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Ethyl Glucuronide (EtG) Assay Development Report

Theranos, Inc.

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

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

This assay is designed to detect Ethyl Glucuronide (EtG) in human urine. The assay has a reportable range of 0.1 to 75 ug/mL (equivalent to 100 – 75,000 ug/L).

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

No predicate methods were available for comparison.

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

A biotin-labeled anti-sheep antibody coated on avidin serves as the capture surface. The sample is diluted and combined with sheep anti-EtG antibody and an enzyme labeled EtG conjugate. This mixture is incubated on the capture surface for 10 minutes. After the incubation, the surface is washed and substrate is incubated on the surface for 10 minutes, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Ethyl b-D-glucuronide (CAS 17685-04-0)	Us Biologicals	E8606-50
Alkaline Phosphatase Substrate	Theranos	T-ALKP-SB01
Low BSA Blocking Buffer (0.03% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Carbonate-bicarbonate buffer	Sigma	C3041
Sheep PAb Anti-EtG	Abcam (original MRF Randox)	ab123950
EtG-Alk Phos Conjugate	YJ Bio	HEG5100-A

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

1.2 Antibody-Conjugate Binding Screen (MTP) [TC "Detection Antibody Conjugate Verification" \f C \l "1"]

Only 3 EtG antibodies were commercially available, 4 were ordered but number 3 and 4 turned out to be the same antibody manufactured by Randox and re-sold by Abcam. The 3 antibodies were coated on a 384 well microtitre plate (MTP) at 10, 1, 0.1 and 0 ug/mL and tested for binding to the commercial EtG-HRP conjugate from Randox at a dilution of 1:1000 from the stock in Stabilzyme Noble (HRP small molecule conjugate stabilizer). All 3 antibodies showed dose dependent binding to the AP conjugate.

Table [SEQ Table * ARABIC]: Antibody Information

Antibody #	Vendor	Catalog #	Clone	Host
1	Mediagnost	M44	2B6	Mouse
2	Mediagnost	M45	2F10	Mouse
3*	Randox life sciences	PAS10109	PAb	Sheep
4*	Abcam	ab123950	PAb	Sheep

* Antibody 3 and 4 turned out to be the same antibody originally made by Randox and re-sold by abcam.

Table [SEQ Table * ARABIC]: Conjugate Information

Antibody #	Vendor	Catalog #	Type
1	Randox	HRP9531	HRP

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

Ab #	[Ab] ug/mL	Mean RLU	CV %	Mod.
1	10	391414	3.1	447
	1	18239	13.1	21
	0.1	1078	1.8	1
	0	876	24.1	
2	10	131107	10.1	129
	1	2379	3.6	2
	0.1	1273	3.5	1
	0	1014	1.1	
3	10	62059	6.5	68
	1	13808	1.0	15
	0.1	1705	7.8	2
	0	915	21.2	

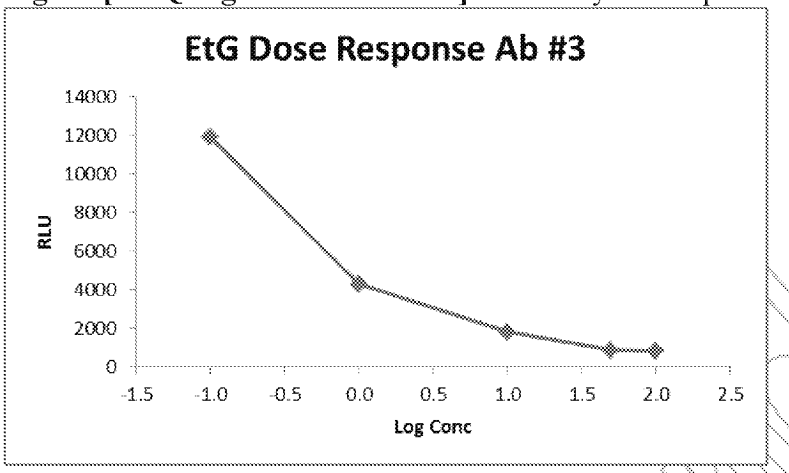
1.3 Competitive Assay Screen (MTP)

The 3 antibodies were coated on an MTP at 10 ug/mL by passive absorption, and tested for response in a competitive assay with EtG calibrators in Low BSA buffer. Only antibody 3 showed a dose response in the competitive assay. Antibodies 1 and 2 showed high binding to the HRP conjugate but no response to the free analyte.

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

Ab #	[EtG] ug/mL	Well 1	Well 2	Mean RLU	CV %	Modulation
1	100	166736	146894	156815	8.9	1
	50	160042	159218	159630	0.4	
	10	175045	166246	170645	3.6	
	1	176588	169235	172912	3.0	
	0.1	170074	169932	170003	0.1	
	0	160990	170602	165796	4.1	
2	100	35104	39914	37509	9.1	1
	50	43012	44233	43623	2.0	
	10	48751	46646	47698	3.1	
	1	46481	44975	45728	2.3	
	0.1	50010	48845	49427	1.7	
	0	48212	46829	47520	2.1	
3	100	820	798	809	2.0	51
	50	869	873	871	0.3	47
	10	1734	1899	1817	6.4	23
	1	4252	4323	4287	1.2	10
	0.1	11740	12014	11877	1.6	3
	0	41229	41240	41235	0.0	

Figure [SEQ Figure * ARABIC]: Antibody 3 Competitive Assay Dose Response



1.4 Theranos System 3.0 Screen

Since only antibody 3 showed a response in the competitive assay, it was tested on the Theranos System 3.0 with anti-sheep coated antibody as the capture surface, unlabeled sheep anti-EtG mixed into the sample along with the EtG-HRP conjugate and a 1:10 sample dilution. The response on the Theranos System was very good, showing sensitivity at the 0.1 ug/mL level needed. A set of 3 level urine controls from Detectabase were also tested, and tracked well with the Low BSA buffer calibrators. New calibrators were made in EtG-negative urine for further assay development.

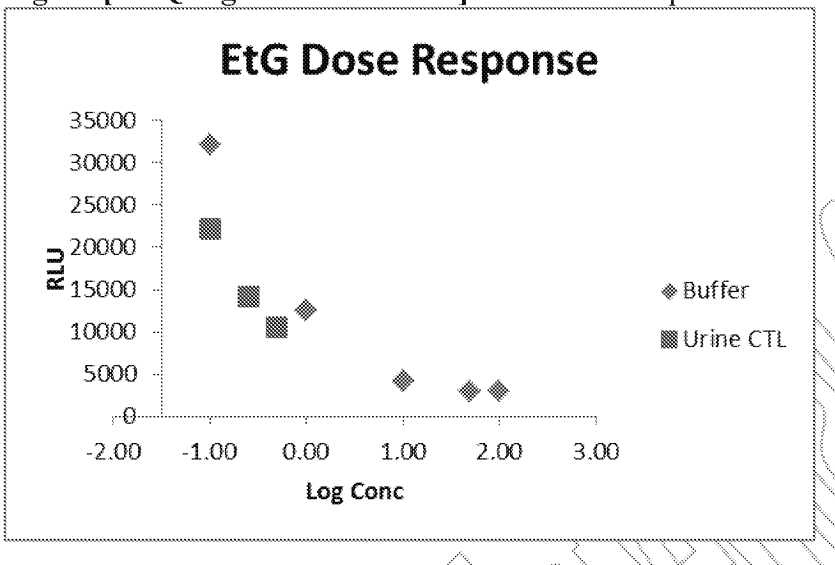
Table [SEQ Table * ARABIC]: Standard Curve in Buffer

[EtG] ug/mL	Mean RLU	CV %	Mod
100	2959	32.0	19
50	3031	9.3	18
10	4170	16.8	13
1	12444	13.8	4
0.1	32218	13.2	2
0	55399	8.4	

Table [SEQ Table * ARABIC]: Detectabase Urine Controls

Reported [EtG] ug/mL	Mean RLU	CV %
0.5	10582	8.9
0.25	14123	14.8
0.1	22170	20.8

Figure [SEQ Figure * ARABIC]: EtG Dose Response on Theranos System 3.0



1.5 Sample Diluents

The assay was tested with Super Block and Low BSA (0.03% BSA in TBS) buffers as sample diluents, using urine calibrators. Low BSA buffer showed the best modulation across the range.

Table [SEQ Table * ARABIC]: Sample Diluents

Sample Diluent	[EtG] ug/mL	Mean RLU	CV %	Mod.
Low BSA	150.00	2432	20.9	18
	75.00	2285	29.7	19
	10.00	4297	5.0	10
	5.00	5201	8.6	8
	1.00	9622	12.5	5
	0.50	10916	11.2	4
	0.10	25660	7.8	2
	0.00	44061	3.1	
Super Block	150.00	6555	5.1	11
	75.00	-	-	-
	10.00	10900	7.1	7
	5.00	12594	6.1	6
	1.00	24698	7.7	3
	0.50	30045	12.7	2
	0.10	59731	8.6	1
	0.00	74509	10.8	

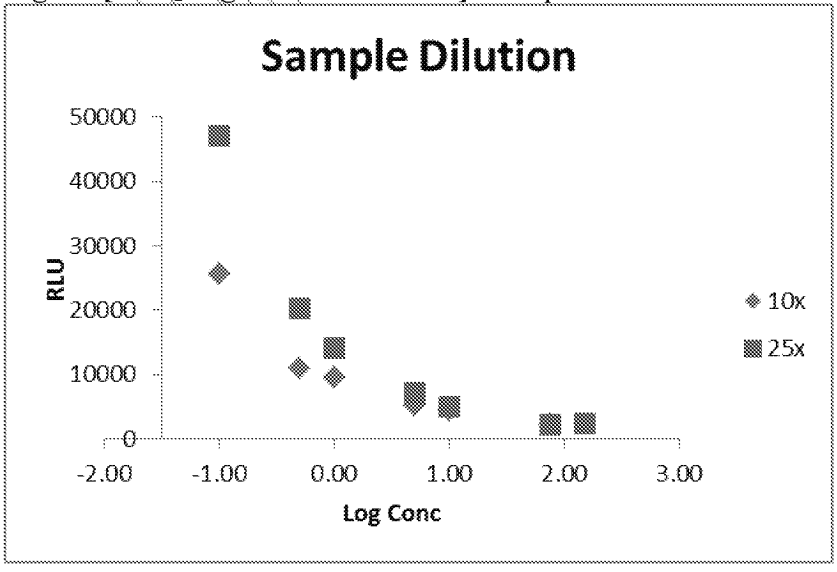
1.6 Sample Dilution

With Low BSA as a sample diluent, a 25-fold sample dilution was tested versus the 10-fold sample dilution. Modulation was improved in the higher end of the range, but sensitivity was lost, as expected. Further assay optimizations were done with the 25-fold sample dilution to restore sensitivity while retaining the higher range.

Table [SEQ Table * ARABIC]: Sample Dilution

Sample Dilution	[EtG] ug/mL	Mean RLU	CV %	Mod.
10x	150.00	2432	20.9	18
	75.00	2285	29.7	19
	10.00	4297	5.0	10
	5.00	5201	8.6	8
	1.00	9622	12.5	5
	0.50	10916	11.2	4
	0.10	25660	7.8	2
	0.00	44061	3.1	
25x	150.00	2298	38.0	26
	75.00	2252	9.0	27
	10.00	4887	13.6	12
	5.00	7135	18.6	8
	1.00	14019	26.3	4
	0.50	20230	13.0	3
	0.10	46989	10.0	1
	0.00	59932	10.3	

Figure [SEQ Figure * ARABIC]: Sample Dilution



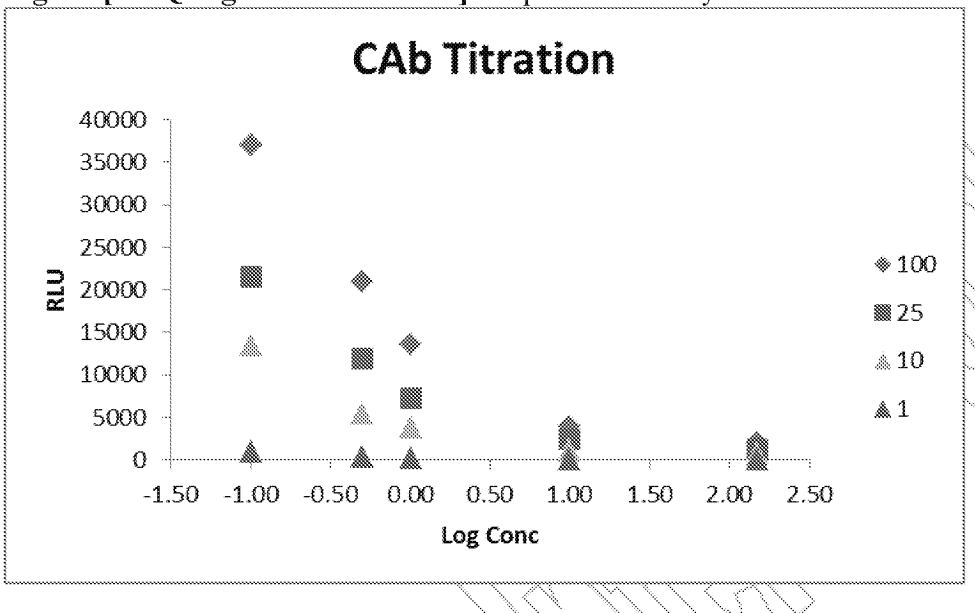
1.7 Capure Antibody Titration

The capture antibody was titrated to determine the optimal concentration. The antibody is diluted 10-fold into the final sample mixture, the loading concentration is shown. A concentration of 10 ug/mL produced the best response with the conjugate at 1:100 lodaing concentration. At this time it was not feasible to titrate the HRP conjugate since it will be replaced by an alkaline phosphatase conjugate, and also because the signal was not high enough with the conjugate more dilute while using a lower dilution than 1:100 would have used up too much reagent. A concentration of 10 ug/mL was chosen – although it appeared that a lwoer concentration of antibody might be preferable, the signal fell too low for reliable PMT measurement. It was also noted that the HRP conjugate showed increased coefficient of variance (CVs) after a few days storage at 4C in the Stabilzyme Noble HRP conjugate stabilizer.

Table [SEQ Table * ARABIC]: Capture Antibody Titration

[CAb] ug/mL	[EtG] ug/mL	Mean RLU	CV %	Mod.
100	150.00	1949	34.3	25.6
	10.00	3882	21.3	12.9
	1.00	13481	24.7	3.7
	0.50	20982	5.4	2.4
	0.10	37065	5.6	1.3
	0.00	49915	13.7	
25	150.00	1162	15.7	26.0
	10.00	2484	6.0	12.2
	1.00	7225	4.8	4.2
	0.50	11845	9.3	2.6
	0.10	21556	3.1	1.4
	0.00	30245	2.9	
10	150.00	680	20.5	25.5
	10.00	1092	38.5	15.9
	1.00	3790	18.3	4.6
	0.50	5489	26.7	3.2
	0.10	13360	12.0	1.3
	0.00	17338	21.1	
1	150.00	46	88.4	36.6
	10.00	90	37.5	18.7
	1.00	194	67.7	8.7
	0.50	364	63.4	4.6
	0.10	913	11.1	1.8
	0.00	1686	14.9	

Figure [SEQ Figure * ARABIC]: Capture Antibody Titration



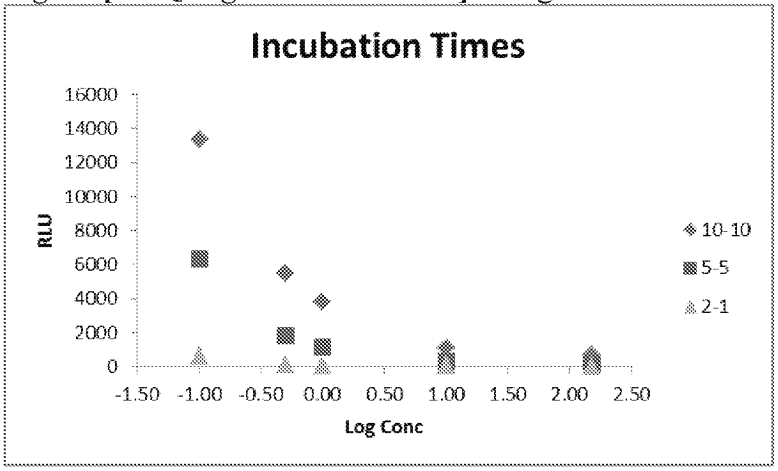
1.8 Reagent Incubation Time

Shorter incubation times were tested compared to the original condition of 10 minute sample mixture and substrate incubations. Five minute and 2, 1 minute incubation times were tested. Without the possibility of using higher concentrations of the HRP conjugate, the signal fell too low for accurate PMT mesasurement with 5, 5 and 2, 1 minute reagent incubations.

Table [SEQ Table * ARABIC]: Reagent Incubation Time

Incubation Time (Min)	[EtG] ug/mL	Mean RLU	CV %	Mod.
10, 10	150.00	680	20.5	25.5
	10.00	1092	38.5	15.9
	1.00	3790	18.3	4.6
	0.50	5489	26.7	3.2
	0.10	13360	12.0	1.3
	0.00	17338	21.1	
5, 5	150.00	151	50.8	73.1
	10.00	336	67.3	32.8
	1.00	1133	54.6	9.7
	0.50	1795	69.8	6.1
	0.10	6327	19.8	1.7
	0.00	11025	16.8	
2, 1	150.00	37	44.7	60.8
	10.00	46	19.9	48.8
	1.00	68	20.5	33.5
	0.50	113	48.0	20.0
	0.10	611	130.7	3.7
	0.00	2261	52.2	

Figure [SEQ Figure * ARABIC]: Reagent Incubation Time



1.9 Clinical Correlation

No predicate method was available to compare to, however urine samples were collected from donors who indicated on a questionnaire whether or not alcohol had been consumed in the past 4 days, and if yes how many drinks and how long the last drink was consumed before the sample was collected. The questionnaire also asked whether alcohol-containing personal care products had been used recently, such as mouthwash or hand sanitizer. The use of these products did not appear to affect the EtG results.

Considering all samples > 0.1 ug/mL as positive, all samples from donors reporting more than 1 drink in past 40 hours were positive. 4 Samples from donors that reported 1-2 drinks greater than 40 hours before donation were negative, and all samples from donors reporting no alcohol use in the past 4 days were negative. These results confirmed that the Theranos EtG assay is sensitive and specific to recent alcohol use.

Table [SEQ Table * ARABIC]: Clinical Correlation

Sample ID	Alcohol Consumption in Past 4 Days?	Total Drinks in 4 Days	Time Since last consumption (H)	Conc, ug/mL	
				Mean Conc	CV %
2	No			0.1	8.8
3	No			0.1	29.0
4	Yes	1	48	0.1	5.9
7	No			0.1	6.8
8	Yes	6	14	0.2	12.0
9	No			0.1	11.3
10	Yes	4	17	20.7	24.2
11	Yes	30	12	1.3	17.8
12	Yes	4	15	2.3	26.4
13	Yes	4	20	0.2	6.3
14	Yes	25	20	4.7	51.9
15	No			0.1	7.6
19	No			OORL	
20	No			OORL	
21	No			OORL	
23	No			OORL	
25	Yes	2	42	OORL	
26	Yes	2	45	OORL	
30	Yes	1	40	0.1	10.6

Pink highlighted results = Positive (> 0.1 ug/mL)
Green highlighted results = Negative (≤ 0.1 ug/mL)

1.10 Calibration Verification

CE-marked Clincheck urine EtG controls (Cat MS 8080 lot 110) and ClinCal urine EtG calibrators (Cat MS8713 lot 110) were obtained from Recipe (Munich, Germany) and tested in the Theranos assay. Recovery was excellent.

Table [SEQ Table * ARABIC]: Recovery of ClinCal EtG Urine Calibrators

Sample ID	[EtG] ug/L	[EtG] ug/mL	Conc, ug/mL		
			Mean	CV %	% Recovery
00	0	0.00	OORL		
01	78.6	0.08	0.09	9.8	115
02	200	0.20	0.18	34.2	90
03	601	0.60	0.68	33.6	113
04	1490	1.49	1.86	50.8	125
05	4900	4.90	3.63	37.8	74
06	9860	9.86	12.05	39.0	122

Table [SEQ Table * ARABIC]: Recovery of ClinCheck EtG Urine Controls

Sample ID	[EtG] ug/L	[EtG] ug/mL	Conc, ug/mL		
			Mean	CV %	% Recovery
Level I	99.8	0.10	0.10	8.4	105
Level II	491	0.49	0.40	24.7	81
Level III	1970	1.97	1.98	42.1	100

1.11 Cross Reactivity

Two potential cross reactants for EtG were tested in the Theranos System. No cross reactivity was seen for glucuronic acid. For Methyl glucuronide as expected some cross reactivity was seen, at 1.4%. This level of cross reactivity should not be significant in clinical results.

Table [SEQ Table * ARABIC]: Cross Reactivity

Test Substance	[Test Substance] ug/mL	Conc, ug/mL		
		Mean Conc	CV %	% Cross Reactivity
Glucuronic Acid	100	OORL		
Methyl Glucuronide	100	1.38	41.5	1.4

1.12 Alkaline Phosphatase Conjugate Stabilizers

An alkaline phosphatase conjugate was ordered and tested in the assay with various AP stabilizers. Theranos Small Molecule AP Stabilizer showed the best modulation.

AP Stabilizer	[Etg] ug/mL	Mean RLU	CV %	Modulation
1:10k in Low BSA	150	10117	16.8	18
	0	182674	10.3	
1:100K in Theranos SM Stab	150	1606	5.1	14
	0	22909	10.4	
1:100k in Stabilzyme AP	150	3680	2.6	4
	0	13435	13.8	
1:100k in Biostab	150	1292	6.5	12
	0	15324	10.0	

1.13 Reagent Titration

To determine the ideal concentration of antibody and EtG-AP, a titration was performed. Modulation was best with either 1:50,000 EtG-AP and 5 ug/mL antibody or 1:5000 EtG-AP and 1ug/mL antibody. Initially, the latter was chosen since it produced higher signal, for tests with shorter reagent incubation time. After the reagent incubation time tests showed that 10 minute incubations were best, the final condition chosen was 1:50,000 EtG-AP and 5 ug/mL antibody.

AP Conjugate, Dilution from Stock	[Ab] ug/mL	[EtG] ug/mL	Mean RLU	CV %	Modulation
1:50k	10	150	1809	13.7	21
		0.5	15943	5.0	2
		0	37376	4.2	
1:10k	5	150	6639	11.9	24
		0.5	85562	3.9	2
		0	160115	7.3	
1:50k	5	150	1514	17.4	25
		0.5	14869	13.0	3
		0	38526	3.9	
1:5k	1	150	4556	4.6	24
		0.5	42961	17.9	3
		0	109336	19.2	

1.14 Reagent Incubation Time

With the higher signal yielded by the AP conjugate, 5, 5 minute and 2, 1 minute sample mixture, substrate incubation times were tested. Although 10 minute incubations were chosen as the final ideal condition, it appeared that 5 minute or 2,1 minute incubation times would still maintain sensitivity of the assay.

Table [SEQ Table * ARABIC]: Reagent Incubation Time

Incubation Time (Min)	[EtG] ug/mL	Mean RLU	CV %	Modulation
10, 10	150	4556	4.6	22
	0.5	42961	17.9	2
	0	98577	27.9	
5, 5	150	1609	24.7	18
	0.5	13518	16.0	2
	0	29364	13.2	
2, 1	150	283	15.6	18
	0.5	2016	18.8	3
	0	5127	12.8	

1.15 Sample Diluent

To determine if various blocking buffers might improve the assay when used as sample diluents, 3 commercial blockers were tested against the Low BSA buffer. An initial screen was performed and it appeared that Pierce Protein Free blocker may improve modulation. However when the full standard curve was tested with low BSA buffer and Protein Free buffer diluents, there was no significant difference.

Table [SEQ Table * ARABIC]: Sample Diluent Screen

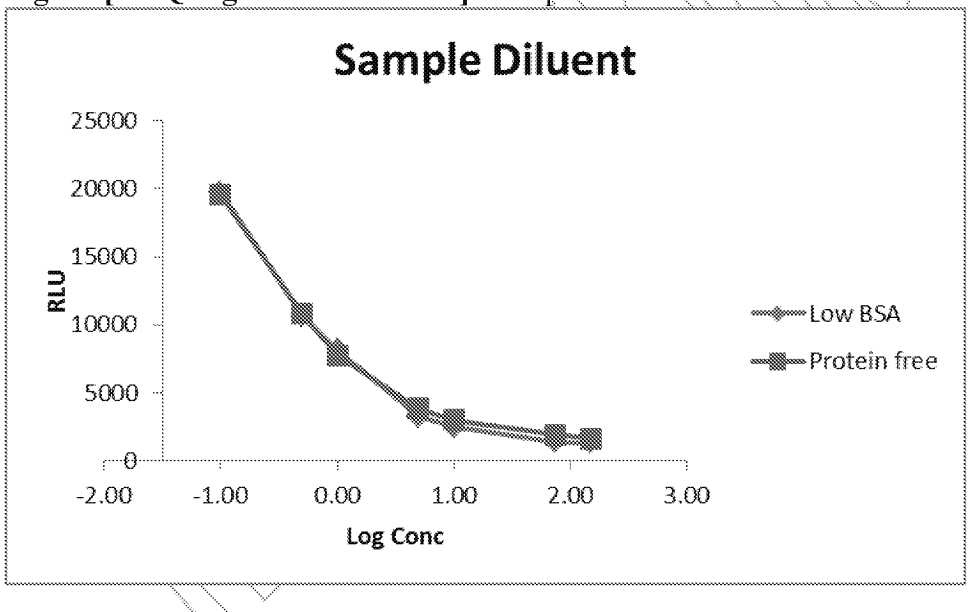
Diluent	[EtG] ug/mL	Mean RLU	CV %	Modulation
Protein Free (Pierce)	75	672	2.6	19
	0	12447	0.3	
Starting Block	75	384	9.3	14
	0	5249	17.8	
Super Block	75	718	8.8	8
	0	5718	8.1	
Low BSA Buffer	75	1395	13.9	17
	0	24122	11.0	

Table [SEQ Table * ARABIC]: Sample Diluent

	Low BSA Diluent			Protein Free Diluent		
[EtG] ug/mL	**Mean RLU**	**CV %**	**Modulation**	**Mean RLU**	**CV %**	**Modulation**
150*	1297	12.0	19	1620	11.8	17
75	1422	12.6	17	1925	7.2	15
10	2481	12.2	10	2999	5.9	9
5	3218	13.2	7	3820	10.5	7
1	8175	18.6	3	7733	19.9	4
0.5	10603	14.6	2	10790	15.8	3
0.1	19765	13.9	1	19540	18.2	1
0	24122	11.0		28229	3.8	

* Anchor point to monitor curve shape, not necessarily included in calibration curve.

Figure [SEQ Figure * ARABIC]: Sample Diluent



1.16 Sample Dilution

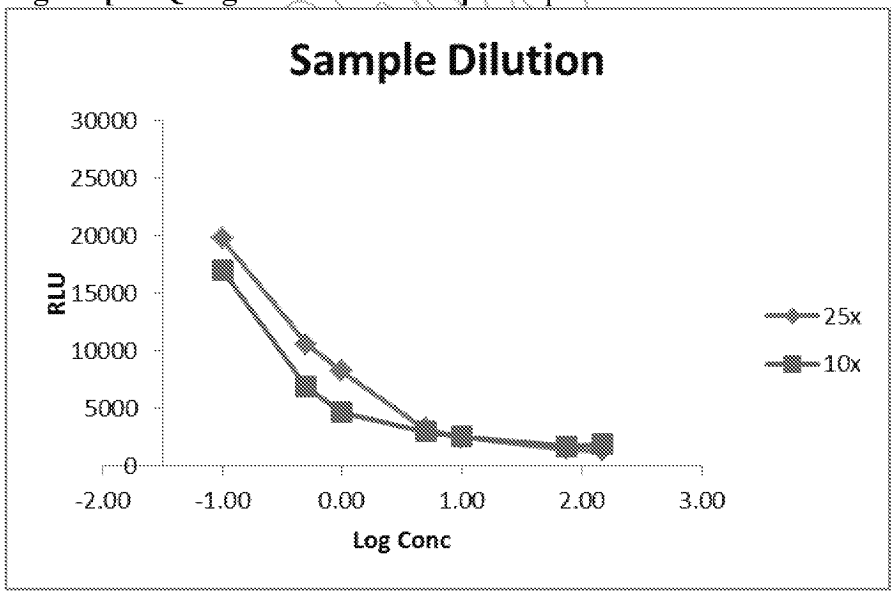
Since the final assay conditions with the AP conjugate were not meeting the sensitivity requirement of 0.1 ug/mL as a cutoff value, a 10-fold sample dilution was re-tested. As expected, the assay showed saturation of response at levels over 75 ug/mL, however the 0.1 ug/mL cutoff was distinguishable. Therefore a 10-fold sample dilution was chosen as the final assay condition.

Table [SEQ Table * ARABIC]: Sample Dilution

[EtG] ug/mL	25x Sample Dilution			10x Sample Dilution		
	Mean RLU	CV %	Modulation	Mean RLU	CV %	Modulation
150*	1297	12.0	19	1844	17.3	15
75	1422	12.6	17	1680	4.1	16
10	2481	12.2	10	2520	14.8	11
5	3218	13.2	7	2972	7.0	9
1	8175	18.6	3	4661	15.4	6
0.5	10603	14.6	2	6858	17.1	4
0.1	19765	13.9	1	17019	3.0	2
0	24122	11.0		27407	10.8	

* Anchor point to monitor curve shape, not necessarily included in calibration curve.

Figure [SEQ Figure * ARABIC]: Sample Dilution



1.17 Determination of LLOQ and ULOQ

A standard curve was run and Theranos calibration software was used to fit the data and determine the LLOQ and ULOQ in urine according to FDA guidelines for calibrating ELISA assays. The LLOQ was 0.1 ug/mL and the ULOQ was 75 ug/mL.

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

[EtG] ug/mL	Signal, RLU		Conc, ug/mL		
	Mean RLU	CV %	Mean Conc	CV %	% Recovery
150*	1269	2.7	OORH	-	-
75	1416	10.0	66.8	17.2	89
10	2391	9.2	10.7	24.1	107
5	2972	7.0	5.0	22.9	101
1	5694	11.3	1.1	28.9	105
0.5	7706	2.6	0.5	4.9	108
0.1	19367	6.1	0.1	10.7	98
0	27407	10.8	OORL		

* Anchor point, used to fit curve but not in assay range.

Table [SEQ Table * ARABIC]: Calibration Parameters

Parameter	Value	Unit
LLOQ	0.10	ug/mL
ULOQ	75.00	ug/mL
LLOQ accuracy	98	%
LLOQ precision	10.7	%
ULOQ accuracy	89	%
ULOQ precision	17.2	%

[SHAPE * MERGEFORMAT]

1.18 Stability

Stability studies are ongoing.

2 REFERENCES

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