800	1.0		15631	1.0	
458	275.9	2.1	193	175.4	2.8	107	218.6	3.2
969	130.3	5.9	548	61.8	6.7	338	69.3	4.8
5703	22.1	22.1	3658	9.3	9.3	1615	14.5	14.5
126270	1.0		33891	1.0		23377	1.0	

Table 3c: Capture antibody titration #3

Final: Ab 10ng/mL			Final: Ab 5ng/mL		
Mean RLU	Modulation	Btw pt	Mean RLU	Modulation	Btw pt
149	113.5	1.9	54	50.4	2.8
283	60.0	9.9	152	17.8	1.9
2798	6.1	6.1	293	9.2	9.2
16938	1.0		2707	1.0	
147	76.5	2.0	106	35.2	0.9
294	38.3	4.3	96	38.7	4.4
1255	9.0	9.0	418	8.9	8.9
11269	1.0		3711	1.0	
114	232.2	1.9	199	29.8	1.3
215	123.3	7.5	265	22.3	2.5
1606	16.5	16.5	652	9.1	9.1
26479	1.0		5914	1.0	

2.4 Detection Titration

For further optimize the assay, the detection conjugate concentration were titrated collaboratively to find the best combination for both signal range and modulation. The assay was performed using a Generic2 50x Competitive 10-10 protocol on the Theranos system. The capture antibody is at 100ng/mL. As the results detection conjugate titration showed that, if the conjugate concentration at 1,000,000 gave the optimal RLU signal and decent signal modulation.

Table 5: Detection Titration

[Heroin-AP] Dilution	Sample [ng/mL]	Average	%CV	Modulation
5000	1000	4279	31	126.51
	250	34956	11	15.49
	25	64388	21	8.41
	0	541339	32	1.00
10,000	1000	18761	23	34.17
	250	45094	20	14.22
	25	208762	20	3.07
	0	641067	28	1.00
1,000,000	1000	302	14	297.71
	250	611	13	147.28
	25	10831	20	8.31
	0	89991	14	1.00

	1000	581	15	238.98
1,500,000	250	1848	11	75.17
	25	11369	29	12.22
	0	138902	17	1.00

2.5 Sample Dilution

To reduce matrix effects, the sample dilution was tested with final sample dilution factors of 1:25, 1:50, and 1:100 on different capture antibody concentration. The assay also evaluated dilution factors on different matrixes. A dilution of 1:50 showed more than adequate sensitivity and higher modulation. The 1:50 sample dilution was chosen for further assay optimization. However, after further optimize the assay, clinical samples and a full standard curve was evaluated, matrix effects were observed. To reduce the potential matrix effects in the assay 100X was chosen as the final sample dilution.

Table 6: Sample dilution

			25X			50X			100X		
Cab Concentration	Sample	[ng/mL]	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation
20ng/mL	Low BSA	1000	110	8	93.2	158	22	149.5	143	13	55.2
		250	199	16	51.7	325	14	72.8	267	23	29.5
		25	679	19	15.1	1635	7	14.5	1876	10	4.2
		0	10262	5	1.0	23636	32	1.0	7876	13	1.0
	Urine	1000	136	7	59.5	196	18	79.6	186	5	65.2
		250	155	60	52.3	244	17	64.1	419	17	28.9
		25	706	8	11.5	1413	21	11.1	2340	11	5.2
		0	8107	19	1.0	15631	15	1.0	12126	17	1.0
	Serum	1000	145	28	117.4	107	21	218.6	197	22	50.7
		250	213	46	80.0	338	31	69.3	257	14	39.0
		25	650	9	26.2	1615	14	14.5	1446	15	6.9
		0	17073	16	1.0	23377	17	1.0	9999	5	1.0
Cab Concentration	Sample	[ng/mL]	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation
50ng/mL	Low BSA	1000	117	1	99.1	411	27	119.2	198	21	58.6
		250	204	17	56.9	592	7	82.7	369	1	31.5
		25	1008	21	11.5	5156	21	9.5	2567	12	4.5
		0	11607	18	1.0	48948	25	1.0	11617	21	1.0
	Urine	1000	144	5	57.2	214	22	129.8	250	10	60.1
		250	239	2	34.4	786	16	35.4	566	18	26.5
		25	1189	15	6.9	5210	13	5.3	3771	6	4.0
		0	8213	18	1.0	27800	24	1.0	15018	8	1.0
	Serum	1000	165	7	133.2	193	31	175.4	221	4	63.6
		250	195	16	112.5	548	0	61.8	414	14	33.9
		25	1051	11	20.9	3658	19	9.3	2442	27	5.7
		0	21937	27	1.0	33891	14	1.0	14032	18	1.0
Cab Concentration	Sample	[ng/mL]	Mean	CV	Modulation	Mean	CV	Modulation	Mean	CV	Modulation
100ng/mL	Low BSA	1000	86	8	110.6	302	14	297.7	459	24	58.7
		250	125	12	76.7	611	13	147.3	823	61	32.7
		25	839	23	11.4	10831	20	8.3	5214	25	5.2
		0	9562	4	1.0	89991	14	1.0	26915	14	1.0
	Urine	1000	188	22	112.2	367	22	221.6	453	22	76.1
		250	306	10	69.0	919	32	88.4	1007	29	34.2
		25	1677	22	12.6	10243	24	7.9	5791	30	6.0
		0	21141	18	1.0	81289	12	1.0	34485	23	1.0
	Serum	1000	238	22	162.7	458	26	275.9	505	31	54.9
		250	356	10	108.6	969	19	130.3	961	1	28.8
		25	1543	12	25.1	5703	17	22.1	6052	33	4.6
		0	38708	22	1.0	126270	17	1.0	27718	69	1.0

2.6 Assay Diluents

In this experiment, four different assay diluents were tested: assay buffer (low BSA), starting block, superblock, and low cross buffer. The protocol is Generic2_50x_Competitive_10-10. Capture antibody at 100ng/mL. Detection at 1,000,000 dilution in low BSA buffer was used.

The results displayed that starting block is yield a high signal, but low BSA assay buffer was chosen to be the final candidate due to later multiplex the assay with other assays.

Table 7: Assay Diluents

Buffers	Sample [ng/mL]	Average	%CV	Modulation
Starting block	1000	260	22	377.83
	250	663	25	148.26
	25	6061	11	16.21
	0	98269	12	1.00
Super block	1000	279	14	346.15
	250	612	5	158.03
	25	6931	20	13.96
	0	96739	12	1.00
Low Cross	1000	372	23	99.71
	250	476	26	78.09
	25	2181	21	17.03
	0	37139	31	1.00
Low BSA	1000	302	14	297.71
	250	611	13	147.28
	25	10831	20	8.31
	0	89991	14	1.00

2.7 Matrix Screen

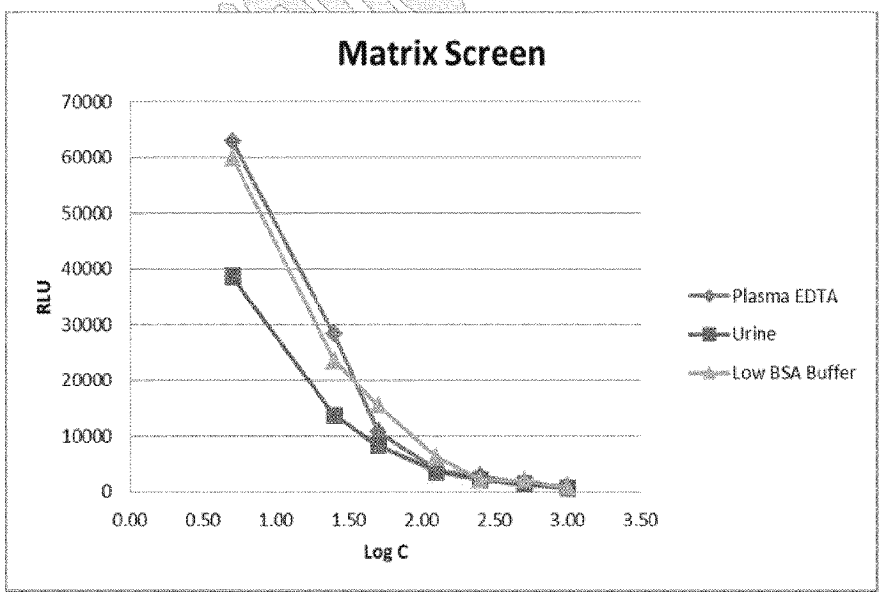
The Theranos opiate assay is intended to target multiple matrixes including urine, serum, an plasma samples. To screen matrix, a pool of donor's urine samples and EDTA plasma samples and low BSA buffer were spiked to make a standard curve to determine the response in the different matrixes. The sample dilution of this screen is 1:50. Plasma and low BSA buffer matrixes showed very similar dose response. However the urine showed a lower signal. Overall plasma and urine matrixes showed similar modulation, but the low BSA buffer showed high modulation. Therefore Plasma and Urine calibrators were used seperate for assay optimization.

Table 8: Matrix screen

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation
Plasma EDTA	1000	1026	3	148.56
	500	1742	13	87.51
	250	2810	24	54.24
	125	3826	27	39.84
	50	10782	8	14.14

	25	28317	27	5.38
	5	62963	21	2.42
	0	152418	25	1.00
Urine	1000	674	23	145.68
	500	1404	20	69.94
	250	2190	23	44.86
	125	3710	23	26.48
	50	8381	27	11.72
	25	13760	16	7.14
	5	38648	22	2.54
	0	98227	19	1.00
Low BSA Buffer	1000	870	29	233.14
	500	2107	25	96.30
	250	2196	21	92.42
	125	6253	23	32.45
	50	15539	27	13.06
	25	23649	20	8.58
	5	59955	15	3.38
	0	202918	14	1.00

Figure 3: Matrix screen



2.8 Detection Stabilizers

To further optimal the assay, the stabilizing buffer for the Heroin-AP conjugate was evaluated including Biostab, Stabilzyme and Theranos in-house Small Molecule AP Conjugate Stabilizer (0.1 mM Zn^{2+}, 5 mM Mg^{2+} and 0.03% BSA with 0.05 % Sodium Azide in 50mM TBS pH 8.0). The protocol is Generic2_competitive-50x_10_10. The capture antibody at 0.10ug/ml and detection conjugate was prepared at 1:100,000 dilution in 3 different stabilizers. All three stabilizers performed well in the assay but the Stabilzyme showed a slightly better modulation and was chosen as the final assay condition.

Table 9: Detection stabilizers

Stabilizer	Sample [ng/mL]	Average	%CV	Modulation
Biostab	1000	1264	23	133.12
	250	1741	10	96.64
	25	22139	18	7.60
	0	168278	17	1.00
Stabilzyme	1000	993	8	193.20
	250	2074	11	92.52
	25	22739	14	8.44
	0	191856	24	1.00
In-house buffer	1000	1143	12	133.93
	250	2061	10	74.26
	25	23412	10	6.54
	0	153027	22	1.00

2.9 Incubation Time

In order to efficiently evaluating the assay, the effect of shorter reagent incubation times was tested with sample, detection conjugate, and substrate incubation times respectively of 10_10 and 5_5 minutes. Assay modulation was excellent at the 10_10 minute incubation times while modulation fell off sharply at the 5_5 incubation time.

Table 10: Incubation time

		5-5 min			10-10 min		
Condition	Sample [ng/mL]	Average	%CV	Modulation	Average	%CV	Modulation
Low BSA Buffer	1000	58	7	68.96	870	29	233.14
	250	117	32	34.25	2196	21	92.42
	25	494	30	8.13	23649	20	8.58
	0	4021	20	1.00	202918	14	1.00
Urine	1000	124	11	38.84	674	23	145.68
	250	314	24	15.33	2190	23	44.86

	25	1253	22	3.84	13760	16	7.14
	0	4810	25	1.00	98227	19	1.00
Plasma EDTA	1000	81	14	86.12	1026	3	148.56
	250	129	6	54.21	2810	24	54.24
	25	720	24	9.75	28317	27	5.38
	0	7016	21	1.00	152418	25	1.00

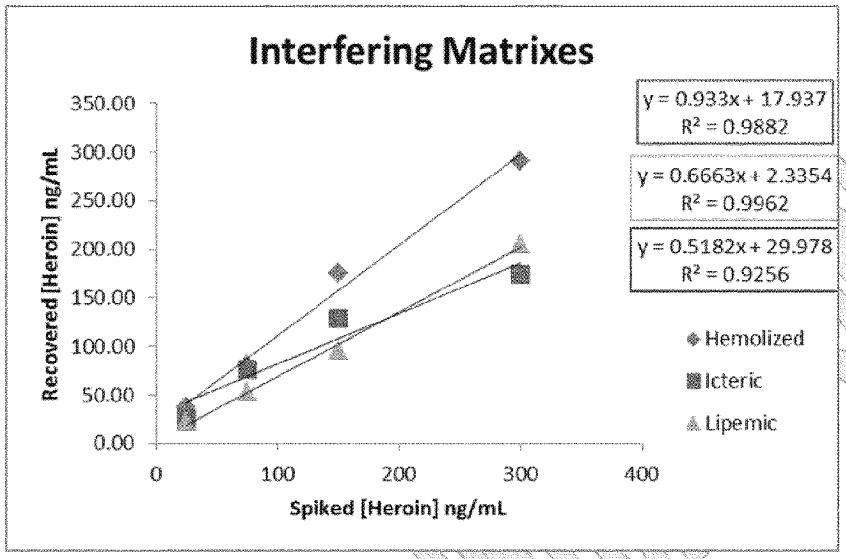
2.10 Interfering Matrixes

Hemolyzed, lipemic, and icteric serum were evaluated for the potential interference. The recovery of Heroin was spiked into these potentially interfering matrixes and was screened on the Theranos System 3.0. Hemolyzed samples showed interference at 25ng/mL cutoff. All spiked samples recovery was on the acceptable range and expected values for icteric and lipemic. Therefore, the assay did show the interference for hemolyzed, but not show any significant matrix effect to interference on lipermic and icteric.

Table 11: Interfering Matrixes

Condition	Concentration [ng/mL]	AVG RLU	CV	Back-Cal [ng/mL]	% Recovery
Hemolyzed	300	2919	16	290.95	97%
	150	4364	30	176.03	117%
	75	8118	22	80.40	107%
	25	14523	7	37.51	150%
	0	135206	7	1.08	NA
Icteric	300	4396	9	174.43	58%
	150	5587	12	129.16	86%
	75	8503	10	75.76	101%
	25	19218	8	25.57	102%
	0	137176	19	1.05	NA
Lipemic	300	3858	16	205.37	68%
	150	7120	20	95.06	63%
	75	11183	19	53.08	71%
	25	21194	29	22.30	89%
	0	138442	7	1.03	NA

Figure 4: Interfering Matrixes



2.11 Standard Curve in Urine, Serum, Plasma, Whole blood, and Buffer in Final Condition

A standard curve in urine, serum, plasma, whole blood, and buffer ranging from 0 – 800ng/mL were run on the final condition to determine the assay range. The final protocol is Generic2_100X_Competitive_10_10. The detection is diluted 1:100,000 as a loading concentration. Capture surface antibody is 100ng/mL. For calibration curve of urine and serum were generated using Dexter software. LLOQ was defined at 10ng/ml and ULOQ was defined at 800ng/ml. These urine and serum final curve was used to calculate the final set of clinical and control samples.

Table 12: Standard Curve in Plasma EDTA

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation	Backcalculated [ng/mL]	% Recovery
Plasma EDTA	800	2254	25	84.99	745	93
	600	2880	26	66.52	591	99
	300	4624	16	41.43	375	125
	150	14680	24	13.05	105	70
	75	19130	20	10.01	74	99
	25	32835	27	5.83	34	135
	10	68872	22	2.78	9	91
	0	191560	14	1.00	1	

Table 13: Standard Curve in Buffer

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation	Backcalculated [ng/mL]	% Recovery
Low BSA Buffer	800	2099	19	82.27	821	103
	600	2350	22	73.49	679	113
	300	3594	25	48.06	379	126
	150	11514	20	15.00	130	87
	75	19626	17	8.80	74	98
	25	33920	26	5.09	31	125
	10	56552	6	3.05	9	94
	0	172732	21	1.00	0	

Table 14: Standard Curve in Whole Blood

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation	Backcalculated [ng/mL]	% Recovery
Whole Blood	800	1754	12	120.35	664	83
	600	1986	4	106.29	758	126
	300	7887	14	26.76	318	106
	150	13350	14	15.81	115	77
	75	15589	7	13.54	84	111
	25	25599	27	8.25	30	120
	10	50467	26	4.18	10	97
	0	211068	19	1.00	#NUM!	

Table 15a: Standard Curve in Urine

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation	Backcalculated [ng/mL]	% Recovery
Urine	800	2136	11	85.80	810	101
	600	3173	18	57.77	575	96
	300	6050	16	30.30	307	102
	150	10783	15	17.00	159	106
	75	21643	4	8.47	63	84
	25	35988	16	5.09	29	114
	10	66011	9	2.78	10	96
	0	183305	15	1.00	1	

Figure 5: Urine Calibration Curve from Dexter Analysis

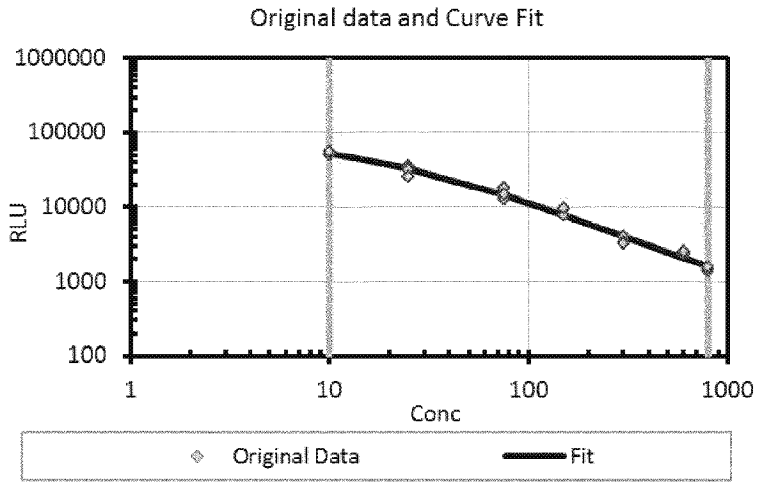


Table 15b: Urine Calibration Curve Parameters from Dexter Analysis

Model Type	4PL
Model Equation	log10(RLU) = b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	Conc = b3 * (((b2 - b1) / (log10(RLU) - b1)) - 1) ^ (1 / b4)
b1	1.733
b2	5.233
b3	451.565
b4	0.423
LLOQ	10ng/ml
ULOQ	800ng/ml
LLOQ accuracy	95%
LLOQ precision	10.40%
ULOQ accuracy	111%
ULOQ precision	18.60%

Table 16a: Standard Curve in Serum

Matrix Condition	Sample [ng/mL]	Average	%CV	Modulation	Backcalculated [ng/mL]	% Recovery
	800	1749	15	98.65	795	99
	600	2540	19	67.91	555	93
	300	3849	21	44.82	355	118

	150	8946	12	19.28	125	83
Serum	75	12748	11	13.53	77	103
	25	26608	9	6.48	27	107
	10	52544	20	3.28	10	96
	0	172495	8	1.00	2	

Figure 6: Serum Calibration Curve from Dexter Analysis

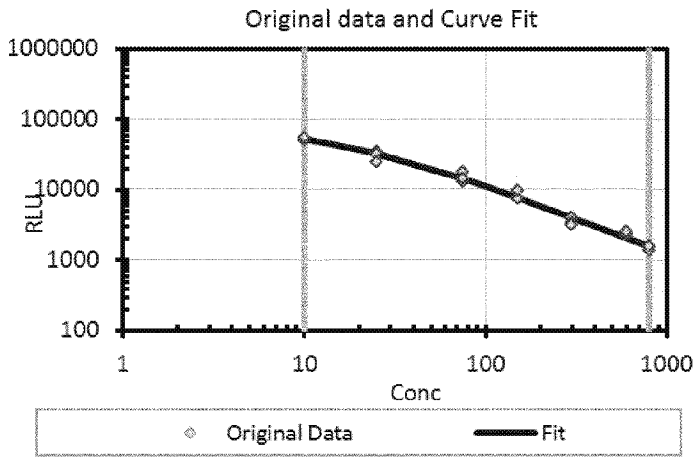


Table 16b: Serum Calibration Curve Parameters from Dexter Analysis

Model Type	4PL
Model Equation	log10(RLU) = b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	Conc = b3 * (((b2 - b1) / (log10(RLU) - b1)) - 1) ^ (1 / b4)
b1	2.668
b2	5.084
b3	208.846
b4	0.660
LLOQ	10ng/ml
ULOQ	800ng/ml
LLOQ accuracy	89%
LLOQ precision	22%
ULOQ accuracy	115%

ULOQ precision	0.00%

2.12 Normal Sample Screen

A set of 8 normal urines and 8 normal EDTA plasma samples were screened on Theranos system 3.0 and reference methods. Urine samples results were calculated on the Urine standard curve. Plasma samples were calculated on the serum standard curve. Three reference methods are Abnova, Neogen, and Advia (urine only). All urine and plasma normal samples were negative in three reference methods and in the Theranos assay. The positive cutoff for opiate assay is urine is 300ng/mL and serum/plasma is 25ng/mL.

Table 17: Normal Plasma Screen

Plasma	Theranos				Abnova	Neogen
Sample ID	Mean RLU	CV	Back-Cal [ng/mL]	Result	Result	Result
M1	144720	24%	1.74	NEG	NEG	NEG
M2	148653	26%	1.63	NEG	NEG	NEG
M3	148903	3%	1.62	NEG	NEG	NEG
M4	151970	11%	1.54	NEG	NEG	NEG
F1	121482	20%	2.66	NEG	NEG	NEG
F2	147265	27%	1.67	NEG	NEG	NEG
F3	139779	19%	1.90	NEG	NEG	NEG
F4	119376	14%	2.77	NEG	NEG	NEG

Table 18: Normal Urine Screen

Urine	Theranos				CLIA (Advia)
Sample ID	Mean RLU	CV	Back-Cal [ng/mL]	Result	Result
BRH668180	112622	29%	3.20	NEG	NEG
BRH668181	147561	13%	1.73	NEG	NEG
BRH668182	131721	9%	2.25	NEG	NEG
BRH668183	117908	16%	2.89	NEG	NEG
BRH668184	91976	3%	4.94	NEG	NEG
BRH668185	145130	24%	1.80	NEG	NEG
BRH668186	132612	8%	2.22	NEG	NEG
BRH668187	141092	17%	1.92	NEG	NEG

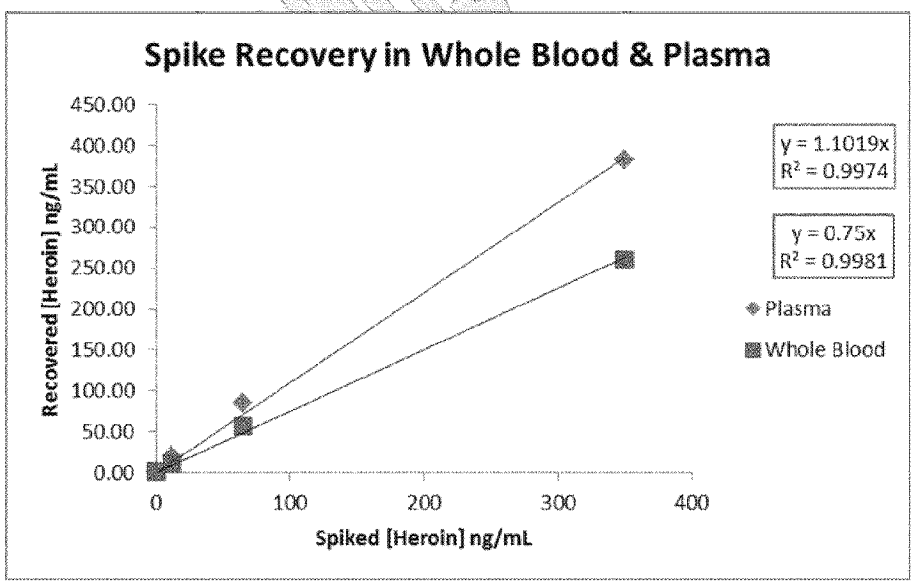
2.13 Whole Blood and Plasma Spike Recovery

The whole blood and EDTA plasma was spiked at four levels for testing the pike recovery. As the result the spike recovery was great in plasma and less in whole blood. It was approximately 75% in whole blood.

Table 19: Spike Recovery in Whole Blood and Plasma

Condition	Concentration [ng/mL]	AVG RLU	CV	Back-Cal [ng/mL]	% Recovery
Whole Blood	350	5007	7	261	75%
	65	15756	22	57.41	88%
	12	48504	7	10.88	91%
	0	142282	16	2.11	
Plasma	350	3597	13	382.92	109%
	65	11892	14	85.09	131%
	12	31572	15	20.80	173%
	0	160901	11	1.75	#DIV/0!

Figure 7: Spike Recovery in Whole Blood and Plasma



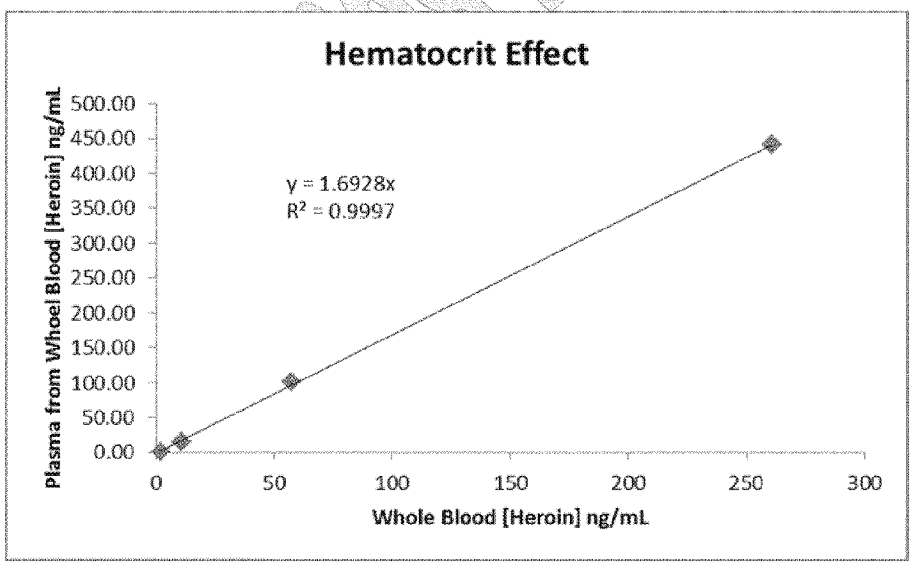
2.14 Hematocrit Effect

The hematocrit effect was screened by spiking whole blood samples and spun down to separate plasma and ran it on the Theranos System. The result showed the plasma were approximately 1.69 times higher than the whole blood. The value was expected based on average blood hematocrit.

Table 20: Hematocrit Effect

Condition	Concentration [ng/mL]	AVG RLU	CV	Back-Cal [ng/mL]	% Recovery
Whole Blood	350	5007	7	261	75%
	65	15756	22	57.41	88%
	12	48504	7	10.88	91%
	0	142282	16	2.11	
Plasma from whole Blood	350	3166	12	440.68	126%
	65	10432	21	101.77	157%
	12	38078	6	15.70	131%
	0	169049	6	1.62	

Figure 8: Hematocrit Effect



2.15 Cross Reactivity and Interference

To test cross reactivity and interference, different substances due to their structural or synthetic molecule that could potentially cross react or interfere with opiate assay were tested. The test levels chosen for each test substance were based on the therapeutic drug levels and an excess of

the clinical ranges. The recovery of opiate was calculated on the control standard curve. No cross reactivity was observed with some substances tested. Other Substances such as dihydrocodeine, hydrocodone, hydromorphone, and Oxycodone and methotrexate were showed cross reactivity/interference. The antibody that was used in this assay is cross reacted to the close family structure (see below table). It shows >12% to morphine, >13% to dihydrocodeine and hydrocodone, and >5% hydromorphone and <1% Morphine-3-glucuroride.

Table 21: Cross reactivity and interference

Sample ID	Concentration [ng/mL]	Mean RLU	CV	Back-Cal [ng/mL]	% Cross Reactivity
Dihydrocodeine	1000	8386	13%	170.92	17%
Hydrocodone	1000	11207	12%	133.11	13%
Hydromorphone	1000	26940	10%	47.03	5%
Morphine-3-glucuroride	1000	82905	10%	2.73	0%
Benzoylecgonine	1000	154636	18%	0.17	0%
Flunitrazepam	1000	136406	18%	0.32	0%
Oxycodone	1328	48304	14%	14.36	1%
Benzodiazepines	1020	120658	20%	0.58	0%
DL-AMP	1000	143108	13%	0.25	0%
Acetaminophen	1000	148785	21%	0.20	0%
Caffeine	1000	134215	12%	0.34	0%
Aspirin	1000	123829	20%	0.51	0%
LSD	1000	159242	3%	0.14	0%
Methadone	1000	154924	4%	0.16	0%
Methamphetamine	1000	104497	23%	1.09	0%
Ibuprofen	1000	165103	12%	0.12	0%

Table 22: Percentage of cross reactivity

Cross-Reactivity	%
Heroin	**155**
6-Monoacetylmorphine	**100**
Codeine	**71.3**
Morphine	**11.2**
Dihydrocodeine	**9.2**
Hydrocodone	**3.9**
Hydromorphone-	**<1.55**
Morphine 3-glucuronide	**<1.55**

2.16 HAMA and RF Screen

Five HAMA positive serum samples and Five RF positive samples from PromedDx were analyzed with final assay condition. The cutoff is 25ng/mL. All samples tested showed negative in Theranos method. It indicates that HAMA positive and RF positive status didn't have any affected on opiate assay.

Table 23: HAMA and RF

Sample ID	Mean RLU	CV	Back-Cal [ng/mL]	Result
HAMA 12	127008	2619%	2.39	NEG
HAMA 13	148787	201%	1.63	NEG
HAMA 14	170228	1759%	1.15	NEG
HAMA 15	113078	2204%	3.15	NEG
HAMA 16	163528	1202%	1.28	NEG
RF 13	153215	1502%	1.51	NEG
RF 16	163361	2017%	1.28	NEG
RF 18	150546	2040%	1.58	NEG
RF 21	175551	1237%	1.06	NEG
RF 22	178948	591%	1.01	NEG

2.17 Clinical Samples

Since positive clinical samples could not be obtained, the 8 normal plasma and urine samples previously screened were spiked with different levels of heroin, 6-AM, and codeine. Also Bio-Rad DOA drug screen urine samples was included to screen on Theranos system 3.0. The samples were measured in the Theranos System, the Abnova ELISA Kit, Neogen, and the urine samples were tested in the Advia system and Neogen.

Positive results are highlighted in pink and negative results are highlighted in green. The Theranos result agreed with the other reference methods.

Theranos Positive Cutoff for opiate:

Plasma cutoff = 25ng/mL
Urine cutoff = 300ng/mL

Table 24: Urine clinical samples

		Theranos		CLIA		Neogen	
Sample [Urine]	[ng/mL]	[ng/mL]	Interpretation	[ng/mL]	Interpretation	Average [OD]	Interpretation
	200	158	NEG	150	NEG	0.719	NEG

Heroin	650	693	POS	535	POS	0.338	POS
	32	35	NEG	40	NEG	1.4495	NEG
	17	12	NEG	NA	NA	1.6125	NEG
	5	5	NEG	NA	NA	1.791	NEG
6-Acetylmorphine	20	26	NEG	24	NEG	1.6025	NEG
	45	46	NEG	55	NEG	1.342	NEG
	677	625	POS	563	POS	0.326	POS
	10	8	NEG	NA	NA	1.6485	NEG
	301	335	POS	256	NEG	0.487	POS
Codeine	75	84	NEG	94	NEG	0.756	NEG
	10	8	NEG	NA	NA	1.538	NEG
	15	13	NEG	33	NEG	1.431	NEG
	505	538	POS	1547	POS	0.225	POS
	29	34	NEG	25	NEG	1.138	NEG
Biorad C2 (low opiate)	225	261	NEG	727	POS	0.253	POS
Biorad C3 (low opiate)	375	487	POS	1426	POS	0.188	POS
Biorad C1	120	143	NEG	271	NEG	0.370	POS
Biorad C2	1500	1129	POS	NA	NA	0.109	POS
Biorad C3	2500	1538	POS	NA	NA	0.097	POS
Biorad C4	4000	1821	POS	NA	NA	0.093	POS
Biorad N (negative)	75	1	NEG	NA	NA	1.917	NEG
Sunnylab	opiate	3040	POS	NA	NA	0.086	POS
Biorad QN	50	3	NEG	NA	NA	NA	NA
Biorad QP	2904	694	POS	3404	POS	NA	NA
Biorad S1S	1500	454	POS	1259	POS	NA	NA
Biorad S2S	2500	615	POS	2273	POS	NA	NA
Biorad S1E (low opiate)	225	81	NEG	216	NEG	NA	NA
Biorad S2E (low opiate)	375	96	NEG	406	POS	NA	NA
Biorad S2	2500	601	POS	2363	POS	NA	NA
Biorad S3	750	214	NEG	776	POS	NA	NA
Heroin	350	395	POS	280	NEG	0.488	POS
	65	84	NEG	95	NEG	1.167	NEG
	12	20	NEG	53	NEG	1.709	NEG
6-Acetylmorphine	400	323	POS	205	NEG	0.697	NEG
	80	102	NEG	139	NEG	1.241	NEG
	20	24	NEG	55	NEG	1.707	NEG

Codeine	380	336	POS	275	NEG	0.438	POS
	62	65	NEG	89	NEG	0.770	NEG
	18	18	NEG	NA	NA	1.534	NEG

Table 25: Plasma clinical samples

		Theranos		Abnova		Neogen	
Sample [Urine]	[ng/mL]	[ng/mL]	Interpretation	Average [OD]	Interpretation	Average [OD]	Interpretation
Heroin	650	638	POS	0.145	POS	0.120	POS
	200	168	POS	0.201	POS	0.245	POS
	17	12	NEG	0.458	NEG	1.107	NEG
	32	29	POS	0.403	NEG	0.815	NEG
	5	4	NEG	0.645	NEG	1.498	NEG
6-Acetylmorphine	677	668	POS	0.127	POS	0.111	POS
	20	19	NEG	0.403	NEG	0.956	NEG
	10	19	NEG	0.532	NEG	1.176	NEG
	45	81	POS	0.285	POS	0.546	POS
	301	411	POS	0.163	POS	0.166	POS
Codeine	10	8	NEG	0.507	NEG	1.023	NEG
	75	58	POS	0.182	POS	0.320	POS
	505	435	POS	0.076	POS	0.096	POS
	200	178	POS	0.459	NEG	0.146	POS
	29	24	NEG	0.359	POS	0.614	POS
Heroin	350	383	POS	0.146	POS	0.133	POS
	65	85	POS	0.272	POS	0.351	POS
	12	21	NEG	0.511	NEG	0.957	NEG
6-Acetylmorphine	400	409	POS	0.136	POS	0.179	POS
	80	85	POS	0.238	POS	0.465	POS
	20	19	NEG	0.417	NEG	0.987	NEG
Codeine	380	273	POS	0.074	POS	0.151	POS
	62	52	POS	0.160	POS	0.423	POS
	18	19	NEG	0.337	POS	0.928	NEG

2.18 Calibration Verification

To verify the Theranos System calibration, five level urine samples from different drugs, heroin, 6-AM, and codeine were spiked at different levels and measured on the Theranos System and

CLIA reference methods. The Theranos result also matched with the Advia system result for all levels.

Table 26: Calibration Verification

Condition	Concentration [ng/mL]	AVG RLU	CV	Back-Cal [ng/mL]	% Recovery	CLIA Result
Heroin	200	10851	27	158	79	150
	650	2568	28	693	107	535
	32	31661	9	35	110	40
	17	58585	6	12	71	No Detectable
	5	91996	11	5	99	No Detectable
6-Acetylmorphine	20	37645	25	26	132	24
	45	26611	9	46	103	55
	677	2892	23	625	92	563
	10	74623	7	8	76	No Detectable
	301	5553	27	335	111	256
Codeine	75	17636	15	84	112	94
	10	70912	17	8	84	No Detectable
	15	55428	15	13	89	33
	505	3416	3	538	107	1547
	29	32212	27	34	118	25

Figure 9: Calibration Verification: Heroin

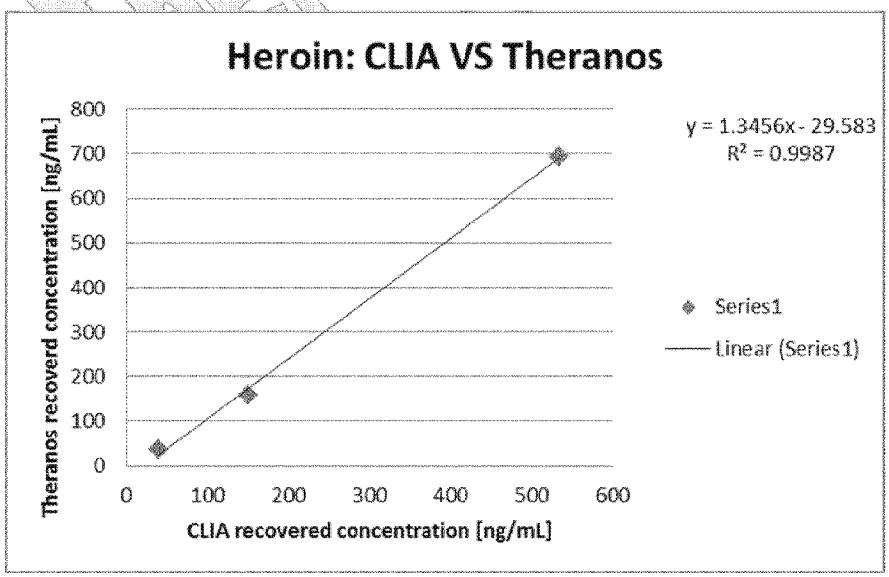


Figure 10: Calibration Verification: 6-Acetylmorphine

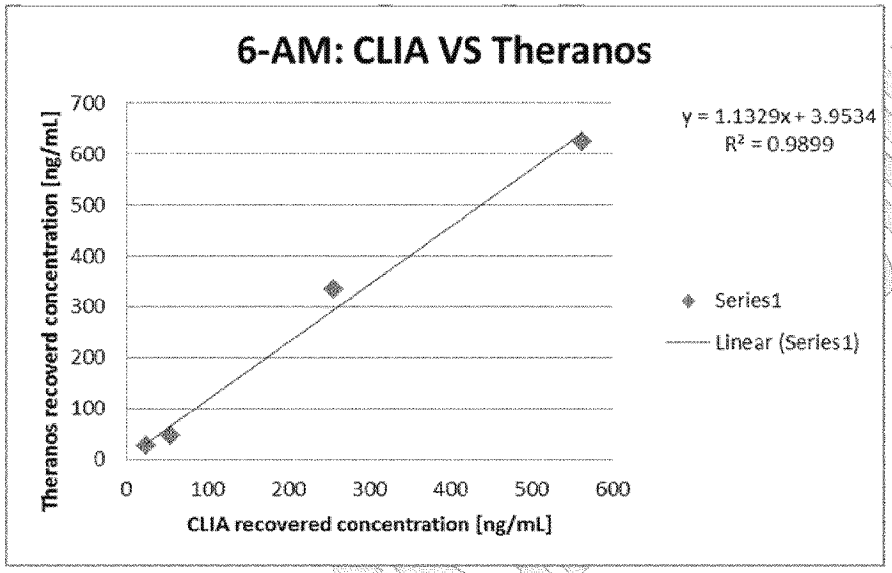
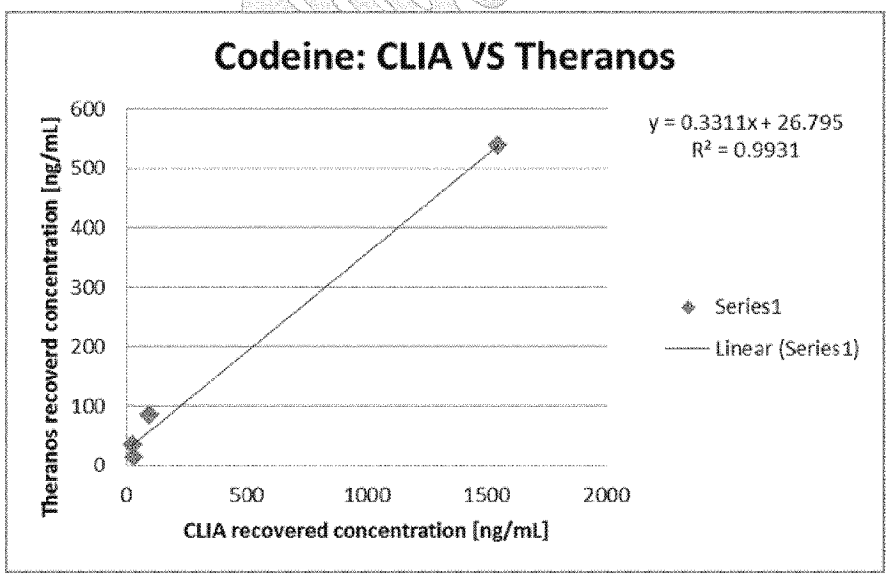


Figure 11: Calibration Verification: Codeine



2.19 Stability

Stability studies are ongoing.

3.0 References