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Aldolase Development Report [Plasma]

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Aldolase Assay Development Report

I) Assay Information

1. Assay Specifications

The assay is designed to measure aldolase in serum and EDTA plasma. The assay has a reportable range of 5.7 U/L to 87.1 U/L. The assay has an expected LLOQ of 5.7 U/L and ULOQ of 87.1 U/L.

2. Analyte Background[XE "Analyte Background"]

Aldolase is an enzyme that plays a role in glycolysis, facilitating in the breakdown of glucose to lactate in muscle. Aldolase converts fructose 1,6-bisphosphate into dihydroxyacetone and glyceraldehyde-3-phosphate. It is a tetrameric enzyme whose secondary structure depends on the tissue from which it originates. There are liver, muscle, and brain forms. Aldolase is used as a marker for muscle damage and disorders, as the enzyme is elevated in various muscle diseases such as polymyositis and Duchenne muscular dystrophy. Aldolase can also be elevated in cases of muscle trauma and liver damage and is known to spike in a similar manner to aspartate transaminase (AST) following myocardial infarction. Aldolase levels can be elevated in prostate cancer as well. Low aldolase is not considered diagnostically significant.

Analyte Range:

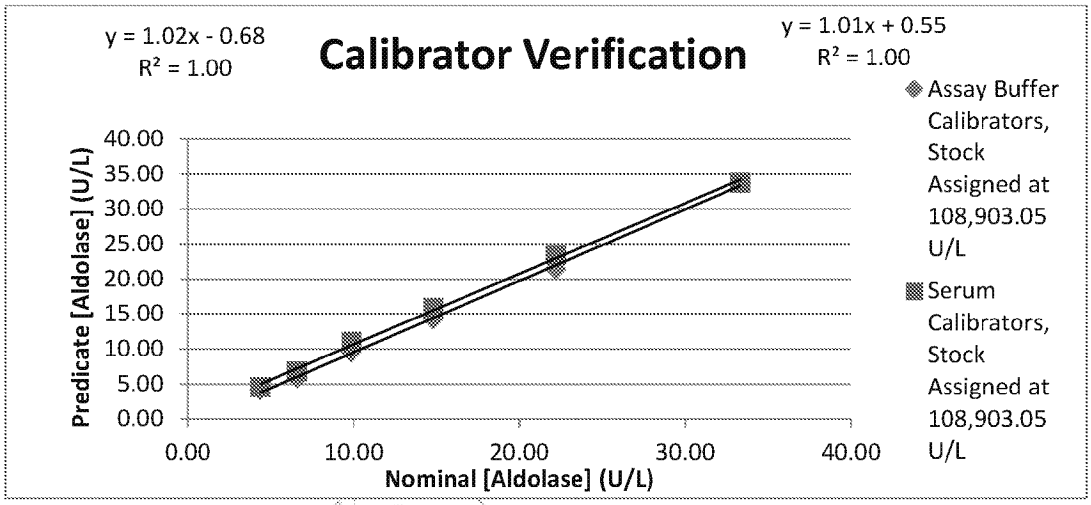
The normal range for aldolase is from 0 to 7.5 U/L or 8 U/L, depending on the reference. The clinical condition of interest is elevated aldolase. In cases of disease, the enzyme may be elevated to levels of 50 U/L or greater.

3. Reference Method[XE "Reference Assays"]

The Aldolase Reagent/Kit from Caldon Biotech cat # CALD 015 was used as a reference during development of this assay.

4. Calibrator Verification

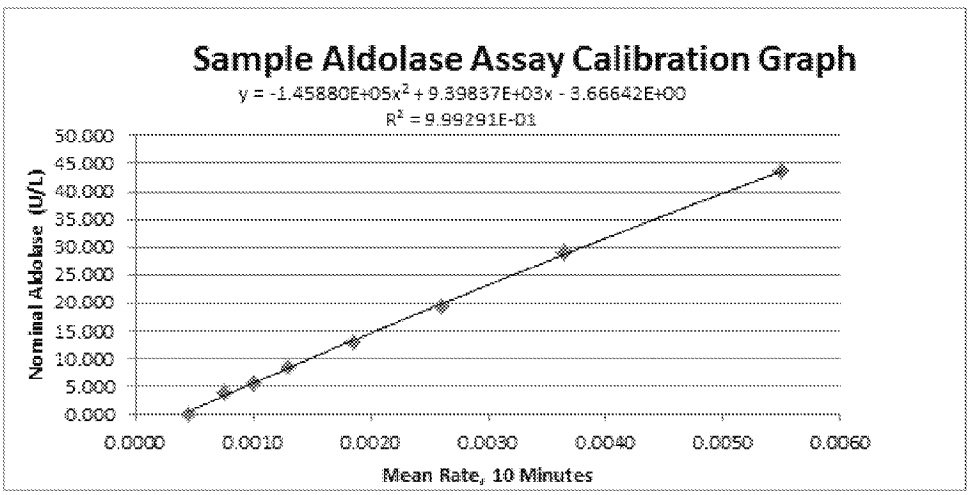
To verify the Theranos calibrator stock, calibrators in assay buffer (3% bovine serum albumin + 0.05% sodium azide in 50mM Tris buffered saline) were made from a frozen stock of aldolase from rabbit muscle and these solutions were tested on the reference method. Testing suggested that the stock was at 87.12% recovery relative to nominal. To confirm the newly assigned activity of 108,903.05 U/L, new sets of calibrator dilutions were made in human serum and in assay buffer (3% bovine serum albumin + 0.05% sodium azide in 50mM Tris buffered saline) from the same aldolase stock using the newly assigned activity and the solutions were tested on the reference method for recovery. The Theranos calibrators in both serum and assay buffer gave roughly 100% recovery on average so the analyte stock was left assigned at 108,903.05 U/L. The experiment showed that the chosen reference method sees the Theranos aldolase calibrators well and does so equally well in assay buffer or serum.



II) Assay Optimization

5. Preferred Calibration

The preferred assay calibration is a second degree polynomial fit to the graph with mean rate ((absorbance at T=0 – absorbance at T=10 minutes)/10) on the X-axis and nominal aldolase activity in U/L on the y-axis.



III) Assay Performance[XE "Assay Optimization"]

6. Spike Recovery

To test the recovery of the analyte in plasma and look at potential cross individual differences, the analyte was spiked at 3 levels into plasma from four different individuals. The spiked solutions and the neat endogenous plasma solutions were tested on the Theranos assay and the recovery of the analyte calculated and compared across individuals. To compare results in lithium heparin vs. EDTA plasma, samples were obtained for 4 patients with blood from each patient drawn into both an EDTA and a

lithium heparin tube to get patient matched samples for each anti-coagulant type. The aldolase spiked into EDTA plasma samples gave recovery that was generally to within 15% of target with CVs of less than 5%, showing good recovery of aldolase in that plasma type on the Theranos assay. For lithium heparin plasma, recovery for samples was good on average but several points gave recovery that was more than 15% off target and CVs were slightly higher. For the tested patient matched samples, the calculated endogenous aldolase was significantly higher in the EDTA plasma than it was in the lithium heparin plasma, showing a clear difference between the two sample types. Because EDTA plasma is presently preferred if possible and in the aldolase spike recovery experiment EDTA plasma gave greater precision and accuracy than lithium heparin plasma, EDTA plasma is the sample of choice for the Theranos aldolase assay.

Spike Recovery Summary for Four Patients: EDTA Plasma

Spiked (U/L)	% Recovery Patient 1	% Recovery Patient 2	% Recovery Patient 3	% Recovery Patient 4	Cross Patient Averages
26.1	114	99	120	106	110
13.1	110	95	113	103	105
6.5	108	91	108	98	101
Average % Signal CV=>	3.0	1.4	3.5	0.7	2.1
Average % Activity CV=>	3.6	1.7	4.2	0.8	2.6

Spike Recovery Summary for Four Patients: Lithium Heparin Plasma

Spiked (U/L)	% Recovery Patient 1	% Recovery Patient 2	% Recovery Patient 3	% Recovery Patient 4	Cross Patient Averages
26.1	90	82	98	82	88
13.1	87	82	97	94	90
6.5	84	89	113	113	100
Average % Signal CV=>	3.1	0.6	5.6	2.7	3.0
Average % Activity CV=>	4.7	0.7	7.5	4.0	4.2

7. Precision

To test accuracy and precision, 5 test samples and an 8 point calibration curve were tested on three separate runs, with each run being calibrated independently and with a combined three run calibration also being performed. Data was analyzed and the results across the three runs compared. For the tested clinical samples, the mean inter-run percent activity CV was 4.8 and the mean intra-run percent activity CV was 3.5, both meeting the goal of

less than 5% CV. The samples also showed good accuracy, with the calculated activity for each run being to within 5% of the mean intra-run calculated value. The average percent activity CV for the calibrators using the three run combined calibration was ~8. This was above the desired 5% target but still lower than 10%. The assay showed good overall accuracy and precision.

Summary Table for Clinical Sample Intra-Run Precision

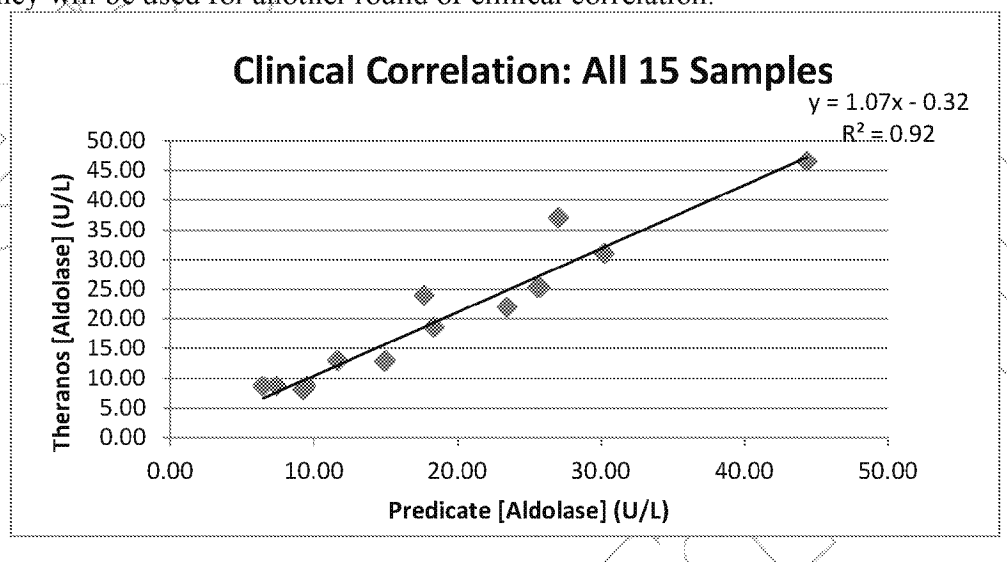
Sample	Mean Inter-Run Calc (u/l)	% Activity CV, Run 1	% Activity CV, Run 2	% Activity CV, Run 3	Mean Inter-Run % CV
ES4 (Spiked EDTA Plasma)	19.13	0.0	0.0	4.0	1.3
ES5 (Spiked EDTA Plasma)	15.61	3.9	0.0	4.8	2.9
Ict 1 (Serum)	24.62	0.0	0.0	0.0	0.0
Ict 2 (Serum)	14.21	8.6	5.1	9.3	7.6
CK 5 (lithium heparin plasma)	46.78	3.7	7.3	5.8	5.6
				Average Intra-Run % Activity CV	3.5
				Average Inter-Run % Activity CV	4.8

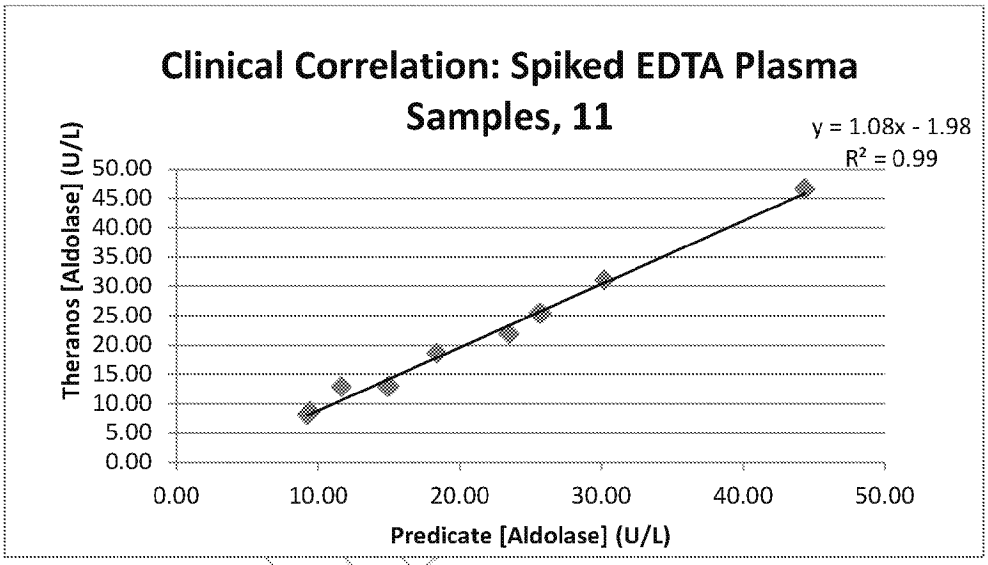
Summary of % Recovery vs. Mean Calculated Value for Tested Samples

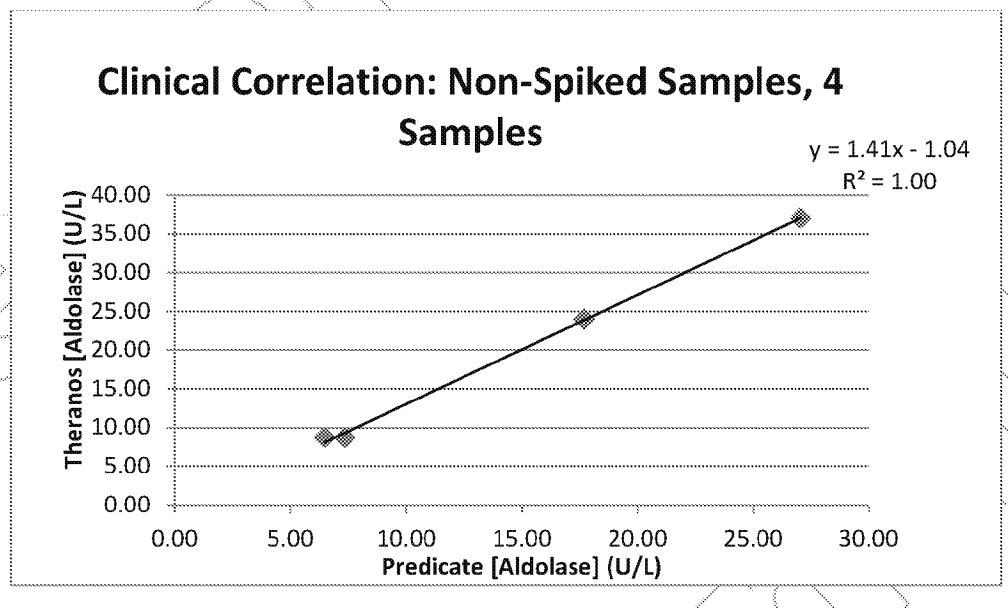
Samples	Run 1 % of Mean Result	Run 2 % of Mean Result	Run 3 % of Mean Result
ES4 (Spiked EDTA Plasma)	99	101	94
ES5 (Spiked EDTA Plasma)	102	98	96
Ict 1 (Serum)	101	99	104
Ict 2 (Serum)	102	98	109
CK 5 (lithium heparin plasma)	96	104	104
Average % Mean Result=>	100	100	101

8. Clinical Correlation

To assess the performance of the Theranos aldolase assay with clinical samples, clinical samples were run on the Theranos Aldolase assay and the reference method and results compared. The Theranos aldolase assay showed good clinical correlation to the reference method in general. For 15 clinical samples consisting of spiked EDTA plasma and non-spiked serum or lithium heparin plasma, the correlation equation was y=1.07x - 0.32 with r-squared value of 0.92. It was notable, however, that the average percent recovery was different between spiked and non-spiked samples, suggesting that there may be some difference between contrived and authentic samples for the assay, most likely stemming from a difference in endogenous human aldolase vs the rabbit muscle aldolase used for spiking. Comparing kit vs. Theranos results for just the 11 spiked samples, the correlation equation was y=1.08x - 1.98, with r-squared of 0.99, showing an acceptable slope. However, comparing just the 4 non-spiked samples, the correlation equation was y=1.41x - 1.04 with r-squared value of 1.0, showing a much higher slope. The results show that the Theranos assay and the predicate method match well but that further testing with un-spiked authentic samples will be needed to exactly how the Theranos assay will correlate with the predicate method using authentic samples and see if correction factors or stock adjustments might be needed. Currently orders have been placed for more clinical samples that should give aldolase levels that span the range. Once these samples arrive, they will be used for another round of clinical correlation.



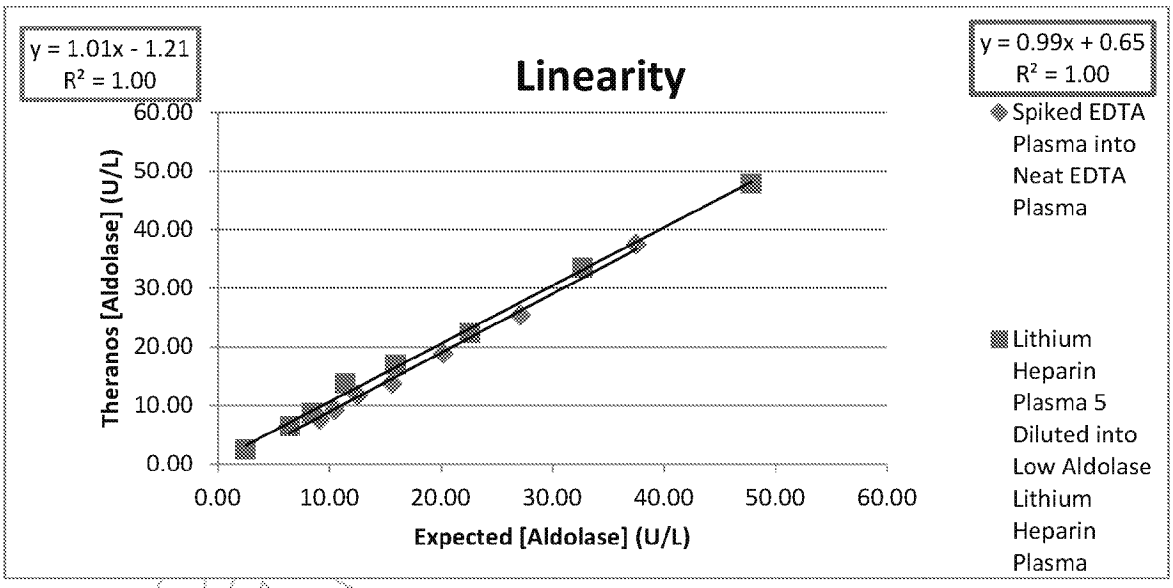




9. Linearity[XE "Dilution Linearity"]

To test dilution linearity, serial dilutions of a high analyte clinical lithium heparin plasma sample (CK 5) into a low analyte clinical lithium heparin plasma sample were made and tested along with the neat high and low samples, and the percent recovery relative to expected was calculated for all dilutions based on the calculated analyte levels for the neat high and low samples and the dilution ratios used. The same thing was done with a high spiked EDTA plasma sample diluting into an unaltered EDTA low aldolase plasma sample to see that both plasma types gave good results. The goal for dilution linearity testing is to ensure that recovery of the analyte is consistent along the range of interest. The assay showed linear recovery and good CVs for the tested solutions for both lithium

heparin and EDTA plasma, with recovery generally to within 10% of target and average concentration CVs of less than 5%.



The following equation was used to determine the recovery percentage: 100*(calculated concentration/expected concentration). Except for the neat high and low samples which were set at 100% recovery by definition.

Summary Data for Dilution Linearity: Lithium Heparin Plasma Pair

Sample Description	Nominal Aldolase (U/L)	Mean Calc (U/L)	% CV	% Recovery
Neat Authentic High Plasma	47.86	47.86	5.1	[100]
1.5X of High into Low	32.74	33.53	5.4	102
2.25 X of High into Low	22.65	22.31	3.8	98
3.38 X of High into Low	15.93	16.96	0.0	106
5.06 X of High into Low	11.45	13.78	2.9	120
7.59 X of High into Low	8.47	8.71	4.5	103
11.39 X of High into Low	6.47	6.51	5.9	101
Neat Low	2.49	2.49	N/A	[100]

Summary Data for Dilution Linearity: EDTA Plasma Pair

Sample Description	Nominal Aldolase (U/L)	Mean Calc (U/L)	% CV	% Recovery
Neat Spiked High Plasma	37.53	37.53	1.1	[100]
1.5X of Spiked	27.15	25.34	0.0	93

High into Low Un-Spiked				
2.25 X of Spiked High into Low Un-Spiked	20.23	18.91	0.0	93
3.38 X of Spiked High into Low Un-Spiked	15.62	13.76	5.2	88
5.06 X of Spiked High into Low Un-Spiked	12.54	11.75	6.0	94
7.59 X of Spiked High into Low Un-Spiked	10.49	9.29	7.4	89
11.39 X of Spiked High into Low Un-Spiked	9.12	7.59	4.5	83
Neat Low Un-Spiked	6.39	6.39	0.0	[100]

10. Interference

The assay was tested by spiking the analyte at three levels into lipemic, icteric, and hemolyzed samples and testing those solutions for spike recovery on the Theranos assay to see if there was any interference. The tested icteric sample with 15.52 mg/dL total bilirubin recovered within 15% of nominal and CVs of less than 10% so no interference is expected for icteric samples with bilirubin levels up to 15.52 mg/dL. The tested lipemic sample with 330 mg/dL triglycerides recovered within 15% of nominal and CVs of less than 10% so no interference is expected for lipemic samples with triglyceride levels up to 330 mg/dL. A hemolyzed sample with 1.83 g/dL hemoglobin was tested and gave recovery of 120% on average and higher CVs than the other sample types. Results suggest that hemolyzed samples with hemoglobin levels of 1.83 g/dL and above are expected to interfere in the Theranos aldolase assay and should not be used. The current plan is to test different levels of hemolysis to see at what level the hemoglobin ceases to cause interference in the assay.

Spiked Icteric Serum (15.52 mg/dL Total Bilirubin)

Spiked (U/L)	% Recovery vs. Nominal	% Activity CV
26.1	87	0.9
13.1	88	1.1
6.5	91	0.0

Spiked Lipemic Serum (330mg/dL Triglycerides)

Spiked (U/L)	% Recovery vs. Nominal	% Activity CV
26.1	103	5.7
13.1	112	0.0
6.5	105	10.8

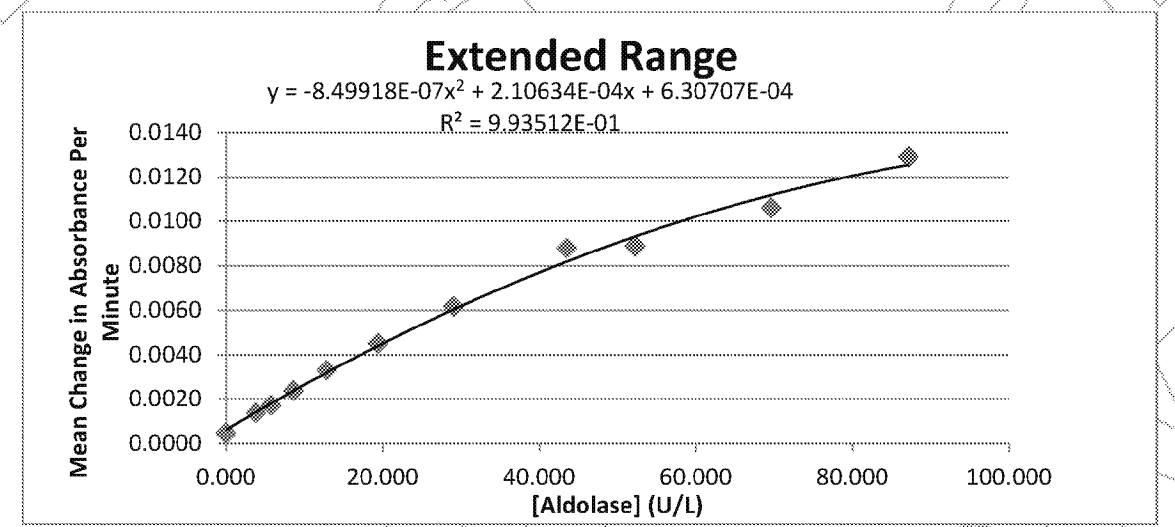
Spiked Hemolyzed Serum Sample (1.83 g/dL Hemoglobin)

Spiked (U/L)	% Recovery vs. Nominal	% Activity CV
26.1	119	6.2
13.1	112	10.2
6.5	129	17.7

11. Extended Range[XE "ULOQ and LLOQ"]

To look at the upper limit of the assay, the assay was calibrated to 43.56 U/L and then test solutions of 52.27, 69.70, and 87.12 U/L were tested as samples. The assay gave good CVs for the tested solutions and the recovery for both the 69.7 and 87.12 U/L points were within ~20% of target so it is likely that the assay ULOQ will be at least 87.12 U/L. When the readings from the extended range points were added onto the assay dose response, the resulting extended range response was slightly curved but not bowed over and still yielded a dose response. Results show that the assay still gives a response out to at least 87.12 U/L and does not hook.

Nominal (U/L)	Mean Calculated (U/L)	% Activity CV	% Recovery
87.12	70.27	1.4	81
69.70	55.03	0.0	79
52.27	44.11	1.0	84



12. Stability

Assay chemistry stability testing is in progress.

13. Conclusions[XE "Conclusions"]

The Theranos Aldolase assay has completed development testing and met the necessary testing criteria. Acceptable precision, accuracy, and response have been demonstrated.