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THERANOS

Creatine Kinase (CK) Development Report

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Development Report
Creatine Kinase (CK) Assay

A) Assay Development

I) Assay Information

1. Analyte Background

Creatine kinase (CK), also known as creatine phosphokinase, is an enzyme that catalyzes the inter-conversion of creatine and phosphocreatine using ADP or ATP. CK is expressed in various parts of the body. There are three different cytosolic isoenzymes: CK-MB (heart muscle), CK-MM (skeletal muscle), and CK-BB (expressed in all tissues). There are also two mitochondrial forms. The enzyme is used as a clinical marker for myocardial infarction, severe muscle damage/breakdown, muscular dystrophy, and acute renal failure (among other things). When tested as part of a cardiac panel, the CK-MB form is often measured along with troponin-I and myoglobin.

Analyte Range:

The normal range for creatine kinase is roughly from 25 - 200 U/L. In disease states, such as with heart attack or other sever muscle damage, however, the enzyme can be present at thousands of units per liter. Low levels are possible but elevated levels are traditionally the condition of diagnostic interest. The chosen Theranos reference assay from Teco, however, has a range that extends out to just 1200 U/L and the commercial kit from BioAssay goes out to 300 U/L.

2. Reference Assay

Creatine Kinase (CK-NAC) Reagent
(UV-Kinetic Method)
Teco Diagnostics
Cat# C512-60
Current Lot: 28691

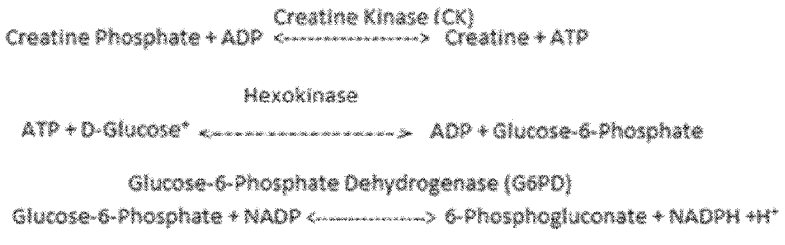
3. Assay Reagents

The chosen chemistry for the Theranos assay involves a series of several chemical reactions started by creatine kinase that result in the reduction of β-nicotinamide adenine dinucleotide phosphate (β -NADP) to β-NADPH, which is measured by monitoring the rate of increase in absorbance at 340nm as the β-NADPH is formed. Please see below for current working reagent specifications and for the assay reaction diagram. Please note that the DTT (dithiothreitol) is added as an activator of creatine kinase because the enzyme has fairly low activity in serum but thiol containing compounds, such as DTT increase that activity to make the enzyme more easily measurable. The AMP (adenosine-5'-monophosphate) is added as an inhibitor to another serum enzyme, adenylate kinase, to help ensure that the adenylate kinase does not interfere with the assay by consuming

needed substrates or products (ADP and ATP, please see Theranos assay reaction diagram below). Adenylate kinase catalyzes the following reaction:

2 ADP ⇔ ATP + AMP.

Theranos Creatine Kinase Assay Reaction Diagram:



Theranos Working Reagent

Substance and Final Working Reagent Level
20mM D-Glucose
10mM Magnesium Acetate
50mM AMP
2.17mM DTT (**6.5mM currently)
90mM Creatine Phosphate
2mM ADP
2mM Beta-NADP
3000 U/L Glucose-6-Phosphate Dehydrogenase
3000U/L Hexokinase
2mM EDTA
in 100mM Tris Buffer

4. Protocols

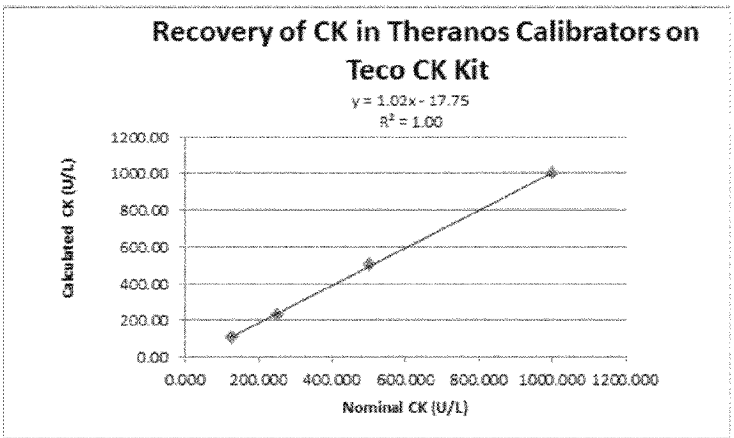
Microtiter Plate (MTP) Testing Protocol:
Dilute the sample 10X using water (for example, 10uL of sample into 90uL of water). In a 96 well MTP, add 60uL of working reagent to each well. Cover the plate and place it at 37C for 4 minutes to bring working reagents up to temperature. Next add 60uL of 10X diluted sample to each well. Mix and incubate in the M5 at 37C for 15 minutes reading kinetic at 340nm at an interval of every minute. Gather data and analyze the rate of change in signal per minute between 0 and 15 minutes and use this to calibrate assay and analyze data.

Assay update: although the assay was released using a 10X sample dilution, follow up testing revealed that a 15X sample dilution can be used if the level of DTT in the working reagent is increased to 6.5mM. Results confirmed with clinical sample testing (see updates in clinical sample section of this report). Thus the current Theranos CK assay uses a 15X sample dilution, with the rest of the protocol being the same as above when testing on a 96 well MTP.

II) Assay Optimization

5. Calibrator Verification

To verify the Theranos CK calibrator stock, the Theranos calibrators were made using dilutions of porcine heart creatine kinase in assay buffer and those solutions were tested on the Teco CK kit for recovery. The Theranos CK stock was at a nominal activity of 500, 000 U/L in assay buffer. The Theranos calibrators as tested on the Teco kit gave 95% recovery on average and when nominal CK (U/L) was plotted against calculated CK (U/L) on the Teco kit, the slope was 1.02, showing average recovery of 102%. Based on the Teco kit testing, the Theranos stock was assigned at nominal activity since it was to within 10% of target. Testing also confirmed that the kit does recognize the Theranos creatine kinase stock.



6. Plasma 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 lithium heparin plasma from four different individuals. The spiked solutions and the neat endogenous plasma solutions were tested on the Theranos assay and the spike recovery of the analyte calculated and

compared across individuals. Spike recovery of CK in plasma was generally to within 15% of nominal in general across the tested range and across all 4 patients and the percent activity/concentration CVs for the spiked samples were generally less than 5%. The conclusion is that the CK assay shows acceptable spike recovery in lithium heparin plasma at the tested spike levels and should be consistent across patients.

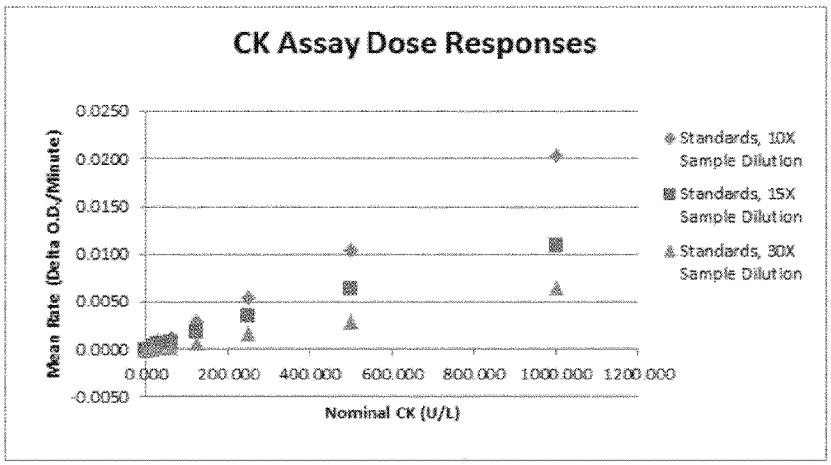
Spike Recovery Summary for Four Patients

CK Spiked (U/L)	% Recovery Patient 1	% Recovery Patient 2	% Recovery Patient 3	% Recovery Patient 4
800.00	88	85	89	84
400.00	85	90	86	84
200.00	94	93	87	91

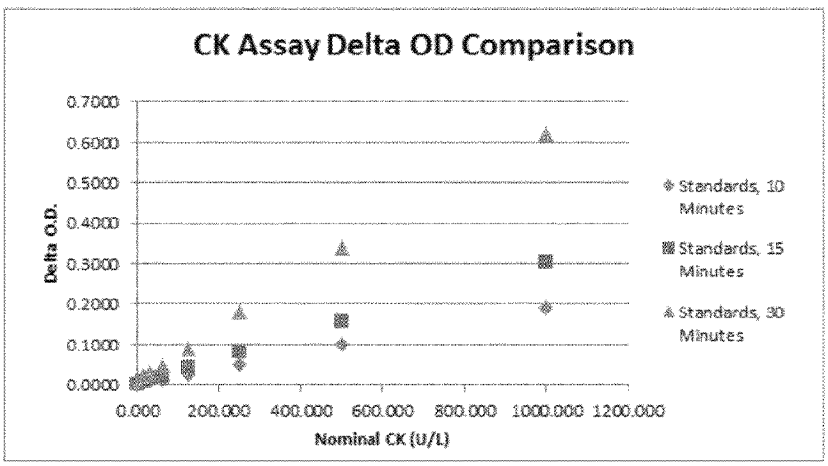
7. **Sample Dilution and Incubation**

To work on optimization and test the assay limits, the assay was run with different sample dilutions and incubation times. For sample dilution testing, the assay was run as a 15 minute assay with 10, 15, and 30X dilution running calibrators and a few clinical samples. In that testing, the 10X sample dilution gave the best overall performance with the highest rate, best lower limit of detection (62.5 U/L), best overall CVs, and slightly better clinical results. The 15X dilution was a close second. The 30X dilution however, gave a very low rate, and although the CVs were not greater than 10% for most solutions, the assay with that dilution did not give the lower limit of sensitivity/detection that the assay needs to see normal samples well and the rate was so low that it was likely to yield high run to run variability. Based on these results, the 10X sample dilution was chosen. Please note, however, that if the assay is allowed to run all the way out to 30 minutes, the rates are stronger and the CVs better for all dilutions and even the 30X gives a good lower limit and good results for clinical samples.

Current assay update: the assay was re-tested and if the concentration of dithiothreitol (DTT) in the working reagent is increased to 6.5mM from the previous 2.17mM, the assay gives good clinical results with a 15X sample dilution and 15 minute incubation so as of 2012, the chosen sample dilution for the Theranos CK assay is 15X (see clinical sample section for 15X sample dilution clinical results).



Assay incubation time was tested running the 10, 15, and 30X dilutions all the way out to 30 minutes, however since the 15 and 30X dilutions required longer incubation times to give good results, the 10X sample dilution was focused on. For incubation times, the 10 minute incubation gave the worst overall results with worse CVs and a worse lower limit of detection. The 30 minute incubation gave the best lower limit of detection, CVs, rate, and correlation with the kit for tested samples. However, since the 15 minute incubation was a close second with acceptable CVs and a shorter incubation time, it was chosen.



8. Precision

To test precision, 6 test samples and an 8 point calibration curve were tested on three separate runs, with each run being calibrated independently and with the a combined three run calibration also being performed. Data was analyzed and the results across the three runs

compared. The assay showed acceptable precision looking at three independent runs making fresh working reagent, fresh calibrators, and fresh 10X dilutions of clinical samples and calibrators for each run. For the calibrators using a three run combined calibration, the assay lower limit of detection was 62.5 U/L. Using the combined calibration, the average concentration and signal CVs for all calibrators at or above the lower limit were less than 7%. For the tested clinical samples the average intra-run CV was 3.2%. The average inter-run CV for calculated concentration of the clinical samples was 5.2%. Overall, the assay showed good precision across runs.

Summary Table for Clinical Sample Intra-Run Precision

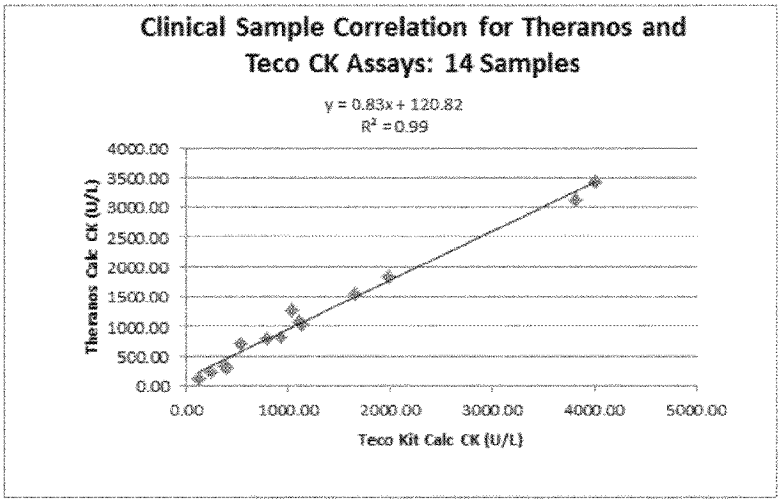
Sample	Nominal or Teco Kit Calc CK (U/L)	% Concentration CV, Run 1	% Concentration CV, Run 2	% Concentration CV, Run 3	Mean Inter-Run % CV
Sample 8	374.43	0.6	11.8	5.0	5.8
Sample 9	790.33	0.0	4.5	2.0	2.2
Sample 11	938.00	0.7	0.5	3.0	1.4
Sample 16	88.16	9.2	5.4	7.5	7.3
Sample V1	1004.00	1.3	0.5	0.9	0.9
Sample V2	505.00	1.3	0.6	2.9	1.6
				Average Intra-Run % Concentration CV	3.2
				Average Inter-Run % Concentration CV	5.2

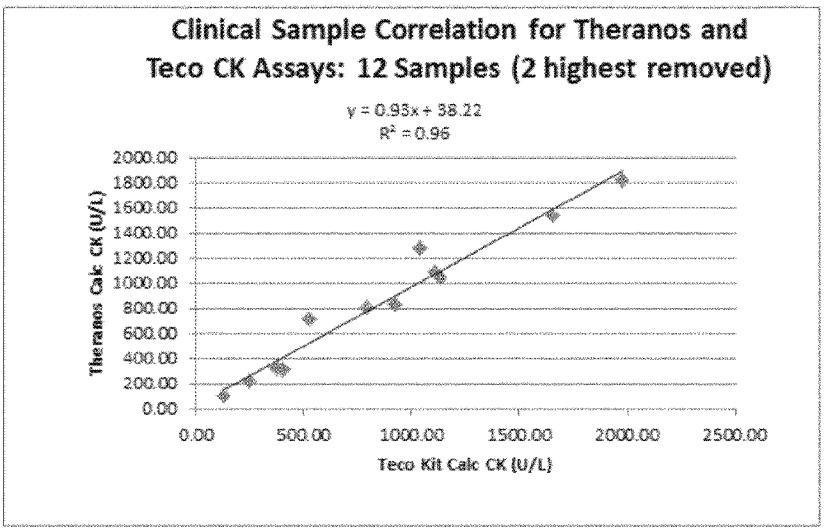
9. Clinical Samples

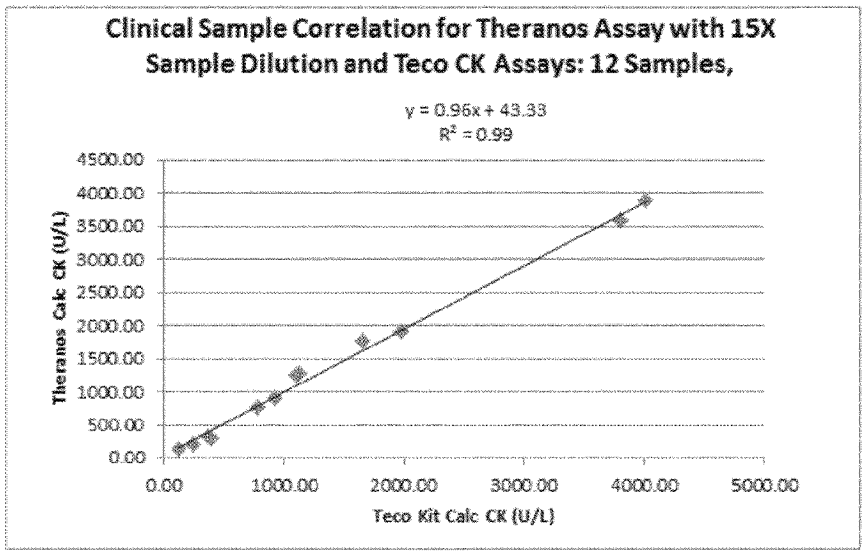
To assess the performance of the Theranos CK assay with clinical samples, the assay with 10X sample dilution and 15 minute incubation was tested running 14 clinical samples and the results were compared to those obtained for the same samples on the Teco CK kit. The correlation equation for the assay testing all 14 samples was y=0.83 + 120.82, r-squared = 0.99. If the two highest samples were removed, the correlation equation became y=0.93 + 38.22, r-squared = 0.96. Based on this testing, the assay performs acceptably relative to our chosen reference.

Current assay update: the CK assay was tested increasing the DTT level to 6.5mM from 2.17mM and testing with 15X sample dilution and the assay and performed well. With the new conditions, testing 12 clinical samples on the new Theranos assay and the same

reference kit, the generated correlation equation was y=0.96 + 43.33, r-squared = 0.99. For this round of sample testing, the Theranos assay gave activity CVs for the samples that were generally less than 5%. Results showed good clinical performance and verified that with the increased DTT level, the Theranos CK assay performs well with 15X sample dilution. Please note that in the graphs the abbreviation "calc" means calculated.







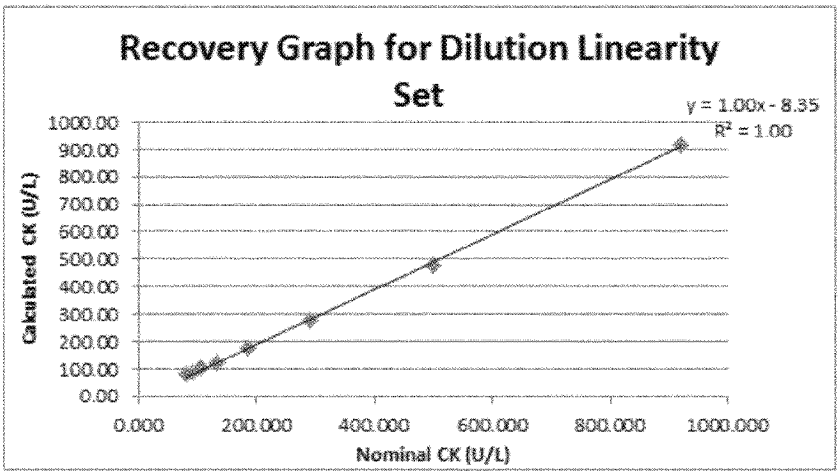
10. Dilution Linearity

To test dilution linearity, serial dilutions of a high CK clinical plasma sample into a low CK clinical 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 goal was to ensure that recovery of analyte is consistent along the range of interest. The assay showed linear recovery and good CVs for the tested solutions, with recovery to within 10% of target and concentrations 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

Sample Description	Nominal CK (U/L)	Mean Calculated (U/L)	% Concentration CV	% Recovery
Neat #7	919.26	919.26	1.5	100
2X of 7 into 16	499.44	478.25	1.5	96
4X of 7 into 17	289.52	274.06	2.8	95
8X of 7 into 18	184.57	173.33	4.7	94
16X of 7 into 19	132.09	120.32	2.2	91
32X of 7 into 20	105.85	102.20	0.7	97
64X of 7 into 21	92.73	88.86	2.5	96
Neat # 16	79.61	79.61	2.7	100



11. Endogenous Interference/Interfering Matrices

The assay was tested 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 from these sample types (the unaltered samples were also run to get the endogenous CK levels). Please note that hemolyzed samples are not recommended for the creatine kinase assay because ATP, glucose-6-phosphate, and an enzyme called adenylate kinase, which takes ATP away from the CK reaction, are all leaked out when red blood cells rupture, which can cause problems with the assay. The ATP and glucose-6-phosphate can fuel the CK assay rate and create falsely high results and the adenylate kinase can take substrates away from the CK assay reactions and create falsely low results. Because of these facts, hemolyzed samples are expected to interfere to varying degrees. For each interfering serum type, two samples were tested. For the two hemolyzed samples tested, the Theranos CK assay gave low recovery, 71% on average, at all tested spike levels (800 – 200 U/L), as such, it is concluded that hemolyzed samples do in fact interfere with the Theranos CK assay, as they do with the predicate method, and this sample type is not suggested for use with the Theranos assay. For icteric samples, interference was seen for total bilirubin at 4.29 mg/dL but not for total bilirubin at 2.39 mg /dL so we expect no interference from icteric samples with total bilirubin levels up to 2.39 mg/dL. Lipemic samples with 210.67 and 212.06 mg/dL total triglycerides were tested with CK spiked into them. Recovery was low for the sample with 212.06mg/dL triglycerides (55% on average), however, the sample with 210.67mg/dL, showed significantly better recovery, giving an average of 79% recovery. Based on testing here, highly lipemic samples are likely to interfere with the assay and are not recommended but since results were close to acceptable for the sample with 210.67mg/dL, it is likely that doing something as simple as adding 0.1% Triton X-100 to the sample diluent or perhaps even the working reagent, will allow the assay to tolerate

mildly lipemic samples so that should be kept in mind for the future. No confirmation testing of the effects of triton addition on the CK assay recovery in lipemic serum have yet been done. At present, lipemic samples with up to 210.67mg/dL total triglycerides interfere to some degree in the Theranos CK assay and should not be used.

Average Results for Two Spiked Hemolyzed Serum Samples (ProMedDX lots 11211891 and 11211903)

Spiked CK (U/L)	Recovered (U/L)	% Recovery vs. Nominal	% Concentration CV
800.000	590.12	74	2.2
400.000	282.17	71	5.1
200.000	135.32	68	7.1

Spiked Icteric Serum (BioReclamation lot BRH459899, 2.39mg/dL total bilirubin)

Spiked CK (U/L)	Recovered (U/L)	% Recovery vs. Nominal	% Concentration CV
800.000	737.30	92	1.5
400.000	365.79	91	0.2
200.000	182.87	91	2.5

Spiked Icteric Serum (BioReclamation lot BRH459900, 4.29mg/dL total bilirubin)

Spiked CK (U/L)	Recovered (U/L)	% Recovery vs. Nominal	% Concentration CV
800.000	558.66	70	1.6
400.000	296.46	74	2.3
200.000	154.64	77	1.7

Spiked Lipemic Serum (ProMedDX lot 11591643, 210.67 mg/dL Total Triglycerides)

Spiked CK (U/L)	Recovered (U/L)	% Recovery vs. Nominal	% Concentration CV
800.000	674.44	84	5.3
400.000	300.49	75	1.5
200.000	153.31	77	4.3

Spiked Lipemic Serum (ProMedDX lot 11427659, 212.06 mg/dL Total Triglycerides)

Spiked CK (U/L)	Recovered (U/L)	% Recovery vs. Nominal	% Concentration CV
800.000	511.27	64	4.5
400.000	244.34	61	1.8
200.000	81.12	41	11.8

12. Extended Range Testing

To look at the upper limit of the assay, the assay was calibrated to 1000 U/L and then test solutions of 2000 and 3000 U/L were run. The assay gave 93% recovery for the 2000 U/L solution with activity/concentration CVs of 2.6% and the 3000 U/L sample gave recovery of 83% with activity/concentration CVs of 1.7%. Testing showed that the Theranos assay still performs well out to 3000 U/L, three times higher than the current top calibrator. The assay still reported the correct result clinically out to 3000 U/L, i.e., the results were still reported as elevated and the response did not bow over, so the Theranos CK assay ULOQ could be as high as 3000 U/L and is at least 2000 U/L.

Nominal CK (U/L)	Mean Calculated (U/L)	% Concentration CV	% Recovery
3000.00	2504.95	1.7	83
2000.00	1854.53	2.6	93

13. Stability

Full assay chemistry stability testing is pending. It is known that the CK working reagent will need stabilization. When the working reagent was stored in solution at 4C, the reagent was down to 37% recovery vs. T=0 after only 1 month of storage. Initial testing of the reagent formulated at 3X the standard concentration with 25mg/mL trehalose and dried down using the Savant SpeedVac showed improved stability over the solution phase stored standard concentration reagent, with 70% recovery relative to T=0 left after 1 month. However, that is a significant loss in activity after 1 month so more work is still needed to stabilize the Theranos CK reagent.

14. Conclusions

The Theranos CK assay has completed evelopment testing and met the necessary testing criteria, excluding stability which is pending. Acceptable precision, accuracy, and response have been demonstrated on the M5. The preferred sample for this assay is lithium heparin plasma. Follow up testing more recently, showed that the assay performs acceptably with 15 minute incubation and 15X sample dilution. This was confirmed with clinical sample testing.