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Theranos, Inc.

Acetaminophen in Plasma Assay Development Report

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Assay Development Report: Acetaminophen in Plasma
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Assay Specifications

This assay is designed to measure acetaminophen in plasma across a range of 20 – 200 ug/ml. The assay uses a 1:1.5 dilution and is completed in 1.5 minutes.

Analyte Background

Acetaminophen is an anti-inflammatory pain reliever commonly known as Tylenol. It targets the cyclooxygenase 3 (COX-3). Acetaminophen is safe at a therapeutic dose (10-30 ug/ml) but at an overdose level (>200 ug/ml) it is hepatotoxic. One of its metabolic pathways in the liver involves cytochrome p450 which produces the toxic metabolite N-acetyl-p-benzoquinone (NAPQI). Under therapeutic conditions glutathione is able to reduce NAPQI and prevent oxidative damage. At overdose levels glutathione becomes depleted and is incapable to accommodate the excess NAPQI which is then free to covalently bind hepatic proteins and cause liver damage.

This assay is designed to measure acetaminophen levels in patients being treated for acetaminophen overdose. The purpose of measuring the serum levels of acetaminophen is to determine and monitor treatment with N-acetyl cysteine, which increases the levels of glutathione and prevents hepatic damage.

Assay Development and Optimization

Assay Chemistry

This assay measures the amount of acetaminophen present in plasma by first cleaving acetaminophen with the enzyme aryl acylamidase to produce p-aminophenol which can then react with O-Cresol and $CuSO_4$ to produce indophenol. Indophenol's presence can be measured at 615 nm as an endpoint reaction. This assay is performed as a 2 step, 2 reagent assay.

Reagent A Component	Concentration
Aryl Acylamidase	10 ug/ml
O-Cresol	0.41%
DI H_2O	N/A

Reagent B Component	Concentration
$CuSO_4$	0.566 mM
NH_4OH	118.34 mM
DI H_2O	N/A

Working reagent A should be kept at 4 °C in an amber coloured bottle. Working reagent B can be kept at room temperature. Calibrators and samples are diluted in 100 mM Tris-HCl, pH 9.

Protocols

1. Dilute calibrators and samples 1:15 (20 ul sample in 280 ul buffer) in 100 mM Tris-HCl, pH 9. Mix by brief vortexing.
2. Add 20 ul of each diluted sample to 384 MTP in duplicate.
3. Under the hood ad 20 ul of Reagent A to each sample.
4. Incubate for 10 minutes at 37 °C.
5. Add 20 ul of Reagent B.
6. Incubate for 5 minutes at 37 °C.
7. Read absorbance as endpoint at 615 nm in spectrophotometer.

Data Analysis

The assay signal is converted to concentration of Acetaminophen by plotting the absorbance of each calibrator on the x-axis and the concentration of each calibrator on the y-axis. The resulting graph can fit a line y=mx+b. Using the slope (m) and the y-intercept (b) the concentration of Acetaminophen in each sample can be calculated by using the following equation:

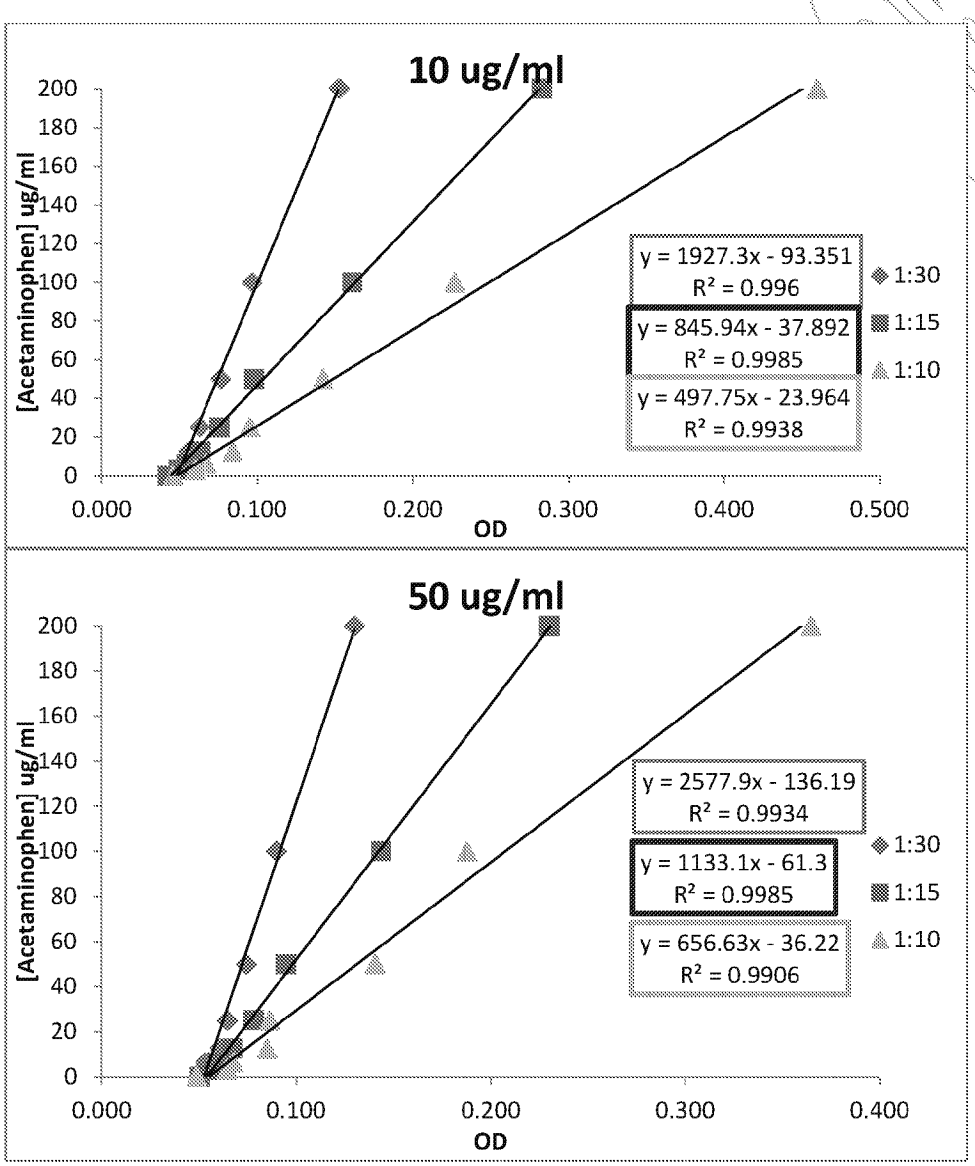
[Acetaminophen] ug/ml = m(sample signal) + b

Reference Assay

In the development of this assay Sigma and Roche Acetaminophen Assays were used as references.

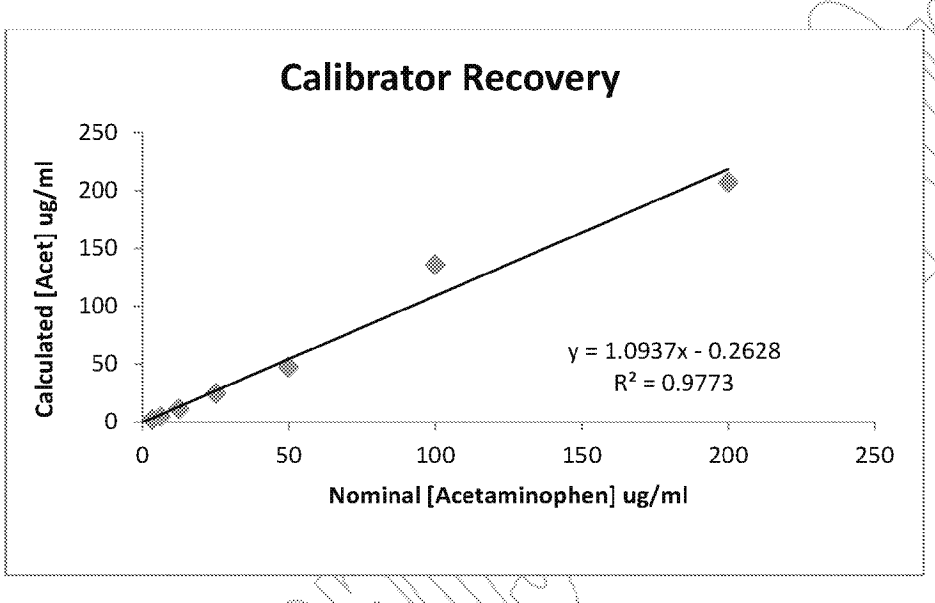
Sample Dilution

The best sample dilution; one with best signal and CVs was 1:15 and the best enzyme dilution was 10 ug/ml.



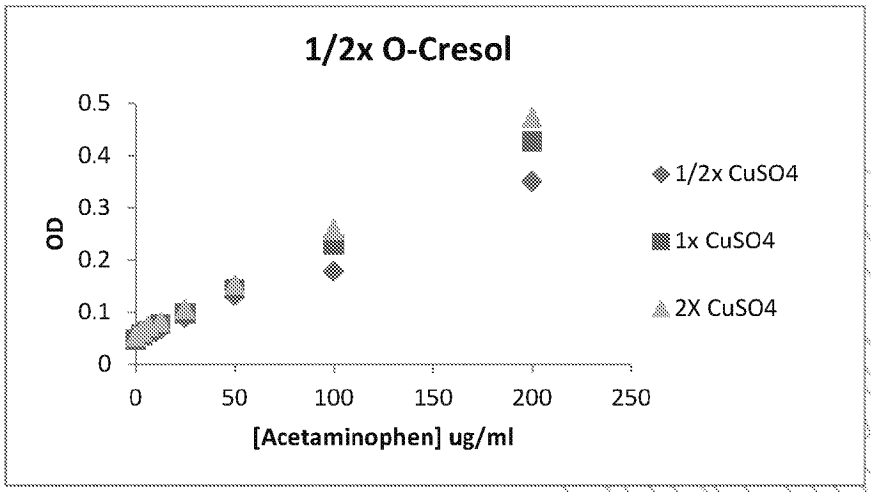
Calibrator Verification

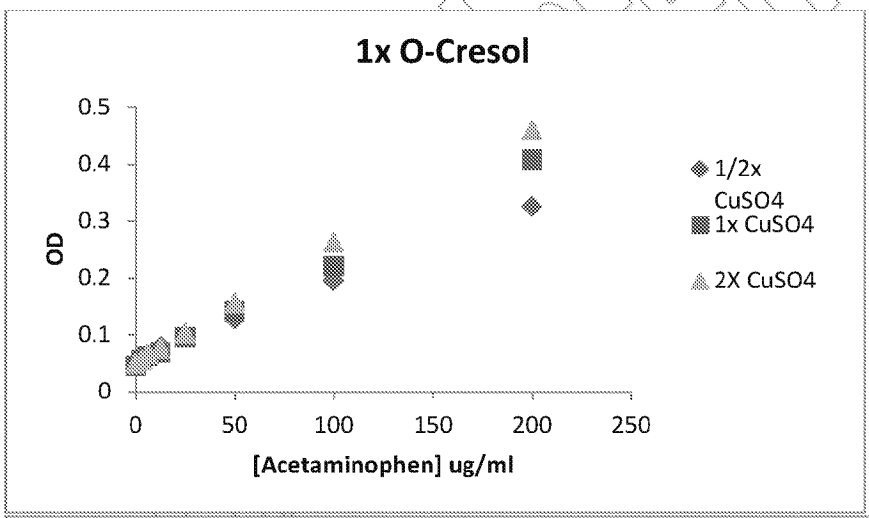
The kit used to verify the calibrators was the Sekisui Diagnostics - Acetaminophen-SL Assay. For the calibrators to work on the kit they had to be diluted in DI water rather than 100 mM Tris-HCl pH 9.

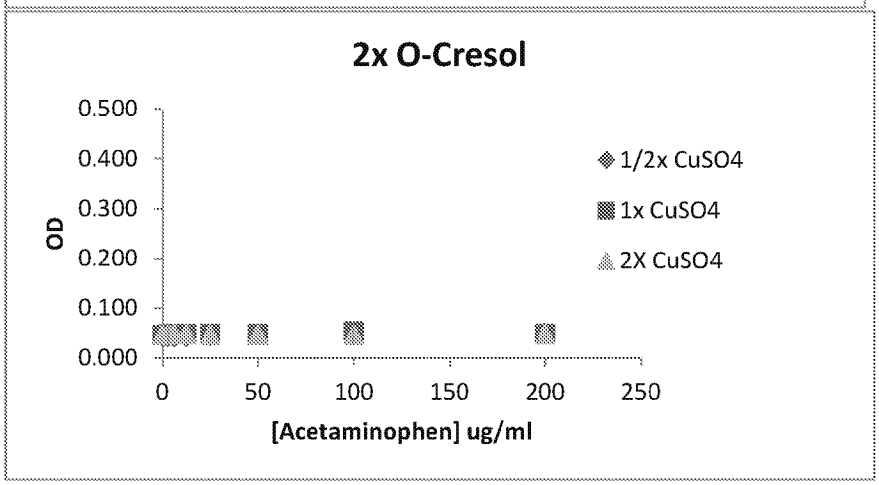


Reagent Titrations

The assay reagents were optimized by titrating different concentrations of O-Cresol and $CuSO_4$ in NH_4OH. Though there appears to be very little variation between 1/2x and 1x O-Cresol, the 2x O-Cresol does not produce any signal at all. This suggests that either the 2x O-Cresol inhibits the enzyme (aryl acylamidase) or the colour reaction with p-aminophenol has an upper limit. Titration of the $CuSO_4$ showed an increase in signal with an increase of reagent. While the 2x concentration had the best signal, the 1x $CuSO_4$ has an acceptable signal and if necessary or more cost effective could be used as the working reagent. This experiment showed that the concentration of $CuSO_4$ has the most influence in variation in signal of the reaction.







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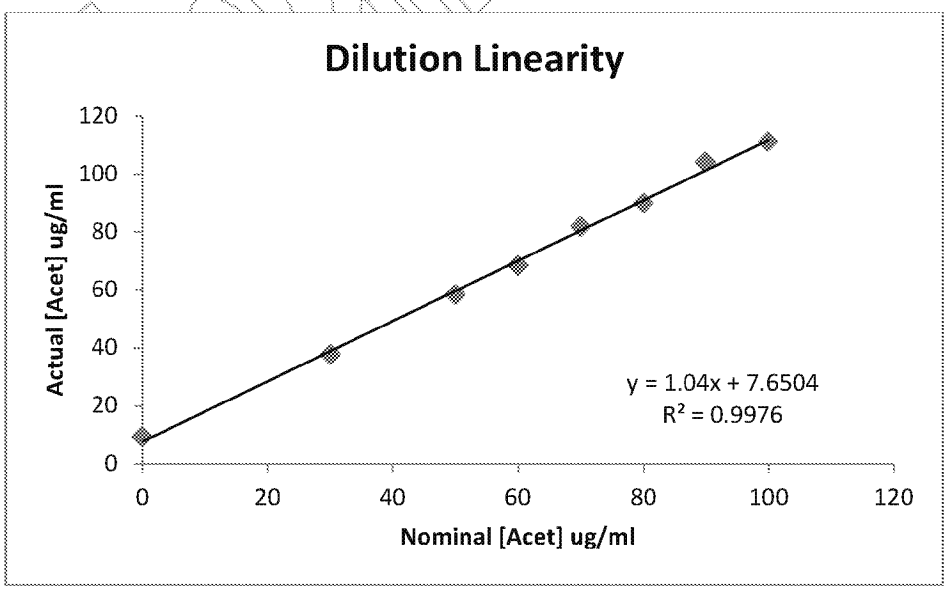
Assay Performance

Dilution Linearity

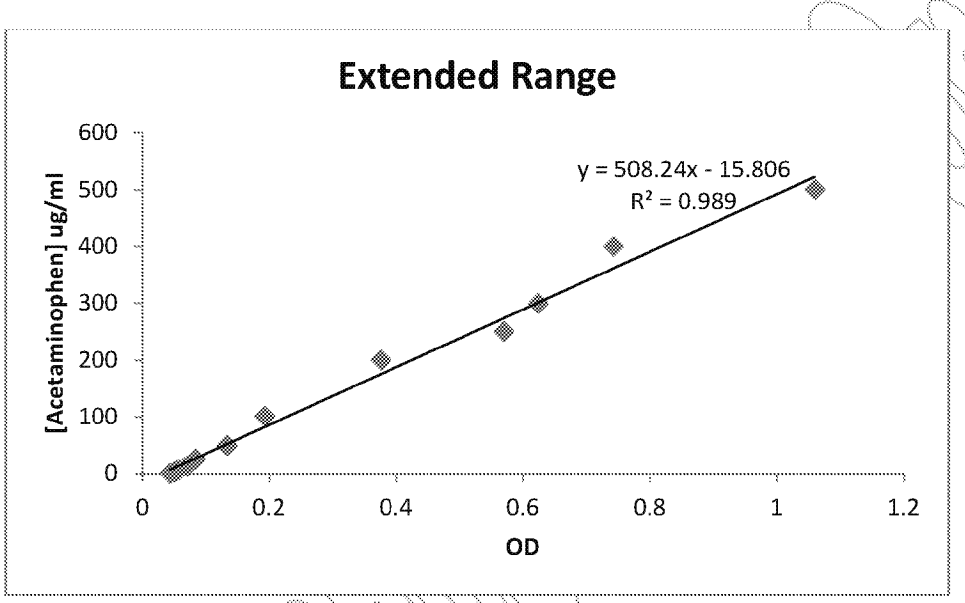
Dilution linearity was performed by mixing spiked (100 ug/ml Acetaminophen) and un-spiked plasma to create a series of samples with varying concentrations. The samples were then diluted 1:15 in 100 mM Tris-HCl, pH 9. Lithium-heparin plasma was used to avoid possible chelation issues that might occur with EDTA plasma. The assay shows good linearity with an R^2= 0.9976. The only issue is the high value seen for the y-int which could be calibrated out in the final assay procedure.

[Acet] ug/ml Spiked	Mean Signal	Std Dev	Signal CV	Mean Value	Recovery
0	0.103	0.005	5%	9.4	-
30	0.162	0.000	0%	37.6	125%
50	0.204	0.004	2%	58.2	116%
60	0.226	0.003	1%	68.4	114%
70	0.253	0.003	1%	81.6	117%
80	0.271	0.001	0%	89.9	112%
90	0.300	0.008	3%	104.0	116%
100	0.315	0.007	2%	111.2	111%



Extended Range

The linear range of this assay was tested by extending the calibration above the clinical range to 500 ug/ml. Additional calibrators were prepared from 100 mM Acetaminophen stock and diluted in 100 mM Tris-HCl, pH 9.



[Acetaminophen] ug/ml	Mean Signal	Std Dev	%CV	Mean Value	Recovery
500	1.060	0.054	5%	522.903	105%
400	0.743	0.067	9%	361.969	90%
300	0.624	0.004	1%	301.514	101%
250	0.570	0.016	3%	273.942	110%
200	0.377	0.013	4%	175.623	88%
100	0.193	0.000	0%	82.335	82%
50	0.134	0.001	1%	52.222	104%
25	0.084	0.001	1%	26.937	108%
12.5	0.070	0.002	3%	19.517	156%
6.25	0.057	0.000	0%	12.986	208%
3.125	0.051	0.001	1%	10.267	329%
0	0.044	0.001	1%	6.658	-

The assay is linear to at least 500 ug/ml.

Plasma Spike Recovery

Charcoal stripped plasma (Sera Con II CD, 3x FT, Lot # 10E1406) was spiked with 4 different therapeutic levels of Acetaminophen using a 50 mM stock. The four resulting samples were subsequently spiked with low, med and high levels of Acetaminophen. Most recoveries are within 10 % of nominal.

[Acetaminophen] Spiked ug/ml	Sample A	Sample B	Sample C	Sample D	Mean % Recovery
0	-	-	-	-	-
25	96%	112%	89%	99%	99%
75	86%	92%	91%	88%	89%
150	90%	91%	91%	93%	91%
Sample Average	91%	98%	90%	93%	93%

Precision

In the development of this assay, precision was tested by spiking one sample of EDTA plasma from Stanford blood bank to produce 5 samples of varying acetaminophen concentration. These samples as well as their calibrators were freshly diluted in 100 mM Tris-HCl, pH 9 each time they were run. The only issue with using EDTA plasma is that the reaction would not reach its end point for 30 min. The data below was collected 30 minutes after the final incubation was completed. This assay demonstrated good precision with average Inter-run CVs at 4% and average Intra-run CVs at 5%.

Reported Acetaminophen (ug/ml)

Sample	Run 1	Run 2	Run 3	Mean	Std. Dev.	CV
1	102.60	99.07	95.45	99.04	3.58	4%
2	70.24	68.70	67.45	68.80	1.40	2%
3	58.17	55.57	55.79	56.51	1.44	3%
4	52.83	46.89	46.84	48.85	3.45	7%
5	34.56	32.12	31.24	32.64	1.72	5%
Average Inter-run Concentration CV					=	4%

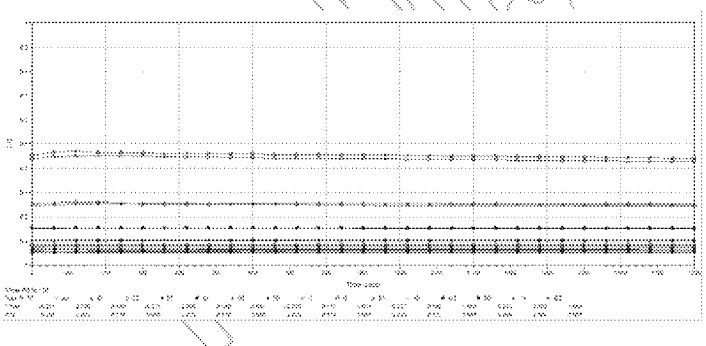
Sample	Run 1	Run 2	Run 3	Mean
1	8%	2%	4%	5%
2	12%	7%	3%	7%
3	2%	1%	5%	3%
4	-	7%	2%	5%
5	1%	5%	5%	4%
	Average Intra-run CV		=	5%

Kinetics

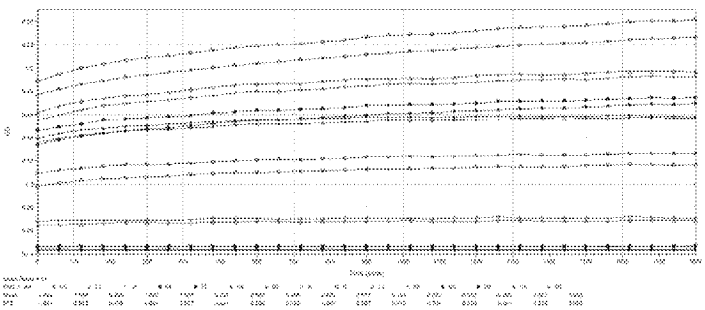
To clarify the late endpoint of the samples in the precision experiment, a kinetic study was done to confirm the endpoint of the assay reaction. Calibrators, and spiked EDTA and Li-Heparin samples were run and their signals were read for 30 minutes after the final incubation step.

The results show that while the calibrators and Li-Heparin samples reach an endpoint immediately, the EDTA samples do not appear to reach their endpoint until 30 minutes after the final incubation step. The Theranos system requires samples to read within 15 minutes and so it is recommended that only Li-Heparin samples are run on the Acetaminophen Assay.

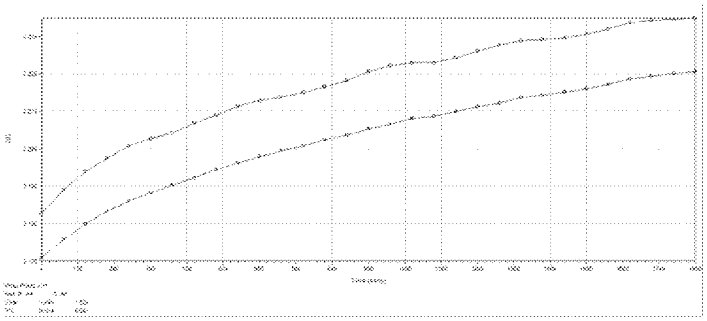
Calibrators



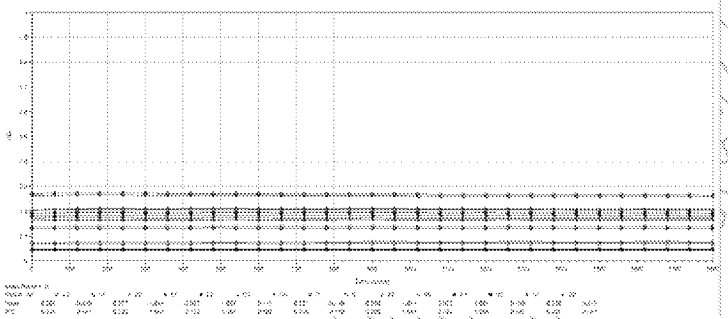
EDTA



EDTA at 100 ug/ml

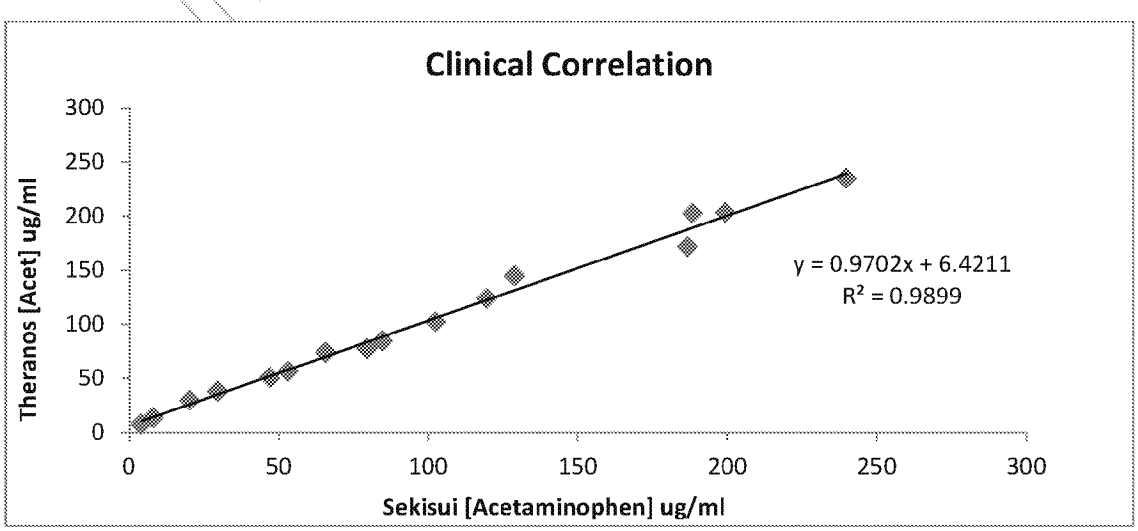


Li-Heparin



Clinical Samples

Clinical samples were prepared by spiking two levels of acetaminophen into Lithium-Heparin plasma from eight different patients. This produced a total of sixteen clinical samples which were then run on both the Theranos and Sekisui assay. The resulting data produces the correlation equation $y = 0.9702x + 6.4211$, $R^2 = 0.9899$ which is more than adequate to our standards



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Interference

Interference was tested by spiking hemolysed, icteric and lipemic plasma samples with different concentrations of Acetaminophen. For each sample spiked with 200 and 100 ug/ml of Acetaminophen recovery was within 10% of nominal but at 50 and 0 ug/ml both have over-recovery for each sample type. Interference is therefore only an issue at lower concentrations of Acetaminophen.

Hemolysed							
[Acetaminophen] ug/ml	1	2	Mean Value	Std Dev	%CV	Mean Value	Recovery
200	0.403	0.410	0.407	0.005	0.013	192.138	96%
100	0.262	0.248	0.255	0.010	0.037	109.708	110%
50	0.189	0.191	0.190	0.002	0.011	74.376	149%
0	0.112	0.115	0.113	0.002	0.014	32.794	-

Icteric							
[Acetaminophen] ug/ml	1	2	Mean Value	Std Dev	%CV	Mean Value	Recovery
200	0.422	0.402	0.412	0.014	0.035	195.128	98%
100	0.232	0.225	0.229	0.005	0.020	95.358	95%
50	0.166	0.164	0.165	0.001	0.008	60.679	121%
0	0.066	0.071	0.069	0.004	0.060	8.361	-

Lipemic							
[Acetaminophen] ug/ml	1	2	Mean Value	Std Dev	%CV	Mean Value	Recovery
200	0.464	0.459	0.461	0.003	0.007	221.898	111%
100	0.256	0.221	0.238	0.024	0.103	100.603	101%
50	0.209	0.187	0.198	0.015	0.077	78.779	158%
0	0.122	0.121	0.121	0.000	0.004	37.142	-

An acetaminophen overdose is treated with N-acetyl cysteine. The dosage of NAC is determined by measuring the amount of acetaminophen in plasma. Once treatment begins, acetaminophen levels are monitored to determine the patient's rate of recovery. It is therefore important that N-Acetyl Cysteine does not interfere with our acetaminophen assay.

To measure the interference of this drug a lithium-heparin plasma sample was spiked with 100 ug/ml acetaminophen. The sample was then split seven ways and each subsequent sample was spike with a different concentration of N-Acetyl Cysteine. The results show that from 500-200 ug/ml of NAC interference is just within 10% and anything below does not face any interference.

[NAC] ug/ml	[Acetaminophen stock] ug/ml	1	2	Mean Value	Std Dev	%CV	Mean Value	Recovery
500	100	0.255	0.256	0.255	0.001	0.003	89.610	90%
400	100	0.257	0.252	0.254	0.003	0.014	89.007	89%
300	100	0.257	0.258	0.258	0.001	0.003	90.817	91%
200	100	0.258	0.255	0.256	0.002	0.010	90.214	90%
100	100	0.282	0.268	0.275	0.010	0.037	99.842	100%
50	100	0.274	0.271	0.273	0.002	0.009	98.688	99%
0	100	0.278	0.268	0.273	0.007	0.025	98.924	99%

Stability

At this time, Reagent A is very unstable and left at room temperature degrades within 30 minutes. Reagent B has proved to be stable for the entirety of the development of this assay.

Conclusion

The Theranos acetaminophen in plasma assay has completed pre-feasibility testing and met the necessary testing criteria, excluding stability which is pending. This assay can measure acetaminophen from 0 - 500 ug/ml at a 1:15 dilution in 15 minutes.