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Marijuana (THC)
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"]

The principle psychoactive constituent of Marijuana (DEA Controlled Schedule I substance) is tetrahydrocannabinol (THC), also known as delta-9-tetrahydrocannabinol (Δ9-THC). 11-*nor*-9-carboxyl-THC, also known as 11-*nor*-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH, THCA) is the major metabolite of THC. Urine contains predominantly THC-COOH, hair contains primarily THC. Blood contains both substances with the relative amounts depends on the recency and extent of usage.

This assay is developed to quantitatively determine the concentration of THC by detecting its metabolite THCA in human urine and serum. The assay can be used as a qualitative screening assay with positive cutoff at 50ng/mL in urine.

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

Siemens ADVIA THC test (urine) provided by CLIA Lab has been used as a predicate method.

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

The assay is an enzyme-linked competitive immunoassay. Goat anti-mouse IgG biotin conjugate coated on ultra avidin serves as the capture surface. Then the primary capture antibody mouse anti-THC monoclonal antibody was coated on capture surface. The sample is diluted and mixed with an alkaline phosphatase-labeled THCA conjugate (THCA-GMBS-AP). The reaction mixture is incubated with the THC antibody coated on capture surface, unconjugated THCA in the sample competes with AP conjugated THCA (THCA-GMBS-AP) for a limited number of antibody binding sites. The surface is then washed and THCA (both AP conjugated and unconjugated) that binds to the anti-THC antibody is bound on the capture surface. Finally, the alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

A greater amount of THCA in the sample results in a lower binding of the alkaline phosphatase-labeled THCA conjugate (THCA-GMBS-AP) to the capture antibody. Thus the signal generated by the assay is inversely proportional to the concentration of THCA in the sample. The higher the RLU indicates the lower level of THCA in the sample.

Table [SEQ Table * ARABIC]: Materials and Reagents

Name	Supplier	Catalog #
Goat Anti-Mouse IgG Biotin Conjugate	Thermo Scientific	31805
Mouse Anti-THC Antibody	US Biological	T2970-01B
Alkaline Phosphatase-labeled THCA Conjugate (THCA-GMBS-AP)	Theranos	Batch# THCA-GMBS-AP_01_030613
Alkaline Phosphatase Substrate	Theranos	Lot#19032013GT-ALP-A
0.03% BSA Block Buffer (0.03% BSA in	Sigma (BSA, Fraction V,	A3059-500G

TBS, 0.05% Sodium Azide)	99% Pure)	
Theranos Small Molecule AP Stabilizer	Theranos	

2 ASSAY DEVELOPMENT FOR URINE TEST [TC "ASSAY OPTIMIZATION" \F C \L "2"]

2.1 Antibody and Conjugate Binding Verification on MTP

To verify that Theranos Alkaline Phosphatase-labeled THC and THCA Conjugates are recognized by the antibodies raised against THC and THCA, two Theranos AP-labeled THC conjugates and two Theranos AP-labeled THCA Conjugates with SMPH and GMBS linkers (Table 2) were tested with 11 anti-THC antibodies (Table 3). The AP conjugate was detected with Theranos Alkaline Phosphatase Substrate.

A Nunc Maxisorb Microtiter plate was coated by passive absorption with 10.0, 1.0, 0.1 and 0 ug/mL of the anti-THC antibodies and binding to a fixed concentration of the conjugates (2.75ng/mL after 1:1000, 000 dilution) was tested. 10 out of the 11 tested antibodies have response to the Theranos AP conjugates except for antibody M5 (Table 4). With THCA-GMBS-AP conjugate, antibodies gave best response. Therefore, all the antibodies (except antibody M5) will be run with competitive format on Theranos Analyzer using THCA-GMBS-AP conjugate.

Table [SEQ Table * ARABIC]: Theranos Alkaline Phosphatase-labeled THC and THCA Conjugates Screened

Manufacturer	Batch #	Description
Theranos	THC-SMPH-AP_01_030613	THC-SMPH-AP Conjugate
Theranos	THC-GMBS-AP_01_030613	THC-GMBS-AP Conjugate
Theranos	THCA-SMPH-AP_01_030613	THCA-SMPH-AP Conjugate
Theranos	THCA-GMBS-AP_01_030613	THCA-GMBS-AP Conjugate

Table [SEQ Table * ARABIC]: Anti-THC Antibodies Screened

Theranos #	Description	Vendor	Cat #	Immunogen
M1	Anti- Tetrahydrocannabinol Sheep Pab	Randox	PAS9750	Δ9-THC-COOH-BSA
M2	Anti- Tetrahydrocannabinol Sheep Pab	Randox	PAS9751	Δ8-THC-COOH-BSA
M3	Anti- Tetrahydrocannabinol Sheep Pab	Randox	PAS9752	THC(9)-BSA
M4	Tetrahydrocannabinol Rabbit Polyclonal Antibody	LSBio	LS-C86324	D 8-THC-KLH
M5	Tetrahydrocannabinol Goat Polyclonal Antibody	LSBio	LS-C86314	Δ9-THC-KLH
M6	Anti-Tetrahydrocannabinol, D9 (THC)(mouse)	USBiological	T2970-05A	11-nor-detla9-THC-acid conjugated at the C-terminus
M7	Anti-Tetrahydrocannabinol (THC)(Sheep)	USBiological	T2970-02	9-THC-COOH-BSA
M8	Anti-Tetrahydrocannabinol (THC)(Sheep)	USBiological	T2970-03	THC(9)-BSA
M9	Anti-Tetrahydrocannabinol (THC)(mouse)	USBiological	T2970-01B	11-nor-detla9-THC-acid conjugated at the C-terminus
M10	Anti-Tetrahydrocannabinol (THC)(mouse)	USBiological	T2970-21D	Tetrahydrocannabinol
M11	Anti-Tetrahydrocannabinol (THC)(mouse)	USBiological	T2970	THC-BSA

Table [SEQ Table * ARABIC]: Antibody Response to Theranos AP Conjugates (MTP)

THC-SMPH-AP

Ab Conc. (ug/ml)	M1		M2		M3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	52351	42	226876	173	31254	10
1	3141	3	10843	8	2219	1
0.1	1328	1	2332	2	1490	0
0	1255		1308		3103	
	M4		M5		M6	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	234720	191	26684	19	88064	70
1	14204	12	1215	1	5721	5
0.1	2285	2	1093	1	1901	2
0	1232		1368		1265	
	M7		M8		M9	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	95046	68	704427	535	639031	480
1	5020	4	55602	42	41243	31
0.1	1427	1	6072	5	4791	4
0	1391		1317		1332	
	M10		M11			
	Mean RLU	Modulation	Mean RLU	Modulation		
10	601990	440	888957	591		
1	41977	31	122713	82		
0.1	5925	4	19400	13		
0	1367		1505			

THC-GMBS-AP

Ab Conc. (ug/ml)	M1		M2		M3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	1483	17	8154	68	1051	7
1	130	1	342	3	137	1
0.1	74	1	193	2	138	1
0	88		120		144	
	M4		M5		M6	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	8567	68	304	3	4181	26
1	494	4	113	1	271	2
0.1	137	1	109	1	109	1
0	126		110		158	
	M7		M8		M9	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	4505	40	33607	243	30004	298
1	293	3	1084	8	628	6
0.1	99	1	169	1	155	2
0	113		138		101	
	M10		M11			
	Mean RLU	Modulation	Mean RLU	Modulation		
10	13432	45	56304	247		
1	640	2	2441	11		
0.1	332	1	571	3		
0	302		228			

THCA-SMPH-AP

Ab Conc. (ug/ml)	M1		M2		M3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	198683	460	228020	593	134434	333
1	9369	22	8631	22	5496	14
0.1	984	2	1488	4	794	2
0	432		385		404	
	M4		**M5**		**M6**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	478202	1015	978	2	312839	828
1	48613	103	907	2	27623	73
0.1	4099	9	451	1	2572	7
0	471		457		378	
	M7		**M8**		**M9**	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	339646	702	687294	1451	630647	1115
1	24746	51	53314	113	22142	39
0.1	2620	5	2632	6	5689	10
0	484		474		566	
	M10		**M11**			
	Mean RLU	Modulation	Mean RLU	Modulation		
10	615689	1040	686803	721		
1	78009	132	144160	151		
0.1	9415	16	22831	24		
0	592		953			

THCA-GMBS-AP

Ab Conc. (ug/ml)	M1		M2		M3	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	233232	728	287051	1074	159419	413
1	11706	37	12974	49	6765	18
0.1	1128	4	1628	6	931	2
0	321		267		386	
	M4		M5		M6	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	582801	1361	938	2	376447	674
1	82822	193	451	1	39041	70
0.1	6202	14	508	1	3648	7
0	428		540		559	
	M7		M8		M9	
	Mean RLU	Modulation	Mean RLU	Modulation	Mean RLU	Modulation
10	348760	299	775208	1215	751848	1215
1	33653	29	80310	126	66851	108
0.1	3700	3	9062	14	6992	11
0	1167		638		619	
	M10		M11			
	Mean RLU	Modulation	Mean RLU	Modulation		
10	722153	1249	838747	971		
1	83895	145	147950	171		
0.1	8316	14	20659	24		
0	578		864			

2.2 Antibody Screen in Competitive Assay Format on Theranos System

Antibodies (at 10ng/mL) were screened using a competitive assay format with antibody in solution, 1:10 sample dilution and Theranos THCA-GMBS-AP conjugate at 1:1000, 000 on Theranos Analyzer. THCA calibrators were prepared in 0.03% BSA Blocking Buffer. Although antibodies M9, M10 and M11 gave the best modulation, the background was very high (Table 5). These three antibodies were then screened at 1:25 sample dilution with the format of antibody on tip at 1, 5, 10 and 25ng/mL (Table 6). Based on the modulation, M9 at 10ng/mL, M10 at 5ng/mL and M11 at 1ng/mL will be selected for further development.

Table [SEQ Table * ARABIC]: Antibody Screen Using Antibody in Solution Format

[THCA] ng/mL	M1			M2			M3		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
250	7164	39	3	8348	18	6	7164	22	6
0	23101	18		51502	20		46278	17	
	M4			M6			M7		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
250	748	9	10	595	24	69	5985	21	2
0	7113	17		40898	17		11025	17	
	M8			M9			M10		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
250	6869	17	4	549	20	492	1934	9	252
0	25695	5		270295	21		487042	9	
	M11								
	Mean RLU	CV%	Modulation						
250	2895	20	264						
0	764621	6							

Table [SEQ Table * ARABIC]: Antibody Screen Using Antibody on Tips Format

Ab Concentration	[THCA] ng/mL	M9			M10			M11		
		Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
1 ng/mL	500	295	10	32	572	15	33	863	16	113
	250	292	36	32	665	36	28	980	18	100
	100	459	20	21	1598	24	12	3018	28	32
	30	1087	20	9	4152	21	5	6171	25	16
	10	2234	17	4	8506	18	2	23474	13	4
	0	9436	21		18916	15		97479	30	
5 ng/mL	500	505	19	105	1264	20	90	1765	27	109
	250	856	19	62	3185	25	36	2220	19	87
	100	2301	13	23	8736	24	13	10901	28	18
	30	5190	17	10	28488	23	4	53986	26	4
	10	13608	19	4	52365	17	2	97153	26	2
	0	53171	12		113398	8		192865	24	
10 ng/mL	500	479	24	139	2332	8	72	2518	20	107
	250	1218	24	55	5813	15	29	6089	9	44
	100	3530	46	19	12349	18	14	29758	24	9
	30	7276	25	9	34990	13	5	55716	15	5
	10	23158	15	3	82846	23	2	166787	21	2
	0	66553	21		166893	10		269796	15	
25 ng/mL	500	3971	24	71	6971	13	53	2487	31	118
	250	5511	21	51	11849	28	31	5504	33	53
	100	14541	28	19	38906	23	9	15377	23	19
	30	73993	22	4	183066	17	2	105786	21	3
	10	149239	55	2	324113	18	1	264938	5	1
	0	281853	21		369392	13		294039	8	

2.3 Matrix Effect on Calibrators

Theranos THC assay was targeted to analyze both urine and serum samples. To evaluate matrix effect, 0.03% BSA buffer, pooled urine and serum samples from healthy donors (negative for THC by reference test) were spiked with THCA at various concentrations and tested on Theranos Analyzer with sample dilution at 1:100 (Table 7, Figure 1-3). The results showed that urine and 0.03% BSA buffer were comparable for all three antibodies. Since the RLU and modulation from serum were quite different from urine and 0.03% BSA buffer, we tested the possibility that higher dilution may reduce the matrix effect with antibodies M9 and M11 (Since the modulation for M10 is low, M10 was exclude from the further development). However, sample dilution at 1:200 and 1:500 didn't change the fact that RLU and modulation from serum were different from urine and 0.03% BSA (Table 8 and 9, Figure 4 and 5). Therefore, we will develop THC assay for urine and serum separately using 0.03% BSA buffer calibrator and serum calibrator respectively.

Table [SEQ Table * ARABIC]: Matrix Effect Test with THCA spiked in Urine, Serum and 0.03% BSA Buffer (sample dilution at 1:100)

M9	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
400	422	17	109	888	9	33	615	29	73
200	806	10	57	1903	16	15	748	5	60
100	1616	19	28	2912	36	10	1339	7	33
50	3111	18	15	4250	17	7	2638	25	17
25	4854	22	9	6801	41	4	4323	24	10
0	45882	26		29419	22		44602	16	

M10	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
400	807	12	26	1663	33	18	714	35	31
200	1221	22	17	2865	9	10	953	15	23
100	2228	20	9	4356	58	7	2319	33	10
50	4662	24	4	7662	10	4	3868	28	6
25	7531	7	3	13687	14	2	6295	25	4
0	20777	42		29543	15		22123	40	

M11	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
400	421	24	92	891	50	33	619	16	65
200	566	29	69	1695	33	17	473	21	85
100	1099	17	35	2155	53	14	956	20	42
50	1853	24	21	3296	17	9	1789	16	22
25	3500	9	11	7141	22	4	3691	23	11
0	38881	21		29536	39		39982	18	

Figure [SEQ Figure * ARABIC]: Matrix Effect Tested with Antibody M9 (sample dilution at 1:100)

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Matrix Effect Tested with Antibody M10 (sample dilution at 1:100)

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Matrix Effect Tested with Antibody M10 (sample dilution at 1:100)

[SHAPE * MERGEFORMAT]

Table [SEQ Table * ARABIC]: Matrix Effect Test with THCA Spiked in Urine, Serum and 0.03% BSA Buffer (sample dilution at 1:200)

M9	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
50	3305	24	11	5978	26	6	2582	17	15
0	34974	9		35147	6		38284	19	

M11	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
50	3260	49	14	4312	18	7	2170	12	20
0	46283	21		30781	9		42518	15	

Table [SEQ Table * ARABIC]: Matrix Effect Test with THCA Spiked in Urine, Serum and 0.03% BSA Buffer (sample dilution at 1:500)

M9	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
100	2327	22	19	5805	18	5	2459	41	18
50	3533	12	13	7964	22	4	3862	18	12
0	45204	22		28778	14		44690	27	

M11	Urine			Serum			0.03% BSA		
Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
100	1609	27	29	4256	21	6	1635	21	29
50	3088	31	15	7350	35	3	2731	22	18
0	47103	40		24589	12		48005	16	

Figure [SEQ Figure * ARABIC]: Matrix Effect Tested with Antibody M9 (sample dilution at 1:500)

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Matrix Effect Tested with Antibody M11 (sample dilution at 1:500)

[SHAPE * MERGEFORMAT]

2.4 Cross Reactivity

Four cross reactants were tested for cross reactivity on Theranos Analyzer using antibody M9 (10ng/mL) and antibody M11 (1ng/mL). The results showed that there is no cross reactivity was observed with both antibodies (Table 10 and 11).

Table [SEQ Table * ARABIC]: Cross Reactivity Test with antibody M9 at 10ng/mL

[THCA] ng/mL	Cross Reactants at 100ng/mL	Mean RLU	CV%	Mean Conc. (ng/mL)
0	Cannabidiol	55769	10	OORL
0	Cannabinol	57089	24	OORL
0	(-)-Δ8-THC	38016	44	OORL
0	(±)-11-Hydroxy-Δ9-THC	38669	14	OORL

Table [SEQ Table * ARABIC]: Cross Reactivity Test with antibody M11 at 1ng/mL

				Back-Calculated Conc, ng/mL	
[THCA] ng/mL	Cross Reactants at 100ng/mL	Mean RLU	CV%	Mean Conc	CV%
0	Cannabidiol	55900	9	25.18	8
0	Cannabinol	54644	16	OORL	
0	(-)-Δ8-THC	48085	15	OORL	

0	(±)-11-Hydroxy-Δ9-THC	29386	12	OORL	

2.5 BIO-RAD Urine Toxicology Controls

To further determine the final antibody for assay development, BIO-RAD Liquicheck Urine Toxicology Controls at different levels were run on Theranos analyzer with both antibody M9 (10ng/mL) and antibody M11 (1ng/mL). Antibody M9 at 10ng/mL was selected for further development due to good recovery and correlation with BIO-RAD urine controls (Table 12 and 13) when 50ng/mL was used as cutoff (Green color indicates negative and pink color indicates positive). Assay optimization will be performed with antibody M9.

Table [SEQ Table * ARABIC]: BIO-RAD Urine Controls Tested with Antibody M9

				Back-Calculated Conc, ng/mL		
Bio-Rad Controls	**Representative Value**	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**	**%Recovery**
C2	11.90	18206	25	OORL		
Negative Control	3.00 (detection limit)	52511	10	OORL		
SIS	34.90	6710	28	33.53	28	96
S2E	59.30	4322	17	51.49	21	87
S3	115.00	2631	13	101.70	18	88

Table [SEQ Table * ARABIC]: BIO-RAD Urine Controls Tested with Antibody M11

Back-Calculated Conc, ng/mL

Bio-Rad Controls	Representative Value	Mean RLU	CV%	Mean Conc	CV%	%Recovery
C2	11.90	15186	24	OORL		
Negative Control	3.00 (detection limit)	45708	16	OORL		
SIS	34.90	4998	17	26.81	13	77
S2E	59.30	3158	15	44.86	21	76
S3	115.00	1739	9	107.49	14	93

2.6 Alkaline Phosphatase Stabilizer

Two commercial and one Theranos alkaline phosphatase stabilizers were tested to find the best one as diluent for Theranos THCA-GMBS-AP conjugate. Theranos Small Molecule AP stabilizer consisting of 5 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 0.03% BSA was selected as final AP stabilizer since the sensitivity with Theranos stabilizer meets the requirement although the commercial AP stabilizers gave slightly higher modulation (Table 14).

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Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizer

AP Stabilizer	Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation
Stabilzyme	400	838	23	54
	200	1391	15	32
	100	2231	12	20
	50	4001	17	11
	25	8786	21	5
	0	45172	3	
BioStab	400	429	14	65
	200	588	22	47
	100	944	12	30

	50	1860	14	15
	25	2855	12	10
	0	27890	14	
Theranos Small Molecule AP Stabilizer	400	912	14	41
	200	2673	20	14
	100	2444	19	15
	50	4511	7	8
	25	8555	14	4
	0	37522	16	

2.7 Alkaline Phosphate Conjugate Titration

Alkaline phosphate conjugate THCA-GMBS-AP was titrated at 1:500,000, 1:1000, 000 and 1:500,000 (Table 15). Although 1:500,000 gave higher modulation at the top end, the background is very high. The modulation at the low end is comparable for 1:500,000 and 1:1,000,000, and the background with 1:1,000,000 dilution is lower. Therefore, 1:1,000,000 was selected as final dilution for Theranos AP conjugate.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Conjugate Titration

AP Conjugate Titration	Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation
1:500,000	400	2128	10	102
	200	4307	34	50
	100	7829	17	28
	50	11285	25	19
	25	30612	18	7
	0	216589	7	
1:1M	400	941	22	53
	200	2234	14	22
	100	2768	36	18
	50	3528	9	14

	25	5594	20	9
	0	49543	10	
1:5M	400	239	25	37
	200	322	24	27
	100	703	26	13
	50	703	26	13
	25	848	14	10
	0	37522	16	

2.8 Sample Dilution

The effect of sample dilution was tested with final sample dilution at 1:50, 1:100 and 1:200. To reduce the potential matrix effect, the sample dilution at 1:100 was selected as the final sample dilution although 1:50 give slightly higher modulation (Table 16). Furthermore, the sensitivity at expected cutoff 50ng/mL was similar for 1:50 and 1:100 dilution.

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

Sample Dilution	Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation
1:50	400	1470	23	136
	200	2426	22	83
	100	4688	28	43
	50	11454	17	17
	25	19675	13	10
	0	200145	15	
1:100	400	2128	10	102
	200	4307	34	50
	100	7829	17	28
	50	11285	25	19
	25	30612	18	7
	0	216589	7	

1:200	400	4288	11	50
	200	6680	19	32
	100	7267	9	30
	50	26507	21	8
	25	41523	8	5
	0	215954	18	

2.9 Incubation Time

The effect of shorter reagent incubation time was tested with sample reaction mixture and substrate incubation time respectively at 10,10 (original condition), 5,5 and 2,1 minutes (Table 17). With 10,10 minute incubation, the modulation was the best. Therefore, 10,10 minute incubation time was selected as the final condition.

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

Incubation Time (min)	Nominal [THCA] ng/mL	Mean RLU	CV%	Modulation
10,10	400	2128	10	102
	200	4307	34	50
	100	7829	17	28
	50	11285	25	19
	25	30612	18	7
	0	216589	7	
5,5	400	834	30	83
	200	2304	24	30
	100	2853	33	24
	50	10470	47	7
	25	15390	21	4
	0	68919	27	
2,1	400	37	2	2

200	27	11	2
100	36	16	2
50	33	15	2
25	28	26	2
0	59	58	

2.10 Determination of LLOQ and ULOQ

A lot of reagents were produced and the calibration was performed using the final assay conditions of 10ng/mL of antibody M9 on tip, 1:100 sample dilution and 10, 10 minute incubation with 3 cartridges per point. The protocol for Theranos 3.0 System is Generic2_100x_Competitive_10_10.

A set of calibrators were run on Theranos Analyzer and Dexter had been used to get back-calculation formula and determine LLOQ and ULOQ using FDA guideline for ELISA assay calibration (Table 18 and 19). The LLOQ was 12.5 ng/mL, and the ULOQ was 400 ng/mL. The back-calculation formula from Dexter was applied for all the following tests.

Table [SEQ Table * ARABIC]: Calibration

Calibrator	Signal, RLU		Back-Calculated Conc, ng/mL		
[THCA] (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
400	942	7	423	10	106
200	1824	13	185	17	92
100	3029	8	98	9	98
50	4815	18	57	19	110
25	9344	16	26	19	105
12.5	17284	6	OORL		
0	81641	15	OORL		

Table [SEQ Table * ARABIC]: Dexter Results for LLOQ and ULOQ

Measurement	Value	Units
LLOQ	12.5	ng/mL
ULOQ	400	ng/mL
LLOQ accuracy	93	%
LLOQ precision	2.7	%
Average Residuals	10	%
Error in prediction: Best case	12	%
Error in prediction: Expected	13	%

2.11 Analysis of Control Samples

Bio-Rad control samples (Table 20), Synerhent Biochem DOA Panel (Table 21) and Zeptometrix UroDetect DOA Panel IV-Cannabinoid (Table 22) were tested on Theranos Analyzer and Siemens ADVIA. The test with these samples could also be treated as a test for cross reactivity and interference with other drugs since each sample contains many drugs at different levels. The results from Theranos Analyzer correlate very well with the reported value and the reference method (Siemens ADVIA) when the assay was used as a qualitative assay with the cutoff at 50ng/mL although sample #3 from Zeptometrix Panel showed negative which is at the border line of the cutoff. Green color indicates negative and pink color indicates positive with the cutoff at 50ng/mL.

Table [SEQ Table * ARABIC]: Bio-Rad Control Samples Tested by Theranos Analyzer and Siemens ADVIA

		Theranos (cutoff at 50ng/mL)		ADVIA (cutoff at 50 ng/mL)
Bio-Rad Controls	Reported (ng/mL)	Mean Conc (ng/mL)	CV%	Mean Conc (ng/mL)
C1	6.24	OORL		OORL
N	3.00	OORL		OORL
S1E	38.80	40	21	36
S1S	34.90	29	17	28
S2E	59.30	54	18	55
S3	115.00	86	19	86

Table [SEQ Table * ARABIC]: Synerhent Biochem DOA Panel Tested by Theranos Analyzer and Siemens ADVIA

		Theranos (cutoff at 50ng/mL)		ADVIA (cutoff at 50 ng/mL)
Synerhent Sample#	**Reported (ng/mL)**	**Mean Conc (ng/mL)**	**CV%**	**Mean Conc (ng/mL)**
S1	386	332	20	207
S2	113	100	21	106
S3	105	88	15	93
S4	40	41	15	37
S5	0	OORL		OORL

Table [SEQ Table * ARABIC]: Zeptometrix UroDetect DOA Panel IV-Cannabinoid Tested by Theranos Analyzer and Siemens ADVIA

		Theranos (cutoff at 50ng/mL)		ADVIA (cutoff at 50 ng/mL)
Zeptometrix Samples	**Reported (ng/mL)**	**Mean Conc (ng/mL)**	**CV%**	**Mean Conc (ng/mL)**
1	57	14	7	29
2	78	OORL		30
3	99	49	25	52
4	Negative (<45)	OORL		17
5	102	14	6	43
6	107	56	17	60
7	107	57	15	78
8	82	51	19	90
9	Negative (<45)	OORL		16
10	97	82	22	100

2.12 Clinical Samples

Urine samples from a collection of healthy donors were screened by Theranos Analyzer and Siemens ADVIA. All the samples are negative for THCA (Table 23). Due to the difficulty in aquiring drug of abuse positive clinical samples, negative urine samples were used as clinical samples after spiked with THCA. The spiked urine samples tested by Theranos Analyzer were in the correct category as Siemens ADVIA using 50ng/mL as cutoff (Table 24). To further confirm the correlation, DRG dipstick for THC test was used to test all the spike urine samples, and the same results were obtained.

Table [SEQ Table * ARABIC]: Urine Samples from Healthy Donors Screened by Theranos Analyzer and Siemens ADVIA

Urine Samples from Healthy Donors	Mean RLU	CV%	Theranos (ng/mL)	ADVIA (ng/mL)
1	73185	16	OORL	OORL
2	66152	8	OORL	OORL
3	70768	14	OORL	OORL
4	75751	14	OORL	OORL
5	67804	6	OORL	OORL
6	75345	10	OORL	OORL
7	82618	8	OORL	OORL
8	75988	5	OORL	OORL

9	81223	7	OORL	OORL
10	20705	3	OORL	12

Table [SEQ Table * ARABIC]: Spiked Urine Samples tested with Theranos Analyzer, Siemens ADVIA and DRG Dipstick

		Theranos (cutoff at 50ng/mL)		ADVIA (cutoff at 50ng/mL)	
Spiked Urine Samples	Nominal [THCA] (ng/mL)	Mean Conc (ng/mL)	CV%	Mean Conc (ng/mL)	DRG
1	280	224	13	249	Positive
2	150	102	25	61	Positive
3	400	254	19	242	Positive
4	70	49	10	35	Negative
5	63	37	24	32	Negative
6	55	31	22	18	Negative
7	50	33	11	25	Negative
8	45	27	17	16	Negative
9	40	21	13	17	Negative
10	38	34	6	25	Negative

3 ASSAY DEVELOPMENT FOR SERUM TEST

Due to matrix effect (See page 14), the decision was made to develop THC assay for urine and serum separately using 0.03% BSA buffer calibrator and serum calibrator respectively.
The serum calibration was performed using the final assay conditions for urine assay (10ng/mL of antibody M9 on tip, 1:100 sample dilution and 10, 10 minute incubation with 3 cartridges per point. The protocol for Theranos 3.0 System is Generic2_100x_Competitive_10_10).

3.1 Serum Calibration and Determination of LLOQ and ULOQ

A set of serum calibrators were run on Theranos Analyzer and Dexter had been used to get back-calculation formula and determine LLOQ and ULOQ using FDA guideline for ELISA assay calibration (Table 25 and 26). The LLOQ was 12.5 ng/mL, and the ULOQ was 400 ng/mL. The back-calculation formula from Dexter was applied for all the following tests.

Table [SEQ Table * ARABIC]: Calibration

Calibrator	Signal, RLU		Back-Calculated Conc, ng/mL		
[THCA] (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	%Recovery
400	1632	6	393	12	98
200	2203	4	233	7	116
100	4811	14	81	17	81
50	5972	9	60	11	121
25	11204	12	26	17	102
12.5	17461	7	OORL		
0	35447	11	OORL		

Table [SEQ Table * ARABIC]: Dexter Results for LLOQ and ULOQ

Measurement	Value	Units
LLOQ	12.5	ng/mL
ULOQ	400	ng/mL
LLOQ accuracy	91	%
LLOQ precision	14.1	%
Average Residuals	10	%
Error in prediction: Best case	16	%
Error in prediction: Expected	17	%

3.2 Whole Blood, plasma and Serum Screen

Ten whole blood samples (samples F1-F5 from female, samples M1-M5 from male) from healthy donors were screened on Theranos Analyzer. EDTA plasma samples, Lithium-Heparin plasma samples and serum samples from the same donors as whole blood samples were also screened on Theranos Analyzer. The results showed that the back-calculation for all the samples is OORL except samples from donor M1 (Table 27-30). The whole blood, plasma and serum samples from donor M1 are consistently positive, suggesting there is no matrix effect.

Table [SEQ Table * ARABIC]: Whole Blood Screen

			Back-Calculated Conc, ng/mL	
Whole Blood Samples	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
M1	3645	15	117	19
M2	77995	5	OORL	
M3	42453	7	OORL	
M4	69958	13	OORL	
M5	61200	15	OORL	
F1	60661	17	OORL	
F2	56957	8	OORL	
F3	274362	12	OORL	
F4	364803	34	OORL	
F5	245413	17	OORL	

Table [SEQ Table * ARABIC]: EDTA Plasma Screen

			Back-Calculated Conc, ng/mL	
EDTA Plasma Sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
M1	2030	4	265	6
M2	70005	10	OORL	
M3	37498	6	OORL	
M4	69767	10	OORL	
M5	54877	11	OORL	
F1	61623	8	OORL	
F2	75431	16	OORL	
F3	63720	17	OORL	
F4	72554	19	OORL	
F5	70512	10	OORL	

Table [SEQ Table * ARABIC]: Lithium-Heparin Plasma Screen

			Back-Calculated Conc, ng/mL	
Lithium-Heparin Plasma Samples	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
M1	1846	16	274	33
M2	65622	16	OORL	
M3	35623	11	OORL	
M4	69731	13	OORL	
M5	53287	3	OORL	
F1	67524	8	OORL	
F2	64157	14	OORL	
F3	64943	17	OORL	
F4	69695	12	OORL	
F5	103277	11	OORL	

Table [SEQ Table * ARABIC]: Serum Screen

			Back-Calculated Conc, ng/mL	
Serum Sample	**Mean RLU**	**CV%**	**Mean Conc**	**CV%**
M1	2131	9	249	15
M2	79333	10	OORL	
M3	27605	25	OORL	
M4	69411	15	OORL	
M5	57686	6	OORL	
F1	59410	11	OORL	
F2	69100	8	OORL	
F3	60404	10	OORL	
F4	69992	6	OORL	
F5	65024	12	OORL	

3.3 Clinical Samples

Due to the difficulty in aquiring drug of abuse positive clinical samples, screened negative serum samples were used as clinical samples after spiked with THCA. The spiked serum samples showed good recovery on Theranos Analyzer (Table 31 and Figure 6).

Table [SEQ Table * ARABIC]: Spiked Serum Samples tested with Theranos Analyzer

				Back-Calculated Conc, ng/mL		
Spiked Serum Samples	Nominal [THCA] (ng/mL)	Mean RLU	CV%	Mean Conc	CV%	Recovery
1	400	1448	17	430	24	108
2	280	2147	11	248	16	88
3	150	3102	9	142	11	95
4	70	4919	15	79	21	113
5	63	5609	12	66	18	106
6	50	6142	9	58	13	117
7	40	10162	14	30	26	75
8	38	8242	21	42	34	111
9	25	13090	6	20	8	80
10	13	19284	8	OORL		

Figure [SEQ Figure * ARABIC]: Recovered vs Spiked Serum THCA

[SHAPE * MERGEFORMAT]

3.4 Whole Blood Spike Recovery and Hematocrit Effect

The whole blood recovery was determined with the whole blood spiked at six levels. The spiked whole blood samples were measured on Theranos Analyzer, and the remaining spiked whole blood was centrifuged and the plasma was collected for testing on Theranos Analyzer to evaluate hematocrit effect. The results suggested that whole blood should not be recommended for Theranos THC test due to low recovery (Table 32, Figure 7). The recovery of THCA from plasma was around 1.3 fold of that from whole blood samples (Figure 8).

Table [SEQ Table * ARABIC]: Whole Blood Spike Recovery and Hematocrit Effect

Whole Blood	Signal, RLU		Back-Calculated Conc, ng/mL		
Nominal [THCA] ng/mL Spiked in Sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
400	1814	15	336	24	84

100	4115	6	97	8	97
50	9407	11	33	16	66
25	11864	5	23	8	92
12.5	25330	10	OORL		
0	70640	4	OORL		

Plasma(from the Whole Blood)	Signal, RLU		Back-Calculated Conc, ng/mL		
Nominal [THCA] ng/mL Spiked in Sample	Mean RLU	CV%	Mean Conc	CV%	%Recovery
400	1168	11	913	30	228
100	3059	17	140	19	140
50	5201	14	73	18	147
25	9049	13	35	19	141
12.5	17730	11	OORL		
0	61333	20	OORL		

Figure [SEQ Figure * ARABIC]: Whole Blood Spike Recovery
[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Hematocrit Effect
[SHAPE * MERGEFORMAT]

3.5 Interference Matrices Screen

Hemolyzed, icteric, and lipemic serum samples were obtained from ProMedDx, and tested on Theranos Analyzer. Some serum samples showed very high concentration of THCA (Table 33).

Although the Siemens ADVIA is intended to be used for urine THC test, we tested the serum samples on ADVIA to get some references. The samples with higher concentrations of THCA on Theranos are all positive on ADVIA. Green color indicates negative and pink color indicates positive using the cutoff for urine at 50ng/mL.

Table [SEQ Table * ARABIC]: Interference Matrices Screen

			Back-Calculated Conc, ng/mL		
Hemolyzed Serum Sample	Mean RLU	CV%	Mean Conc	CV%	ADVIA (ng/mL)
#3	60831	12	OORL		OORL
#5	98903	5	OORL		OORL
#6	19555	6	OORL		OORL
#7	30233	3	OORL		OORL
#9	2706	10	285	14	203

			Back-Calculated Conc, ng/mL		
Icteric Serum Sample	Mean RLU	CV%	Mean Conc	CV%	ADVIA (ng/mL)
#15	1108	19	OORH		275
#17	81848	24	OORL		OORL
#18	32545	13	OORL		OORL
#19	2782	5	271	7	237
#20	91877	17	OORL		OORL

			Back-Calculated Conc, ng/mL		
Lipemic Serum Sample	Mean RLU	CV%	Mean Conc	CV%	ADVIA (ng/mL)
#1	1649	10	OORH		160
#2	99189	22	OORL		OORL
#3	55116	20	OORL		7
#4	75370	7	OORL		OORL
#5	31201	9	OORL		OORL

3.6 Specificity

Five RF positive serum samples and five HAMA positive serum samples were obtained from Bioreclamation and tested for specificity on Theranos Analyzer, the results showed OORL for all the tested RF positive samples (Table 34). HAMA positive samples were sent to CLIA lab to test on Siemens ADVIA since some of the samples showed high concentration of THCA on Theranos Analyzer. Consistently, those samples are THC positive on Siemens ADVIA. Green color indicates negative and pink color indicates positive using the cutoff for urine at 50ng/mL (Table 34).

Table [SEQ Table * ARABIC]: Specificity Test with RF and HAMA samples

RF Positive Samples	Mean RLU	CV%	Mean Conc
#3	58743	13	OORL
#6	69822	17	OORL
#7	65405	13	OORL
#8	77049	16	OORL
#9	77606	15	OORL

			Back-Calculated Conc, ng/mL		
HAMA Positive Samples	Mean RLU	CV%	Mean Conc	CV%	ADVIA (ng/mL)
#4	70742	31	OORL		OORL
#29	2200	18	396	24	204
#30	97520	14	OORL		OORL
#31	70879	12	OORL		OORL
#32	4884	11	122	16	167

3.7 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light.