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Methadone
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 methadone in human serum, plasma and urine. The assay has a reportable range of 40 to 1000 ng/mL. For urine samples the cut off is established at 300 ng/mL and for serum/plasma samples the cutoff is 40 ng/ml. The methadone assay is calibrated using the Certified Reference Material (±)-Methadone (Cat#M-007) from Cerilliant.

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

The reference assay available for the urine matrix is the Siemen Advia Methadone_2 (MDN_2).

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

The capture surface is made up of a stack comprising avidin, biotin-labeled goat anti-mouse secondary antibody and an anti-methadone antibody. The sample is diluted and combined with the tracer enzyme labeled methadone 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 #
(±)-Methadone	Cerilliant	M-007
Goat Anti mouse secondary Antibody	Pierce	31805
Mouse monoclonal Anti-Methadone	Lifespan Biosciences	LS-C72467
Methadone-Alk Phos Conjugate" A"	Theranos	
Carbonate-bicarbonate buffer	Sigma	C3041
Low BSA Blocking Buffer (0.03% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Alkaline Phosphatase Substrate	Theranos	T-ALKP-SB01

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"]

30 anti-methadone antibodies (Table 2 and Table 3) were commercially available and ordered. All 30 antibodies were coated on a 384 well microtitre plate (MTP) at 10,1,0.1 and 0 ug/mL and tested for binding to the commercial Methadone-HRP conjugate at a dilution of 1:1000 from the stock in Stabilzyme Noble (HRP small molecule conjugate stabilizer). The data are summarized in Table 4 and 5. 10 out of the 30 antibodies (#s 1, 2, 10, 11, 12 16, 18 22, 23 and 26) showed binding affinities to the commercial methadone HRP conjugate. All 10 antibodies were chosen for further evaluation on the competitive format on the Theranos system.

Table [SEQ Table * ARABIC]: Antibody Information

Ab#	Vendor	Cat #	Clone #	Description
1	biorbyt	orb24614	BID902	Monoclonal Antibody to Methadone
2	biorbyt	orb24615	BID905	Monoclonal Antibody to Methadone
3	mybiosource	MBS530781	M610245	Monoclonal Antibody to Methadone
4	mybiosource	MBS530516	M081030	Monoclonal Antibody to Methadone
5	mybiosource	MBS310801	B658M	Monoclonal Antibody to Methadone
6	Arista Biologicals	ABMTD-0401	clone 1	Monoclonal Antibody to Methadone
7	Lifespan biosciences	LS-C72465	Mab	Monoclonal Antibody to Methadone
8	Lifespan biosciences	LS-C56380	sheep	Sheep polyclonal against methadone
9	Lifespan biosciences	LS-C55835	Met 7F5	Monoclonal Antibody to Methadone
10	Lifespan biosciences	LS-C56379	Met 2A7	Monoclonal Antibody to Methadone
11	Lifespan biosciences	LS-C72466		Monoclonal Antibody to Methadone
12	Lifespan biosciences	LS-C72467		Monoclonal Antibody to Methadone
13	Lifespan biosciences	LS-C130728		Monoclonal Antibody to Methadone
14	Lifespan biosciences	LS-C130729		Monoclonal Antibody to Methadone
15	Lifespan biosciences	LS-C130732		Monoclonal Antibody to Methadone

Table [SEQ Table * ARABIC]: Antibody Information (contnd.)

Ab#	Vendor	Cat #	Clone #	Description
16	ARP	13-2029		Monoclonal Antibody to Methadone
17	Us biol	M3010-02	4A144	Monoclonal Antibody to Methadone
18	Us biol	M3010-03	1.BB.906	Monoclonal Antibody to Methadone
19	Us biol	M3010-03A	9L467	Monoclonal Antibody to Methadone
20	Us biol	M3010-03B	9L468	Monoclonal Antibody to Methadone
21	East coast Bio	P11-99-08M-P		Monoclonal Antibody to Methadone
22	East coast Bio	P11-99-05M-P		Monoclonal Antibody to Methadone
23	East coast Bio	P11-99-09M-P		Monoclonal Antibody to Methadone
24	Calbioreagents	M199		Monoclonal Antibody to Methadone
25	Calbioreagents	M263		Monoclonal Antibody to Methadone
26	Biospacific	A53140174P		Monoclonal Antibody to Methadone
27	Biospacific	A53141503P		Monoclonal Antibody to Methadone
28	Biospacific	A53143501P		Monoclonal Antibody to Methadone
29	Biospacific	A53144501P		Monoclonal Antibody to Methadone
30	Biospacific	A53151146P		Monoclonal Antibody to Methadone

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

Randox Methadone HRP Conjugate									
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**	**Ab#**	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
1	10	612344	0.1	221	10	10	1142326	0.2	404
	1	14033	12.7			1	6029	15.7	
	0.1	3570	5.5			0.1	3709	14.2	
	0	2771	12.7			0	2826	27.8	
2	10	919190	2.5	407	11	10	833687	6.8	290
	1	11439	3.5			1	60525	20.6	
	0.1	2641	2.9			0.1	6210	11.4	
	0	2260	16.6			0	2874	7.2	
3	10	2535	3.4	1	12	10	1127097	12.9	313
	1	1568	1.1			1	37299	9.7	
	0.1	1488	16.6			0.1	4813	10.7	
	0	1795	6.8			0	3601	4.3	
4	10	4035	20.6	3	13	10	10104	4.6	4
	1	2147	19.3			1	2369	7.7	
	0.1	2107	34.7			0.1	2814	0.9	
	0	1557	14.6			0	2768	4.5	
5	10	4695	7.3	2	14	10	1886	1.1	1
	1	2992	7.1			1	2266	4.2	
	0.1	3119	11.3			0.1	1832	16.6	
	0	2742	3.9			0	2371	20.2	
6	10	4522	6.7	1	15	10	2249	6.8	1
	1	3877	32.7			1	2062	15.2	
	0.1	3624	20.6			0.1	2257	4.5	
	0	3593	33.3			0	2739	4.5	
7	10	2658	9.1	1	16	10	666174	7.5	230
	1	2714	0.2			1	4562	16.2	
	0.1	2593	8.9			0.1	4123	2.4	
	0	2903	6.7			0	2898	16.1	
8	10	98172	2	42	17	10	2831	4.7	1
	1	28441	3.9			1	2851	0.5	
	0.1	2573	15.6			0.1	2878	0.5	
	0	2338	5.8			0	3164	2.1	
9	10	41971	5.3	21	18	10	658926	8.3	170
	1	2170	3			1	27631	8.4	
	0.1	1516	32.6			0.1	4511	11.6	
	0	1961	3.1			0	3868	10	

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

Randox Methadone HRP Conjugate									
Ab#	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**	**Ab#**	**[Ab] ug/mL**	**Mean**	**CV%**	**S/B**
19	10	13072	1.2	3	25	10	3117	3.1	1
	1	3329	9.8			1	3109	0.1	
	0.1	3640	1.6			0.1	2411	9.6	
	0	3796	2.7			0	2764	6.2	
20	10	5249	5.8	2	26	10	721392	0.1	283
	1	2304	0.5			1	16663	3.8	
	0.1	2419	12.8			0.1	3231	0.4	
	0	3013	0.1			0	2546	12.3	
21	10	334679	29.1	162	27	10	2961	10.7	1
	1	5963	0.3			1	2535	3.6	
	0.1	2306	1.3			0.1	2297	7.1	
	0	2069	1.8			0	2320	7.5	
22	10	1878476	3.3	809	28	10	2634	8.1	1
	1	116465	11.3			1	2673	14.3	
	0.1	10680	63			0.1	2416	2.6	
	0	2323	25			0	2668	6.4	
23	10	1024954	5.4	379	29	10	10759	7.1	4
	1	24932	10.1			1	2984	4.5	
	0.1	3688	2.7			0.1	2902	16.7	
	0	2702	6.8			0	2827	14.3	
24	10	25623	2.1	7	30	10	3912	1	1
	1	5115	15.4			1	3372	0.6	
	0.1	3833	22			0.1	3446	13.9	
	0	3900	18.1			0	3792	22.1	

1.3 Competitive Assay Screen (Theranos 3.0 System)

The ten anti-methadone monoclonal antibodies were evaluated for their performance in the competitive format by coating the capture surface with an avidin-biotin labeled goat anti-mouse secondary antibody combination. Following this each of the unlabeled anti-methadone antibodies were mixed into the sample along with the Theranos methadone -AP conjugate at a 1:10 sample dilution and tested for response in a competitive assay using BioRad urine toxicology controls. Antibody 12 and 23 showed a dose response in the competitive assay and were chosen for further evaluation. Data summarized in Table 6.

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

Ab#	[Methadone] ng/ml	Inter-Cartridge Mean	Inter-Cartridge CV%	S/B	Ab#	[Methadone] ng/ml	Inter-Cartridge Mean	Inter-Cartridge CV%	S/B
1	750	1627	8.8	1.9	18	750	1611	14.5	4.6
	375	1751	3			375	1558	21.4	
	225	1696	10.6			225	1758	5.9	
	0	3166	57.6			0	7382	1.7	
2	750	1150	4.8	1.6	21	750	1172	1.7	6.5
	375	1281	6			375	1512	10	
	225	1358	4.3			225	1417	0	
	0	1854	30.5			0	7567	2.7	
10	750	1012	0.6	1.0	22	750	1399	24.4	1.2
	375	903	0.2			375	2950	14.4	
	225	1309	3.9			225	2616	2.9	
	0	969	19.5			0	1613	2	
11	750	1309	28.5	6.2	23	750	1340	2	12.8
	375	1182	12.9			375	1375	4.9	
	225	1178	33.2			225	1507	3.1	
	0	8168	6.2			0	17196	3.8	
12	750	1610	9.4	12.0					
	375	1554	22.2						
	225	1625	5.4						
	0	19306	7.5						

1.4 Test coating formats

Three different coating formats were tested : (i) Anti-mouse secondary antibody direct coat followed by anti-methadone antibody, (ii) Avidin followed by biotin labeled anti mouse secondary antibody followed by anti-methadone antibody and (iii) Avidin followed by biotin labeled anti methadone antibody. These three methods were compared to mixing the secondary antibody in solution in a homogenous competitive assay. The best dose reposnse was seen when the anti-methadone antoibody was placed in solution, followed by coating method 2 and 1 on the surface. In order to simplify the assay format for easier multiplexing in the drugs screening panels method 2 was finalized as the coating format. Ab#12 was chosen as the capture antibody for further optimization. Ab#23 would be a candidate backup. The Theranos methadone AP conjugate was used at a 1:50,000 fold dilution for each of these evaluations. The calibrators used were methadone buffer calibrators. Results are summarized in Table 7 and Table 8 for Ab 12 and Ab23 respectively.

Table [SEQ Table * ARABIC]: Results of different coating formats for Ab#12

Ab#12	Method 1				Method 2				Method 3				Ab in solution			
[Methadone] ng/ml	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.
1000.0	45227	2	24	1.6	55758	1	28	1.8	545121	26	3	1.1	13724	3	81	1.3
750	71001	28	15.2	1.6	100701	0.6	15	1.5	581249	7	2.5	1.7	18255	41	61	1.4
300	111488	15	9.7	1.9	153881	1.2	10	2.1	983907	15	1.5	1.2	24727	14	45	1.7
150.0	211901	3	5.1	2.2	315733	10	4.9	2.0	1220005	17	1.2	1.2	42462	6	26	3.2
40	473674	6	2.3	2.3	635690	13	2.4	2.4	1522390	7	1.0	1.0	134880	15	8	8.3
0	1081825	17	1.0	0.0	1540450	2	1.0	0.0	1469616	4	1.0	0.0	1116511	8	1	0.0

Table [SEQ Table * ARABIC]: Results of different coating formats for Ab#23

Ab#23	Method 1				Method 2				Method 3				Ab in solution			
[Methadone] ng/ml	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.
1000.0	144390	1	10	1.2	61243	4	25	1.3	760869	18.1	2.4	1.2	14542	10	66	1.0
750	178623	26	8	2.1	78846	8.6	19	2.0	893426	1	2.0	1.5	15177	25	63	2.0
300	369240	14	4.1	1.1	154814	0.0	10	2.1	1305123	6	1.4	1.3	30083	4	32	1.9
150.0	418647	33	3.6	2.8	324526	13	4.6	2.0	1647503	3	1.1	1.1	56741	1	17	2.6
40	1169743	14	1.3	1.3	646341	23	2.3	2.3	1818245	4	1.0	1.0	147214	36	7	6.5
0	1504821	12	1.0	0.0	1506272	3	1.0	0.0	1811938	3.5	1		958826	13	1	0.0

1.5 Checkerboard titration of Primary antibody on surface and methadone AP conjugate

Ab#12 was coated following method 2 outlined in section 1.4 at 10, 25, 50 and 100 ng/mL. The dose response and point to point modulation were tested at several combinations of the capture antibody and the tracer, Theranos methadone AP as outlined in Table 9. The highest S/B and modulation was seen at the lowest capture antibody concentration of 10 ng/mL and a dilution of 1:50,000 of the Theranos methadone AP conjugate.

Table [SEQ Table * ARABIC]: Checkerboard titration

		10 ng/mL				25 ng/ml				50 ng/mL				100 ng/ml			
[Methadone] ng/ml	Theranos Methadone AP conjugate	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.
1000.0		3388	39	87	3.2	9172	24	62	2.3								
300	1:25,000	10732	9	27	4.3	21170	22	27	6.9	nd				nd			
40.0		46095	30	6	6.4	146618	33	4	3.9								
0		294392	17	1	0.0	572913	1	0	0.0								
1000.0		1719	0	151	4.4	7344	12	60	2.4	13148	16	57	2.6	27499	13	40	1.3
300	1:50,000	7511	12	35	4.5	17725	0	25	3.7	34215	7	22	5.6	36745	8	30	9.0
40.0		33601	2	8	7.7	65827	11	7	6.7	190895	14	4	3.9	330078	18	3	3.4
0		259714	20	1	0.0	440991	38	1	0.0	753361	25	0	0.0	1107867	3	1	0.0
1000.0										1870	14	63	2.0	1490	53	68	2.3
300	1:500,000	nd				nd				3679	2	32	5.8	3465	16	29	8.0
40.0										21482	33	5	5.5	27868	18	4	3.6
0										117593	18	1	0.0	101308	19	1	0.0

1.6 Comparison of two Theranos Methadone AP conjugates

Two versions of the methadone AP conjugates were tested to check which one provided the best S/B and overall modulation in the assay in its currently optimized form. Conjugate A had a F/P ratio of 3.8 and conjugate B had a F/P ratio of 7.0. Two working concentrations of the each conjugate at 1:50,000 and 1:100,000 were tested. The concentration of the anti-methadone antibody was 10 ng/mL on the surface. Methadone assay buffer calibrators were used and the overall sample dilution as kept at 25 –fold. Data are summarized in Table 10. Conjugate A provided the best overall modulation and S/B at both the above working concentrations. This conjugate was finalized for the rest of the assay optimization.

Table [SEQ Table * ARABIC]: Comparison of two Theranos Methadone AP conjugates

Conjugate A	**1:50,000 dilution**				**1:100,000 dilution**			
[Methadone] ng/ml	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Modn.**	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Modn.**
1000.0	707	37	148	2.4	480	12	121	2.3
300	1693	12	62	10.4	1106	28	53	6.0
40.0	17659	5	6	5.9	6625	18	9	8.8
0	104720	15	1	0.0	58137	34	1	0.0
Conjugate B	**1:50,000 dilution**				**1:100,000 dilution**			
[Methadone] ng/ml	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Modn.**	**Inter-Cartridge Mean**	**CV%**	**S/B**	**Modn.**
1000.0	1394	21	105	2.4	1195	7	87	1.8
300	3376	8	43	7.7	2137	5	48	6.6
40.0	26063	28	6	5.6	14052	39	7	7.4
0	145708	22	1	0.0	103438	0	1	0.0

1.7 Effect of sample dilution using different matrices

The Theranos Methadone assay needs to be able to detect methadone in serum, plasma and urine. The target cut off for serum /plasma is 40 ng/ml and for urine matrix is 300 ng/ml. Given the different matrices that will be sampled and cutoff requirements for each matrix, the effect of sample dilution was tested individually in buffer, plasma and urine matrices. The goal of the experiment was to find a sample dilution factor that would nullify any matrix effects thereby enabling the use of a single matrix (example, buffer) for calibration. Data are summarized in Table 11. Sample dilutions of 25-fold, 50-fold and 100-fold were tested. The modulation and S/B at 100 fold sample dilution was still high enough so this was picked as the final sample dilution. All three matrices performed equally in the assay at this condition so the assay buffer matrix was finalized for calibration purposes.

Table [SEQ Table * ARABIC]: Effect of sample dilution using different matrices

		25x sample dilution				50x sample dilution				100x sample dilution			
[Methadone] ng/ml	Matrix	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.	Inter-Cartridge Mean	CV%	S/B	Mod.
1000.0	Buffer	1719	0	151	4.4	2495	6	66	2.7	5022	26	36	2.9
300		7511	21	35	4.5	6753	9	24	5.5	14775	32	12	5.0
40.0		33601	2	8	7.7	37384	37	4	4.4	74369	4	2	2.4
0		259714	20	1	0.0	163735	16	1		180179	11	1	
1000.0	Urine	1783	21	123	2.8	3067	14	71	2.5	5032	6	41	2.4
300		5079	1	43	5.1	7515	2	29	7.3	11880	9	17	5.5
40.0		25678	2	9	8.5	54974	11	4	4.0	65211	20	3	3.1
0		219022	38	1		219727	9	1		205402	2	1	
1000.0	Serum	1592	38	152	2.5	3277	33	67	2.4	4708	3	59	2.6
300		4000	23	60	4.6	6211	37	35	7.1	12292	5	23	5.7
40.0		18471	7	13	13.1	44218	2	5	6.4	70023	20	4	4.0
0		241466	12	1		284337	4	1		227129	8	1	

1.8 Effect of conjugate stabilizer

The Methadone AP conjugate was formulated in 3 different AP conjugate stabilizer and the dose response was evaluated as shown in Table 12. The Theranos Small Moleculae AP Conjugate Stabilizer showed the best modulation between the top two calibrator levels. It was finalized as the AP conjugate stabilizer.

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

	Theranos Small molecule AP conjugate stabilizer				StabilZyme				Biostab			
[Methadone] ng/ml	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.
1000.0	5022	26	36	2.9	8677	11	46	1.8	5229	3	49	2.4
300	14775	32	12	5.0	15318	19	26	7.6	12537	1	21	6.5
40.0	74369	4	2.4	2.4	116137	45	3	3.4	81290	20	3	3.2
0	180179	11	1		395703	18	1		258621	16	1	

1.9 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 slightly lower at shorter incubation times and the relative counts were also low. The control condition of 10x10 reagent incubation times was finalized.

Table [SEQ Table * ARABIC]: Effect of reagent incubation time

	10'x10'				5'x5'				2'x1'			
[Methadone] ng/ml	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.	Inter-Cartridge Mean	CV%	S/B	Modn.
1000.0	5022	26	36	2.9	1435	4	18	2.1	262	9	25	2.2
300	14775	32	12	5.0	3038	10	8	6.4	581	11	11	4.6
40.0	74369	4	2.4	2.4	19380	11	1.3	1.3	2647	6	3	2.5
0	180179	11	1		25205	31	1		6659	11	1	

1.10 Determination of LLOQ and ULOQ and comparison of matrices

Methadone was spiked into assay buffer, normal serum and normal urine to construct 8 point standard curves. Table 14 describes the assay buffer calibration curve details and the calibration parameters are summarized in Table 15. Figure 1 shows the calibration curve plot with the curve fit. The target LLOQ and ULOQ of 40 ng/ml and 1000 ng/mL were met and the accuracies and precision were within the FDA criteria.

Table [SEQ Table * ARABIC]: Assay buffer calibration for Methadone assay

Conc = b3 * (((b2 - b1) / (RLU - b1)) - 1) ^ (1 / b4)

[Methadone] ng/ml	RLU Inter-Cartridge Mean	RLU Inter-Cartridge CV%	S/B	Mod.	Conc. ng/ml Inter-Cartridge Mean	Conc. ng/ml Inter-Cartridge CV%	% Recovery
1000	4303	6.2	51	1.1	1008	7.9	101
750	4863	3.5	45	1.9	867	4.0	116
500	9295	16.0	23	1.2	410	18.4	82
300	11022	13.4	20	2.1	329	15.6	110
150	22804	26.0	10	2.3	143	25.0	95
40	51577	15.8	4.2	1.8	45	23.4	113
20	93241	6.0	2.3	2.3	17	16.3	86
0	218020	35.9	1	0.0	OORL		

Table [SEQ Table * ARABIC]: Calibration curve parameters

LLOQ	40	ng/mL
ULOQ	1000	ng/mL
LLOQ accuracy	113	%
LLOQ precision	23.4	%
ULOQ accuracy	101	%
ULOQ precision	7.9	%

Using the buffer based calibration curves the recoveries for a spiked methadone urine and normal serum curves were computed. These results are summarized in Tables 16 and 17. In both cases the back calculated concentrations were within 20% of nominal. The Theranos assay will be a qualitative assay. For urine , results > and = 300 ng/mL would be positive (shaded in red) and < 300 ng/ml would be negative (shaded in green). For serum samples, results of 40 ng/mL and greater would be positive (shaded in red) and < 40 ng/ml would indicate a negative result (shaded in green). It is clear from this exercise that a buffer based calibration could be applied to calculate results in both serum and urine matrix.

Figure [SEQ Figure * ARABIC]: Methadone Standard Curve in Assay Buffer

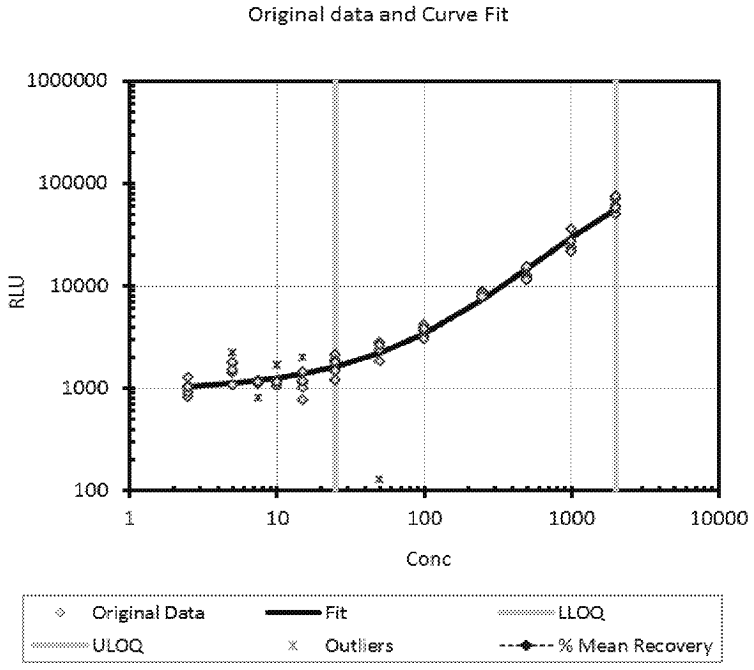


Table [SEQ Table * ARABIC]: Methadone spiked urine standard curve

[Methadone] ng/ml	RLU Inter-Cartridge Mean	RLU Inter-Cartridge CV%	S/B	Mod.	Conc. ng/ml Inter-Cartridge Mean	Conc. ng/ml Inter-Cartridge CV%	% Recovery	Theranos Result
1000	3913	10.1	60	1.4	1150	12.7	115	Pos
750	5474	7.6	43	1.7	747	9.9	100	Pos
500	9237	10.7	26	1.2	403	15.5	81	Pos
300	10938	19.0	22	2.1	340	23.1	113	Pos
150	23260	3.9	10	2.5	129	3.6	86	Neg
40	58546	14.6	4.0	1.9	38	22.3	94	Neg
20	114129	34.2	2.1	2.1	OORL			Neg
0	236347	6.1	1	0.0	OORL			Neg

Conc = b3 * (((b2 - b1) / (RLU - b1)) - 1) ^ (1 /b4)

Table [SEQ Table * ARABIC]: **Methadone spiked serum standard curve**

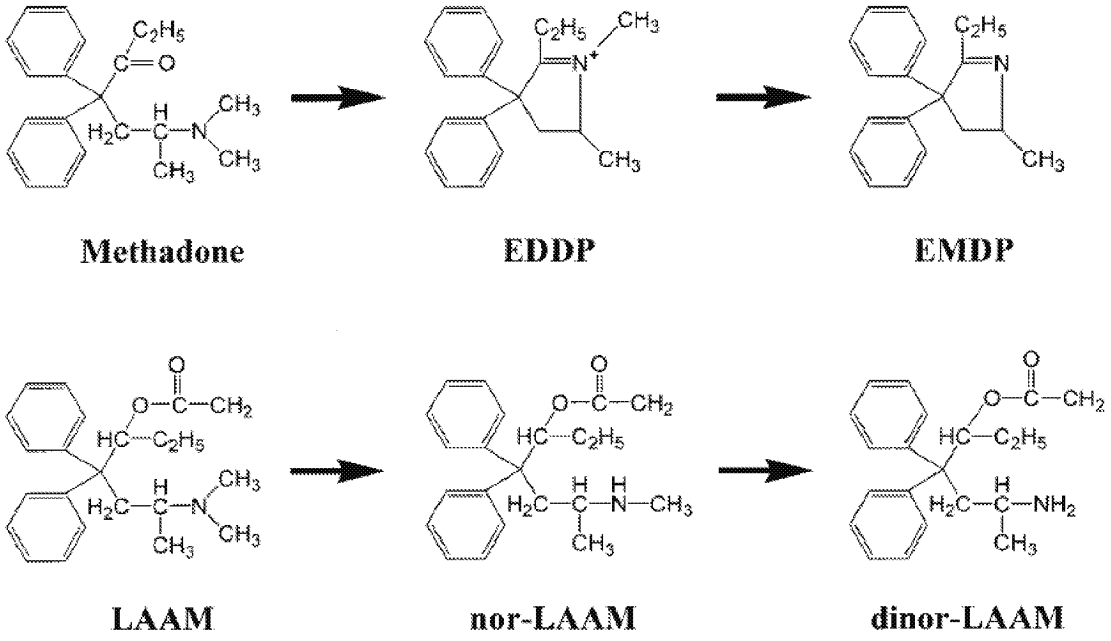
[Methadone] ng/ml	RLU Inter-Cartridge Mean	RLU Inter-Cartridge CV%	S/B	Mod.	Conc. ng/ml Inter-Cartridge Mean	Conc. ng/ml Inter-Cartridge CV%	% Recovery	Theranos Result
1000	5113	13.8	40	1.1	830	17.6	83	Pos
750	5867	24.0	35	1.6	730	22.5	97	Pos
500	9306	9.2	22	1.4	402	7.8	80	Pos
300	13285	11.2	15	1.5	257	11.3	86	Pos
150	19693	27.2	10	2.8	166	28.0	111	Pos
40	54901	4.1	4	2.7	39	5.6	98	Pos
20	148612	17.6	1	1.4	OORL			Neg
0	204396	15.2	1	0.0	OORL			Neg

Conc = b3 * (((b2 - b1) / (RLU - b1)) - 1) ^ (1 / b4)

1.11 Cross reactivity with Methadone analogs

Methadone is predominantly *N*demethylated and undergoes spontaneous cyclization to produce 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine(EDDP), which is subsequently *N*-demethylated to 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) (Fig. 2) These two metabolites are devoid of opiate activity. *l*-α-Acetylmethadol (LAAM) is an analog of methadone, characterized by longer duration of effectiveness when administered every 2 or 3 days. LAAM is sequentially *N*-demethylated to form *l*-α-acetyl-*N*-normethadol (nor-LAAM) and *l*-α-acetyl-*N,N*-dinormethadol (dinor-LAAM) (Fig. 2). The Theranos Methadone assay was tested for cross reactivity against EDDP and EMDP. LAAM and its metabolites were not available for testing.

Figure [SEQ Figure * ARABIC]: Methadone metabolites



The Methadone metabolites EDDP and EMDP were first tested at 1000 ug/mL to see the upper limit of cross reactivity. At this level both analogs tested positive on the Theranos methadone assay. The analogs were then titrated to several levels below 1000 ug/mL to see at which concentration they would test negative for the 300 ng/ml cut off. The data are summarized in Table 18 and Table 19. Concentrations of EMDP and EMDP showing a negative response to the 300 ng/ml cut off were 50 and 100 ug/mL.

Table [SEQ Table * ARABIC]: Cross reactivity with EMDP

		RLU		Conc. ng/mL			
[EMDP] ug/ml	[EMDP] ng/ml	Inter-Cartridge Mean	CV%	Mean	CV%	% Cross reactivity	Theranos Result
1000	1000000	1033	1.7	7338	4.1	OORH	Pos
500	500000	1473	9.1	4336	7.6	OORH	Pos
100	100000	7042	18.2	573	23.5	0.6	Pos
50	50000	12843	8.0	275	10.5	0.6	Neg
5	5000	85455	26.7	25	39.2	0.5	Neg
1	1000	132382	18.0	OORL			Neg
0	0	218020	35.9	OORL			Neg

Table [SEQ Table * ARABIC]: Cross reactivity with EDDP

		RLU		Conc. ng/mL			
[EDDP] ug/ml	[EDDP] ng/ml	Inter-Cartridge Mean	CV%	Mean	CV%	% Cross reactivity	Theranos Result
1000	1000000	2744	17.1	1866	26.9	0.2	Pos
500	500000	4242	6.7	1027	8.4	0.2	Pos
100	100000	11999	12.5	294	14.2	0.3	Neg
50	50000	22899	10.6	131	15.1	0.3	Neg
5	5000	102549	0.0	14	0.3	0.3	Neg
1	1000	177977	8.8	OORL			Neg
0	0	218020	35.9	OORL			Neg

1.12 Cutoff verification and Cross reactivity with other drugs-of-abuse controls

In order to verify cut offs and calibration commercially available urine drugs-of-abuse controls were tested. All these controls had reported values for methadone. As can be seen from Table 20 there was good correlation between the Theranos results and the reported values of methadone for these controls with recoveries within 20% of the reported values. A separate set of commercially available controls that included several other classes of drugs (amphetamines, cannabinoids, cocaine etc.) were also tested in the assay to check for cross reactivity. The results are presented in Table 21. No cross reactivity was detected against any of these drug panels except the cocaine panel which had high levels of methadone for which the results were positive.

Table [SEQ Table * ARABIC]: Cut off verification

Control	Reported Level of Methadone	RLU Inter-Cartridge		Conc. ng/mL Inter-Cartridge		% Recovery	Theranos Result
	ng/ml	Mean	CV%	Mean	CV%		
BioRad Urine toxicology S1	225	17503	13.7	183	17.3	81	Neg
BioRad Urine toxicology S2	375	8285	11.0	453	13.8	121	Pos
BioRad Urine toxicology S3	750	5301	9.9	779	11.9	104	Pos
BioRad Urine toxicology Neg	50	188042	15.2	OORL			Neg
BioRad Urine Ctrl Q. Positive	694	5417	2.8	781	6.1	113	Pos
Synerhent controls S1	207	13770	4.2	242	4.8	117	Neg
Synerhent controls S2	394	10170	12.3	356	14.4	90	Pos
Synerhent controls S3	418	8414	15.3	480	21.6	115	Pos
Synerhent controls S4	897	4385	12.5	1073	19.1	120	Pos
Synerhent controls S0	0	136689	14.2	OORL			Neg

Table [SEQ Table * ARABIC]: Cross reactivity with other classes of drugs

		DOA Levels from Zeptometrix Datasheet							Theranos	
Name of Panel	Panel Member #	Amphetamines	Barbiturates	Benzodiazepines	Cannabinoid	Cocaine	Oxycodone	Methadone	[Methadone] ng/mL	Result
Amphetamine	1	5,304 (Pos)	Neg (<270)	Neg (<270)	Neg (<45)	Neg (<125)	1,286(Pos)	Neg (<270)	OORL	Neg
	4	1,500 (Pos)	Neg (<270)	Neg (<270)	Neg (<45)	Neg (<125)	Neg	Neg (<270)	193	Neg
	6	5,118 (Pos)	Neg (<270)	Neg (<270)	Neg (<45)	Neg (<125)	Neg	Neg (<270)	90	Neg
Benzodiazepine	9	Neg (<450)	Neg (<270)	3,032 (Pos)	Neg (<45)	Neg (<125)	1,460 (Pos)	Neg (<270)	OORL	Neg
Barbiturate	5	Neg (<450)	1,267(Pos)	Neg (<270)	46(Pos)	Neg (<125)	Neg	Neg (<270)	252	Neg
Oxycodone	1	Neg (<450)	Neg (<270)	Neg (<270)	Neg (<45)	Neg (<125)	1,316 (Pos)	Neg (<270)	OORL	Neg
Cocaine	2	Neg (<450)	Neg (<270)	Neg (<270)	Neg (<45)	1,468 (Pos)	Neg	1,279 (Pos)	OORH	Pos
	4	4,659 (Pos)	Neg (<270)	3,465 (Pos)	Neg (<45)	721(Pos)	1, 231 (Pos)	751(pos)	756	Pos
	5	Neg (<450)	Neg (<270)	Neg (<270)	Neg (<45)	903(Pos)	Neg	Neg (<270)	OORL	Neg
	9	Neg (<450)	Neg (<270)	Neg (<270)	Neg (<45)	1,984 (Pos)	Neg	1,476 (Pos)	OORH	Pos
	10	Neg (<450)	Neg (<270)	Neg (<270)	Neg (<45)	Neg (<125)	Neg	1,329 (Pos)	OORH	Pos

1.13 Normal Donor Screen

10 normal donor urine samples and 10 normal EDTA plasma samples were screened on the Theranos methadone assay. All 20 samples were OORL (out of range low) and yielded a negative result on the Theranos assay (Table 22 and 23).

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

Urine Sample ID	Inter-Cartridge RLU		[Methadone] ng/mL	Theranos Result
	Mean	CV%		
U1	203088	10.8	OORL	Neg
U2	162161	15.9	OORL	Neg
U3	145606	16.8	OORL	Neg
U4	201981	18.4	OORL	Neg
U5	177154	11.9	OORL	Neg
U6	182445	33.5	OORL	Neg
U7	165293	19.1	OORL	Neg
U8	214530	5.4	OORL	Neg
U9	227228	22.0	OORL	Neg
U10	191554	4.9	OORL	Neg

Table [SEQ Table * ARABIC]: Normal donor EDTA plasma sample screen

EDTA plasma Sample ID	Inter-Cartridge RLU		[Methadone] ng/mL	Theranos Result
	Mean	CV%		
F1	202914	8.8	OORL	Neg
F2	177657	2.3	OORL	Neg
F3	141508	21.5	OORL	Neg
F4	163636	4.3	OORL	Neg
F5	201730	13.6	OORL	Neg
M22	146175	29.7	OORL	Neg
M23	195616	19.9	OORL	Neg
M24	205756	35.1	OORL	Neg
F16	258448	15.4	OORL	Neg
F17	228770	20.6	OORL	Neg

1.14 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 methadone does not concentrate into plasma. The result measured in the plasma is about the same as the one measured in whole blood. Data are summarized in Table 24.

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

	Spiked whole blood recovery						
[Methadone] ng/ml	Inter-Cartridge RLU		S/B	Modn.	Inter-Cartridge Conc.		% Recovery
	Mean	CV%			Mean	CV%	
1000	9320	9.0	27	1.3	826	9.8	83
750	11825	9.3	21	1.4	633	10.8	84
500	16166	9.6	16	1.2	448	10.0	90
300	19877	8.8	13	2.2	356	9.9	119
150	42943	18.5	5.9	2.6	148	19.1	99
40	109683	7.4	2.3	1.9	37	13.9	93
20	203906	15.3	1.2	1.2	OORL		
0	253549	11.8	0	0.0	OORL		
	Plasma Generated from Spiked whole blood recovery						
[Methadone] ng/ml	Inter-Cartridge RLU		S/B	Modn.	Inter-Cartridge Conc.		% Recovery
	Mean	CV%			Mean	CV%	
1000	8313	4.8	38	1.2	936	5.3	94
750	9682	3.7	33	1.4	785	4.0	105
500	13737	3.7	23	1.7	534	4.1	107
300	22938	8.6	14	1.7	310	8.1	103
150	39927	7.8	8	2.7	158	9.5	105
40	108546	4.9	3	1.3	38	9.6	95
20	137463	25.7	2.3	2.3	OORL		
0	319680	12.9	0	0.0	OORL		

1.15 Final calibration

At this stage of assay development the coating format was changed from Method 2 to Method 1 as described in section 1.4. This constituted a minor change in the presentation of the secondary antibody on the surface. As can be seen from Table 7 the two methods had identical modulation. A new set of reagents was manufactured and was used to produce a calibration curve described in Table 25 and Figure 3. The target LLOQ and ULOQ of 40 and 1000 ng/ml were met. The accuracy and precision data are summarized in Table 26. This lot of reagents was used for the remaining sets of experiments.

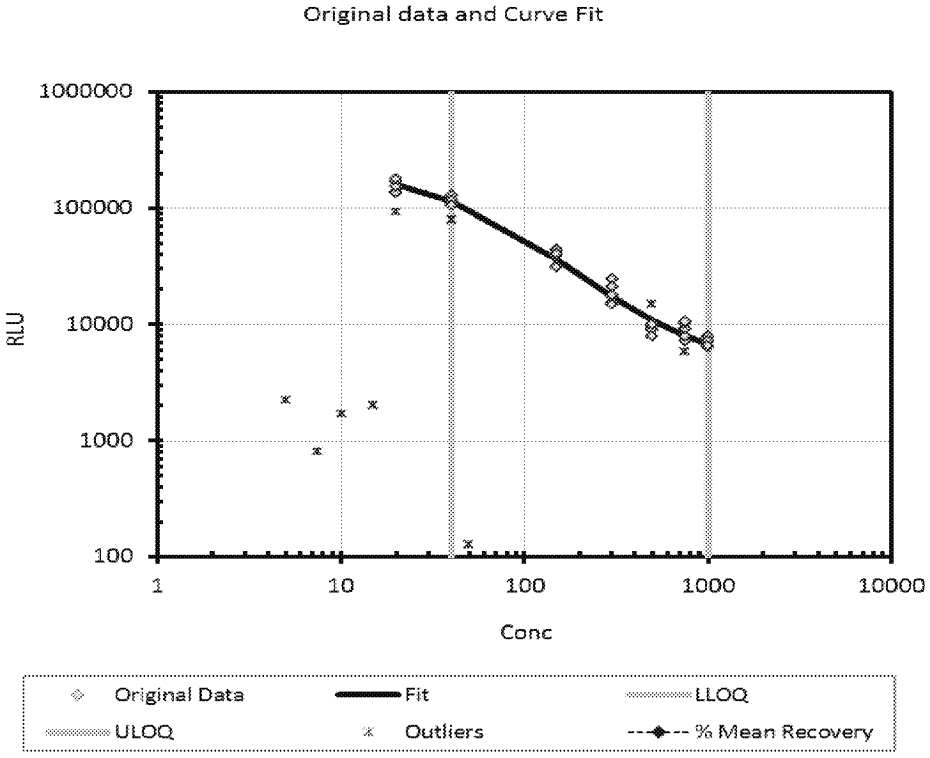
Table [SEQ Table * ARABIC]: Final calibration curve data

[Methadone]	Inter-Cartridge RLU		S/B	Modn.	Back calc.Conc.		%
ng/ml	Mean	CV%			Mean	CV%	Recovery
1000	7148	9.8	34	1.2	928	17.6	93
750	8723	15.4	28	1.2	701	23.5	93
500	10120	17.9	24	1.8	582	18.6	116
300	18574	20.3	13	2.1	290	17.5	97
150	38337	15.7	6	3.0	145	16.0	97
40	115326	9.3	2.1	1.3	40	15.2	100
20	151900	12.6	1.6	1.6	OORL		
0	240835	9.1	1		OORL		

Table [SEQ Table * ARABIC]: Final calibration curve parameters

LLOQ	40	ng/mL
ULOQ	1000	ng/mL
LLOQ accuracy	100	%
LLOQ precision	15.2	%
ULOQ accuracy	93	%
ULOQ precision	17.6	%

Figure [SEQ Figure * ARABIC]: Methadone final calibration curve



1.16 Interference Test for RF and HAMA positive samples

The Theranos methadone assay was tested for interference from RF positive and HAMA positive samples. 5 samples of each type were tested on the Theranos system. The results for all 10 samples were negative. The data are summarized in Table 27 and Table 28.

Table [SEQ Table * ARABIC]: RF positive samples

RF Pos samples	Inter-Cartridge RLU		% Recovery	Theranos Result
	Mean	CV%		
R1	315317	13.9	OORL	Negative
R2	377093	14.2	OORL	Negative
R3	274733	2.5	OORL	Negative
R4	292511	3.0	OORL	Negative
R5	379756	12.0	OORL	Negative

Table [SEQ Table * ARABIC]: HAMA positive samples

HAMA Pos samples	Inter-Cartridge RLU		% Recovery	Theranos Result
	Mean	CV%		
H1	358838	19.5	OORL	Negative
H2	344812	14.5	OORL	Negative
H3	306179	21.4	OORL	Negative
H4	358458	15.5	OORL	Negative
H5	341186	13.1	OORL	Negative

1.17 Interference matrices test

Hemolyzed, lipemic and icteric serum samples were obtained from a commercial source. The recovery of methadone spiked into these potentially interfering matrices was evaluated on the Theranos System (Table 29). The assay did not show any interference from icteric and lipemic samples. The assay showed lower than optimal recoveries (68-86%) with the hemolyzed sample suggesting that grossly hemolyzed samples might interfere with the assay.

Table [SEQ Table * ARABIC]: Interfering matrices test

Nominal Spiked [Methadone] ng/mL	Lipemic						Hemolyzed						Icteric					
	RLU		Conc. ng/ml		%	Theranos	RLU		Conc. ng/ml		%	Theranos	RLU		Conc. ng/ml		%	Theranos
	Mean	CV%	Mean	CV%	Rec.	Result	Mean	CV%	Mean	CV%	Rec.	Result	Mean	CV%	Mean	CV%	Rec.	Result
1000	7775	6.3	812	12.4	81	Positive	7444	1.7	856	2.2	86	Positive	7760	11.9	816	18.8	82	Positive
300	19394	4.1	270	3.9	90	Positive	23528	18.5	229	16.3	76	Positive	22159	9.3	239	8.1	80	Positive
40	130355	11.4	33	21.4	82	Negative	142345	10.0	27	22.5	68	Negative	109674	8.7	44	14.6	111	Positive
0	373575	19.8			OORL	Negative	250187	16.9			OORL	Negative	321656	16.0			OORL	Negative

1.18 Cross reactivity with more drugs

The following drugs: aspirin, ibuprofen, caffeine, cotinine, chlorpromazine, trimipramine, imipramine and acetaminophen were tested on the assay. None of them displayed any cross reactivity with the assay (Table 30).

Table [SEQ Table * ARABIC]: Cross reactivity testing with more drugs

Drugs	Concentration	Inter-Cartridge		%	Theranos
Conc tested	tested, ug/mL	Mean	CV%	Recovery	Result
Acetaminophen	1000	273372	7.7	OORL	Negative
Caffeine	1000	258230	11.2	OORL	Negative
Ibuprofen	1000	257519	10.3	OORL	Negative
Aspirin	1000	286151	9.2	OORL	Negative
Cotinine	100	198263	7.9	OORL	Negative
Chlorpromazine	100	193872	1.4	OORL	Negative
Trimipramine	100	167180	12.9	OORL	Negative
Imipramine	100	282711	3.6	OORL	Negative

1.19 Clinical Sample Correlation

Due to the non-availability of samples that were positive for methadone from a commercial source, methadone was spiked into individual urine or plasma samples across the range of the assay above and below the cutoff values for the respective matrices. A set of 20 samples were constructed in this manner for the urine matrix and a similar set was made using plasma/serum. The urine samples were sent to the CLIA lab for testing on the Siemens Advia methadone assay. There was excellent correlation of the back calculated concentrations to the reported values from the Siemens Advia (Table 31). The Theranos assay being a qualitative assay the results (positive or negative) also tracked well. For the plasma/serum samples no predicate was available. The recoveries were within 20% of the nominal spiked concentration for all the samples tested (Tables 32 and 33).

Table [SEQ Table * ARABIC]: Clinical sample correlation for urine samples

Sample ID	CLIA Advia Results ng/ml	Theranos Result Mean ng/mL		% Recovery
U1	1286	OORH	Positive	OORH
U2	913	OORH	Positive	OORH
U3	700	898	Positive	128
U4	671	762	Positive	114
U5	551	575	Positive	104
U6	386	416	Positive	108
U7	289	248	Negative	86
U8	271	284	Negative	105
U9	217	216	Negative	100
U10	199	236	Negative	119
U11	685	727	Positive	106
U12	587	577	Positive	98
U13	446	471	Positive	105
U14	454	370	Positive	81
U15	406	372	Positive	92
U16	334	321	Positive	96
U17	184	196	Negative	107
U18	157	170	Negative	108
U19	151	163	Negative	108
U20	130	91	Negative	70
U21		749	Positive	75
U22		355	Positive	118

Table [SEQ Table * ARABIC]: Spike recovery of N=10 EDTA plasma samples

Sample ID	Nominal [Methadone] ng/ml	Inter-Cartridge RLU		Inter-Cartridge Conc. ng/ml		% Recovery	Theranos Result
		Mean	CV%	Mean	CV%		
P1	1200	6684	7.9	1048	13.8	87	Positive
P2	950	8297	11.0	742	15.2	78	Positive
P3	700	10397	8.4	534	10.0	76	Positive
P4	550	12294	6.8	433	8.1	79	Positive
P5	375	17688	5.4	295	5.3	79	Positive
P6	225	27217	10.2	198	10.2	88	Positive
P7	155	41133	31.4	140	26.4	90	Positive
P8	65	88034	13.4	61	15.7	93	Positive
P9	40	100276	2.5	50	3.6	124	Positive
P10	15	172327	16.8			OORL	Negative

Table [SEQ Table * ARABIC]: Spike recovery of N=10 EDTA plasma samples

Sample ID	Nominal [Methadone] ng/ml	Inter-Cartridge RLU		Inter-Cartridge Conc. ng/ml		% Recovery	Theranos Result
		Mean	CV%	Mean	CV%		
S1	1000	7565	11.4	852	17.2	85	Positive
S2	835	8619	7.1	687	10.6	82	Positive
S3	650	8382	9.9	721	15.0	111	Positive
S4	425	13018	22.7	427	22.4	100	Positive
S5	250	20770	23.6	265	26.0	106	Positive
S6	100	48983	3.1	113	2.9	113	Positive
s7	75	61940	9.0	90	9.5	120	Positive
S8	60	76236	6.3	71	6.9	118	Positive
S9	40	126787	14.3	34	26.7	86	Negative
S10	35	116431	4.1	39	6.8	113	Negative
S11	25	136997	6.3	29	14.5	OORL	Negative
S12	10	195638	27.0			OORL	Negative