D-Amphetamine Assay Development Report

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1 ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

1.1 [TC "Assay Specifications" \f C \l "3"]Analyte information

Amphetamines are potent central nervous system stimulants. These drugs are originally prescribed for treatment of narcolepsy and are found helpful for treatment of attention deficit hyperactivity disorder (ADHD), and obesity. However, because of the central nervous system and cardiovascular stimulation effect that produce wakefulness, alertness, and increased energy, they are commonly sold illicitly and abused. Some studies show high dose abuse of amphetamines cause physiologic dependence and permanent damage of central nerve structures in the brain. In the United States, amphetamines are Schedule II controlled substances, which are classified as having a high potential for abuse and a high possibility of severe psychological and physiological dependence.

The term of "Amphetamines" refers to a group of drugs including D-Amphetamine, L-Amphetamine, D,L,--Methamphetamine, Methylenedioxyamphetamine (MDA), Methylenedioxymethamphetamine (MDMA) and Methylenedioxyethylamphetamine (MDEA). Amphetamines can be taken orally, intravenously, or by inhalation. After administration, they are easily absorbed from the gastrointestinal tract and are then either deactivated by the liver or excreted unchanged in the urine. They can be detected in urine within three hours after administration and for 24 to 48 hours after the last dose. Amphetamine is excreted mostly unchanged. Methamphetamine is partially metabolized into Amphetamine by demethylation. Thus, Amphetamine is the primary urinary marker for detection of amphetamine use.

Measurement of amphetamines in urine is included in "drug test" for the diagnosis of amphetamines use. Current specimen positive cut-offs are established by the Substance Abuse Mental Health Services Administration (SAMSHA) as screening cut-off at 500ng/ml for urine, confirmation cut-off at 200ng/ml for urine, screening cut-off at 30ng/ml for serum and confirmation cut-off at 20ng/ml for serum. Immunoassay methods have been used for screening tests. GC/MS and LC-MS/MS methods are identified by SAMSHA as confirmatory methods. Screen test provides preliminary analytical data. A more specific, alternative method is required to obtain a confirmed positive result.

1.2 Assay specifications

This Theranos assay is developed to quantitatively determine D-Amphetamine in urine, serum, plasma and whole blood. The assay has a quantification range of 30ng/ml – 10,000ng/ml, in the above mentioned matrices. The assay can be used as a qualitative screening assay with positive cutoff for urine at 500ng/ml or positive cutoff for serum/plasma at 30ng/ml.

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

The following assays were used as reference methods:

(1). Amphetamine test card, DRG, Cat#RAP-2583

(2). Amphetamine test card, Rapidtest/Diagnostic Automation, Cat#121020-1
(3). Amphetamine ELISA kit, MyBiosource, Cat#MBS580057

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

A competitive immunoassay using anti-amphetamine antibody was developed for the quantitative determination of D-Amphetamine in urine, serum, plasma and whole blood.

In this assay, a mouse anti-amphetamine antibody was used as capture agent. Reaction tips were coated with Ultra-avidin first, and followed by coating of secondary antibody biotinylated goat anti-mouse IgG Fc. Then primary capture antibody mouse anti-amphetamine monoclonal antibody was coated in the third coating step. Urine, serum, plasma or whole blood samples were diluted 100 folds with sample diluent and mixed with amphetamine-alkaline phosphatase conjugate. The mixture was incubated with capture antibody coated tips. Amphetamine in sample and Amphetamine-AP conjugate competitively bind to anti-amphetamine antibody on tips. After incubation, the tips were washed with wash buffer and incubated with AP substrate. The chemiluminescence results were measured and reported as Relative Light Units (RLU). A calibration curve was generated by plotting the measured response (RLU) vs. concentration of each calibrator. D-Amphetamine concentration of unknown sample was calculated from calibration curve.

Table [SEQ Table * ARABIC]: D-Amphetamine assay materials in final assay procedure

Name	Supplier	Catalog number
Amphetamine	Cerriliant	A-008
Biotinylated Goat anti-mouse IgG Fc	PIERCE	31805
Mouse anti-Amphetamine monocloncal antibody	US Biological	A1510-02
Tris buffer (powder)	Sigma	T6664
Tris buffer with Tween (powder)	Sigma	T9079
Bovine serum albumin	Sigma	A3059
Sucrose	Sigma	S5016
5% Sodium Azide solution	VWR	101320-516
Carbonate-bicarbonate buffer	Sigma	C3041
1M Magnesium chloride solution	Sigma	M1028
0.1M Zinc Chloride solution	Sigma	39059
Wash buffer	In house	
UltraAvidin	Leinco	A110
AP substrate buffer	In house	Current Lot 19122012 NGB-ALP

Amphetamine-AP conjuagte	In house	Current Lot OK-0448-75-p-3

1.5 Raw data storage

Raw data of assay development were stored in Elog #836 and Theranos notebook #429.

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

2.1 Initial antibody screening on MTP

2.1.1 Antibody screen with AMP-AP conjugate direct binding

During initial assay development stage, eighteen anti-Amphetamine antibodies were screened on multi-titer plate (MTP) with antibody direct coating format. A commercial Amphetamine-AP conjugate obtained by special order from the vendor "YJ Bio-product" was first used for antibody screen before Theranos in-house Amphetamine-AP conjugate was available. Because the analyte Amphetamine is a small molecule (M.W. 135Da), the assay is targeted to be a competitive immunoassay format.

The initial screen was conducted for AMP-AP conjugate direct binding to anti-AMP antibodies on MTP.

Methods:

The MTP was coated with anti-AMP antibodies at 10, 1, 0.1, 0ug/ml in coating buffer. AMP-AP conjugate was diluted in low BSA blocking buffer to 100ng/ml and incubated on MTP. After incubation and wash, AP substrate was added to each well and Relative Luminescence Unit (RLU) was measured by a plate reader. Modulations for each antibody were calculated using RLU of each coating concentration level divided by the RLU of background (buffer blank, no antibody coating).

Results:

Many antibodies showed good modulations. Because the assay will be developed as a competitive format, all eighteen antibodies were proceeded to screen with Amphetamine competition.

Table [SEQ Table * ARABIC]: Antibody screened on MTP

anti-Amphetamine antibody #					
	Name	**Supplier**	**Cat#**	**Lot#**	**Clone #**
1	anti-AMP McAB	Calbioreagents	M264	MA1047	
2	anti-AMP McAB	Calbioreagents	M327	1050	
3	anti-AMP McAB	Biosellsolutions	AMP1026	#070606	
4	anti-AMP McAB	Biosellsolutions	C03-99-72-P	J24	
5	anti-AMP McAB	US Biological	A1510-02A	L12081054	10B153
6	anti-AMP McAb	MyBioSource	MBS310890	6F16402	BD1088
7	anti-AMP McAB	LS Bio	LS-C84265	37916	M9905225
8	anti-AMP McAB	RayBiotech	DS-MB-00072	240811A	
9	anti-AMP McAB	MyBiosource	MBS530543	6546	M994299
10	anti-AMP McAB	MyBiosource	MBS530743	2202	M610256
11	anti-AMP McAB	MyBiosource	MBS531419	8552	M8122021
12	anti-AMP McAB	US Biological	A1510-02	L12082001	10B152
13	anti-AMP McAB	Fitzgerald	10-A44E	202	M610256
14	anti-AMP McAB	Fitzgerald	10-A44C	6546	M994299
15	anti-AMP McAB	Fitzgerald	10-A44F	8552	M8122021
16	anti-AMP McAB	antibody-online	ABIN283158	1129	M410195
17	anti-AMP McAB	antibody-online	ABIN283159		M5010513
18	anti-AMP McAB	Fitzgerald	10-IA52	1351	J24

Table [SEQ Table * ARABIC]: Results of initial screen on MTP

Antibody	#1	#2	#3	#4	#5	#6	#7	#8	#9
Modulation									
Antibody	#10	#11	#12	#13	#14	#15	#16	#17	#18
Modulation									

	Excellent modulation (>4000)
	Good modulation (>2000)
	Moderate modulation (>100)

2.1.2 Antibody screening with amphetamine competition

Because antibodies #13, #14, #15 have the same clone number as antibodies #9, #10, #11, these three antibodies were not continued in further tests. Fifteen anti-amphetamine antibodies were tested on MTP for Amphetamine and AMP-AP conjugate competitively binding to antibody.

Methods:

The MTP was coated with anti-AMP antibodies at 1ug/ml in coating buffer. AMP-AP conjugate was diluted in low BSA blocking buffer to working solution at 100ng/ml. D-AMP calibrators were prepared from 100ng/ml to 10,000ng/ml in low BSA blocking buffer and then further diluted 50-fold with low BSA blocking buffer. Diluted D-Amphetamine calibrators were mixed with AMP-AP conjugate working solution at 1:1 volume ratio in MTP wells to incubate with coated antibodies. After incubation and wash, AP substrate was added to each well and Relative Luminescence Unit (RLU) was measured by a plate reader. Modulations for each antibody were calculated using RLU of each calibrator concentration level divided by the RLU of background (buffer blank).

L-Amphetamine was also prepared in low BSA blocking buffer at 1000ng/ml to be tested for cross reactivity.

Bio-Rad drugs-of-abuse Liquichek Urine Toxicology Screen Controls (Catalog # 460, 461, 462, 463) were used at the same condition to assist antibody selection. These controls contain multiple drugs-of-abuse other than Amphetamine, thus these were served as part of cross reactivity and interference screening.

Results:

Among fifteen antibodies being tested, fourteen antibodies showed Amphetamine competition except antibody#3. L-Amphetamine didn't show significant cross reactivity at testing condition. With the considerations of modulations of Amphetamine competition, L-AMP cross reactivity and recovery of Bio Rad controls, four antibodies (#4, #6, #10, #12) were selected for further evaluation.

Table [SEQ Table * ARABIC]: Antibody screening with Amphetamine competition

		Ab#1				Ab#2		
Calibrator	Conc (ng/ml)	Mean	%CV	Modulation		Mean	%CV	modulation
C1	10000	38246	5	3.8		27200	6	10.1
C2	3000	74640	2	1.9		58966	11	4.7
C3	1000	114927	9	1.3		136197	3	2.0
C4	300	145076	0	1.0		203242	1	1.4
C5	100	156544	5	0.9		238449	2	1.2
C6	0	145062	2	1.0		274779	7	1.0
L-AMPH	1000	137316	10	1.1		289438	2	0.9
		Ab#3				Ab#4		
Calibrator	Conc	Mean	%CV	modulation		Mean	%CV	Modulation

	(ng/ml)							
C1	10000	268	5	1.1		3005	1	62.2
C2	3000	326	0	0.9		7602	2	24.6
C3	1000	355	3	0.8		22349	22	8.4
C4	300	294	16	1.0		55853	8	3.3
C5	100	281	9	1.0		92060	7	2.0
C6	0	282	15	1.0		186985	6	1.0
L-AMPH	1000	441	48	0.6		151724	1	1.2
		Ab#5				Ab#6		
Calibrator	Conc (ng/ml)	Mean	%CV	modulation		Mean	%CV	Modulation
C1	10000	733	22	54.6		1465	18	54.2
C2	3000	1768	8	22.7		3690	44	21.5
C3	1000	4635	7	8.6		9323	7	8.5
C4	300	9970	7	4.0		18690	8	4.3
C5	100	17619	9	2.3		37641	5	2.1
C6	0	40064	15	1.0		79479	19	1.0
L-AMPH	1000	33149	15	1.2		72649	2	1.1
		Ab#7				Ab#8		
Calibrator	Conc (ng/ml)	Mean	%CV	Modulation		Mean	%CV	Modulation
C1	10000	3887	16	62.5		4690	15	59.3
C2	3000	8148	2	29.8		12244	22	22.7
C3	1000	24487	4	9.9		26736	17	10.4
C4	300	61274	5	4.0		54934	14	5.1
C5	100	135422	6	1.8		156733	20	1.8
C6	0	242981	4	1.0		277875	30	1.0
L-AMPH	1000	276554	0	0.9		215669	6	1.3
		Ab#9				Ab#10		
Calibrator	Conc (ng/ml)	Mean	%CV	Modulation		Mean	%CV	modulation
C1	10000	3839	2	43.7		2033	12	56.6
C2	3000	8479	8	19.8		4274	7	26.9
C3	1000	19979	2	8.4		9460	4	12.2
C4	300	43857	4	3.8		20250	0	5.7
C5	100	72852	3	2.3		53635	6	2.1
C6	0	167697	10	1.0		115046	23	1.0
L-AMPH	1000	137543	18	1.2		119277	1	1.0
		Ab#11				Ab#12		
Calibrator	Conc (ng/ml)	Mean	%CV	modulation		Mean	%CV	modulation
C1	10000	40450	9	4.1		2247	20	58.1
C2	3000	68272	9	2.5		4415	1	29.6

C3	**1000**	122350	13	1.4		11157	11	11.7
C4	**300**	127882	11	1.3		29195	11	4.5
C5	**100**	171737	1	1.0		72965	4	1.8
C6	**0**	167461	11	1.0		130494	1	1.0
L-AMPH	**1000**	177887	19	0.9		145720	18	0.9
		Ab#16				**Ab#17**		
Calibrator	**Conc (ng/ml)**	**Mean**	**%CV**	**modulation**		**Mean**	**%CV**	**modulation**
C1	**10000**	148088	2	1.6		960916	8	1.2
C2	**3000**	179228	2	1.3		1032387	2	1.1
C3	**1000**	213486	7	1.1		1079616	4	1.1
C4	**300**	239522	10	1.0		1065417	2	1.1
C5	**100**	264916	5	0.9		1091870	4	1.1
C6	**0**	234438	8	1.0		1163721	8	1.0
L-AMPH	**1000**	250137	3	0.9		1077837	5	1.1
		Ab#18						
Calibrator	**Conc (ng/ml)**	**Mean**	**%CV**	**modulation**				
C1	**10000**	3334	11	42.6				
C2	**3000**	7499	32	18.9				
C3	**1000**	20753	3	6.8				
C4	**300**	36913	14	3.8				
C5	**100**	71672	8	2.0				
C6	**0**	141991	10	1.0				
L-AMPH	**1000**	123000	5	1.2				

Table [SEQ Table * ARABIC]: Recovery of Bio Rad controls in antibody initial screening

		Ab#1			**Ab#2**			**Ab#4**	
Bio Rad control #	**Conc (ng/ml)**	**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**
460	**0**	81			13			5	
461	**750**	723	96		637	85		859	115
462	**1250**	1115	89		1381	110		1256	100
463	**2000**	1743	87		2092	105		2425	121
		Ab#5			**Ab#6**			**Ab#7**	
Bio Rad control #	**Conc (ng/ml)**	**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**
460	**0**	0			1			0	

461	**750**	356	47		544	73	437	58
462	**1250**	765	61		1137	91	697	56
463	**2000**	1735	87		2147	107	1624	81
		Ab#8			**Ab#9**		**Ab#10**	
Bio Rad control #	**Conc (ng/ml)**	**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**	**Cal conc (ng/ml)**	**%Recovery**
460	**0**	0			0		32	
461	**750**	485	65		442	59	478	64
462	**1250**	1128	90		938	75	939	75
463	**2000**	1991	100		1477	74	1861	93
		Ab#11			**Ab#12**		**Ab#18**	
Bio Rad control #	**Conc (ng/ml)**	**Cal conc (ng/ml)**	**%Recovery**		**Cal conc (ng/ml)**	**%Recovery**	**Cal conc (ng/ml)**	**%Recovery**
460	**0**	337			105		240	
461	**750**	895	119		594	79	740	99
462	**1250**	1590	127		923	74	970	78
463	**2000**	2167	108		1943	97	2978	149

2.1.3 Antibody screening on MTP with the second format

In order to find the best assay format, four antibodies were selected to be tested on MTP with other formats. The second format was to conjugate antibodies with Biotin using Theranos in-house biotinylation kit and to coat biotinylated antibody on UA coated surface.

Methods:

The biotinylated antibody and conjugate direct binding was tested by MTP coated with UA at 20ug/ml in coating buffer and then Biotin labeled antibodies at various concentrations in blocking buffer. AMP-AP conjugate was then diluted in Theranos in-house small molecule AP buffer to 100ng/ml and incubated with coated antibody for 10min. The MTP was then washed and incubated with AP substrate for 10min. RLU was measured for each tip.

Amphetamine competition was tested in the second coating format following the same procedure as in initial screen with antibody direct coating format. Biotinylated antibodies were coated at 1ug/ml. AMP-AP conjugate working solution was 10ng/ml. Bio Rad controls were also used for antibody evaluation.

Results:

In the second format (coating with UA and Biotinylated antibody), all four antibodies showed good modulation of Amphetamine competition. Ab#12 gave the highest modulation and best recovery of Bio Rad controls.

Table [SEQ Table * ARABIC]: Antibody screening with Amphetamine competition from the second coating format: UA + Bioting-anti-AMP antibody

		Ab#4				Ab#6		
Calibrator	Conc (ng/ml)	Mean	%CV	Modulation		Mean	%CV	modulation
C1	10000	27200	6	10.1		23010	2	30.5
C2	3000	58966	11	4.7		63270	3	11.1
C3	1000	136197	3	2.0		151949	5	4.6
C4	300	203242	1	1.4		308452	0	2.3
C5	100	238449	2	1.2		472828	1	1.5
C6	0	274779	7	1.0		701915	2	1.0
L-AMPH	1000	289438	2	0.9		689660	2	1.0

		Ab#10				Ab#12		
Calibrator	Conc (ng/ml)	Mean	%CV	modulation		Mean	%CV	modulation
C1	10000	48715	7	22.7		4401	7	109.1
C2	3000	125519	2	8.8		11490	2	41.8
C3	1000	323612	1	3.4		38963	12	12.3
C4	300	609450	4	1.8		97740	1	4.9
C5	100	859953	2	1.3		214669	3	2.2
C6	0	1105909	2	1.0		480100	1	1.0
L-AMPH	1000	1091624	2	1.0		471037	1	1.0

Table [SEQ Table * ARABIC]: Recovery of Bio Rad controls in antibody screening using the second coating format

		Ab#4			Ab#6		Ab#10		Ab#12	
Sample #	AMP Conc (ng/ml)	Cal conc (ng/ml)	% Recovery		Cal conc (ng/ml)	% Recovery	Cal conc (ng/ml)	% Recovery	Cal conc (ng/ml)	% Recovery
460	0	0			19		30		19	
461	750	155	21		514	69	590	79	673	90
462	1250	500	40		709	57	856	69	915	73
463	2000	1497	75		1396	70	1622	81	1582	79

2.1.4 Antibody screening on MTP with the third format

The third format for antibody screen was using secondary antibody to coat MTP first and unconjugated primary anti-amphetamine antibody on the "second layer" of coating.

Methods:

In conjugate direct binding screening, the MTP was coated with goat anti-mouse IgG Fc at 20ug/ml in coating buffer and then coated with primary anti-amphetamine antibody at various concentrations in blocking buffer. AMP-AP conjugate was then diluted in Theranos in-house small molecule AP buffer to 100ng/ml and incubated with coated antibody for 10min. The MTP was then washed and incubated with AP substrate for 10min. RLU was measured for each tip.

Amphetamine competition was tested in the third coating format following the same procedure as in initial screen with antibody direct coating format. Anti-amphetamine antibodies were coated at 1ug/ml. AMP-AP conjugate working solution was 5ng/ml. Bio Rad controls were also used for antibody evaluation.

Results:

In the third format (coating with secondary antibody goat anti-mouse IgG Fc and anti-amphetamine antibody), all four antibodies showed good modulation of Amphetamine competition. Ab#12 gave the highest modulation and reasonable recovery of Bio Rad controls.

Table [SEQ Table * ARABIC]: Antibody screening with Amphetamine competition from the third coating format: Goat anti-mouse IgG Fc + mouse anti-amphetamine antibody

		Ab#4				Ab#6		
Calibrator	Conc (ng/ml)	Mean	%CV	Modulation		Mean	%CV	modulation
C1	10000	9235	4	37.3		7756	6	40.1
C2	3000	25217	1	13.7		22883	0	13.6
C3	1000	71215	1	4.8		58380	0	5.3
C4	300	139214	0	2.5		117101	2	2.7
C5	100	244019	4	1.4		223987	1	1.4
C6	0	344427	5	1.0		311252	2	1.0
L-AMPH	1000	340979	3	1.0		333146	1	0.9

		Ab#10				Ab#12		
Calibrator	Conc (ng/ml)	Mean	%CV	modulation		Mean	%CV	modulation
C1	10000	12454	3	38.0		1311	0	134.2
C2	3000	37948	4	12.5		3531	6	49.8
C3	1000	93337	2	5.1		8756	5	20.1
C4	300	199795	4	2.4		23486	3	7.5
C5	100	344051	2	1.4		69631	6	2.5
C6	0	473656	2	1.0		175974	4	1.0

L-AMPH	1000	483832	1	1.0		175990	4	1.0

Table [SEQ Table * ARABIC]: Recovery of Bio Rad controls in antibody screening using the third coating format

		Ab#4		Ab#6		Ab#10		Ab#12	
Sample #	Conc (ng/ml)	Cal conc (ng/ml)	% Recovery	Cal conc (ng/ml)	% Recovery	Cal conc (ng/ml)	% Recovery	Cal conc (ng/ml)	% Recovery
460	0	26		35		32		47	
461	750	576	77	507	68	547	73	536	71
462	1250	806	65	855	68	870	70	791	63
463	2000	1724	86	1550	78	1798	90	1405	70

2.2 Antibody screen on readers

From antibody screening on MTP, four antibodies were chosen for further evaluation on Edison readers. In order to find the best assay format, all three coating formats tested on MTP were used for screening on readers. Edison protocol Generic2_competitive_100x_10_10 was used. In this protocol, sample and conjugate were mixed first and then co-incubated with antibody coated tips. AMP-AP conjugate was prepared at 10x of final concentration and loaded into cartridges. During sample preparation on board, sample was diluted 1:100 and conjugate was diluted 1:10 in final mixture to co-incubate with antibody.

Bio Rad DOA controls were also tested in this stage serving as "training set" and evaluated for positive or negative determination in a qualitative approach using calibrator at 1000ng/ml as positive cutoff. (1000ng/ml was the cutoff value SAMSHA used for urine screen for a long time and was used in most commercial methods including Bio Rad DOA control preparation).

2.2.1 Antibody evaluation on readers with coating format-1: antibody direct coating

Methods:

In format-1, antibody was direct coated on tips at 1ug/ml in coating buffer. AMP-AP conjugate final concentration was 100ng/ml. AMP calibrators prepared in low BSA assay buffer were used at sample dilution 1:100.

Results:

All four antibodies had similar modulation in Amphetamine competition assay. Bio Rad controls evaluated in the qualitative approach showed good separation between positive and negative results. The results correlated well with Bio Rad data. Antibody #12 gave the best results among four antibodies.

Table [SEQ Table * ARABIC]: Antibody screening on readers: format-1

		Ab#4			Ab#6		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C1	10000	2093	14	28.2	2187	71	24.6
C2	3000						
C3	1000	8535	7	6.9	7523	22	7.1
C4	300	20737	15	2.8	14019	15	3.8
C5	100	37064	23	1.6	32089	15	1.7
C6	20	43926	4	1.3	41087	13	1.3
C7	0	58973	22	1.0	53720	20	1.0
L-AMPH	1000	47406	1	1	44671	6	1

		Ab#10			Ab#12		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C1	10000	1493	22	37.7	1931	12	30.3
C2	3000	4573	6	12.3	4550	22	12.9
C3	1000	6562	19	8.6	7195	13	8.1
C4	300	21266	10	2.7	18539	12	3.2
C5	100	31177	20	1.8	35577	11	1.6
C6	20	47165	25	1.2	57309	12	1.0
C7	0	56361	6	1.0	58491	5	1.0
L-AMPH	1000	72953	6	1	70909	11	1

Table [SEQ Table * ARABIC]: Bio Rad DOA controls on readers: format-1

			Ab#4			Ab#6		
Bio Rad #	Conc. (ng/ml)	Bio Rad preparation	Mean	%CV	Use Cutoff1000	Mean	%CV	Use Cutoff1000
460	0	Negative control	48964	3	NEG	49009	13	NEG
461	750	25% lower than cutoff	9868	23	NEG	11786	10	NEG
462	1250	25% higher than cutoff						
463	2000	two-fold higher than	5434	22	POS	5027	24	POS

		cutoff (positive control)						
			Ab#10			Ab#12		
Bio Rad #	Conc. (ng/ml)	Bio Rad preparation	Mean	%CV	Use Cutoff1000	Mean	%CV	Use Cutoff1000
460	0	Negative control	54219	14	NEG	55269	28	NEG
461	750	25% lower than cutoff	11037	4	NEG	9000	9	NEG
462	1250	25% higher than cutoff	7390	10	NEG	6677	5	POS
463	2000	two-fold higher than cutoff (positive control)	4751	6	POS	3919	14	POS

2.2.2 Antibody screening on readers with format-2

Methods:

In coating format-2, tips were coated with UA 20ug/ml in coating buffer and then Biotin labeled anti-amphetamine antibody 1ug/ml in blocking buffer. AMP-AP conjugate final concentration was 10ng/ml. Same Edison protocol was used as in testing coating format-1. Bio Rad DOA control samples were also tested.

Results:

In this format, very high %CV (>25%) among 6 tips in three cartridges was seen for all calibrators and Bio Rad controls with all four antibodies. It indicated that the format might not be optimal for antibodies in this assay. Although Bio Rad controls had good correlation with reported values, this format was not chosen for any more tests.

Table [SEQ Table * ARABIC]: Antibody screening on readers: format-2

		Ab#4			Ab#6		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	27301	38	6.0	22278	60	11.4
C4	300	27354	12	6.0	50940	31	5.0
C6	30	140502	53	1.2	134930	50	1.9
C7	0	164576	32	1.0	254793	54	1.0
		Ab#10			Ab#12		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	35341	39	12.1	12799	17	8.1
C4	300	112809	35	3.8	23115	34	4.5

C6	30	467350	15	0.9	136959	28	0.8
C7	0	426228	34	1.0	103183	20	1.0

Table [SEQ Table * ARABIC]: Bio Rad controls on readers: format-2

			Ab#4			Ab#6		
Bio Rad #	**Conc. (ng/ml)**	**Bio Rad preparation**	**Mean**	**%CV**	**Use Cutoff1000**	**Mean**	**%CV**	**Use Cutoff1000**
460	0	Negative control	118926	32	NEG	176587	35	NEG
461	750	25% lower than cutoff	33676	13	NEG	66228	71	NEG
462	1250	25% higher than cutoff	23351	25	POS	27223	48	NEG
463	2000	two-fold higher than cutoff (positive control)	22935	28	POS	22240	14	POS
			Ab#10			Ab#12		
Bio Rad #	**Conc. (ng/ml)**	**Bio Rad preparation**	**Mean**	**%CV**	**Use Cutoff1000**	**Mean**	**%CV**	**Use Cutoff1000**
460	0	Negative control	383196	46	NEG	99451	37	NEG
461	750	25% lower than cutoff	64482	47	NEG	17633	11	NEG
462	1250	25% higher than cutoff	31357	34	POS	9337	13	POS
463	2000	two-fold higher than cutoff (positive control)	34655	18	POS	6815	49	POS

2.2.3 Antibody screening on readers with format-3

Methods:

In coating format-3, tips were coated with secondary antibody goat anti-mouse IgG Fc 20ug/ml in coating buffer and then mouse anti-amphetamine antibody 1ug/ml in blocking buffer. AMP-AP conjugate final concentration was 1ng/ml. Same Edison protocol was used as in testing coating format-1. Bio Rad DOA controls were also tested.

Results:

In format-3, all four antibodies gave good modulation and Bio Rad controls had excellent correlation with all four antibodies. Because primary coating concentration was able to be

reduced to 1ug/ml and AMP-AP conjugate was used at 1ng/ml, this condition would be very helpful for increasing assay sensitivity and for saving materials. Format-3 was chosen as final format for further optimization.

Table [SEQ Table * ARABIC]: Antibody screening on readers: format-3

		Ab#4			Ab#6		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	98449	18	3.8	102865	20	3.8
C4	300	181062	5	2.0	176941	8	2.2
C6	30	269856	14	1.4	296936	10	1.3
C7	0	369233	10	1.0	394175	16	1.0

		Ab#10			Ab#12		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	81474	8	4.0	22286	21	10.1
C4	300	195962	13	1.6	61689	17	3.7
C6	30	305068	21	1.1	170437	23	1.3
C7	0	322178	11	1.0	225972	13	1.0

Table [SEQ Table * ARABIC]: Bio Rad controls on readers: format-3

			Ab#4			Ab#6		
Bio Rad #	Conc. (ng/ml)	Bio Rad preparation	Mean	%CV	Use Cutoff1000	Mean	%CV	Use Cutoff1000
460	0	Negative control	309753	7	NEG	323974	7	NEG
461	750	25% lower than cutoff	116510	5	NEG	106730	21	NEG
462	1250	25% higher than cutoff	94135	18	POS	97863	10	POS
463	2000	two-fold higher than cutoff (positive control)	54158	23	POS	54248	16	POS
			Ab#10			Ab#12		
Bio Rad #	Conc. (ng/ml)	Bio Rad preparation	Mean	%CV	Use Cutoff1000	Mean	%CV	Use Cutoff1000
460	0	Negative control	314700	23	NEG	207650	14	NEG
461	750	25% lower than cutoff	108729	3	NEG	30674	10	NEG
462	1250	25% higher than cutoff	66276	2	POS	19458	8	POS

463	2000	two-fold higher than cutoff (positive control)	44396	8	POS	11618	17	POS

2.2.4 Matrix comparison for antibody screening with format-3

This assay targeted to analysis both urine and serum/plasma samples. In order to evaluate matrix suitability, several urine samples and EDTA plasma samples from healthy donors were screened by reference methods (Amphetamine test cards or ELISA kit). A few negative samples were pooled together to make urine matrix or EDTA plasma matrix for testing matrix effect on these antibody candidates. Amphetamine was spiked into pooled urine or pooled EDTA plasma or low BSA assay buffer at various concentrations and analyzed with tips coated using format-3.

Methods:

All four antibodies were tested for matrix effect. Amphetamine calibrators prepared in low BSA buffer, spiked urine samples, and spiked EDTA plasma samples were analyzed with tips coated using format-3. All procedures were the same as antibody screening with format-3.

Results:

Urine and EDTA plasma were quite compatible for all four antibodies. Spiked urine samples and spiked EDTA plasma samples had similar signal and modulation as calibrators in buffer. Antibody #12 showed the highest modulation and the closest signal among different matrix.

Table [SEQ Table * ARABIC]: Matrix comparison with antibodies on format-3

Ab#4	Assay buffer			Urine			EDTA plasma		
AMP Conc. (ng/ml)	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1000	98449	18	3.8	87399	4	3.1	60883	21	4.2
300	181062	5	2.0	181840	5	1.5	151609	14	1.7
100				197001	5	1.4			
30	269856	14	1.4				211256	10	1.2
0	369233	10	1.0	269196	5	1.0	254137	4	1.0
Ab#6	Assay buffer			Urine			EDTA plasma		
AMP Conc. (ng/ml)	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1000	102865	20	3.8	87261	17	3.1	71647	11	4.3
300	176941	8	2.2	166137	13	1.6	174892	6	1.8
100				241546	12	1.1			

30	296936	10	1.3				261518	2	1.2
0	394175	16	1.0	267409	9	1.0	307205	19	1.0
Ab#10	Assay buffer			Urine			EDTA plasma		
AMP Conc. (ng/ml)	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1000	81474	8	4.0	88807	24	3.2	66445	9	5.3
300	195962	13	1.6	167840	23	1.7	190899	3	1.8
100				272736	16	1.0			
30	305068	21	1.1				272416	5	1.3
0	322178	11	1.0	284533	15	1.0	351698	18	1.0
Ab#12	Assay buffer			Urine			EDTA plasma		
AMP Conc. (ng/ml)	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**	**Mean RLU**	**%CV**	**Modulation**
1000	22286	21	10.1	23164	2	8.4	15444	30	12.8
300	61689	17	3.7	64391	3	3.0	54861	10	3.6
100				98715	9	2.0			
30	170437	23	1.3				156452	2	1.3
0	225972	13	1.0	194593	17	1.0	197166	16	1.0

2.2.5 Adjustment of antibody coating concentration for final selection

During antibody coating format evaluation, the first time measurement of Ab#12 stock solution concentration was found not accurate. Because all coating concentrations of Ab#12 in coating format evaluation were calculated from the first measurement, the actual coating concentration was 0.3ug/ml instead of 1ug/ml. In order to compare all antibodies at the same coating concentrations, all four antibodies were re-coated at 0.3ug/ml in format-3 to normalize coating condition.

Methods:

In format-3, tips were coated with goat anti-mouse IgG 20ug/ml in coating buffer and then mouse anti-amphetamine antibody at 0.3ug/ml in coating buffer. The following procedure was the same as antibody screening with format-3.

Results:

After coating concentration was adjusted to 0.3ug/ml for all four antibodies, Ab#12 still showed the highest modulation. Ab#12 was chosen as the final capture antibody to be used on format-3 for complete assay development.

Table [SEQ Table * ARABIC]: Comparison of antibodies and adjusted coating concentration

		Ab#4			Ab#6		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	21475	11	6.6	19323	6	6.8
C4	300	68718	19	2.1	59423	28	2.2
C6	30	145032	8	1.0	104594	5	1.3
C7	0	141806	22	1.0	131082	7	1.0

		Ab#10			Ab#12		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C3	1000	38085	21	6.2	20996	5	10.6
C4	300	72031	29	3.3	45550	40	4.9
C6	30	201494	12	1.2	157453	12	1.4
C7	0	237354	2	1.0	223020	8	1.0

2.3 Assay condition optimization

Ab#12 was chosen as the capture antibody. Antibody coating format-3 was selected as the final format. Further optimization was done to finalize assay conditions.

2.3.1 Antibody coating concentration titration and AMP-AP conjugate concentration titration

Because the assay was in a competitive format, titrations of coating concentration and detection conjugate concentration were conducted collaboratively to find the best combination for both signal range and modulation.

Methods:

Using assay format-3, tips were coated with goat anti-mouse IgG Fc at 20ug/ml in coating buffer, and then with Ab#12 in blocking buffer at concentrations 0.3ug/ml, 0.15ug/ml and 0.06ug/ml. AMP-AP detection conjugate was prepared as 10x stock at different dilutions to be sued with different coating conditions respectively. Amphetamine calibrators in buffer were used to calculate signal modulations. Edison protocol Generic2_competitive_100x_10-10 was used for sample and conjugate co-incubation and sample dilution of 1:100.

Results:

The results of coating concentration titration and detection conjugate titration showed that, if the conjugate concentration kept the same, the lower coating concentration gave the lower RLU

signal but the higher signal modulation. At the same coating concentration, the lower conjugate concentration gave the lower RLU signal but didn't change the signal modulation. To achieve the best modulation and also to keep the optimal RLU signal range, two coating conditions of 0.15ug/ml and 0.06ug/ml were taken to the next step to evaluate the effect of AP conjugate stabilizers.

Table [SEQ Table * ARABIC]: Titration of coating concentration and detection conjugate concentration

		AP conj=1:500,000 (1ng/ml)								
Ab#12 Coating conc.		0.3ug/ml			0.15ug/ml			0.06ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Mod.	Mean	%CV	Mod.	Mean	%CV	Mod.
C3	1000	20996	5	10.6	8620	24	14.5	1796	34	20.2
C4	300	45550	40	4.9	24400	5	5.1	6569	17	5.5
C6	30	157453	12	1.4	84756	19	1.5	26470	27	1.4
C7	0	223020	8	1.0	125082	23	1.0	36345	32	1.0

		AP conj=1:1,000,000 (0.5ng/ml)								
Ab#12 Coating conc.		0.3ug/ml			0.15ug/ml			0.06ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Mod.	Mean	%CV	Mod.	Mean	%CV	Mod.
C3	1000	8703	21	12.0	3916	25	13.7	911	4	20.3
C4	300	21523	15	4.9	9087	10	5.9	3431	7	5.4
C6	30	67802	0	1.5	32555	17	1.6	10216	7	1.8
C7	0	104517	13	1.0	53459	20	1.0	18534	8	1.0

		AP conj=1:2,000,000 (0.25ng/ml)					
Ab#12 Coating conc.		0.3ug/ml			0.15ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Mod.	Mean	%CV	Mod.
C3	1000	4592	9	10.5	2046	24	12.6
C4	300	15546	3	3.1	6339	11	4.1
C6	30	43447	8	1.1	21625	21	1.2
C7	0	47998	11	1.0	25805	25	1.0

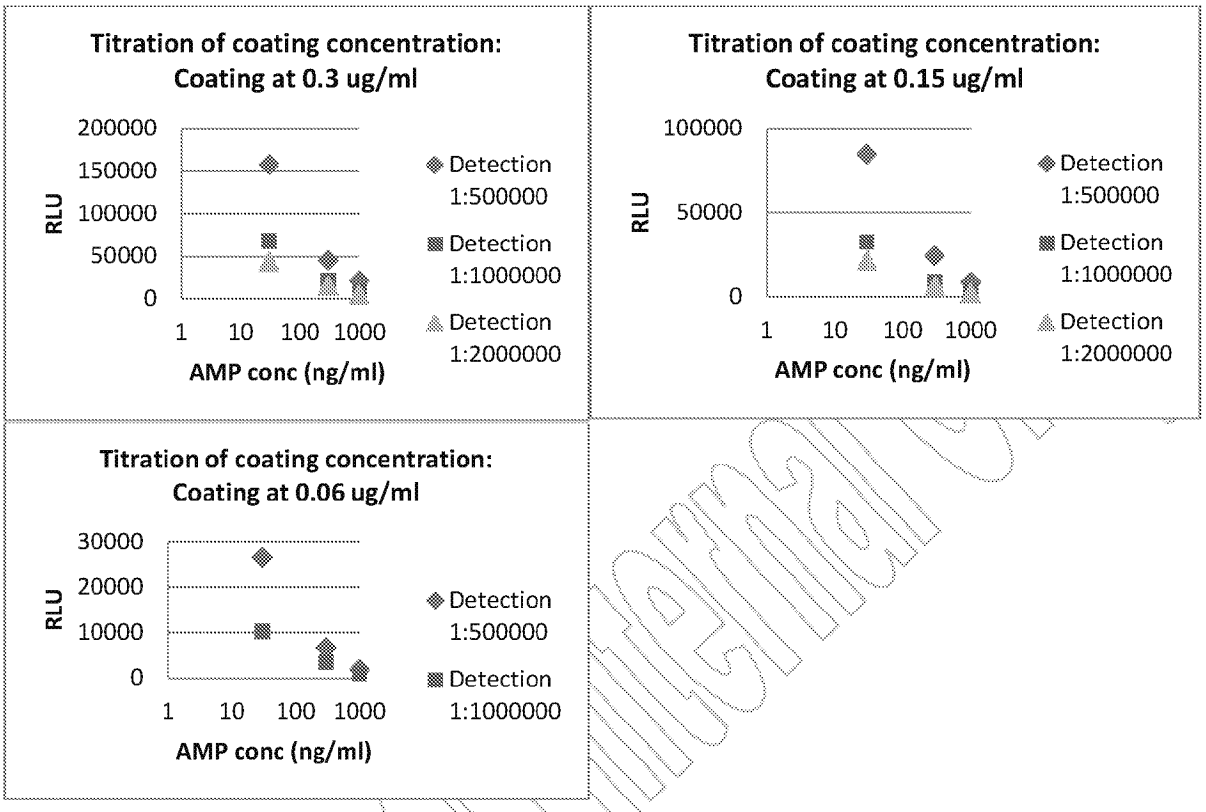
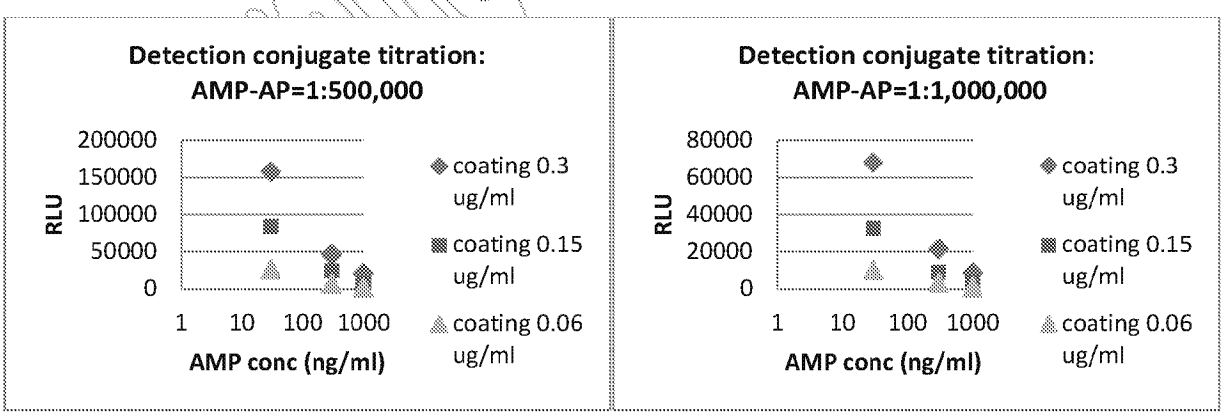


Figure [SEQ Figure * ARABIC]: Titration of coating concentration



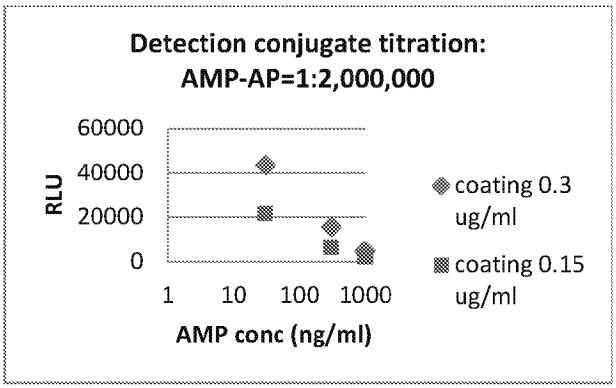


Figure [SEQ Figure * ARABIC]: Titration of detection conjugate

2.3.2 Selection of detection conjugate stabilizer

Methods:

With capture antibody at 0.15ug/ml or 0.06ug/ml in blocking buffer, detection conjugate was prepared at 1ng/ml in Sigma BioStab AP stabilizer, or Surmodics StabilZyme AP stabilizer, or Theranos in-house AP stabilizer. All conditions were tested with protocol Generic2_competitive-100x_10_10 to compare the effect of AP stabilizers.

Results:

All three AP stabilizers gave the similar signal modulations. However, BioStab and StabilZyme had much lower RLU signal range. Theranos in-house AP stabilizer was chosen as final reagent.

Table [SEQ Table * ARABIC]: Results of detection conjugate stabilizer comparison

AP stabilizer		In-house AP buffer, conj 1:500,000					
Coating conc.		0.15ug/ml			0.06ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Modulation	Mean	%CV	Modulation
C3	1000	8620	24	14.5	1796	34	20.2
C4	300	24400	5	5.1	6569	17	5.5
C6	30	84756	19	1.5	26470	27	1.4
C7	0	125082	23	1.0	36345	32	1.0

AP stabilizer		BioStab, conj 1:500,000					
Coating conc.		0.15ug/ml			0.06ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Modulation	Mean	%CV	Modulation
C3	1000	1080	5	15.5	358	16	21.9
C4	300	2822	22	5.9	1156	27	6.8
C6	30	9696	14	1.7	3148	40	2.5
C7	0	16761	19	1.0	7847	28	1.0

AP stabilizer		StabilZyme, conj 1:500,000					
Coating conc.		0.15ug/ml			0.06ug/ml		
Sample	Conc. (ng/ml)	Mean	%CV	Modulation	Mean	%CV	Modulation
C3	1000	945	24	13.9	265	20	21.1
C4	300	2340	15	5.6	721	21	7.7
C6	30	8183	4	1.6	2582	11	2.2
C7	0	13091	15	1.0	5590	18	1.0

2.3.3 Determination of sample dilution factor

2.3.3.1 Comparison of sample dilution of Amphetamine calibrators in assay buffer

Methods:

Capture antibody was coated at 0.15ug/ml or 0.06ug/ml in blocking buffer. Detection conjugate was used at 1ng/ml in Theranos in-house AP stabilizer. Samples were tested at 1:100, 1:50 or 1:25 dilutions. Theranos low BSA assay buffer was used as sample diluent for all dilution conditions. The following Edison protocols, Generic2_competitive_100x_10_10, Generic2_competitive_50x_10_10, or Geberic2_competitive_25x_10_10, were used for each sample dilution condition respectively.

Results:

At both coating concentrations, the less the sample being diluted gave the higher modulation of the signal of each calibrator to buffer background. With the concern of matrix effect at different sample dilutions, spiked urine and plasma samples were tested further to compare with calibrators in buffer.

Table [SEQ Table * ARABIC]: Results of sample dilution factor determination with calibrators in assay buffer

		coating at 0.15ug/ml, AP conj 1:500,000 in in-house buffer								
		sample dilution 100x			sample dilution 50x			sample dilution 25x		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
C3	1000	8620	24	**14.5**	4040	17	**29.6**	1694	18	**80.6**
C4	300	24400	5	**5.1**	15375	17	**7.8**	6352	2	**21.5**
C6	30	84756	19	**1.5**	57691	2	**2.1**	42318	10	**3.2**
C7	0	125082	23	**1.0**	119425	2	**1.0**	136583	12	**1.0**

		coating at 0.06ug/ml, AP conj 1:500,000 in in-house buffer								
		sample dilution 100x			sample dilution 50x			sample dilution 25x		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.	Mean RLU	%CV	Mod.
C3	1000	1796	34	**20.2**	1216	7	**32.9**	461	18	**125.0**
C4	300	6569	17	**5.5**	3722	29	**10.8**	2428	33	**23.7**
C6	30	26470	27	**1.4**	24335	27	**1.6**	17439	27	**3.3**
C7	0	36345	32	**1.0**	40042	17	**1.0**	57655	23	**1.0**

2.3.3.2 Comparison of matrix effect at 50x and 100x sample dilutions

With the objectives of keeping the final assay procedure less complicated and keeping the possibility to use one generic protocol for multiple DOA assays, the effect was to minimize matrix effect so that one protocol would be used for testing both urine and plasma samples. To make it possible to use calibration curve prepared in assay buffer to calculate both urine and plasma samples, a group of spiked urine samples and plasma sample were prepared to the targeting clinical concentration range. Spiked samples were analyzed using both 50x and 100x protocols and calculated from a calibration curve at the same condition.

Methods:

Spiked urine and plasma samples prepared using pooled urine matrix or pooled plasma matrix were tested using 50x or 100x sample dilution protocols to confirm matrix compatibility. All spiked samples were calculated from a calibration curve at the same condition.

Results:

By comparing RLU signal and modulations, both 50x and 100x sample dilutions had minimum matrix effect among buffer, urine and EDTA plasma. However, when spiked samples were calculated from calibration curve made in assay buffer, sample dilution at 50x had less accurate concentration recovery. Sample dilution at 100x showed satisfied accuracy and recovery for when calculating urine and plasma samples from calibration curve prepared in assay buffer. To simplify the assay condition, calibration curve prepared assay buffer could be used for analyzing both urine and plasma samples at sample dilution 1:100. Sample dilution of 100-fold was chosen for final procedure.

Results of 50x sample dilution:

Table [SEQ Table * ARABIC]: Calibration curve with 50x sample dilution

Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	back cal. Conc. (ng/ml)	% accuracy
C1	10000	289	2	177.6	10354	104

C2	3000	795	21	64.5	2268	76
C3	1000	1236	26	41.5	1356	136
C4	300	5889	11	8.7	270	90
C5	100	13220	28	3.9	99	99
C6	30	26288	14	2.0	32	107
C7	10	46607	11	1.1	10	97
C7	0	51279	11	1.0		

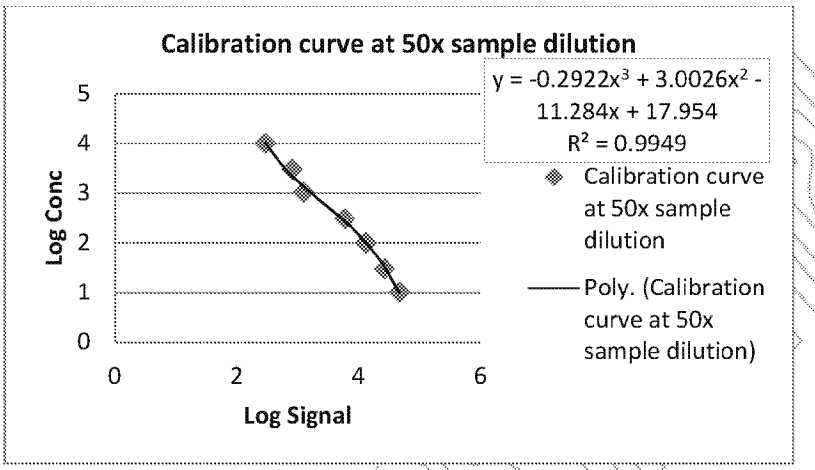


Figure [SEQ Figure * ARABIC]: Calibration curve of 50x sample dilution

Table [SEQ Table * ARABIC]: Spiked urine and plasma samples analyzed at 50x sample dilution

Sample Matrix	Conc. (ng/ml)	Mean RLU	%CV	back cal. Conc. (ng/ml)	% accuracy
urine	1250	1694	11	971	78
urine	1000	2141	13	765	76
urine	750	2373	10	690	92
urine	375	4340	25	375	100
urine	300	5127	18	314	105
urine	225	7613	13	202	90
urine	100	9198	22	160	160
urine	0	39469	7	14	--
EDTA plasma	1000	987	6	1748	175
EDTA plasma	300	4008	24	407	136
EDTA plasma	37.5	25735	15	33	89
EDTA plasma	30	24321	14	37	124
EDTA plasma	22.5	25192	8	35	155
EDTA plasma	0	46546	13	10	--

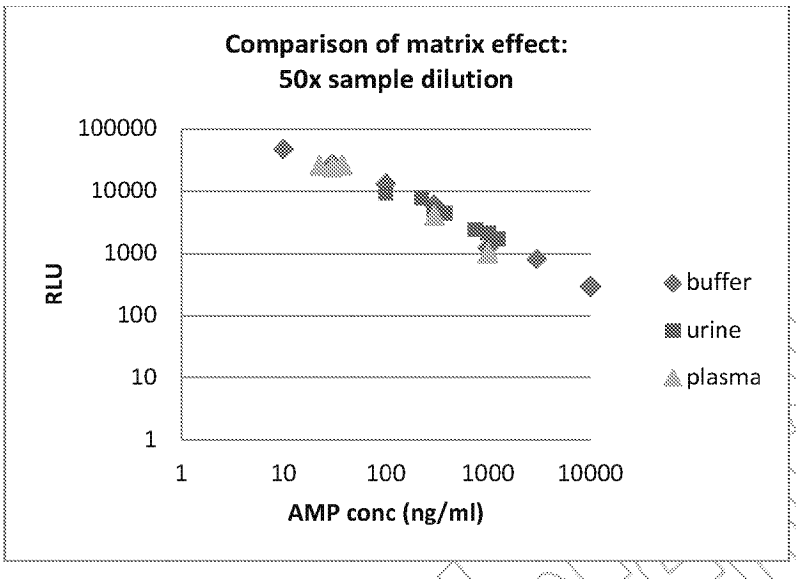


Figure [SEQ Figure * ARABIC]: Matrix comparison at 50x sample dilution

Results of 100x sample dilution:

Table [SEQ Table * ARABIC]: Calibration curve with 100x sample dilution

Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	back cal. Conc. (ng/ml)	%accuracy
C1	10000	845	9	119.85	10510	105
C2	3000	2524	7	40.13	2571	86
C3	1000	6678	23	15.17	1152	115
C4	300	22163	11	4.57	330	110
C5	100	46403	3	2.18	88	88
C6	30	73896	18	1.37	27	89
C7	10	96576	8	1.05	12	116
C8	0	101278	16	1.00	OORL	--

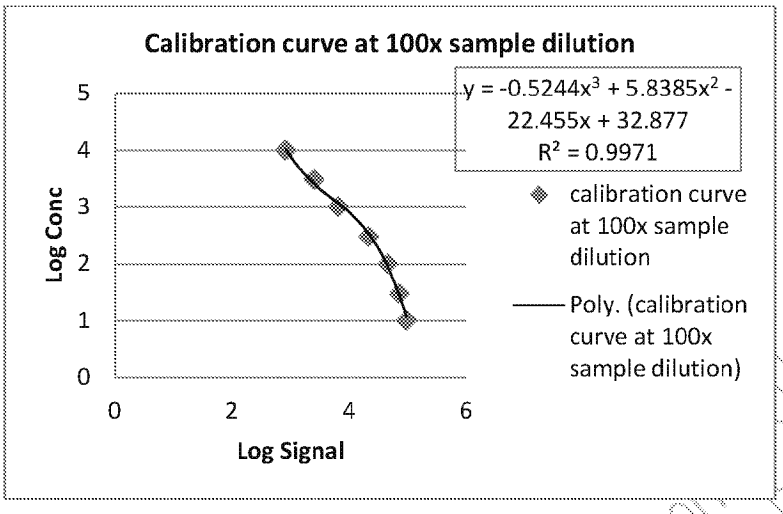


Figure [SEQ Figure * ARABIC]: Calibration curve with 100x sample dilution

Table [SEQ Table * ARABIC]: Spiked urine and plasma samples analyzed at 100x sample dilution

Sample matrix	Conc. (ng/ml)	Mean RLU	%CV	back cal	%accuracy
Urine	3000	2404	7	2692	90
Urine	1250	4611	19	1544	124
Urine	1000	6943	3	1116	112
Urine	750	8726	12	921	123
Urine	375	16769	13	475	127
Urine	300	21006	8	355	118
Urine	225	26413	9	253	113
Urine	0	87098	14	OORL	--
EDTA plasma	300	20158	21	376	125
EDTA plasma	100	44165	4	98	98
EDTA plasma	37.5	67937	11	29	98
EDTA plasma	30	71542	10	34	90
EDTA plasma	22.5	76193	20	24	109
EDTA plasma	0	104541	17	OORL	--

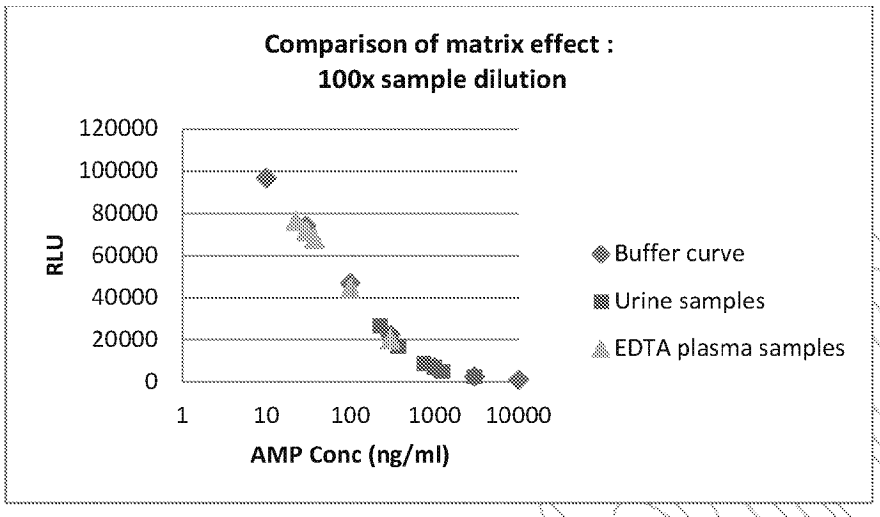


Figure [SEQ Figure * ARABIC]: Matrix comparison at 100x sample dilution

2.3.4 Comparison of incubation time

Methods:

After sample dilution was selected at 100x, sample and substrate incubation time was tested using Edison protocols Genric2_competitive_100x_10_10, Genric2_competitive_100x_5_5, and Genric2_competitive_100x_2_1.

Results:

Incubation 5_5 or 2_1 gave lower modulation and affected assay sensitivity. If incubation time 5_5 or 2_1 would be using, the optimization of coating concentration and detection conjugate concentration might be needed. Without further optimization, incubation time10_10 was chosen for analyzing more samples.

Table [SEQ Table * ARABIC]: Comparison of incubation time

Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C1	10000	845	9	120	417	8	83	110	8	60
C2	3000	2524	7	40	1142	12	30	236	27	28
C3	1000	6678	23	15	3237	29	11	495	21	13
C4	300	20431	17	5.0	11257	34	3.1	1751	5	3.8
C5	100	46403	3	2.2	18402	21	1.9	2932	12	2.3
C6	30	73896	18	1.4	31882	1	1.1	5019	15	1.3
C7	10	96576	8	1.0	35926	9	1.0	6698	6	1.0
C8	0	101278	16	1.0	34550	22	1.0	6598	11	1.0

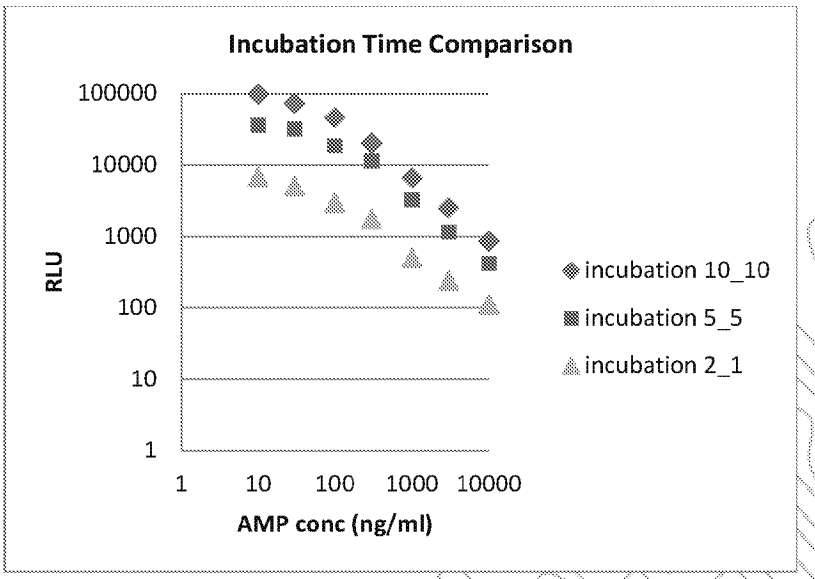


Figure [SEQ Figure * ARABIC]: Comparison of incubation time

2.4 Analysis of clinical samples and control samples with AMP-AP conjugate from commercial vendor

After assay condition optimization, the final procedure was set as:

- Tip coating with goat anti-mouse IgG Fc at 20ug/ml in coating buffer first, then mouse anti-Amphetamine monoclonal antibody (Ab#12) at 0.15ug/ml in blocking buffer
- Sample dilution 100-fold in Theranos low BSA assay buffer
- AMP-AP conjugate from YJ-Bioproducts was mixed with diluted sample at final working concentration 0.1ng/ml to co-incubate antibody
- Edison protocol: Generic2_competitive_100x_10_10

Because of the limited availability of drugs-of-abuse positive clinical samples, some DOA positive control sample panels were used to verify the assay performance. Besides Bio Rad DOA controls used in early assay development, the following samples were ordered and tested with final assay protocol:

- DOA positive urine panel I (AMPs) from Zeptometrix, Cat#KZMC014, Lot# 1112-272-00005
- DOA urine control from Synerhent Biochem, Cat#371, Lot#1224703
- DOA Amphetamine positive urine and saliva pair from Sunnylab, Cat#U105, Lot#1454 and 1555

Urine samples and plasma samples from healthy donors were used to make spiking samples.

2.4.1 Clinical and control samples verification by reference methods

The following commercial methods were used as reference assays for Amphetamine assay development:

- Amphetamine test card, DRG, Cat#RAP-2583
- Amphetamine test card, Rapidtest/Diagnostic Automation, Cat#121020-1
- Amphetamine ELISA kit, MyBiosource, Cat#MBS580057

Test card from DRG is FDA approved method for qualitative urine sample screening at positive cut-off of 1000ng/ml. Test card from Rapidtest/Diagnostic Automation is not FDA approved method, and it is also a qualitative method for urine sample only with positive cut-off at 1000ng/ml. ELISA kit from MyBiosource has a positive control sample with reported value of 50ng/ml. Suggested sample dilution is 20-fold to make positive cut-off at 1000ng/ml. When this ELISA kit was used for Theranos assay development, an alternative quantitative measurement was conducted by diluting positive control to a series of concentration and to be used as calibration curve. The LLOQ of ELISA kit was 20ng/ml of Amphetamine in urine or plasma with sample minimum dilution of 1:20.

Urine control samples, urine sample from healthy donors and spiked urine samples were tested by DRG test card and Rapidtest test Card at no dilution. Some urine samples were also tested by ELISA kit at sample dilution of 1:20 or more. Plasma samples from healthy donors and spiked samples were tested by ELISA kit only with sample dilution of 1:20 or more.

Results of samples measured by reference methods are listed in Tables in section 2.4.3.

2.4.2 Calibrator verification by reference methods

Amphetamine calibrators were analyzed by reference methods to verify prepared concentration.

Table [SEQ Table * ARABIC]: Calibrator verification by reference methods

			DRG test card (cutoff 1000ng/ml)	Rapidtest test card (cutoff 1000ng/ml)	My Biosource ELISA kit (cutoff 1000ng/ml, LLOQ 20ng/ml)	
	Sample	*Nominal conc. (ng/ml)*	+/-	+/-	ng/ml	+/-
Calibrators D-AMP in low BSA buffer	C1	***10000***	+	+	10323	+
	C2	***3000***	+	+	3018	+
	C3	***1000***	+	+	1058	+
	C4	***300***	-	+	313	-

	C5	***100***	-	-	103	-
	C6	***0***	-	-	OORL	-
L-AMP	L-AMP	***1000***	-	-	OORL	-

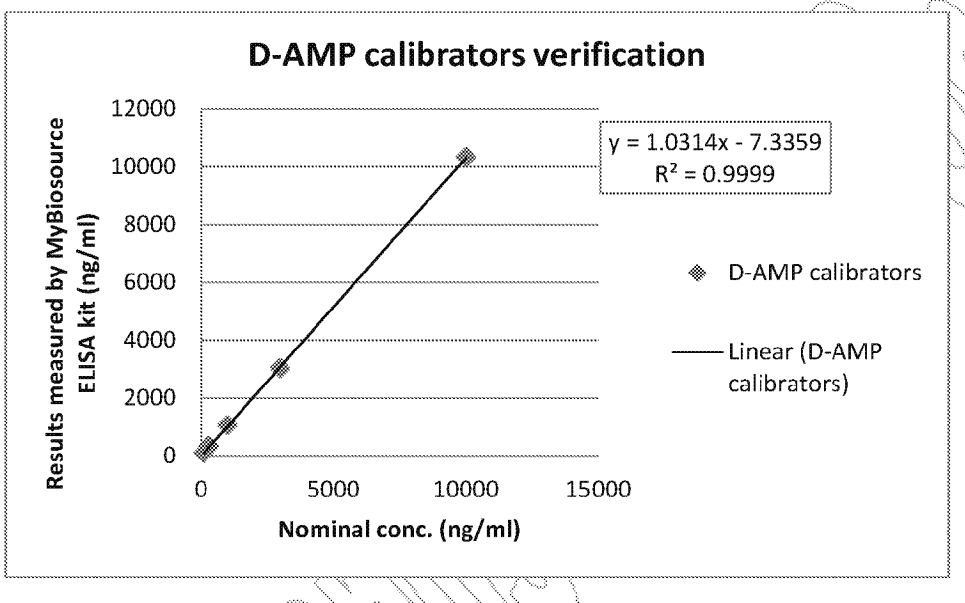


Figure [SEQ Figure * ARABIC]: Calibrator verification by ELISA kit

2.4.3 Urine clinical samples and control samples analyzed by Theranos method

2.4.3.1 DOA Amphetamine urine control panel from Zeptometrix

Zeptometrix DOA positive control panel I, which is Amphetamine positive controls containing 10 samples (8 positive and 2 negative), was analyzed by Thersnos method and results were compared with results from reference methods.

Although Theranos results of some samples (sample #2, #4, #5, #7) did not match with reported values from Zeptometrix, Thersnos results were correlated with reference methods well. The discrepancy might come from the antibody specificity to difference Amphetamine analogs in different methods.

Samples from Zeptometrix all contained a panel of DOA substances other than D-Amphetamine. These samples could also be treated as cross reactivity and interference test.

Table [SEQ Table * ARABIC]: Sample information of Zeptometrix DOA Panel I

UroDetect Drugs of Abuse Panel I – Amphetamine

Lot Number: 1112-272-00005

Panel Member	Amphetamines Ref Range*: 450-550 ng/mL	Barbiturates Ref Range*: 270-330 ng/mL	Benzodiazepine Ref Range*: 270-330 ng/mL	Cannabinoid Ref Range*: 45-55 ng/mL	Cocaine Ref Range*: 125-175 ng/mL	Oxycodone Ref Range*: 0 ng/mL	Methadone Ref Range*: 270-330ng/mL
1	5,304 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	1,286 (Pos)	Negative (<270)
2	859 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	1,385 (Pos)
3	10,596 (Pos)	Negative (<270)	2,294 (Pos)	Negative (<45)	Negative (<125)	Negative	846 (Pos)
4	1,500 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	Negative (<270)
5	857 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	1,281 (Pos)
6	5118 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	Negative (<270)
7	1,032 (Pos)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	1,068 (Pos)
8	5,663 (Pos)	Negative (<270)	3,272 (Pos)	Negative (<45)	Negative (<125)	1,208 (Pos)	Negative (<270)
9	Negative (<450)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	Negative (<270)
10	Negative (<450)	Negative (<270)	Negative (<270)	Negative (<45)	Negative (<125)	Negative	Negative (<270)

Table [SEQ Table * ARABIC]: Results of Zeptometrix DOA Panel measured by Theranos method and reference methods

			Results from commercial methods				Theranos Results (LLOQ 30ng/ml)			
		Reported value	DRG test card (cutoff 1000ng/ml) FDA approved IVD	RapidTest Test card (cutoff 1000ng/ml)	My Biosource ELISA kit (cutoff 1000ng/ml, LLOQ 20ng/ml)					
	Sample	*AMP conc. (ng/ml)*	+/-	+/-	ng/ml	+/-	Mean RLU	%CV	AMP conc (ng/ml)	cutoff 1000ng/ml
Zeptometrix DOA positive urine panel I (AMPs) Lot# 1112-272-00005	Z1	***5304***	+	not tesetd	6445	+	1413	11	4906	+
	Z2	***859***	-	not tesetd	OORL	-	83329	5	OORL	-
	Z3	***10596***	+	not tesetd	5760	+	1698	14	3943	+
	Z4	***1500***	-	not tesetd	84.7	-	51011	5	71	-
	Z5	***857***	-	not tesetd	OORL	-	82412	3	OORL	-
	Z6	***5118***	+	not tesetd	7948.5	+	1851	14	3586	+
	Z7	***1032***	-	not tesetd	108	-	50005	33	74	-
	Z8	***5663***	+	not tesetd	17085	+	2295	32	2880	+
	Z9	***-ve***	-	not tesetd	OORL	-	100187	9	OORL	-
	Z10	***-ve***	-	not tesetd	OORL	-	86865	11	OORL	-

2.4.3.2 DOA Amphetamine urine control panel from Synerhent Biochem

DOA urine control panel from Synerhent Biochem contains 5 control samples. They all have reported values by GC-MS for a panel of DOA substances. These samples could also be used for evaluating cross reactivity and interference.

All 5 samples were analyzed by Theranos method and reference methods. Amphetamine recovery for all 5 control levels were correlated well with the reported value as measured by GC-MS, and correlated with results from reference methods. No cross reactivity or interference from other DOA substances in these samples was observed.

Table [SEQ Table * ARABIC]: Sample information of Synerhent Biochem DOA urine control samples

	Control Level (sample #)	571-1	570-1	570-2	571-2	571-3
Non-Target Drug Levels by GC-MS, ng/mL	11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid	0	40.4	104.5	113	386
	Amitriptyline	0	831	1470	1910	3510
	Benzoylecgnine	0	229	362	428	776
	d-Amphetamine	0	714	1086	1350	2827
	Methadone	0	207	394	418	897
	Methaqualone	0	238	368	428	945
	Morphine	0	268	406	480	951
	Oxazepam	0	142	266	306	611
	Propoxyphene	0	230	358	464	938
	Secobarbital	0	223	356	468	892

Table [SEQ Table * ARABIC]: Results of Synerhent Biochem DOA Panel measured by Theranos method and reference methods

			Results from commercial methods				Theranos Results (LLOQ 30ng/ml)			
		AMP Reported value	DRG test card (cutoff 1000ng/ml) FDA approved IVD	RapidTest (cutoff 1000ng/ml)	My Biosource ELISA kit (cutoff 1000ng/ml, LLOQ 20ng/ml)					
	Sample	*conc. (ng/ml)*	+/-	+/-	ng/ml	+/-	Mean RLU	% CV	AMP conc (ng/ml)	cutoff 1000ng/ml
Synerhent Biochem DOA urine control	SU-0	*-ve*	-	-	OORL	-	100032	17	<30	-
	SU-1	***714***	+	+	698.1	-	9563	8	868	-
	SU-2	***1086***	+	+	1014.4	+	7091	13	1124	+
	SU-3	***1350***	+	+	1276.2	+	5502	20	1379	+

	SU-4	***2827***	+	+	3054	+	2686	8	2488	+

2.4.3.3 Paired urine and saliva samples from Sunnylab

A paired urine and saliva samples from the patient positive for Amphetamine were obtained from Sunnylab. These paired samples do not have reported Amphetamine value from Sunnylab and are only known being collected from Amphetamine positive patient. The samples were analyzed by Theranos method and reference methods. Results from Theranos methods correlated with results from reference methods very well.

Table [SEQ Table * ARABIC]: Results of Sunnylab Amphetamine positive paired urine and saliva samples by Theranos method and reference methods

			Results from commercial methods				Theranos Results (LLOQ 30ng/ml)			
	Sample	*Reported value*	DRG test card (cutoff 1000ng/ml) FDA approved IVD	RapidTest (cutoff 1000 ng/ml)	My Biosource ELISA kit (cutoff 1000 ng/ml, LLOQ 20ng/ml)					
	Sample	*AMP*	+/-	+/-	ng/ml	+/-	Mean RLU	% CV	AMP conc (ng/ml)	cutoff 1000ng/ml
Sunnylab paired sample U105	Urine 1454	From positive patient	+	+	5411	+	1664	2	4034	+
	Saliva 1455	From positive patient	-	-	332	-	20141	10	381	-

2.4.3.4 Urine sample screening and analysis of spiked urine samples

Urine samples from a collection of healthy donors were screened by Theranos methods and reference methods. Because of the difficulty of obtaining DOA positive clinical samples, a few of these negative urine samples were used to prepare spiked urine positive. SAMSHA urine screening cutoff and confirmation cutoff have been updated a few times and different cutoff values have been mentioned in different documents, so the spiked samples were prepared to target different cutoff values. Following the requirement of DOA screening test, samples containing Amphetamine concentrations at 25% higher than cutoff or 25% lower than cutoff were prepared as quality controls of qualitative determination.

The results of analyzing spiked samples by Theranos methods showed very good recovery to the expected values. When using qualitative determination, all samples were in the correct category as expected.

Table [SEQ Table * ARABIC]: Urine samples screening by Theranos method and reference methods

		Results from commercial methods				Theranos Results (LLOQ 30ng/ml)			
		DRG test card (cutoff 1000ng/ml)FDA approved IVD	RapidTest (cutoff 1000ng/ml)	My Biosource ELISA kit (cutoff 1000ng/ml, LLOQ 20ng/ml)					
	Urine Sample	+/-	+/-	ng/ml	+/-	Mean RLU	%CV	AMP conc (ng/ml)	cutoff 1000ng/ml
Healthy donor collection	U1	NEG	NEG	OORL	NEG	103044	1	OORL	NEG
	U2	NEG	NEG	OORL	NEG	107366	2	OORL	NEG
	U3	NEG	NEG	OORL	NEG	77550	5	OORL	NEG
	U4	NEG	NEG	OORL	NEG	77058	1	OORL	NEG
	U5	NEG	NEG	OORL	NEG	111448	16	OORL	NEG
	U6	NEG	NEG	OORL	NEG	87768	13	OORL	NEG
	U7	NEG	NEG	OORL	NEG	59045	2	49	NEG
	U8	NEG	NEG	OORL	NEG	105279	22	OORL	NEG
	U9	NEG	NEG	OORL	NEG	88828	9	OORL	NEG
	U10	NEG	NEG	OORL	NEG	88162	15	OORL	NEG
	U11	NEG	NEG	OORL	NEG	103394	1	OORL	NEG
	U12	NEG	NEG	OORL	NEG	110709	14	OORL	NEG
	U13	NEG	NEG	OORL	NEG	87412	5	OORL	NEG
	U14	NEG	NEG	OORL	NEG	106307	6	OORL	NEG
	U15	NEG	NEG	OORL	NEG	106016	14	OORL	NEG
	U16	NEG	NEG	OORL	NEG	105913	5	OORL	NEG
	U17	NEG	NEG	OORL	NEG	106833	14	OORL	NEG
	U18	NEG	NEG	OORL	NEG	108054	8	OORL	NEG
	U19	NEG	NEG	OORL	NEG	104850	6	OORL	NEG

Table [SEQ Table * ARABIC]: Analysis of spiked urine samples by Theranos method

Sample #	Nominal conc. (ng/ml)	Mean RLU	%CV	Cal. Conc. (ng/ml)	% Recovery	Conc Target	cutoff 1000	cutoff 500	cutoff 200
spiked 1	3000	2721	19	2695	90	3x of cutoff 1000ng/ml	POS	POS	POS

spiked 2	1250	6935	18	1092	87	+25% of cutoff 1000ng/ml	POS	POS	POS
spiked 3	1000	9788	6	816	82	at cutoff 1000ng/ml	cutoff	POS	POS
spiked 4	750	12162	7	673	90	-25% of cutoff 1000ng/ml	NEG	POS	POS
spiked 5	625	12402	15	661	106	+25% of cutoff 500ng/ml	NEG	POS	POS
spiked 6	500	15133	13	545	109	at cutoff 500ng/ml	NEG	POS	POS
spiked 7	375	20425	5	393	105	-25% of cutoff 500ng/ml	NEG	cutoff	POS
spiked 8	250	25550	4	296	118	+25% of cutoff 200ng/ml	NEG	NEG	POS
spiked 9	200	35243	18	183	91	at cutoff 200ng/ml	NEG	NEG	cutoff
spiked 10	150	39826	4	148	99	-25% of cutoff 200ng/ml	NEG	NEG	NEG

2.4.4 Blood samples analyzed by Theranos method

2.4.4.1 Anticoagulant effect

Whole blood, serum, EDTA plasma and heparin plasma samples from seven donors (5 male and 2 female) were obtained in pairs from Stanford Blood Center. All samples were analyzed with final assay procedure. All results came as negative as expected and there were no significant differences of signal among whole blood, EDTA plasma, heparin plasma and serum. If plasma is going to be collected for analysis, both EDTA and Li-heparin could be used as anticoagulant.

Table [SEQ Table * ARABIC]: Blood sample matrix effect and anticoagulant effect

Donor #	Whole blood				Serum			
	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Cutoff 30ng/ml	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Cutoff 30ng/ml
M1	78860	4	OORL	NEG	74357	10	OORL	NEG
M2	63039	15	16.9	NEG	75310	14	OORL	NEG
M3	78755	27	OORL	NEG	73295	6	10.18	NEG
M4	75110	3	OORL	NEG	80876	1	OORL	NEG
M5	77038	12	OORL	NEG	85929	14	OORL	NEG

F1	77280	8	OORL	NEG	60703	15	19.00	NEG
F2	66842	12	13.9	NEG	80580	20	OORL	NEG

Donor #	EDTA plasma				Heparin plasma			
	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Cutoff 30ng/ml	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Cutoff 30ng/ml
M1	84803	10	OORL	NEG	61959	0	17.8	NEG
M2	66468	8	14.2	NEG	60710	16	19.0	NEG
M3	67184	10	13.7	NEG	53780	14	27.4	NEG
M4	52845	1	28.8	NEG	75870	4	OORL	NEG
M5	63501	6	16.5	NEG	72503	16	10.6	NEG
F1	67177	0	13.7	NEG	68600	25	12.8	NEG
F2	65789	16	14.7	NEG	75922	8	OORL	NEG

2.4.4.2 Analysis of spiked plasma samples

Spiked plasma samples were prepared with different Amphetamine concentration levels and analyzed by Theranos method. The recovery of each sample to the expected values was within the acceptable range. When using qualitative determination, all samples were in the correct category as expected.

Table [SEQ Table * ARABIC]: Analysis of spiked plasma samples

	Nominal conc. (ng/ml)	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	% recovery	Conc. target	Cutoff 30ng/ml
SP1	1000	3259	18	1189	118.9	very high conc.	POS
SP2	100	29598	14	116	115.6	3x of cutoff	POS
SP3	37.5	44749	16	45	120.9	+25% of cutoff	POS
SP4	30	50454	20	33	109.4	cutoff	cutoff
SP5	22.5	54687	2	26	115.8	-25% of cutoff	NEG
blank	no spike	70709	5	OORL			NEG

2.4.4.3 Effect of positive HAMA and RF factor

Three HAMA positive serum samples and three RF positive samples from PromedDx were analyzed with final assay condition. All samples were also analyzed by MyBiosource ELISA kit.

All samples tested showed negative in Theranos method, agreeing with referecen method. The results indicated that HAMA positive and RF positive status didn't affect Amphetamine analysis in Theranos method.

Table [SEQ Table * ARABIC]: Results of analysis of HAMA positive samples and RF positive samples

	MyBiosource ELISA kit		Theranos method			
Sample #	Conc. (ng/ml)	Cutoff 30ng/ml	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Cutoff 30ng/ml
HAMA +ve 1	OORL	NEG	79636	11	OORL	NEG
HAMA +ve 2	OORL	NEG	60878	3	19	NEG
HAMA +ve 3	OORL	NEG	74957	7	OORL	NEG
RF +ve 1	OORL	NEG	86836	23	OORL	NEG
RF +ve 2	OORL	NEG	97085	25	OORL	NEG
RF +ve 3	OORL	NEG	88292	12	OORL	NEG

2.4.4.4 Effect of interfering matrixes

Hemolyzed serum, lipemic serum, and icteric serum were tested to evaluate potential interference. Amphetamine was spiked into these matrixes at various levels and spiked samples were analyzed for calculating spiking recovery.

All spiked samples had acceptable recovery to the expected values. Hemolyzed serum, lipemic serum, and icteric serum didn't show matrix effect to interfere Amphetamine measurement in this assay.

Table [SEQ Table * ARABIC]: Results of evaluating interfering matrixes

	Nominal conc. (ng/ml)	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	% recovery	Conc. target	Cutoff 30ng/ml
Hemolyzed serum	100	34186	5	86	85.9	3x of cutoff	POS

	37.5	50775	16	32	86.0	+25% of cutoff	POS
	22.5	60573	28	19	85.0	-25% of cutoff	NEG
	no spike	76965	16	OORL			NEG
Lipemic serum	100	32904	9	93	93.2	3x of cutoff	POS
	37.5	46489	6	41	109.4	+25% of cutoff	POS
	22.5	56635	18	23	104.4	-25% of cutoff	NEG
	no spike	74867	11	OORL			NEG
Icteric serum	100	41593	24	111	111.2	3x of cutoff	POS
	37.5	65580	12	37	99.7	+25% of cutoff	POS
	22.5	66067	17	27	121.3	-25% of cutoff	NEG
	no spike	90484	14	OORL			NEG

2.4.4.5 Hematocrit effect

The hematocrit effect was tested by spiking Amphetamine into whole blood samples. The spiked whole blood samples were analysis by Theranos method, and processed to collect plasma. The plasma samples were also analyzed by Theranos method. The results in plasma and the results in whole blood were compared for calculating hematocrit effect.

Amphetamine concentrations measured in plasma and in whole blood were close to 1:1 ratio in the assay. No concentration adjustment is needed when analysis whole blood sample in the method.

Table [SEQ Table * ARABIC]: Hematocrit effect analysis

	Whole blood				EDTA plasma		
Sample #	Nominal conc. (ng/ml)	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml	Mean RLU	%CV	Cal conc. (ng/ml) LLOQ 10ng/ml
1	1000	3891	36	1046	4676	6	919
2	300	12767	13	400	14294	15	353
3	100	26859	8	139	30902	34	106
4	30	47228	12	39	49212	29	35

5	no spike	78814	1		70709	5	

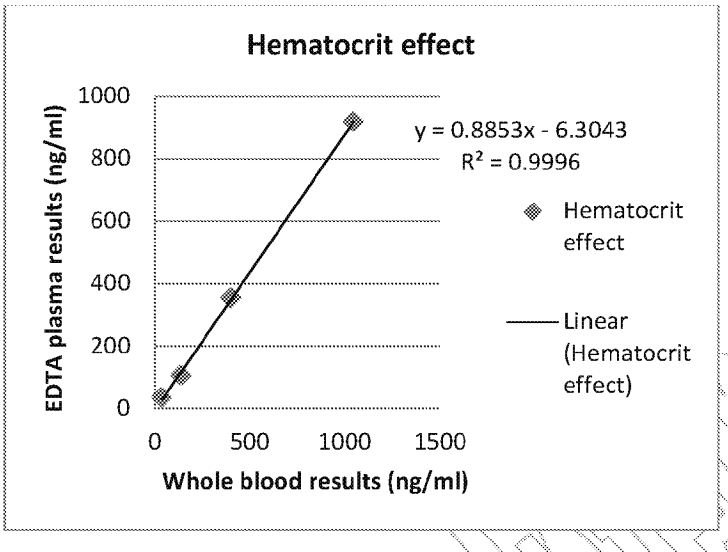


Figure [SEQ Figure * ARABIC]: Hematocrit effect analysis

2.5 Assay finalization with in-house Amphetamine-AP conjugate

2.5.1 Transition to in-house Amphetamine-AP conjugate

After the assay was developed with commercial AMP-AP conjugate, Theranos in-house AMP-AP conjugate became available. In order to replace commercial conjugate with in-house conjugate, conjugates made in-house were titrated with antibody direct binding and evaluated with Amphetamine competition using the same protocol as commercial conjugate.

2.5.1.1 In-house AP conjugate titration

Two lots of Amphetamine-AP conjugates, AMP-p-AP (Lot#OK-0448-63-p-1) and AMP-m-AP (Lot # OK-0448-63-m-1), were first received and evaluated.

Antibody direct binding was done to titrate conjugate concentration. Conjugates were prepared to the concentration 10 times of the final working solution and protocol Generic2_competitive_100x_10_10 was used without adding AMP calibrators.

Table [SEQ Table * ARABIC]: In-house AMP-AP conjugate titration

Sample	Conj Conc. (Loading at 10x sln)	Mean RLU	%CV

AMP-p-AP	10x=100ng/ml	2347688	14
AMP-p-AP	10x=10ng/ml	502041	14
AMP-p-AP	10x=1ng/ml	66529	24
AMP-p-AP	10x=0.1ng/ml	7540	4
AMP-m-AP	10x=100ng/ml	2019258	7
AMP-m-AP	10x=10ng/ml	458050	6
AMP-m-AP	10x=1ng/ml	53835	9
AMP-m-AP	10x=0.1ng/ml	5136	17

Amphetamine competition with conjugate was done by choosing the optimal conjugate concentration to compete with AMP calibrators. L-AMP was also checked to make sure the new conjugates didn't affect specificity.

Table [SEQ Table * ARABIC]: Amphetamine competition with in-house AMP-AP conjugates

AP conjugate	Sample Conc. (ng/ml)	Mean RLU	%CV	Modulation
AMP-p-AP 10x= 2ng/ml	D-AMP 1000	14038	15	10.6
	D-AMP 30	106398	24	1.4
	buffer	148917	10	1.0
	L-AMP 1000	140895	15	
AMP-m-AP 10x= 2ng/ml	D-AMP 1000	10938	10	10.2
	D-AMP 30	92577	24	1.2
	buffer	111192	5	1.0
	L-AMP 1000	115514	2	
Control: commercial AMP-AP 10x= 10ng/ml	D-AMP 1000	6678	23	15.2
	D-AMP 30	73896	18	1.4
	buffer	101278	16	1.0
	L-AMP 1000	114678	5	

Comparing with commercial AP conjugate, the first two lots of in-house AMP-AP conjugate had slightly lower modulation and slightly decreased sensitivity. A third batch of conjugate was requested with the requirement of lower AMP to AP molecule ratio in order to increase sensitivity.

2.5.1.2 Selection of the final in-house conjugate working condition

The third batch of in-house AMP-AP conjugate (AMP-AP-p-3, Lot # OK-0448-75-p-3) was produced with AMP to AP ratio of 3:1. Three batches of in-house conjugates were prepared at the same concentration to compare by running AMP calibration curve using protocol Generic2_competitive_100x_10_10.

AMP-AP-p-3 was finally chosen to use for its higher modulation and better sensitivity.

Table [SEQ Table * ARABIC]: Comparison of three batches in-house AP conjugates

		AMP-p-AP 10x=1.5ng/ml			AMP-m-AP 10x=1.5ng/ml			AMP-AP-p-3 10x=1.5ng/ml		
Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation	Mean RLU	%CV	Modulation
C1	10000	1116	7	101.2	742	24	129.3	758	18	146.4
C2	3000	3378	15	33.4	2551	31	37.6	2246	31	49.4
C3	1000	9343	11	12.1	8467	12	11.3	7855	18	14.1
C4	300	28583	2	4.0	23186	15	4.1	24323	5	4.6
C5	100	46572	36	2.4	42442	16	2.3	47866	6	2.3
C6	30	77894	20	1.5	65762	20	1.5	72662	16	1.5
C7	10	117633	10	1.0	93306	11	1.0	85738	12	1.3
C8	0	112947	6	1.0	95950	20	1.0	110915	26	1.0

2.5.1.3 Sample analysis with in-house AP conjugate

Calibration curve was established with in-house AP conjugate AMP-AP-p-3. Some control and clinical samples which were analyzed using commercial AP conjugate were measured again with in-house AP conjugate. The results showed the measurements from two conjugates were very consistent.

Table [SEQ Table * ARABIC]: Calibration curve with AMP-AP-p-3

Sample	Nominol Conc. (ng/ml)	Mean RLU	%CV	Modulation	back cal. Conc (ng/ml)	% accuracy
C1	10000	758	18	146	10513	105
C2	3000	2246	31	49	2635	88
C3	1000	7855	18	14	1144	114
C4	300	24323	5	5	340	113
C5	100	47866	6	2	84	84
C6	30	72662	16	2	24	81
C7	10	85738	12	1	13	135
C8	0	110915	26	1		

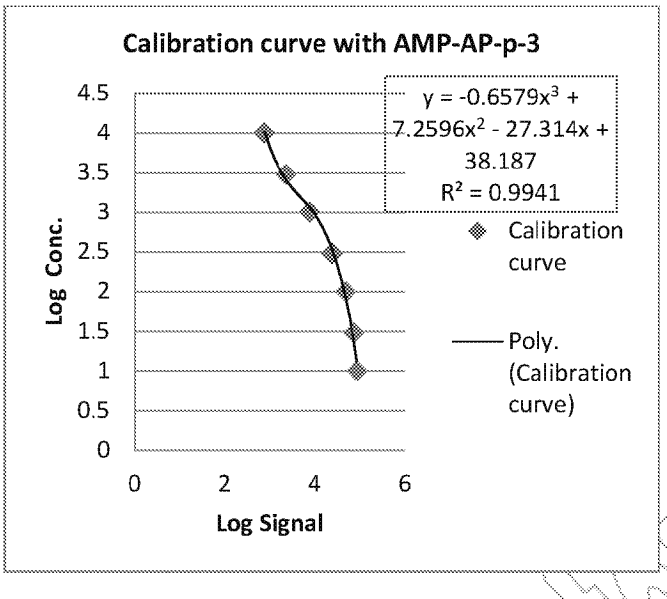


Figure [SEQ Figure * ARABIC]: Calibration curve with AMP-AP-p-3

Table [SEQ Table * ARABIC]: Bio Rad DOA controls measured with AMP-AP-p-3

Sample	ID	AMP Reported Value (ng/ml)	MEAN RLU	%CV	Theranos results (ng/ml)	% recovery
Bio Rad DOA controls	Bio Rad C2LO (468)	375	21411	16	413	110
	Bio Rad C3LO (469)	625	11418	8	851	136
	Bio Rad S1LO (466)	750	11007	45	879	117
	Bio Rad S2LO (467)	1250	6303	20	1326	106
	Bio Rad S1 (461)	750	11074	7	874	117
	Bio Rad S2 (462)	1250	6190	18	1341	107
	Bio Rad S3 (463)	2000	4504	21	1633	82
	Bio Rad Neg (460)	-	97811	13	OORL	

Table [SEQ Table * ARABIC]: DOA control and clinical samples from Zeptometrix, Synerhent Biochem, and Sunnylab

Sample	ID	Reported Value	MEAN RLU	%CV	Theranos results (ng/ml)	Previous results with commercial AP conj (ng/ml)
Zeptometrix Panel I	Z1	5304	1292	19	4622	4906
	Z2	859	72009	25	25	OORL
	Z3	10596	1678	3	3441	3943
	Z4	1500	55236	7	57	70
	Z5	857	76889	0	20	OORL
	Z6	5118	1755	28	3291	3586
	Z7	1032	63078	9	38	74
	Z8	5663	2443	5	2464	2880
	Z9	-	84516	15	14	OORL
	Z10	-	92268	16	OORL	OORL
Synerhent Biochem DOA control	SU0	-	96595	4	OORL	OORL
	SU1	714	12539	21	780	868
	SU2	1086	6759	4	1267	1124
	SU3	1350	5871	14	1387	1379
	SU4	2827	2588	18	2358	2488
Sunnylab paired sample	Urine 1454	from AMP positive patient	1708	21	3380	4034
	Saliva 1455		16756	3	571	381

2.5.2 Final coating format optimization

After the assay condition was finalized, 24 trays of tips were coated for quality control test of large scale coating. Two concentration levels of AMP calibrators were analyzed using 6 tips from each tray for each level in 3 cartridges. For QC purpose, percentage of CV was calculated from 6 tips from each tray, and the difference between each individual tray and mean of 24 trays was also calculated. When 24 trays were coated with the format of secondary antibody goat anti-mouse IgG Fc in coating buffer first and then mouse anti-Amphetamine antibody in blocking buffer, %CV higher than 20% was observed in many trays. Variation of signal from tray to tray was also large than expected. Thus, further optimization of coating format was considered.

2.5.2.1 Coating with UA and Biotin labeled mouse anti-Amphetamine antibody

Ab#12 was labeled with Biotin using in-house Biotin labeling kit. Tips were coated 20ug/ml UA in coating buffer and then biotin labeled Ab#12 in blocking buffer. The format was tested

previously when choosing of assay format was conducted in early development stage. At that time, this format was not chosen because high %CV was observed. When this format was evaluated again, the issue of high %CV remained the same. Therefore, this format was not preceded anymore.

2.5.2.2 Coating with UA and Biotin labeled secondary antibody goat anti-mouse IgG Fc

In this format, tips were coated with 20ug/ml UA in coating buffer and 10ug/ml of biotin labeled goat anti-mouse IgG Fc in blocking buffer. Ab#12 was not coated on tips. In protocol Generic2_competitive_100x_10_10, Ab#12 was prepared in blocking buffer as 10x solution. During sample dilution, Ab#12 was diluted 1:10 and mixed with diluted sample and AMP-AP conjugate to co-incubate with reaction tips. The working concentration of Ab#12 and AMP-AP conjugate were kept unchanged.

When calibration curve was generated in this format, high modulations and tight %CV in all calibrators were got satisfactorily. However, in order to make the possible assay multiplexing easier, antibody in solution was not the priority format. So, this format was kept as a backup plan.

2.5.2.3 "3-stack" coating format

A "3-stack" coating format was also tested. In this format, tips were coated as the followings:

1. UA at 20ug/ml in coating buffer
2. Biotin labeled goat anti-mouse IgG Fc at 10ug/ml in blocking buffer
3. Ab#12 (mouse anti-Amphetamine antibody) at 0.15ug/ml in blocking buffer

Protocol Generic2_competitive_100x_10_10 was used for mixing diluted sample and AMP-AP conjugate for co-incubation.

This format gave the best %CV, modulation and sensitivity among all formats tested. The coating format was finally updated to "3-stack" coating.

Table [SEQ Table * ARABIC]: Calibration curve with "3-stack" coating format

Calibrator	Nominal Conc. (ng/ml)	Mean RLU	%CV	Modulation	back cal. Conc. (ng/ml)	% accuracy
C1	10000	1537	6	83	10607	106
C2	3000	3303	6	39	2697	90
C3	1000	9972	25	13	1089	109
C4	300	32628	19	4	350	117
C5	100	64424	7	2	83	83
C6	30	89300	10	1	29	98
C7	10	113238	7	1	12	117
C8	0	127900	10	1		

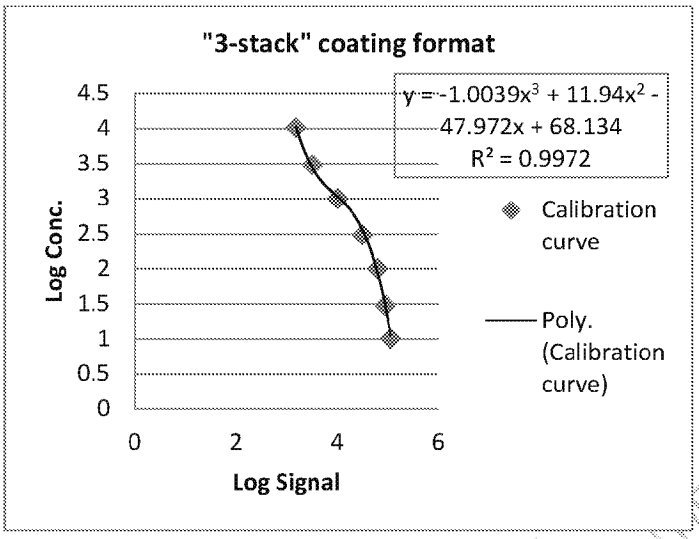


Figure [SEQ Figure * ARABIC]: Calibration curve with "3-stack" coating format

2.5.3 Completion with "3-stack" coating format

After the assay format was finalized as "3-stack" coating, large scale tips were coated with "3-stack" coating format. A final calibration curve was generated and LLOQ and ULOQ of this assay were calculated using Dexter. Some clinical and control samples were repeatedly tested to confirm the assay performance with the final assay format.

2.5.3.1 Dexter calculation of final calibration parameters

Calibration curve was generated using Dexter software. LLOQ was defined at 30ng/ml and 10ng/ml calibrator was included at anchor point. ULOQ was defined at 10000ng/ml. The final curve was used to calculate the final set of clinical and control samples.

Table [SEQ Table * ARABIC]: Final calibration curve

Sample	Conc. (ng/ml)	Mean RLU	%CV	Modulation	Cal from Dexter (ng/ml)	% Accuracy
C1	10000	1453	12	92	10307	103
C2	3000	3959	14	34	2757	92
C3	1000	9052	8	15	1079	108
C4	300	26113	15	5	315	105
C5	100	63162	20	2	86	86
C6	30	92529	5	1	34	114
C7	10	121983	6	1	OORL	
C8	0	132985	6	1	OORL	

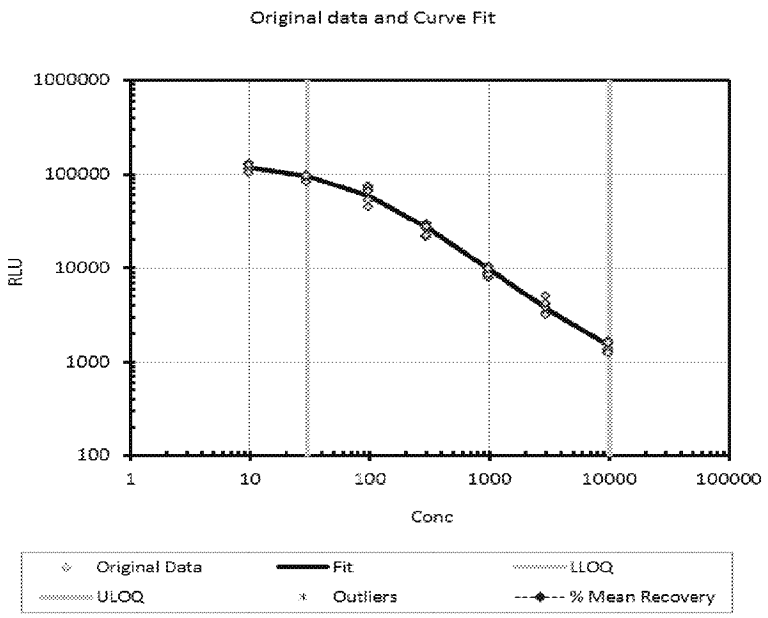


Figure [SEQ Figure * ARABIC]: Calibration curve from Dexter analysis

Table [SEQ Table * ARABIC]: Calibration curve parameters

Model Type	4PL
Model Equation	RLU = b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	conc=77.203*(((132827.687-538.291)/(RLU-538.291))-1)^(1/1.105)
b1	538.291
b2	132827.697
b3	77.203
b4	1.015
LLOQ	30ng/ml
ULOQ	10,000ng/ml
LLOQ accuracy	116%
LLOQ precision	17.80%
ULOQ accuracy	106%
ULOQ precision	19.40%

2.5.3.2 Re-confirmation of specificity

More amphetamine analogs were tested in final assay format to evaluate cross reactivity and re-confirm the assay specificity. At concentration 100ng/ml (commonly used urine cut-off), D,L,-Amphetamine showed around 50% recovery and L-amphetamine had no cross reactivity. (±)-3,4-Methylenedioxyamphetamine (MDA) showed about 60% cross reactivity. Other amphetamine analogs had no cross reactivity in this assay.

Table [SEQ Table * ARABIC]: Cross reactivity of Amphetamine analogs

Sample	Conc. (ng/ml)	Mean RLU	%CV	Cal. Conc. (ng/ml)	% cross reactivity
DL-AMP	1000	18875	21	511	51
L-AMP	1000	125157	18	OORL	--
(+/-) MDMA	1000	123620	23	OORL	--
(+/-) MDA	1000	16129	21	600	60
(+/-) MDEA	1000	128281	21	OORL	--
(+) Methamphetamine	1000	114115	7	OORL	--
(-) Methamphetamine	1000	117200	5	OORL	--

Although interference information was obtained from Zepitometrix, Synerhaent Biochem and Bio Rad controls, several other drugs-of-abuse were prepared at or higher than their cut-off values to re-confirm no cross reactivity in this assay. Some common drugs were also prepared at concentrations higher than clinical values to confirm they had no effect in this assay.

Table [SEQ Table * ARABIC]: Common drugs and other drugs-of-abuse measured in this assay

Sample	Conc.	Mean RLU	%CV	Cal. Conc. (ng/ml)	% cross reactivity
Ibuprofen	100ug/ml	146249	11	OORL	--
Aspirin	100ug/ml	139541	14	OORL	--
Acetaminophen	100ug/ml	147315	5	OORL	--
Caffeine	100ug/ml	127320	5	OORL	--
Codeine	1000 ng/ml	135021	4	OORL	--
Morphine	1000 ng/ml	144995	13	OORL	--
Heroin	1000 ng/ml	126916	26	OORL	--
Methadone	1000 ng/ml	136682	6	OORL	--

2.5.3.3 Use of Bio Rad DOA controls for final checking

Bio Rad DOA control samples were analyzed one more time with final assay format for confirmation. All samples had recovery within 80-120% range of reported value. When using qualitative determination, all samples were in correct categories. The results confirmed the performance of "3-stack" coating format.

Table [SEQ Table * ARABIC]: Analysis results of Bio Rad DOA control samples

Sample #	AMP reported Conc. (ng/ml)	Target value	Inter-cartridge Ave.	%CV-inter Cartridge	Cal from Dexter	% Recovery	Qualitative determination cut-off 500ng/ml	Qualitative determination cut-off 1000ng/ml
Bio Rad C2LO (468)	375	25% lower than cut-off 500ng/ml	26382	17	311	83	NEG	NEG
Bio Rad C3LO (469)	625	25% higher than cut-off 500ng/ml	17630	22	506	81	POS	NEG
Bio Rad S1LO (466)	750	25% lower than cut-off 1000ng/ml	13160	10	708	94	POS	NEG
Bio Rad S2LO (467)	1250	25% higher than cut-off 1000ng/ml	8724	14	1124	90	POS	POS
Bio Rad S1 (461)	750	25% lower than cut-off 1000ng/ml	15859	16	610	81	POS	NEG
Bio Rad S2 (462)	1250	25% higher than cut-off 1000ng/ml	8062	8	1228	98	POS	POS
Bio Rad S3 (463)	2000	2-fold higher than cut-off 100ng/ml	5060	34	2076	104	POS	POS
Bio Rad Neg (460)		negative control	121499	24	OORL		NEG	NEG

2.5.3.4 Urine and plasma samples analyzed using final "3-stack" coating format

Five urine samples from healthy donors and five spiked urine samples were analyzed using final assay format. All results came as expected. Spiked samples had good recovery when being calculated from calibration curve and were in correct categories when being determined by cut-off values.

EDTA plasma samples from ten donors (5 male and 5 female) were screened using final assay format. Spiked samples were prepared in a wide concentration range. All samples had expected results.

Analysis of urine and plasma samples confirmed the final assay format worked for both urine and plasma sample matrix.

Table [SEQ Table * ARABIC]: Results of urine sample analysis[LINK Excel.Sheet.12 "\\\\srw006pa\\users\\rhu\\Amphetamine\\tables and figures for report.xlsx" "final Dexter calculation!R149C20:R159C26" \a \f 4 \h * MERGEFORMAT]

Table [SEQ Table * ARABIC]: Results of EDTA plasma sample analysis

Sample #	Nominal Conc. (ng/ml)	Target value	Mean RLU	%CV	Cal. Conc. (ng/ml)	% Recovery
Stanford EDTA plasma M1			113565	10	OORL	
Stanford EDTA plasma M2			117045	11	OORL	
Stanford EDTA plasma M3			124957	11	OORL	
Stanford EDTA plasma M4			118763	5	OORL	
Stanford EDTA plasma M5			100975	10	OORL	
Stanford EDTA plasma F1			96887	6	OORL	
Stanford EDTA plasma F2			130915	19	OORL	
Stanford EDTA plasma F3			108403	3	OORL	
Stanford EDTA plasma F4			133629	9	OORL	
Stanford EDTA plasma F5			123932	14	OORL	
P1000	1000	high positive	8274	9	1193	119
P300	300	high positive	23160	21	366	122
P100	100	high positive	53604	14	115	115
P37.5	37.5	25% higher than cut-off 30ng/ml	87953	13	40	107
P30	30	cut-off	97074	14	29	97
P22.5	22.5	25% lower than cut-off 30ng/ml	99314	3	27	118

2.5.3.5 Cut-off values for qualitative determination

All assay development had been done in quantitative determination format. The clinical screening assay for Amphetamines is in qualitative format. This Theranos assay can also be used in qualitative determination format. Currently, a few different urine cut-off values are seen in commercial methods. Screening cut-off of 1000ng/ml in urine and confirmation cut-off of 500ng/ml or 300ng/ml in urine are commonly used. In the most recent SAMSHA document, screening cut-off in urine is defined as 500ng/ml and confirmation cut-off is 200ng/ml. In order for this method to be used as a qualitative assay, "cut-off calibrators" were prepared at different concentration levels to reflect various cut-off values. These calibrators were analyzed in multiple cartridges in multiple runs. The results were consistent from all experiments.

Table [SEQ Table * ARABIC]: Cut-off value evaluation for qualitative determination

				Theranos Method

Sample Type	Target	Reference method	Cut-off	Mean RLU from multiple runs	%CV from multiple runs
Urine	Screening	Immunoassay	1000ng/ml	9052	7.7
Urine	Screening	Immunoassay	500ng/ml	19313	6.4
Urine	Confirmation	LC-MS/MS or GC/MS	300ng/ml	26113	14.5
Urine	Confirmation	LC-MS/MS or GC/MS	200ng/ml	41487	14.4
Serum/Plasma	Screening	LC-MS/MS or GC/MS	30ng/ml	92529	5.4
Serum/Plasma	Confirmation	LC-MS/MS or GC/MS	20ng/ml	doesn't meet in this assay	

2.6 Stability

Assay stability monitoring was done with reagents and coated tips stored at 4˚C for 12 weeks. All reagents showed stable performance over the period of 12 weeks.

Table [SEQ Table * ARABIC]: Summary of stability monitoring for 12 weeks

		week 0			week 1			week 2		
Calibrator	Nominal conc (ng/ml)	RLU	%CV	Back calculation	RLU	%CV	Back calculation	RLU	%CV	Back calculation
1	500	19204	6.8	457.6	16825	22.1	534.2	19037	9.8	462.3
2	200	40437	11.6	176.6	41368	9.8	170.9	38445	4.4	189.7
3	30	93366	11.9	33.2	99601	12.8	26.3	97128	5.9	29.0
4	blank	132985	6.1		129286	12.3		131758	16.6	
					% Signal Change		% Accuracy	% Signal Change		% Accuracy
1	500				-12.4		106.8	-0.9		92.5
2	200				2.3		85.4	-4.9		94.8
3	30				6.7		87.7	4.0		96.5
4	blank				-2.8			-0.9		
		week 4			week 8			week 12		
Calibrator	Nominal conc (ng/ml)	RLU	%CV	Back calculation	RLU	%CV	Back calculation	RLU	%CV	Back calculation
1	500	15720	19.2	577.8	16609	9.0	542.2	15622	13.3	582.0

2	200	39408	2.8	183.2	33322	9.7	230.5	36871	4.3	201.0
3	30	93041	12.3	33.6	91427	9.2	35.6	92334	4.6	34.5
4	blank	116606	13.8		116335	10.8		122275	9.3	
		%Signal Change		% Accuracy	**% Signal Change**		**% Accuracy**	**% Signal Change**		**% Accuracy**
1	500	-18.1		115.6	-13.5		108.4	-18.7		116.4
2	200	-2.5		91.6	-17.6		115.3	-8.8		100.5
3	30	-0.3		112.1	-2.1		118.6	-1.1		114.9
4	blank	-12.3			-12.5			-8.1		

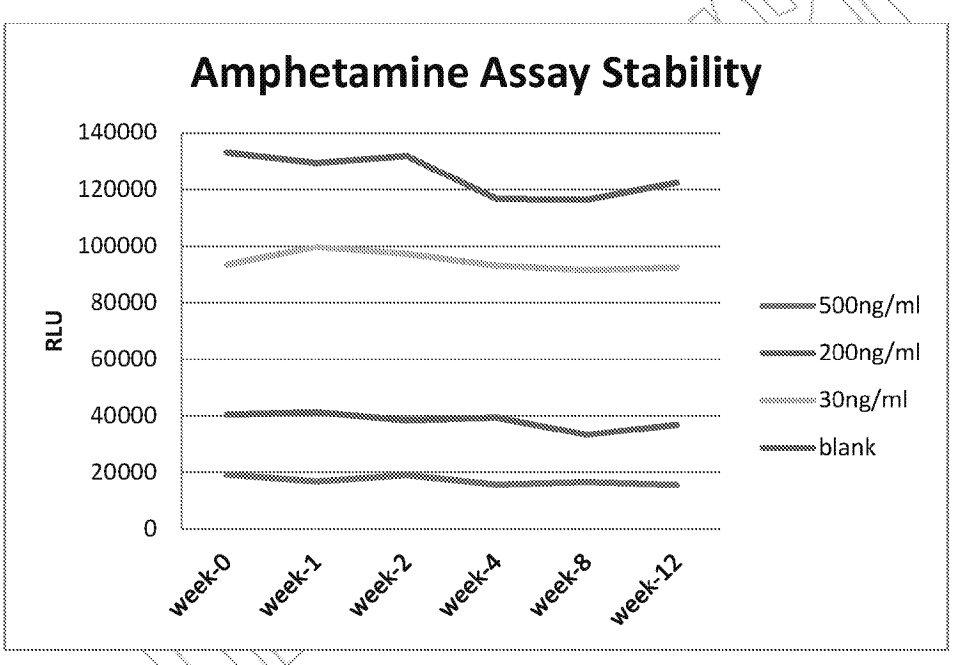


Figure [SEQ Figure * ARABIC]: Stability monitoring for 12 weeks

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