



# Creatine Assay for Use on Bench Top Chemistry Analyzer: Quick Analysis of Creatine in Human Serum Samples

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Creatine Assay for Use on Bench Top Chemistry Analyzer: Quick Analysis of Creatine  
in Human Serum Samples.

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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## Abstract

Creatine (Cr) was initially discovered in the 1830s by a Frenchman named Chevreul. In subsequent years, it was discovered that creatine was utilized by the muscular system, which fueled research on its overall use as a metabolite in the human body. Investigation into the role of this chemical in the human body led to its current use as a sports supplement and potential treatment option for individuals with muscular disorders such as Parkinson's and Huntington's. With creatine's increased use as a sports supplement there is also a strong interest in its long term effects, as well as its potential as a treatment option. There is need for further investigation as well as a means for dosage determination, and a method that will allow for accurate concentration determination. The current methods used to determine creatine concentration are time consuming and complicated analytical methods such as high pressure liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GS-MS). A single reagent enzymatic assay (Cr assay) that can be used on any bench top chemistry analyzer for accurate and precise measurement of Cr in human serum and aqueous samples was developed and is summarized here. The assay can measure samples in 10 minutes, is linear from 0-15mg/dL of Cr, the reagent has a shelf life of 45 days refrigerated. The Cr assay is within 98% accuracy, run to run CV from 1.6-5.3% for clinical samples and a sample-to-sample CV of less than 0.5%. This assay can easily be adapted for any bench top chemistry analyzer that has the capability of a programmable channel, and can read clinical samples with accuracy, precision and reproducibility.

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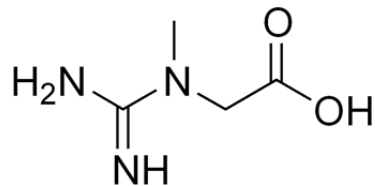
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## Chapter I

### Introduction



Creatine (Cr) was initially discovered in the 1830s by a French man named Chevreul (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). Studies of this chemical have been ongoing ever since, the first of which was done in foxes by Lieberg in 1847, who noted that foxes killed during a fox chase had much higher concentrations of Cr than those killed in captivity (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). With the discovery of creatinine (crea) in urine by Heintz and Pettenkofer speculations began that the crea was derived by the cr stored in muscles (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994).

This eventually led to studies on cr ingestion, which showed that not all cr ingested could be recovered in urine, indicating that it was being utilized by the body. Although there have been a multitude of studies completed to investigate the metabolism of cr, few investigated the effect and role of cr supplementation on exercise and performance in humans. Only recently have these studies been gaining popularity, and with the gain in interest in cr, the scientific community has experienced an increased need

for a method to detect cr concentration in the human circulatory and muscular systems that is quick and reliable. (Schilling 2001).

#### Potential Beneficial Impacts of Cr

As the knowledge of the muscular system and its function increases so are the possible treatment options for patients with muscular dysfunctions. The role of cr in the muscular system has made it a key target for treatment of disorders such as Parkinson's, Huntington's disease (HD) and sclerosis (Ferrante 2000; Andrew 1993; Taylor 1995; Bender 2006). HD is a genetic neurodegenerative disease that has a very late onset of mid to late life (Ferrante 2000). Symptoms of the disease include but are not limited to loss of motor function, mental function and behavioral symptoms (Ferrante 2000; Andrew 1993). Ferrante tested cr supplementation in an HD mouse model in 2000; the data suggested that cr could be used as a novel therapeutic agent (Ferrante 2000). Testing showed that cr supplementation in the HD mouse model significantly increased survival, slowed the development of brain atrophy, and delayed atrophy of striatal neurons and the formation of huntingtin-positive aggregates, therefore delaying the onset and severity of symptoms of HD (Ferrante 2000). Parkinson's disease is also a degenerative disorder but impacts the central nervous system, which in turn affects motor function (Taylor 1995). In 2006, a randomized placebo-control study was conducted at the University of Munich by Bender et. al. using 60 Parkinson's disease patients to determine the effects of cr supplementation on the progression of the disease symptoms (Bender 2006). Although the study results did not prove promising for the motor function of the patient population, patients did experience an increase in positive feelings,

outlook and overall mood, allowing for the reduction of dopaminergic therapy dosage (Bender 2006). A summary of the potential benefits of Cr supplementation was published by Tarnopolski in 2000 which indicates clear evidence that Cr monohydrate has potential neuroprotective effects in animal models of Parkinson's disease, Alzheimer's disease, after ischemia and amyotrophic lateral sclerosis (Tarnopolsky 2000). These are only a few examples of the current research being conducted to determine a beneficial impact for Cr supplementation. As many researches mentioned in their publications, there is an increasing need for further research in the impact of Cr as a supplement (Becque 2000; Farquhar 2002; Yoshizumi 2004). In addition to investigation on beneficial implication of Cr, there is also ongoing investigation on the long-term impact of cr supplementation on healthy individuals (Becque 2000; Farquhar 2002; Yoshizumi 2004).

#### Current Assays for Cr Detection

Currently, research on the effects of Cr supplementation on kidney function relies on more complicated analytical methods such as HPLC and GC-MS (Yoshimuzi 2004). In this growing research field there is a need for a method to measure cr with high throughput and without complicated separation techniques. Arias, et al. published a paper in 2006 explaining the current and very complicated procedure of Cr detection (Arias 2006). High pressure liquid chromatography-tandem mass spectrometry (HPLC/MS) is a process which combines the capabilities of liquid chromatography to physically separate chemical compounds and the capability of mass spectrometry to detect the mass of a compound (Aebersold 2003; Jonsson 2004). A sample that potentially contains Cr is passed through a liquid chromatography column filled with adsorbent material, and

chemical components that are in the sample each react differently with the adsorbent material. The time it takes for each compound to pass through the column is different based on that interaction (Aebersold 2003). Each chemical passes through the column at different rates allowing for the separation and identification of each chemical based on the interaction with the adsorbent material (Aebersold 2003). A very complicated method that also has other limitations, such as interfering substances such as creatinine, and other metabolites such as glucose which will not allow for proper separation with creatinine molecule and therefore will give incorrect or no results at all (Aebersold 2003). Mass spectrometry enables identification of amount and type of chemicals present in a solution (Schilling 2001). This is done by separating the chemicals based on the mass to charge ratio in their gas-phase (Jonsson 2004). The second method described by Arias is gas chromatography mass spectroscopy (GC/MS). Similar to liquid chromatography, gas chromatography separates chemicals, but does so by vaporization, and is then combined with mass spectrometry to detect the presence and concentration of each chemical (Arias 2006; Rawson 2011). As Arias et. al. concludes, in comparing creatinine to creatinine ratios in patient samples there is a constant and proportional error and therefore the creatinine to creatinine ration should be evaluated with caution, especially when using GC/MS (Arias 2006). Finally BioVision (Milpitas, CA) has a colorimetric/fluorometric assay kit, which uses a colorimetric or fluorometric intensity change to detect the concentration of creatinine in samples (Rawson 2011). The assay is relatively easy to use according to the manufacturer's instructions – but the reaction requires 1 hour for incubation and no separation is required. However, the assay is for research purposes only and is not traceable to

standard methods so it cannot be used for clinical testing. The methods currently available to test for cr in clinical samples are either very complicated to use such as GC/MS and HPLC/MS or not approved for analysis of patient samples. There is an increasing need for the ability to quickly analyze cr in patient samples to be able to evaluate cr deficiency syndroms (Arias 2006). There is also an increased need for evaluation of long-term cr supplementation (Yoshizumi 2004).

### Assay Requirements

To be able to detect an unknown concentration of a chemical, the assay must fulfill some basic requirements. The method of detection must be sensitive enough to respond to the lowest concentrations that are to be measured. The detection method must be accurately and easily calibrated against a known standard concentration that is within the range in which is to be detected, and the reaction must be linear in response to the concentration ranges of measurement. For clinical sample analysis, the impact of biological sample matrix on accuracy and precision should be part of the critical criteria of the assay. This is applicable to any measurement system that is sensitive to a specific chemical in any background matrix. Currently to calculate accurate estimated glomerular filtration rate (eGFR) hospitals must have a system that can accurately measure concentrations of crea in either blood or plasm over a period of time from the same patient (Levey 1999). Therefore a hospital must have an instrument that can accurately measure the Crea level from a patient's blood or serum. Instruments that are utilized for these purposes in hospitals are either large, slow bench top chemistry analyzers, or

critical care instruments which are smaller and easier with much quicker throughput. These instruments are capable of measuring crea using enzymatic methods via detection of colorimetric changes or current changes (Pamidi 2010). Pamidi explains that by taking advantage of the natural breakdown process of crea via enzymatic methods, one can develop a sensor that has the capability to detect the final breakdown product, hydrogen peroxide, which can be detected by a spectrophotometer or by a current detector. There has not been a method developed that utilizes a similar concept for the accurate detection of Cr in human blood.

#### Proposed Enzymatic Creatine Assay from Adapting Enzymatic Creatinine Assay

For the measurement of crea in clinical samples, a quick assay has been developed utilizing creatinine amidohydrolase (creatininase), creatine amidohydrolase (creatinase) and sarcosine oxidase as can be seen in Figure 1 to convert crea to cr then creatine to urea and sarcosine. Sarcosine is then converted to formaldehyde and hydrogen peroxide. Hydrogen peroxide will interact with a specific dye or chromophore to give a colorimetric change, which can be detected by a spectrophotometer. This assay has been made available by several different manufacturers such as Roche, Sekisui, and Horriba. The available assays are two-step, two reagent assays, in which reagent one contains creatinase, which converts cr to sarcosine and urea. This is done to eliminate all cr present in the sample as it causes interference with the crea detection. Sarcosine oxidase is in turn needed to convert the produced sarcosine to glycine, formaldehyde and hydrogen peroxide. The time delay between the addition of the first and second reagent



allows for the degradation of hydrogen peroxide which is determined by the concentration of the enzymes present in the reagent. The second reagent contains creatininase which converts Crea to Cr and the creatinase that is still present in reagent one converts Cr to sarcosine and the same cascade reaction will follow until hydrogen peroxide is formed. The hydrogen peroxide will interact with the 4-aminoantipyrine dye in reagent two, which is catalyzed by horse radish peroxidase to give a color change that can be detected by photospectrometry. We are proposing a similar enzymatic based method to allow for the breakdown of Cr to hydrogen peroxide. The enzymatic method that we are suggesting only requires a single reagent as the Cr molecule can be directly converted to sarcosine by the creatinase enzyme that is in reagent one of crea assay and the produced sarcosine is converted to hydrogen peroxide, which will react with 4-aminoantipyrine in the presence of horse radish peroxidase to allow for detection via uv-vis on any photospectrometer. All chemicals can be maintained in one reagent as no time delay is needed.

Having a reference material such as National Institute of Standard and Technology (NIST) traceable standard allows for accurate clinical assay development and is recommended by FDA regulations. Unlike crea, there is no NIST traceable Cr standard available making the assay development more challenging. As there are very limited testing assays currently available on the market for Cr and most of them are for laboratory research purposes only, there is a need for a method with quicker results, which can be utilized for clinical sample measurement.

## Goals for Creatine Assay

Our ultimate goal is to create an accurate and convenient creatine assay, which is quick and easy to use. It is based on a single part reagent via enzymatic conversion of creatine with colorimetric end point detection of 4-aminoantipyrine at 547 nm. In contrast to the purchasable manual microtiter plate based assay, our assay incubation time is much faster so it can be fully automated by easily adapting it to any clinical chemistry analyzer which provides customizable assay channels. The cr in clinical samples can be analyzed simultaneously with other commercially available assays like crea, glucose, lactate, etc.

Using a bench top chemistry analyzer, the goal is to develop a cr assay through optimizing assay conditions and procedures with a specific small (<300uL) sample and reagent volume, and an incubation time of less than 20 minutes. Methods and procedures will be developed for calibration standards and reagents that can measure the cr concentrations in clinical samples. The assay will achieve linearity of 0-15mg/dL, accuracy within 2%, recovery within +/- 3% from gravimetric values. In addition, coefficient of variation (CV), which is a standardized measure of dispersion of a probability distribution or frequency distribution, will be applied to analyze variability from samples and runs. It is defined as the ratio of the standard deviation to the mean. The target set for run to run is CV of less than 2% and sample to sample of 0.5%.

## Chapter II

### Materials and Methods

Materials and methods utilized for the development of the enzymatic creatine assay include participants who provided human blood and serum samples. To measure the outputs of our assay instruments such as a benchtop chemistry analyzer and a spectrophotometer were utilized. Reagents, calibration material and standard (control) material were developed along with parameters for specific use on bench top chemistry analyzers with an open channel feature. Finally data processing and statistical outputs were utilized to determine the success of our enzymatic creatine assay.

#### Participants

Participants are individuals that have in some form contributed to the development on the cr enzymatic assay; individuals from who blood and serum were used are participants. For the purposes of our study human biological samples such as blood and serum were needed to test the capability of the enzymatic cr assay. Human blood and serum were chosen as there are easy samples to collect from patients and in addition the enzymatic cr assay must have the capability to detect cr concentrations regardless of matrix and specifically in serum samples. These patient samples were collected by Lahey Hospital and Medical Center in Burlington Massachusetts. These samples were collected as de-identified pathologic discard whole blood and serum samples.

## Measure

To aid in the development of the enzymatic cr reagent a spectrophotometer was used to detect the optimal absorption value and the optimal incubation time. A bench top chemistry analyzer was used to conduct detailed evaluation of the parameters selected for the assay along with over all evaluation of the assay reagent, and calibration solutions. The instruments used are as follows.

UV-vis spectrometer, Evolution 6000 (Hitachi Ltd, Chiyoda, Japan) with quartz cells for optimization of wavelength for assay conditions. The spectrometer was used to determine the optimal absorption value for the photophor selected which was the 4-aminoantiparine, as well as to determine the optimal incubation time for the assay. Data shown in Figures 2a and 2b.

Bench top chemistry analyser, Roche Cobas C311 analyzer (Roche Diagnostics, Indianapolis, IN, USA), with programmable channel capability (open channel). Using the open channel function for programmability this instrument allowed for the programming of the optimized parameters for the enzymatic cr assay and all optimization and final analysis was completed using this instrument.

## Reagent

To develop a enzymatic cr reagent to use in the analysis of the enzymatic cr assay a commercially available assay for crea (Sekisui Diagnostics LLC, Lexington MA) was obtained and modified by adding 4-amynoantipyrene and horse radish peroxidase to

reagent one of the crea assay. Enzymatic Cr reagent includes the following chemicals and enzymes: buffer (pH 7.5) >12000 U/L creatine amidinohydrolase, > 4000 U/L sarcosine oxidase, > 2 U/mL horse radish peroxidase, > 0.24 mmol/L N-ethyl-N-sulfopropyl-m-toluidine, > 0.16mg/mL 4-aminoantipyrine, ascorbate oxidase, stabilizers, surfactants, and preservatives. (22)

To determine whether the reagent would be satisfactory, a predetermined concentration of 4-aminoantipyrin and horse radish peroxidase were added to the Sekisui reagent, for a total of 250mL of reagent. Based on the parameters of the Sekisui Creatinine assay, a ratio of one part sample to five parts reagent was tested. Total volumes tested were 1.5mL of reagent and 0.3mL of sample. The sample that was chosen was a 5mg/dL concentration of cr in deionized water. The reagent and sample were mixed and allowed to incubate for 5 and 30 minutes, at which point absorption spectra was taken using a UV-Vis spectrometer (the Evolution 6000) with quartz cell. A blank was taken as well which included only the reagent with deionized water (Fig. 2).

#### Reference method

HPLC analysis was conducted by an outside laboratory (Organix Inc, Woburn MA, USA) as an independent check to verify the correct concentrations of our prepared standards S1, S2, S3 and S4.

Isotope Dilution Mass Spectroscopy was conducted to measure cr values in plasma samples.

## Data Process and Statistical Analysis

Microsoft excel and Minitab were used for statistical analysis along with figure and table generation.

### Calibration Material

The cr assay is calibrated using a two point calibration at concentrations 0mg/dL and 5mg/dL of cr. The 0mg/dL concentration also serves as a blank measurement for the reagent. A 5mg/dL standard was prepared to calibrate the assay. Initially the standard was prepared using the gravimetric procedure by dissolving 5mg/dL of cr in deionized water at pH 11.0. As part of optimization another procedure was found to be more suitable as it showed less variability. This procedure required preparing a high concentration of cr, 1000mg/dL, and the standard was then prepared by diluting to 5mg/dL.

As seen in Figure 3, the gravimetric method was conducted by weighing out 44.0mg and 58.7mg of creatine monohydrate and then dissolving in 1L of deionized water to make a 3.9mg/dL and 5.2mg/dL cr solution respectively. A 1000mg/dL Cr stock solution was prepared by weighing 1.131g of creatine monohydrate and dissolving it in 100mL of deionized water. This stock solution was then used to make the two lower concentration solutions. The 5.2mg/dL solution was made by adding 52uL of the 1000mg/dL solution to 10mL of deionized water and the 3.9mg/dL solution was made by adding 39uL of the 1000mg/dL solution to 10mL deionized water.

### Standard material (Control Standards)

For the development of standards and calibration materials for the enzymatic cre assay samples of known concentration were used to ensure proper function of assay and assay calibration. These samples were expected to recover the same value over time as they are stable materials and are stored in a refrigerator (2-8C). These materials were prepared and values were determined independently so these materials can be used as quality control material, HPLC analysis was conducted to assign their value by an outside lab, Organix. The target value of cr is 5.2mg/dL.

When developing calibration standard and procedures these Control Standards were used as calibration standards, and other investigational test solutions were tested as samples on the cr assay.

### Assay Parameters

As shown in Table 1, the parameters for this assay are easily programmable for any bench top chemistry analyzer that has the capability of an open channel. The required sample volume is 30uL, the reagent volume is 150uL, an incubation time of 10 minutes and a wavelength of 546nm are required to read the final absorbance.

## Chapter III

### Results

To determine the proper enzymatic cr reagent formulation, the reagent was first formulated. Reagent formulation as described in Chapter II was executed by modifying a crea reagent purchased by Sekisui, modification was completed by adding HRP and 4-aminoantipyrine. The enzymatic cr reagent was then tested on a spectrometer to determine optimal absorption values and incubation times.

To determine the sensitivity of the proposed enzymatic reagent we measured the absorbance change of the reagent in the presence of 5mg/dL Cr using UV-Vis spectrophotometry. Measurements were taken at various incubation times from 0 to 30 minutes. The data in Figure 2 shows that the proposed reagent was undergoing a chemical change in the presence of Cr which was detected via UV-Vis at absorbance peak of 540nm. As shown in Figure 2 the dye selected was the proper dye as the interaction with hydrogen peroxide in the presence of horseradish peroxidase caused a peak in the spectral scan at 540nm. Furthermore the presence of a peak indicated that the concentrations of enzymes and the dye were appropriate. The absorbance data also showed that the reaction was fully completed within 10 minutes from the plateau, so the incubation time was then set for 10 minutes for the assay.



## Calibration Solution Development

To develop a precise, accurate and stable calibration standard solution, two methods of preparation were tested. These calibration solutions were run as samples on the Cobas C311 (Roche Diagnostics) using the single reagent enzymatic cr assay after it was calibrated with an independent calibration solution. Standards were prepared via dilution method and gravimetric method. Gravimetric method was conducted by weighing out cr to directly make a 3.9mg/dL and 5.0mg/dL solution, while the dilution method was conducted by weighing out cr to first make a 1000mg/dL cr stock solution, then diluting that solution to 3.9mg/dL and 5.0mg/dL. As seen in Figure 3 the data shows that there is less variability in the preparation of the calibration solution by following the dilution method.

## Linearity

For proper performance of the assay linearity to substrate must be established and determined, if assay is non-linear a mathematical formulation must be established to correct to non-linearity.

To test for linearity of the assay, concentrations of 0, 1, 2.5, 5,10 and 15mg/dL of Cr were prepared via serial dilution. The samples were run on the Cobas C311 using the single reagent cr assay after it was calibrated. As seen in Figure 4 the  $R^2$  value is  $> 0.999$  indicating that the assay is linear within the range of 0-15mg/dL cr.

## Reagent Stability

To evaluate the stability of the enzymatic cr assay reagent over its shelf life, reagent was prepared and stored under refrigeration (2-4C) and tested after 1, 5, 10, 20, 30 and 45 days of storage. The stability of the reagent was evaluated by completing a three point calibration using 0mg/dL, 3.9mg/dL and 5.2mg/dL of Cr, and correlate the absorbance values of each calibration sample versus gravimetric cr concentrations. The absorbance value increases as the reagent ages but the linearity of the calibration does not change, indicating that the reagent can maintain functionality after 45 days of refrigerated storage, giving the assay a shelf life of 45 days (Figure 5).

## Precision and Accuracy

To determine the accuracy and precision of the assay, four individual samples were prepared and run after three separate calibrations. Samples included crea as well as cr to allow for the comparison of the two enzymatic reagent methods which were both run simultaneously on the Cobas C311. These Cr results were compared to crea data to ensure comparability. As seen in Figure 6a the total variability of recovery for the cr assay is minimal and comparable to the crea assay as shown in Figure 6b. As shown in Table 2 the crea assay is capable of precision within 1.3% and accuracy within 1.7% and 0.5% and 1.2% respectively for the enzymatic cr assay, proving that the cr assay is comparable to a commercially available crea assay. As can be seen in Figures 7a and 7b, day to day, run to run and sample to sample variability are shown for each individual sample cr and crea assay respectively.

## Creatine Assay Performance in Plasma Samples

To complete determination of successful enzymatic cr assay clinical samples were tested and evaluated using all optimized parameters, reagents (assay reagent and calibration samples).

To establish the cr assay capability for clinical sample recovery, plasma samples of known cr concentration were measured on the Cobas C311 using the cr assay. This was done to determine the linearity, accuracy and precision of the method in the clinical sample matrix, as opposed to aqueous samples. As can be seen in Figure 8a samples were run over a 4 day period and for each day data were used to establish correlation between the Isotope Dilution Mass Spectrometry (IDMS) measured values and the cr recovery using the cr assay. Data are summarized in Figure 8b and a single formula was calculated to show correlation of the two methods. The formula for correlation of Cr assay vs. IDMS (to correct the matrix effect) was then used to correct subsequent clinical samples as shown in Figure 9 that were run on the Cobas C311 to calculate cr recovery using the cr assay

## Chapter IV

### Discussion

Cr has been investigated since the 1830s and research has revealed its role in the proper functioning of the muscular system (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). With this knowledge the use of cr as a potential drug for treatment of muscular disorders such as Parkinsons, Huntington's disease and sclerosis, along with other muscular system disorders are subjects of research (Ferrante 2000; Andrew 1993; Taylor 1995; Bender 2006). The sport nutrition/supplementation market quickly realized that cr can be used as a supplement and as such can increase strength, fat free mass, and muscle morphology in humans (Cooper 2012). It increases crea storage, which in turn increases muscle mass and promotes a faster regeneration of ATP between high intensity exercises (Cooper 2012). These improved outcomes will increase performance and promote greater training adaptations (Cooper 2012). Although short term use of Cr is fairly well understood and considered safe and ethical in the sports world, the perception of safety cannot be assumed, that it is safe and ethical especially in long term users from diverse population background. To summarize there is an increased need for investigation of the impact of cr on potentially beneficial implications as well as long term impact of its use as a supplement (Cooper 2012; Yoshizumi 2004).

Currently to determine the concentration of cr researchers must rely on methods such as HLPC and GC-MS, which are complicated to conduct and require a high level of

expertise (Arias 2006; Aebersold 2003; Jonsson 2004; Rawson 2011). Furthermore as discussed by Arias et. al. there are also issues with using human serum with such techniques that do not allow for accurate measurements due to interference from other components that are present in serum (Arias 2006). There is an increasing need for the capability of quick analysis of Cr in patient samples to be able to evaluate long term impact of supplementation or potential dosage management for use of cr as a drug (Arias 2006).

#### Cr Assay Parameter Optimization

An accurate and convenient enzymatic based Cr assay was developed, which is easy to use and reports results much quicker than the current methods. The single part reagent uses enzymatic conversion of cr with a colorimetric end point detection of 4-aminoantipyrine at 547nm. The method has been optimized and demonstrated on a bench top chemistry analyzer and has a run time of 10 minutes, using only 30uL of sample.

The enzymatic reagent is adapted from a commercially available Creatinine assay from Sekisui which is then modified by adding 4-aminoantipyrine and horse radish peroxidase. To determine whether the reagent would be satisfactory a predetermined concentration of 4-aminoantipyrin and horse radish peroxidase were added to the Sekisui reagent. Based on the parameters of the Sekisui Creatinine assay and optimization a final formulation was reached along with appropriate assay parameters adaptable to a bench top chemistry analyzer.

Using a Roche Cobas C311 analyzer, which is a bench top chemistry analyzer typically used in hospitals for measurements of electrolytes, and metabolites such as crea,

the assay and its parameters were evaluated. The assay parameters were programmed into the instrument's open channel, which allows for user programmable assays. The assay parameters are simple and easy to program as can be seen in Table 1. For assay development the proposed assay was initially calibrated with a 5mg/dL cr in deionized water.

### Stability of the Reagent

To then study the stability of the reagent as it ages a fresh calibration material was made and a three point calibration was executed to determine the linearity at multiple test points of the reagent shelf life. Testing was executed on the day that the reagent was prepared and 5,10,20,30 and 45 days after that with the reagent being stored in a refrigerator (2-4 C). As can be seen in Figure 5, all data points show linearity and more importantly very similar slopes indicating that the linearity of the assay has not been changed over time. This data shows that the cr reagent is stable for at least 45 days when stored in the refrigerator.

### Optimize Procedure for Calibration Standards

To develop a precise, accurate and stable assay, an equally as accurate and reproducible calibration solution is necessary. To achieve this, two different methods of making calibration solutions were evaluated. Initial experiments tested concentrations of 3.9 and 5.2 mg/dL cr. These two standard concentrations were prepared by two different methods. The gravimetric method in which the appropriate concentration of Cr is weighed out and dissolved in deionized water. The dilution method, by which a high

concentration of cr stock solution is prepared and then diluted down to the final concentration was the other method tested. Based on calibration standard preparation optimization the dilution method was determined to be the most reproducible and accurate preparation method as can be seen in Figure 3.

#### Linearity of Assay

With optimized assay parameters and calibration standards, we then evaluated the linear range of the assay. To do so a high concentration sample of 1000mg/dL cr was prepared using creatine monohydrate. This sample was then diluted to 1, 2.5, 5, 10 and 15mg/dL cr. All samples were tested in triplicate on the Roche Cobas C311 using the Cr assay. The data in Figure 3 shows an  $R^2 > 0.999$ ; therefore our assay shows linearity within the range of 0-15mg/dL.

#### Accuracy and Precision

To determine precision and accuracy of the cr assay, three samples from four individual cr test solutions were run in triplicate. The test was repeated three times with three separate calibrations, giving a total of 27 measurements. Detailed data is presented in Figures 6a & 7a. These data were compared to a commercially available Roche crea assay to ensure comparability (Figures 6b & 6b). As can be seen in Figure 6a the accuracy of the cr assay is comparable to that of the crea assay shown in Figure 6b. This experiment summarized replicate to replicate variability, sample to sample variability and run to run variability. Also shown in Figures 7a and 7b, no outlier was observed.

Statistical analysis is shown in Table 2, which indicates that the CV is consistently better

for the cr assay as opposed to the crea assay with CVs of 0.28-0.49% and 0.74-1.26% respectively. In addition the analysis indicated better accuracy with the cr assay ranging from 0.4-1.2% as compared to the crea assay ranging from 0.8-1.7%. The data also show that there is no interference from crea as all solutions recover very close to their gravimetric values with and without the presence of crea. Samples S1, S3 and S4 are mixtures of both crea and cr and the presence of these chemicals together does not impact the recovery of the cr assay, as seen in Table 2. Furthermore, the data shown in table 2 indicated that the cr assay gives both better precision and accuracy than the commercially available crea assay. This could be explained by the volume ratio of sample to assay reagent as the commercially available crea assay has a ratio of 1:32 in comparison to that of the cr assay of 1:5. The higher sample volume that the cr assay has could explain the improvement in the accuracy and precision over the commercially available crea assay.

#### Recovery in Clinical Samples

Performance of the cr assay on the Cobas C311 in plasma samples was evaluated by testing plasma samples with cr values determined by IDMS, as seen in Figure 8. Three levels of plasma samples were tested at 0.5mg/dl, 1.8mg/dL and 4.5mg/dL. All samples were tested in duplicates over four separate days under four separate calibrations. The data was correlated against the IDMS measured values as can be seen in Figures 8a and b, showed good linearity  $R^2 > 0.99$  for samples between 0.5-4.5mg/dL cr. These data show that regardless of the sample matrix the cr assay developed on the Cobas C311 has



good linearity ( $R^2 > 0.99$ ). The bias at these three levels and the CV from run to run are well within our original goals, as can be seen in Table 3 and Table 4.

There was a bias of +0.2479 as can be seen in Figure 8b against the IDMS which is likely due to plasma sample matrix effect versus aqueous calibration. The effect can be corrected if sample measurements are normalized against IDMS serum samples. As can be seen in Figure 9 clinical samples that were tested on the Cobas C311 using the cr assay and then normalized versus IDMS fall within the described clinical ranges (0.2-1.7 mg/dL +/- 0.3) with some high samples that were spiked to increase the cr concentration. Clinical samples tested using the enzymatic Cr show recovery of 0.26 -1.26 mg/dL +/- 0.24 (excluding the spiked samples), showing that the cr assay developed is capable of measuring cr in clinical samples (Straseski 2011).

Based on the data presented, a enzymatic cr assay that can be used to measure clinical samples on a bench top chemistry analyzer was developed and successfully met all of the requirements that we set out in Table 4. The assay is a single part reagent that uses enzymatic conversion of cr with colorimetric end point detection of 4-aminoantipyrine at 547nm. The assay uses a small sample size of 30uL, and takes 10 minutes (Table 2 and 4). Furthermore the enzymatic cr assay has proven linearity from 0-15mg/dL, accuracy within 2%, run to run CV from 1.6-5.3% for clinical samples and finally sample to sample CV of less than 0.5%. This assay can easily be adapted for any bench top chemistry analyzer that has the capability of a programmable channel, and can analyze clinical samples with accuracy, precision and reproducibility. In comparison to other methods of cr detection such as HPLC and GS/MS the enzymatic cr assay has less

interference from sample matrix and the accuracy and precision is comparable to both methods, with the enzymatic cr assay having precision and accuracy within 2%, recovery of 0.5% as compared to HPLC which has better precision and accuracy which is as low as 0.5% but has many interference issues from samples matrix, GS/MS has similar issues and recovery. This enzymatic cr assay could have future application for the potential beneficial implication of cr, as well as investigation on the long term impact of cr supplementation of healthy individuals.

## Appendix 1. Tables

Table 1. Cr assay parameters programmed on the open channel option on the Roche Cobas C311.

Creatine Assay on Roche C311	
Parameter	
Sample Volume (uL)	30
Reagent Volume (uL)	150
Assay type	1-point
Time (minutes)	10
Wavelength (nm)	546
Absorbance Limit	Increase
Measurement taken (cycle)	57
Reagent Used	IL-Cr_Sek

Table 2: Results of Precision study of new cr assay vs commercial Roche crea assay on

Roche Cobas C311

Sample ID	S1		S2		S3		S4	
Analyte	Cr	Crea	Cr	Crea	Cr	Crea	Cr	Crea
Gravimetric Value (mg/dL)	5.2	3.0	3.8	0.0	5.0	2.9	3.6	1.0
Average Recovery (mg/dL)(	5.18	2.97	3.77	0.14	5.06	2.85	3.63	1.08
SD	0.01	0.02	0.02	0.01	0.02	0.04	0.01	0.01
Precision (%CV)	0.28	0.74	0.44	n/a	0.49	1.26	0.41	1.00
Inaccuracy (%)	0.4	1.0	0.8	n/a	1.2	1.7	0.8	8.0

Table 3: Summary data of Cr recovery from plasma samples

Plasma Samples	Level1	Level2	Level3
IDMS Value (mg/dL)	0.5	1.8	4.5
Average Cr assay recovery (mg/dL)	0.7	1.7	3.9
SD	0.035	0.029	0.062
CV run to run	5.3	1.7	1.6
CV sample to sample	0.5	0.4	0.2
Inaccuracy (%)	+30	-8	-13.0

Table 4: Summary of Goals and performance for Cr assay

Goal	Achieved Performance	Reference	Pass/Fail
Sample and reagent volume < 300uL	180uL	Table 1	Pass
Incubation time < 20 minutes	10 minutes	Table 1	Pass
Linarity 0-15mg/dL	0-15mg/dL	Figure 4	Pass
Accuracy within 2%	1.20%	Table 2	Pass
Run to run CV < 2%	5.3%, 1.6% and 1.7%	Table 3	Pass
Sample to sample CV < 0.5%	0.5%, 0.4% and 0.2%	Table 3	Pass

Appendix 2. Figures

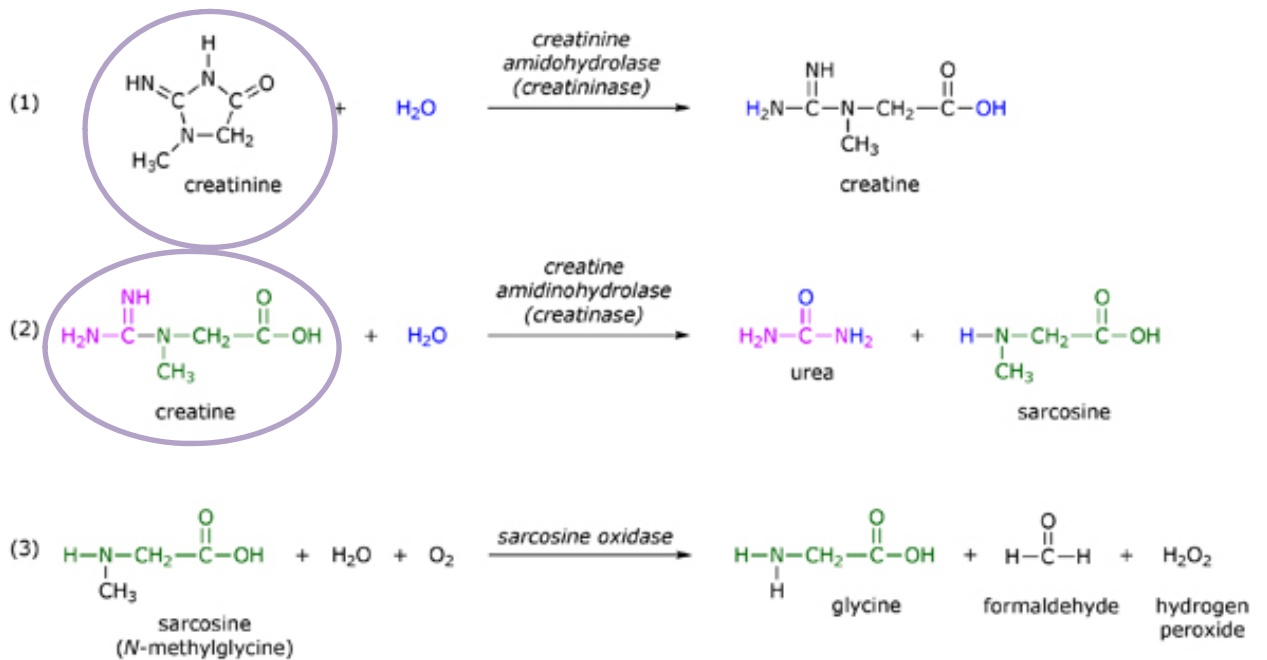


Figure 1. Enzymatic Cascade from creatinine to hydrogen peroxide.

Pictorial description of the change that occurs for the Crea enzymatic reaction for the conversion of creatinine to hydrogen peroxide. Which allows for the interaction of hydrogen peroxide with 4-aminoantipyrine to give a colorimetric change allowing for the detection of Crea concentration.

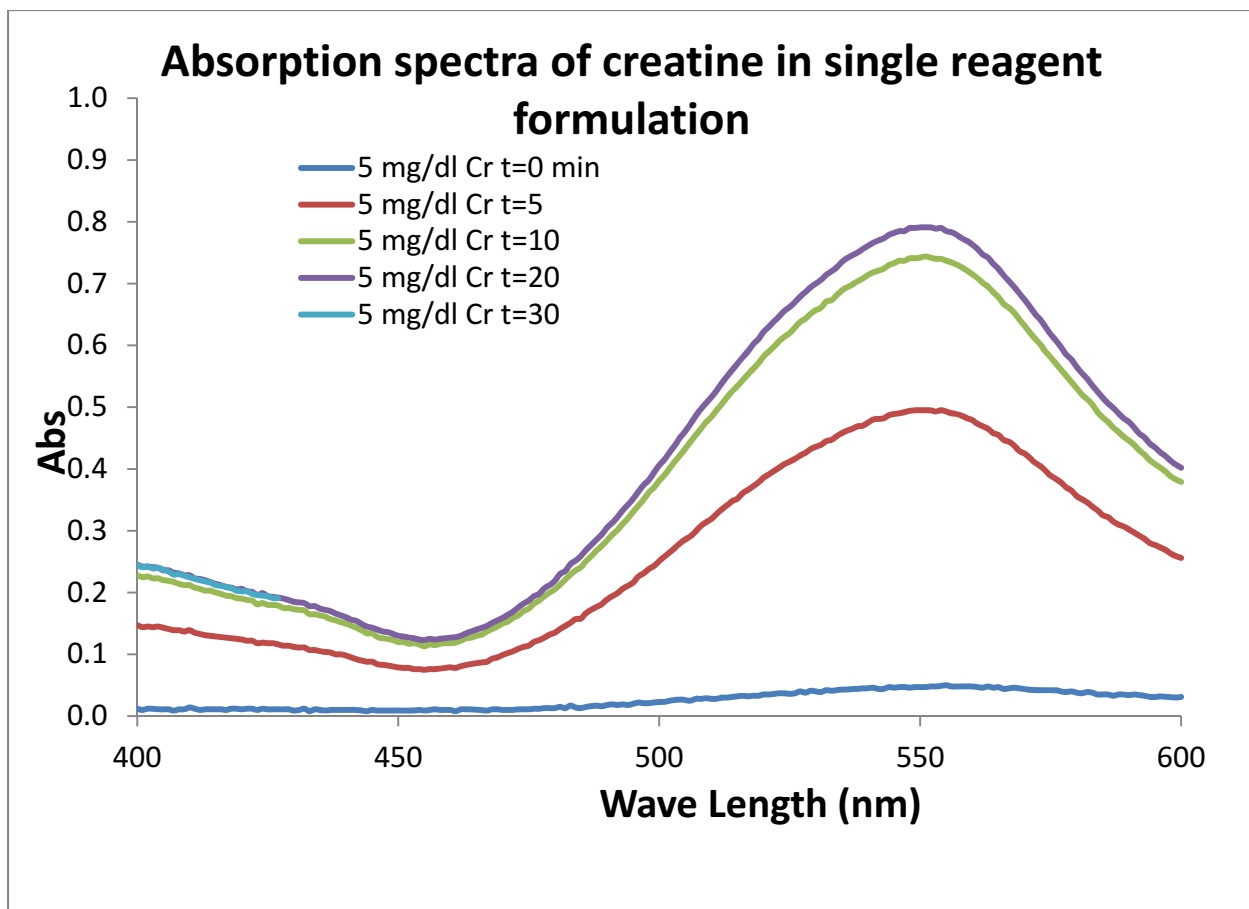


Figure 2a. UV Absorption spectra of enzymatic cr reagent.

Uv-vis spectrometer: Evolution 6000 with quartz cells. Sekisui Reagent RI modified with 2U/mL HRP and 16 mg/dL 4-AMP, 2.5 mL. Sample: 5 mg/dL Cr solution, 0.5 mL. Blank: DI water. Absorbance collected at 0, 5, 10, 20, and 30 minutes of incubation time.



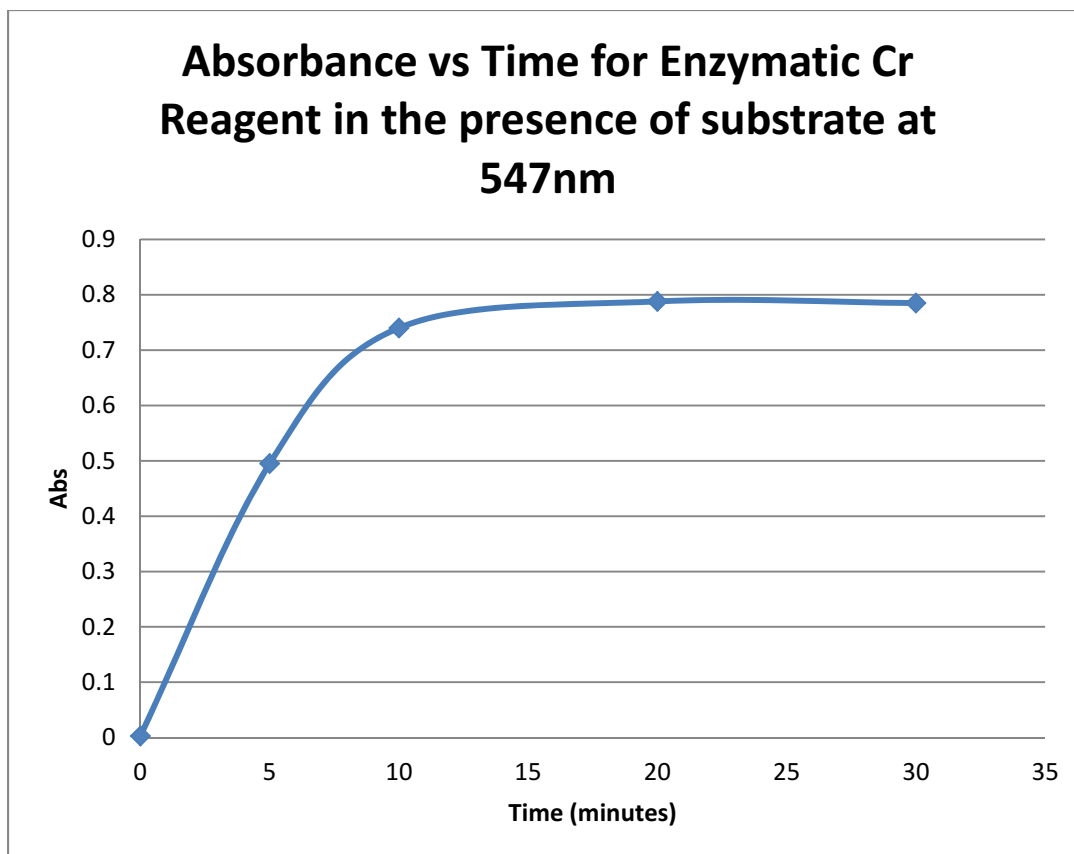


Figure 2b. Absorbance versus time profile for enzymatic single cr assay reagent.

Uv-vis spectrometer: Evolution 6000 with quartz cells. Sekisui Reagent RI modified with 2U/mL HRP and 16 mg/dL 4-AMP, 2.5 mL. Sample: 5 mg/dL Cr solution, 0.5 mL. Blank: DI water. Absorbance collected at 0, 5, 10, 20, and 30 minutes of incubation time at 547nm.

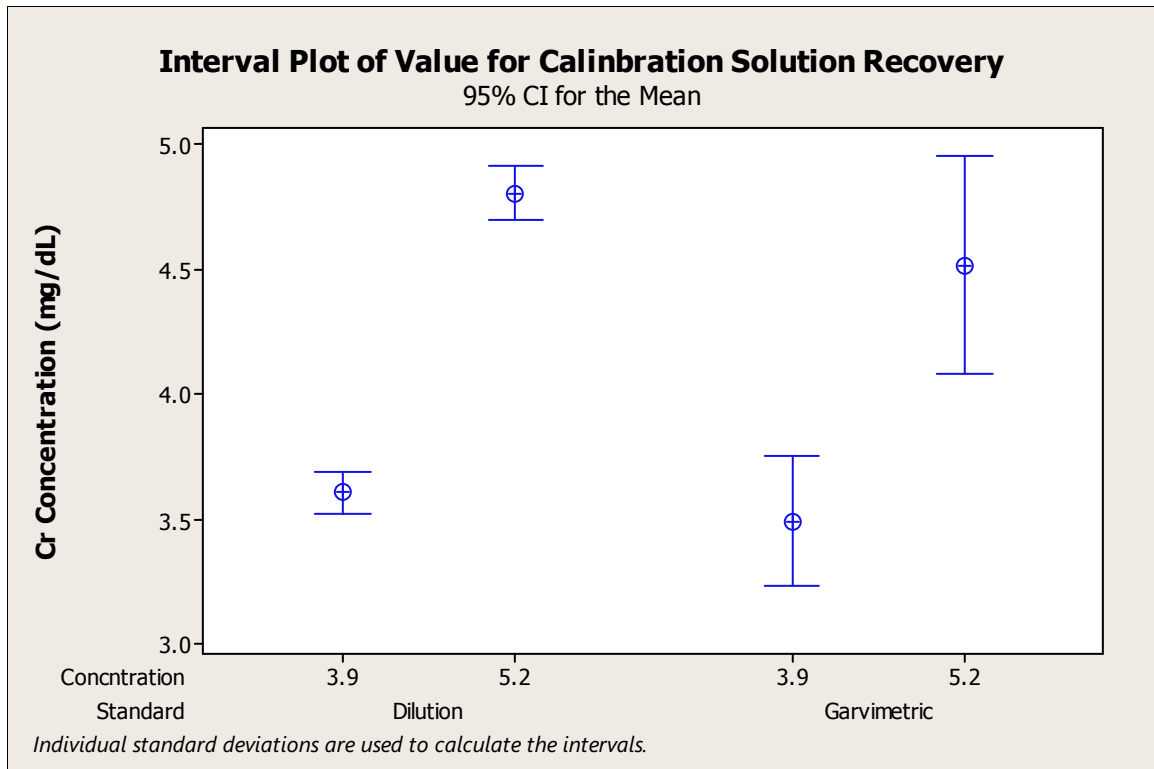


Figure 3. Comparison of two calibration standard prep methods.

Two different concentrations, 3.9mg/dL and 5.2mg/dL were tested by both methods.

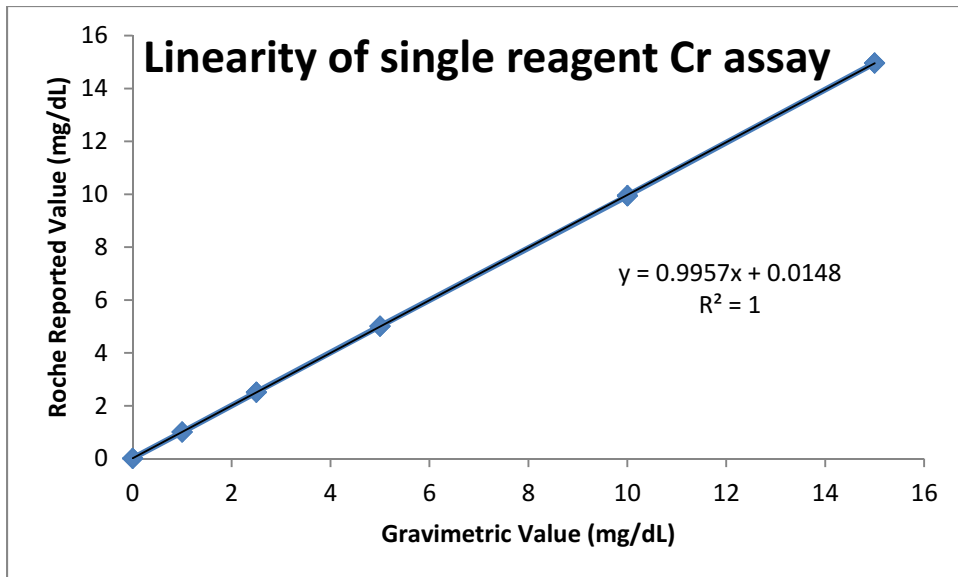


Figure 4. Linearity of Cr assay.

Total of 6 samples at gravimetric values of 0, 1.5, 2.5, 5, 10 and 15mg/dL cr were run in replicated of five on the Roche Cobas C311 using the single reagent cr assay. Gravimetric Values of Cr solution against the reported cr values by the cr assay adapted to the Cobas C311.

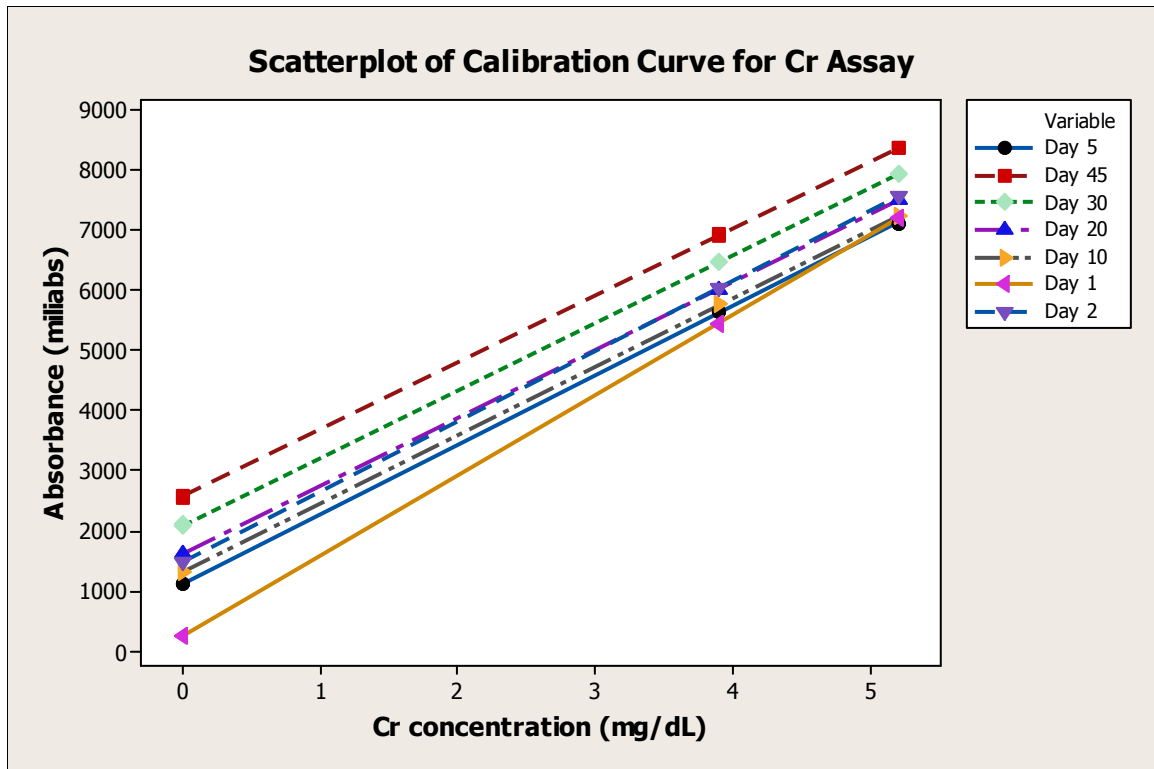


Figure 5. Study of single Reagent shelf life stability.

Scatterplot for cr assay linearity of fresh standards in miliabsorbance over a period of 45 days of reagent shelf life. Each data point represent duplicated samples of each concentration point of each calibration.

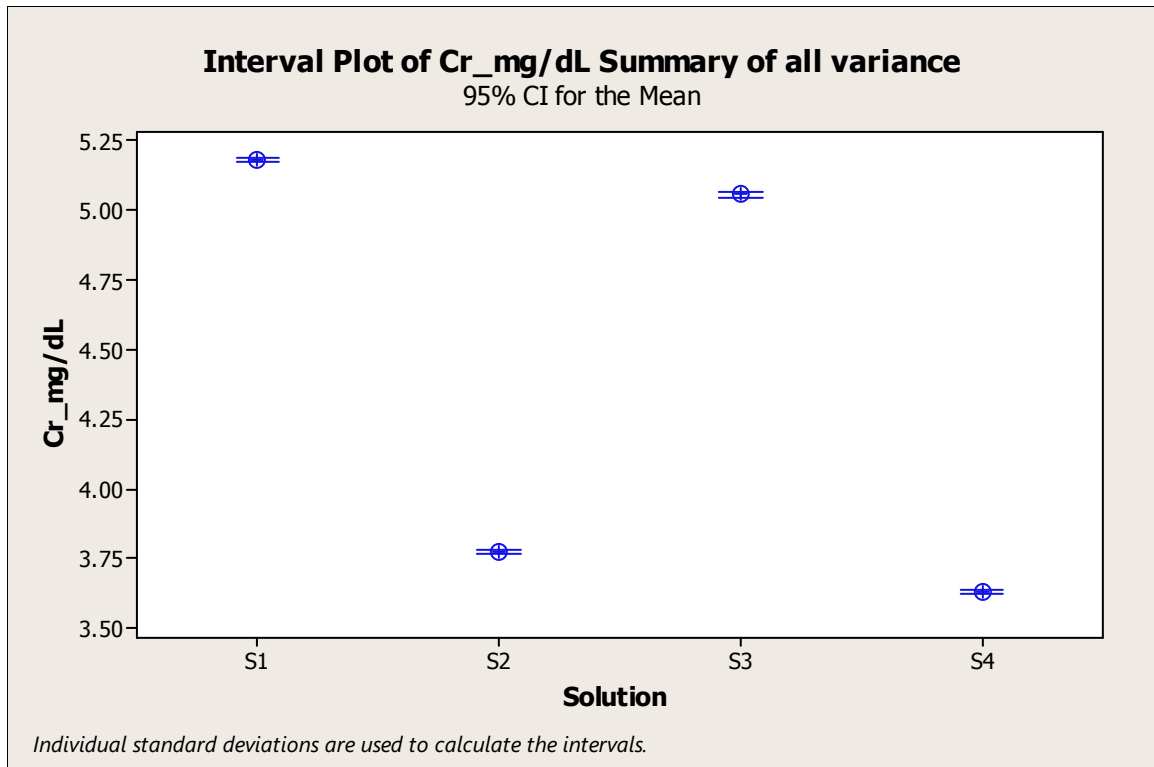


Figure 6a. Summary of all variability for Cr assay.

Summary of all variability on four different test solutions for Cr results by cr assay tested on the Cobas C311. The average along with the 95% confidence interval showed the overall variability of the assay. Source of variability includes calibration to calibration, and sample imprecision. Data point representing each sample includes the average and 95% confidence interval of a total of 27 measurements. Test were completed on three different days, on each day a fresh calibration using fresh calibration standards was conducted, three different preparations of Samples 1- 4 were made and tested in triplicated using the cr assay on the Roche Cobas C311.

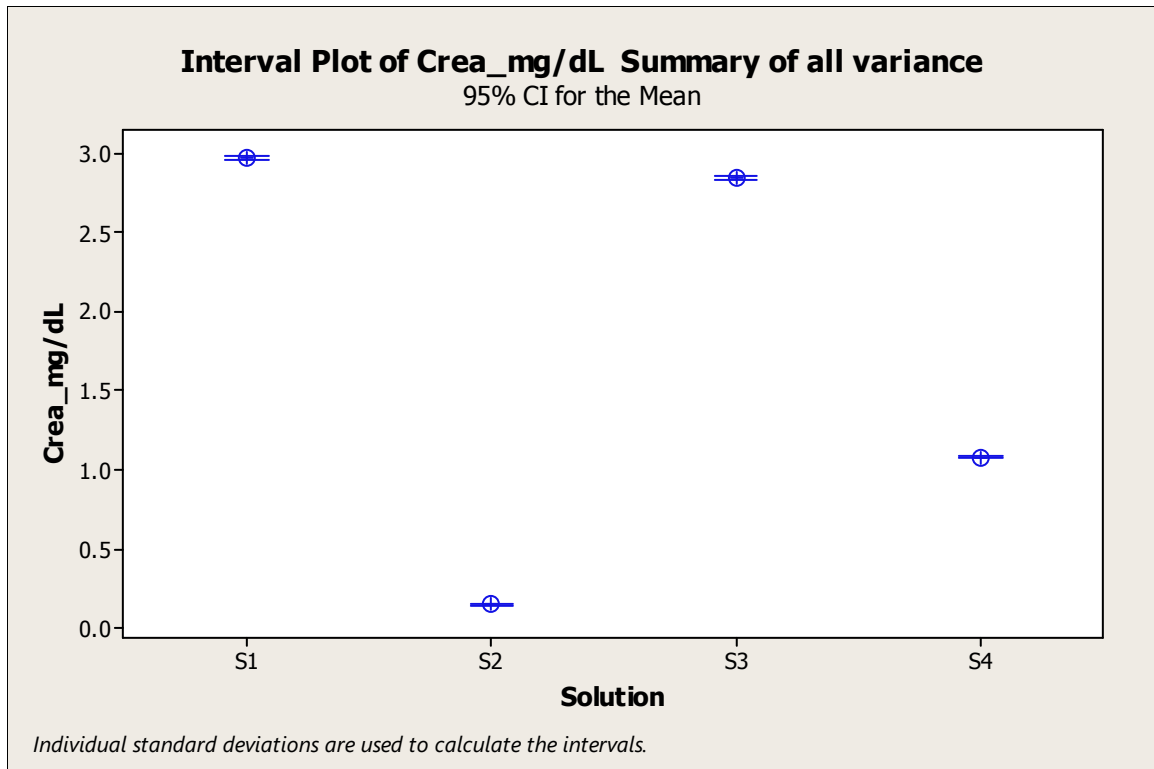


Figure 6b. Summary of all variability for Crea precision study

Summary of all variability on four different test solutions for Crea results by Roche crea assay tested on the Cobas C311. The average, shown with the 95% confidence interval showed the overall variability of the assay. Source of variability includes calibration to calibration, and sample imprecision. Data point representing each sample includes the average and 95% confidence interval of a total of 27 measurements. Tests were completed on three different days. On each test day a fresh calibration solution was prepared and used to calibrate the instrument, this was repeated twice. Samples 1- 4 were made and tested in triplicated using the crea assay on the Roche Cobas C311.

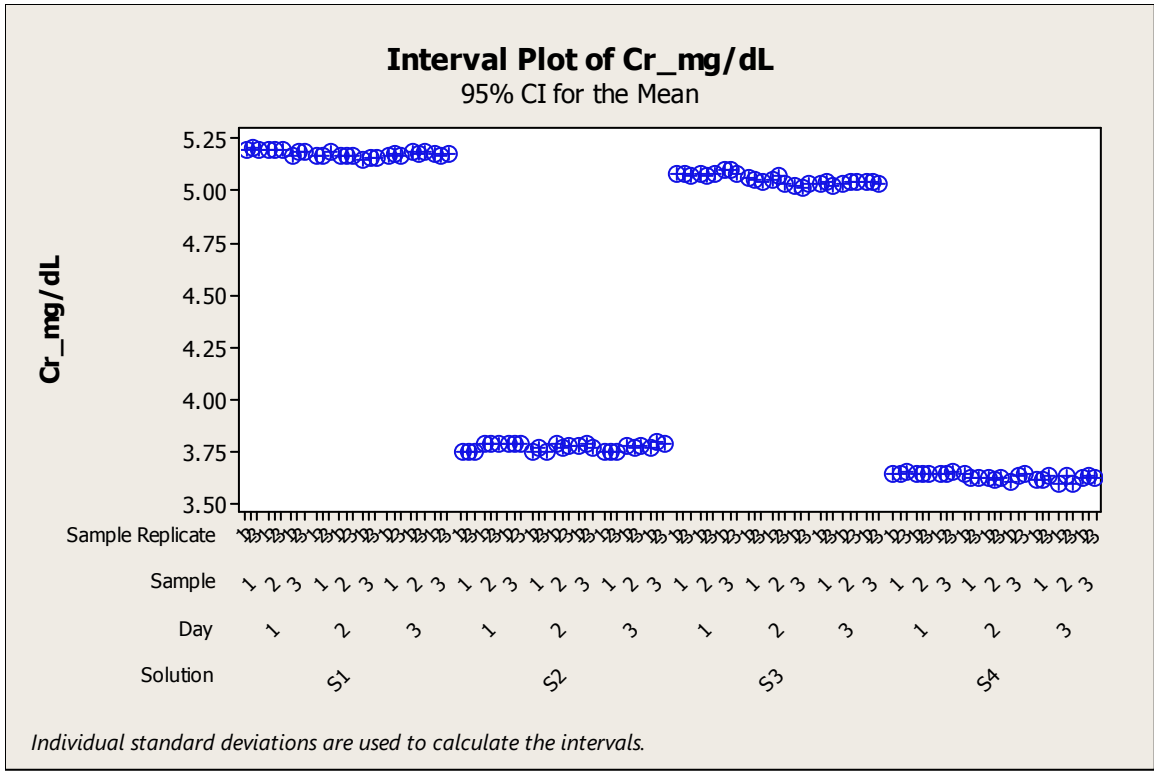


Figure 7a. Day to Day, Run to run and sample to sample variability of Cr assay precision study.

All individual data points are presented to show the overall change when presented with different variables. Variability of run to run, sample to sample and replicate to replicate for Cr recovery on Roche C311 Cr assay. These are all individual measurements shown summarized in Fig 6a.

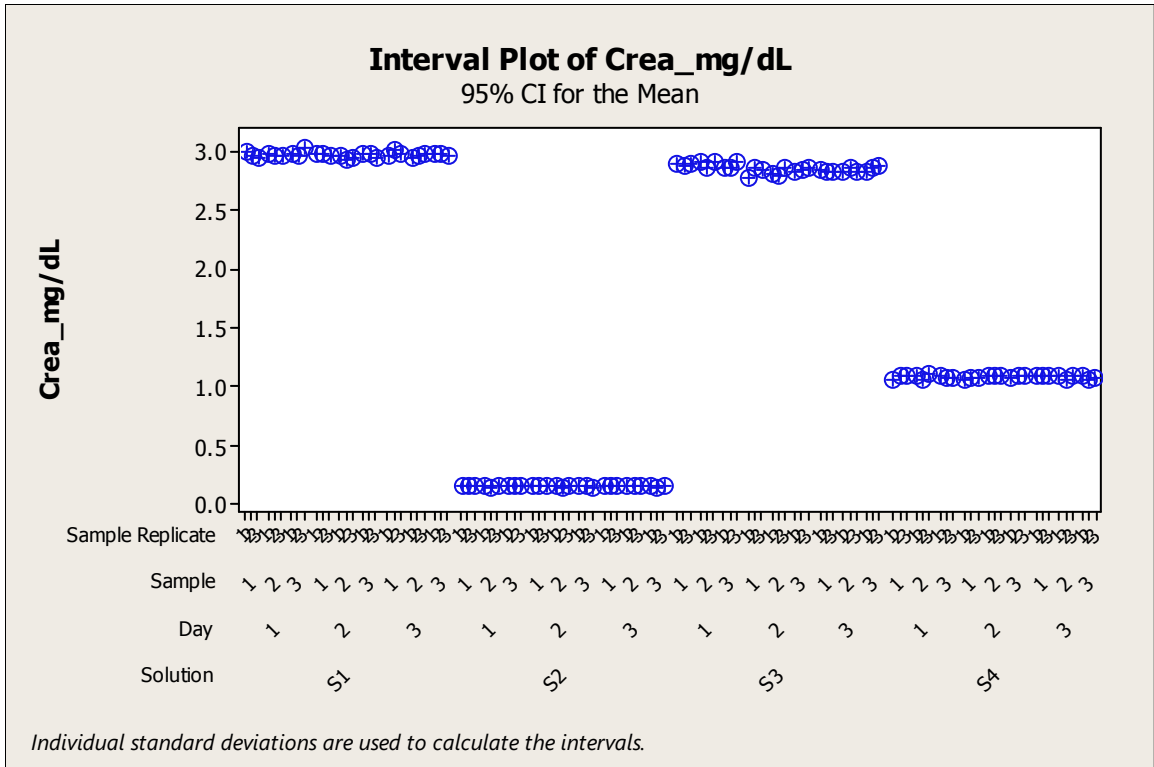


Figure 7b. Day to Day, Run to run and sample to sample variability of Crea assay.

All individual data points are presented to show the overall change in value when presented with different variables. Variability of run to run, sample to sample and replicate to replicate for Crea on Roche C311crea assay. These are all individual data points shown summarized in Fig 6b.



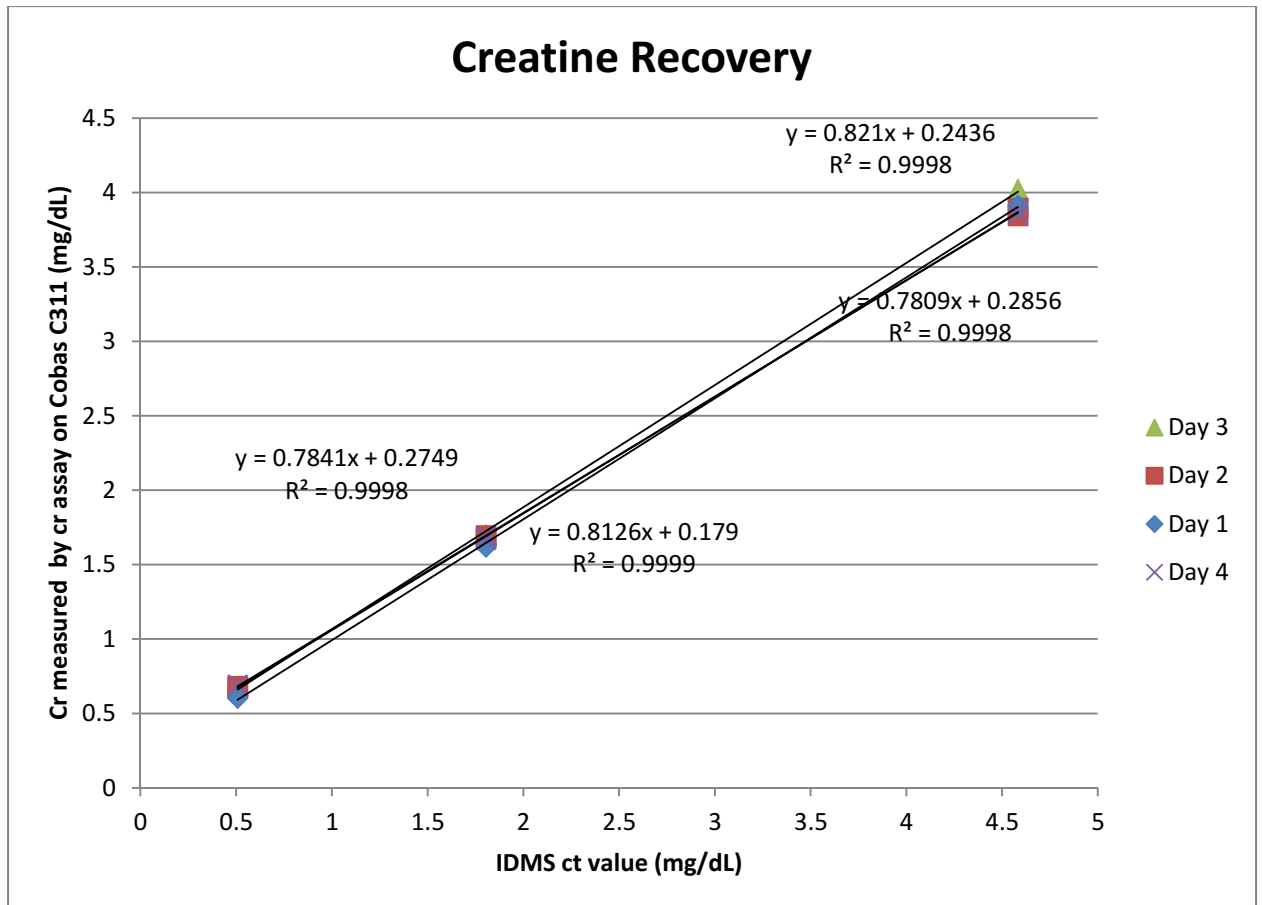


Figure 8a. Cr recovery from plasma samples (enzymatic assay on Cobas C311 vs IDMS)

Plasma samples were tested on the Cobas C311 to determine cr recovery. Data was correlated to the Isotope Dilution Mass Spectrometry (IDMS) measured values to determine the correlation. Data were collected in duplicate over four different days to account for run to run variations.

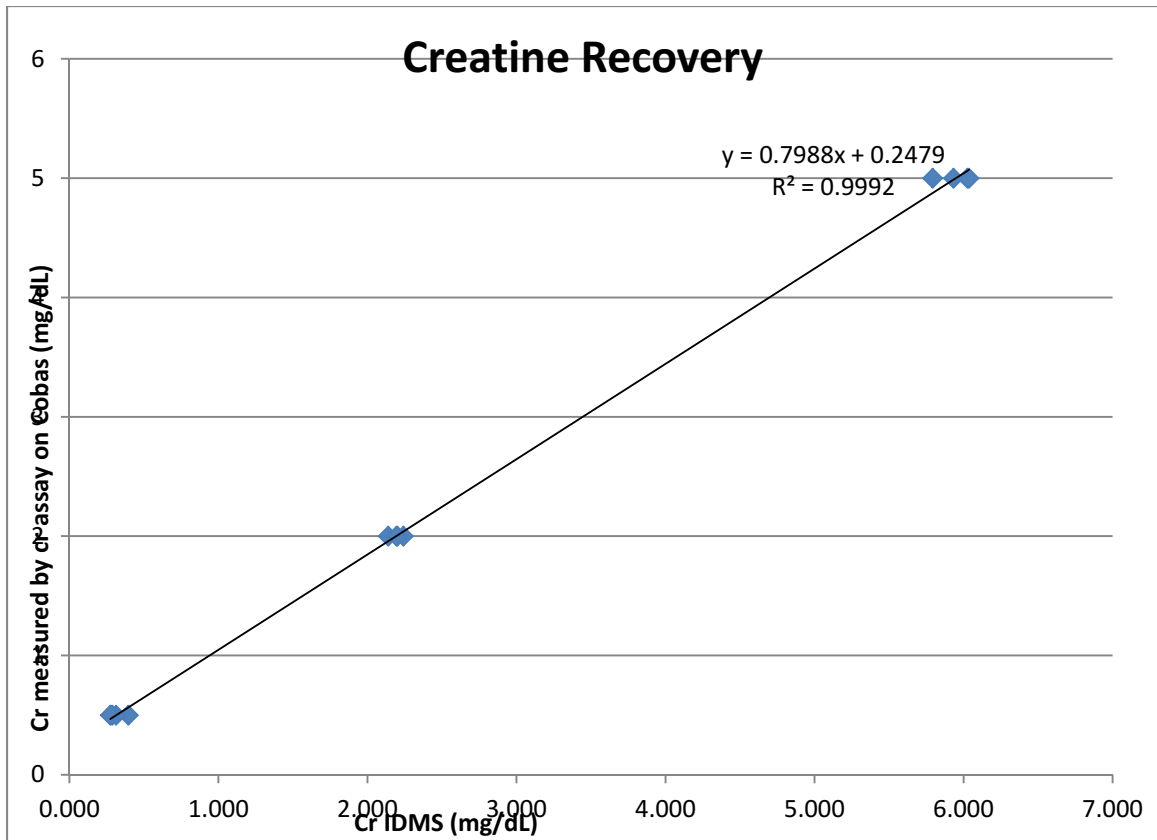


Figure 8b. Average Cr recovery from plasma samples (enzymatic assay on Cobas C311 vs IDMS)

Using all data collected in duplicate over a period of four days a single formula was calculated to determine the bias of the two methods - the cr assay on the Cobas C311 vs IDMS.

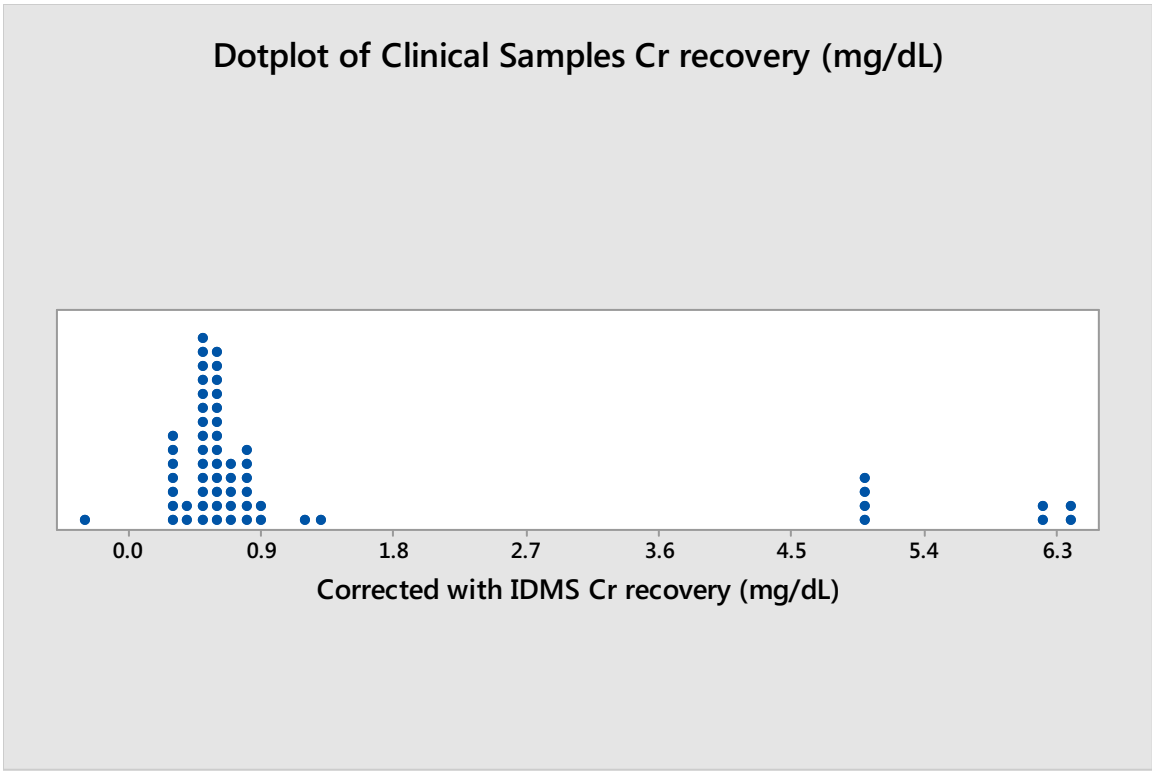


Figure 9. Distribution of cr values from 60 clinical patient samples run on the Cobas C311 with normalization to IDMS samples.

Dot plot of Clinical sample recovery using the cr assay after correction of bias the calculated formula from IDMS samples.

### Appendix 3. Raw Data

#### Absorbance Data

nm	A	nm	A	nm	A	nm	A
400	0.088	400	0.1	400	0.235	400	0.316
401	0.086	401	0.096	401	0.23	401	0.311
402	0.081	402	0.093	402	0.226	402	0.307
403	0.081	403	0.093	403	0.225	403	0.304
404	0.078	404	0.089	404	0.223	404	0.301
405	0.076	405	0.087	405	0.219	405	0.296
406	0.073	406	0.084	406	0.214	406	0.292
407	0.075	407	0.084	407	0.214	407	0.292
408	0.069	408	0.08	408	0.208	408	0.283
409	0.069	409	0.08	409	0.206	409	0.281
410	0.066	410	0.08	410	0.205	410	0.278
411	0.066	411	0.078	411	0.201	411	0.274
412	0.063	412	0.073	412	0.196	412	0.269
413	0.064	413	0.075	413	0.195	413	0.267
414	0.062	414	0.073	414	0.192	414	0.265
415	0.061	415	0.072	415	0.19	415	0.261
416	0.06	416	0.071	416	0.188	416	0.258
417	0.058	417	0.068	417	0.185	417	0.253
418	0.058	418	0.07	418	0.184	418	0.252
419	0.058	419	0.07	419	0.183	419	0.249
420	0.056	420	0.067	420	0.18	420	0.246
421	0.056	421	0.068	421	0.178	421	0.244
422	0.056	422	0.068	422	0.178	422	0.243
423	0.057	423	0.067	423	0.175	423	0.238
424	0.054	424	0.066	424	0.173	424	0.237
425	0.056	425	0.067	425	0.174	425	0.236
426	0.054	426	0.065	426	0.172	426	0.234
427	0.052	427	0.063	427	0.168	427	0.231
428	0.055	428	0.066	428	0.169	428	0.23
429	0.052	429	0.063	429	0.166	429	0.227
430	0.055	430	0.064	430	0.167	430	0.228
431	0.053	431	0.063	431	0.164	431	0.225
432	0.051	432	0.063	432	0.162	432	0.222
433	0.054	433	0.062	433	0.161	433	0.219

434	0.055	434	0.066	434	0.162	434	0.22
435	0.052	435	0.062	435	0.157	435	0.215
436	0.054	436	0.063	436	0.158	436	0.215
437	0.053	437	0.063	437	0.156	437	0.21
438	0.054	438	0.064	438	0.154	438	0.209
439	0.054	439	0.064	439	0.154	439	0.206
440	0.054	440	0.064	440	0.152	440	0.204
441	0.055	441	0.065	441	0.15	441	0.201
442	0.054	442	0.064	442	0.146	442	0.197
443	0.057	443	0.066	443	0.147	443	0.196
444	0.057	444	0.065	444	0.145	444	0.191
445	0.057	445	0.067	445	0.145	445	0.191
446	0.058	446	0.067	446	0.142	446	0.186
447	0.058	447	0.067	447	0.141	447	0.184
448	0.059	448	0.068	448	0.141	448	0.184
449	0.059	449	0.068	449	0.139	449	0.182
450	0.059	450	0.068	450	0.138	450	0.179
451	0.059	451	0.068	451	0.137	451	0.179
452	0.059	452	0.068	452	0.137	452	0.176
453	0.06	453	0.069	453	0.136	453	0.178
454	0.059	454	0.069	454	0.135	454	0.175
455	0.06	455	0.069	455	0.135	455	0.173
456	0.059	456	0.069	456	0.135	456	0.175
457	0.058	457	0.069	457	0.134	457	0.173
458	0.058	458	0.068	458	0.135	458	0.175
459	0.056	459	0.066	459	0.134	459	0.174
460	0.057	460	0.067	460	0.136	460	0.175
461	0.058	461	0.066	461	0.136	461	0.177
462	0.056	462	0.067	462	0.137	462	0.178
463	0.056	463	0.067	463	0.139	463	0.182
464	0.055	464	0.066	464	0.139	464	0.182
465	0.054	465	0.064	465	0.14	465	0.184
466	0.054	466	0.064	466	0.141	466	0.186
467	0.055	467	0.064	467	0.143	467	0.192
468	0.054	468	0.065	468	0.147	468	0.195
469	0.054	469	0.065	469	0.149	469	0.199
470	0.053	470	0.063	470	0.152	470	0.203
471	0.054	471	0.064	471	0.156	471	0.207
472	0.054	472	0.064	472	0.159	472	0.213
473	0.053	473	0.064	473	0.162	473	0.216

474	0.051	474	0.062	474	0.163	474	0.221
475	0.052	475	0.063	475	0.166	475	0.226
476	0.05	476	0.062	476	0.17	476	0.232
477	0.052	477	0.064	477	0.175	477	0.239
478	0.05	478	0.063	478	0.177	478	0.244
479	0.05	479	0.063	479	0.182	479	0.25
480	0.05	480	0.063	480	0.185	480	0.255
481	0.048	481	0.063	481	0.189	481	0.261
482	0.047	482	0.06	482	0.193	482	0.268
483	0.047	483	0.064	483	0.198	483	0.276
484	0.047	484	0.061	484	0.204	484	0.283
485	0.047	485	0.06	485	0.205	485	0.288
486	0.044	486	0.059	486	0.211	486	0.295
487	0.042	487	0.058	487	0.214	487	0.301
488	0.042	488	0.059	488	0.219	488	0.31
489	0.042	489	0.058	489	0.223	489	0.317
490	0.038	490	0.056	490	0.227	490	0.323
491	0.037	491	0.056	491	0.231	491	0.33
492	0.036	492	0.054	492	0.235	492	0.337
493	0.033	493	0.052	493	0.239	493	0.344
494	0.032	494	0.05	494	0.243	494	0.352
495	0.031	495	0.051	495	0.247	495	0.361
496	0.027	496	0.049	496	0.253	496	0.368
497	0.025	497	0.046	497	0.256	497	0.375
498	0.025	498	0.046	498	0.263	498	0.387
499	0.023	499	0.045	499	0.266	499	0.393
500	0.021	500	0.044	500	0.272	500	0.402
501	0.02	501	0.043	501	0.278	501	0.41
502	0.018	502	0.043	502	0.283	502	0.419
503	0.017	503	0.043	503	0.289	503	0.429
504	0.016	504	0.043	504	0.296	504	0.438
505	0.015	505	0.041	505	0.301	505	0.448
506	0.014	506	0.039	506	0.305	506	0.458
507	0.012	507	0.04	507	0.311	507	0.467
508	0.011	508	0.039	508	0.319	508	0.476
509	0.01	509	0.039	509	0.324	509	0.486
510	0.011	510	0.039	510	0.33	510	0.495
511	0.01	511	0.038	511	0.337	511	0.505
512	0.009	512	0.039	512	0.344	512	0.514
513	0.009	513	0.039	513	0.35	513	0.525

514	0.007	514	0.038	514	0.355	514	0.533
515	0.009	515	0.041	515	0.361	515	0.543
516	0.007	516	0.04	516	0.368	516	0.551
517	0.007	517	0.039	517	0.373	517	0.56
518	0.007	518	0.04	518	0.379	518	0.569
519	0.007	519	0.04	519	0.385	519	0.578
520	0.005	520	0.04	520	0.391	520	0.587
521	0.005	521	0.04	521	0.396	521	0.595
522	0.004	522	0.04	522	0.401	522	0.602
523	0.004	523	0.041	523	0.406	523	0.611
524	0.005	524	0.042	524	0.413	524	0.619
525	0.006	525	0.042	525	0.418	525	0.626
526	0.004	526	0.041	526	0.42	526	0.633
527	0.004	527	0.044	527	0.426	527	0.642
528	0.005	528	0.043	528	0.431	528	0.648
529	0.003	529	0.044	529	0.435	529	0.654
530	0.002	530	0.042	530	0.438	530	0.659
531	0.005	531	0.044	531	0.444	531	0.666
532	0.003	532	0.045	532	0.448	532	0.674
533	0.006	533	0.047	533	0.453	533	0.679
534	0.005	534	0.047	534	0.458	534	0.686
535	0.002	535	0.045	535	0.46	535	0.691
536	0.003	536	0.046	536	0.465	536	0.697
537	0.003	537	0.047	537	0.468	537	0.701
538	0.002	538	0.046	538	0.471	538	0.706
539	0.003	539	0.048	539	0.473	539	0.711
540	0.003	540	0.048	540	0.478	540	0.717
541	0.002	541	0.048	541	0.482	541	0.72
542	0.003	542	0.047	542	0.484	542	0.724
543	0.004	543	0.048	543	0.486	543	0.728
544	0.002	544	0.049	544	0.489	544	0.732
545	0.003	545	0.049	545	0.492	545	0.736
546	0.002	546	0.049	546	0.492	546	0.739
547	0.003	547	0.05	547	0.495	547	0.74
548	0.003	548	0.049	548	0.497	548	0.744
549	0.002	549	0.049	549	0.497	549	0.743
550	0.004	550	0.051	550	0.499	550	0.746
551	0.003	551	0.05	551	0.498	551	0.747
552	0.002	552	0.05	552	0.497	552	0.744
553	0.003	553	0.051	553	0.496	553	0.743

554	0.001	554	0.05	554	0.496	554	0.741
555	0.001	555	0.051	555	0.494	555	0.738
556	0.003	556	0.051	556	0.493	556	0.737
557	0.001	557	0.049	557	0.49	557	0.732
558	0.001	558	0.049	558	0.487	558	0.727
559	0.002	559	0.05	559	0.484	559	0.723
560	0.002	560	0.05	560	0.481	560	0.717
561	0.001	561	0.048	561	0.474	561	0.71
562	0.003	562	0.049	562	0.472	562	0.704
563	0.002	563	0.05	563	0.467	563	0.697
564	0.002	564	0.048	564	0.46	564	0.688
565	0	565	0.047	565	0.455	565	0.678
566	0.002	566	0.046	566	0.449	566	0.669
567	0.002	567	0.047	567	0.446	567	0.663
568	0	568	0.046	568	0.438	568	0.653
569	0.001	569	0.046	569	0.43	569	0.643
570	0.001	570	0.045	570	0.426	570	0.633
571	0.001	571	0.044	571	0.419	571	0.622
572	0.003	572	0.045	572	0.413	572	0.614
573	0	573	0.042	573	0.405	573	0.602
574	0.001	574	0.043	574	0.398	574	0.591
575	0.001	575	0.043	575	0.39	575	0.582
576	0.001	576	0.043	576	0.385	576	0.571
577	0.002	577	0.042	577	0.38	577	0.563
578	0.002	578	0.041	578	0.372	578	0.552
579	0	579	0.04	579	0.365	579	0.541
580	0.003	580	0.041	580	0.359	580	0.534
581	0.002	581	0.039	581	0.353	581	0.522
582	0.001	582	0.04	582	0.347	582	0.514
583	0.001	583	0.039	583	0.34	583	0.505
584	0.002	584	0.037	584	0.335	584	0.494
585	0.004	585	0.039	585	0.329	585	0.487
586	0.002	586	0.037	586	0.324	586	0.478
587	0.002	587	0.035	587	0.317	587	0.469
588	0.002	588	0.037	588	0.312	588	0.462
589	0.001	589	0.036	589	0.308	589	0.453
590	0.001	590	0.035	590	0.303	590	0.447
591	0	591	0.035	591	0.297	591	0.439
592	0.002	592	0.036	592	0.294	592	0.432
593	0.001	593	0.034	593	0.288	593	0.424



594	0.003	594	0.034	594	0.283	594	0.417
595	0.001	595	0.032	595	0.278	595	0.409
596	0.001	596	0.033	596	0.274	596	0.403

Calibration Standard Method Development

S_ID	Value	Run	SampleState
5_1	4.860	1	1 gravimetric
5_1	4.842	1	1 gravimetric
5_1	4.854	1	1 gravimetric
5_1	4.835	1	1 gravimetric
5_1	4.851	1	1 gravimetric
5_2	5.169	1	2 gravimetric
5_2	5.173	1	2 gravimetric
5_2	5.164	1	2 gravimetric
5_2	5.186	1	2 gravimetric
5_2	5.172	1	2 gravimetric
5_3	4.795	1	3 gravimetric
5_3	4.778	1	3 gravimetric
5_3	4.786	1	3 gravimetric

5_3	4.791	1	3	gravimetric
5_3	4.788	1	3	gravimetric
5_1	4.881	2	1	gravimetric
5_1	4.894	2	1	gravimetric
5_1	4.886	2	1	gravimetric
5_1	4.901	2	1	gravimetric
5_1	4.901	2	1	gravimetric
5_2	5.146	2	2	gravimetric
5_2	5.140	2	2	gravimetric
5_2	5.149	2	2	gravimetric
5_2	5.138	2	2	gravimetric
5_2	5.137	2	2	gravimetric
5_3	4.777	2	3	gravimetric
5_3	4.784	2	3	gravimetric
5_3	4.793	2	3	gravimetric
5_3	4.779	2	3	gravimetric

5_3	4.780	2	3	gravimetric
5_1	4.870	3	1	gravimetric
5_1	4.855	3	1	gravimetric
5_1	4.855	3	1	gravimetric
5_1	4.860	3	1	gravimetric
5_1	4.870	3	1	gravimetric
5_2	5.100	3	2	gravimetric
5_2	5.121	3	2	gravimetric
5_2	5.111	3	2	gravimetric
5_2	5.114	3	2	gravimetric
5_2	5.108	3	2	gravimetric
5_3	4.769	3	3	gravimetric
5_3	4.754	3	3	gravimetric
5_3	4.767	3	3	gravimetric
5_3	4.764	3	3	gravimetric
5_3	4.776	3	3	gravimetric

5	4.896	1	1	dilution
5	4.907	1	1	dilution
5	4.908	1	1	dilution
5	4.901	1	1	dilution
5	4.899	1	1	dilution
5	4.899	1	2	dilution
5	4.896	1	2	dilution
5	4.911	1	2	dilution
5	4.889	1	2	dilution
5	4.908	1	2	dilution
5	4.868	1	3	dilution
5	4.901	1	3	dilution
5	4.879	1	3	dilution
5	4.898	1	3	dilution
5	4.873	1	3	dilution
5	4.918	2	1	dilution

5	4.906	2	1	dilution
5	4.918	2	1	dilution
5	4.902	2	1	dilution
5	4.926	2	1	dilution
5	4.905	2	2	dilution
5	4.910	2	2	dilution
5	4.914	2	2	dilution
5	4.924	2	2	dilution
5	4.904	2	2	dilution
5	4.896	2	3	dilution
5	4.894	2	3	dilution
5	4.905	2	3	dilution
5	4.906	2	3	dilution
5	4.901	2	3	dilution
5	4.907	3	1	dilution
5	4.904	3	1	dilution

5	4.907	3	1	dilution
5	4.894	3	1	dilution
5	4.899	3	1	dilution
5	4.894	3	2	dilution
5	4.902	3	2	dilution
5	4.882	3	2	dilution
5	4.903	3	2	dilution
5	4.893	3	2	dilution
5	4.884	3	3	dilution
5	4.876	3	3	dilution
5	4.894	3	3	dilution
5	4.880	3	3	dilution
5	4.894	3	3	dilution

### Linearity Data

S_ID	Value	V_Unit
0	0.012	mg/dL
0	0.009	mg/dL
0	0.011	mg/dL
1	1.006	mg/dL
1	1.007	mg/dL
1	1.006	mg/dL
2.5	2.506	mg/dL
2.5	2.513	mg/dL
2.5	2.517	mg/dL
5	5.012	mg/dL
5	5.006	mg/dL
5	5.009	mg/dL
10	9.933	mg/dL
10	9.955	mg/dL
10	9.948	mg/dL
15	14.9615	mg/dL
15	14.964	mg/dL
15	14.9605	mg/dL

### Reagent Shelf Life Data

Cr_Cal	Cnc	Bad_Cal	Cal_1	Cal_2	Cal_3	Cal_4	Cal_New_reag
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Average for all Cals

0.0	1128	2571	2106	1617	1310	260	1498.67
0.0	1128	2564	2077	1617	1306	247	1489.83
3.9	5645	6921	6482	6022	5778	5451	6049.83
3.9	5634	6902	6461	6019	5771	5431	6036.33

5.2 7112 8363 7948 7497 7237 7214 7561.83

5.2 7113 8355 7921 7489 7232 7213 7553.83

Crea and Cr assay comparison

S_ID	Cr_mg/dL	Crea_mg/dL	Run	SampleReplicate
wca	5.202	3.00	1	1
wca	5.207	2.97	1	2
wca	5.195	2.95	1	3
wca	5.198	2.98	1	2
wca	5.202	2.96	1	2
wca	5.200	2.96	1	2
wca	5.168	2.98	1	3
wca	5.185	2.97	1	3
wca	5.193	3.03	1	3
wca	5.170	2.98	2	1
wca	5.168	2.98	2	1



wca	5.185	2.97	2	1	3
wca	5.170	2.97	2	2	1
wca	5.173	2.93	2	2	2
wca	5.166	2.94	2	2	3
wca	5.153	2.99	2	3	1
wca	5.161	2.99	2	3	2
wca	5.161	2.94	2	3	3
wca	5.168	2.97	3	1	1
wca	5.175	3.01	3	1	2
wca	5.171	2.99	3	1	3
wca	5.185	2.95	3	2	1
wca	5.183	2.97	3	2	2
wca	5.185	2.99	3	2	3
wca	5.175	2.99	3	3	1
wca	5.166	2.99	3	3	2
wca	5.178	2.97	3	3	3

Cal A	5.084	2.90	1	1	1
Cal A	5.084	2.88	1	1	2
Cal A	5.069	2.90	1	1	3
Cal A	5.079	2.91	1	2	1
Cal A	5.074	2.86	1	2	2
Cal A	5.084	2.91	1	2	3
Cal A	5.106	2.86	1	3	1
Cal A	5.104	2.86	1	3	2
Cal A	5.086	2.91	1	3	3
Cal A	5.060	2.78	2	1	1
Cal A	5.052	2.87	2	1	2
Cal A	5.040	2.85	2	1	3
Cal A	5.050	2.81	2	2	1
Cal A	5.072	2.80	2	2	2
Cal A	5.035	2.87	2	2	3
Cal A	5.023	2.82	2	3	1

Cal A	5.020	2.85	2	3	2
Cal A	5.037	2.87	2	3	3
Cal A	5.038	2.84	3	1	1
Cal A	5.040	2.82	3	1	2
Cal A	5.023	2.82	3	1	3
Cal A	5.038	2.82	3	2	1
Cal A	5.048	2.86	3	2	2
Cal A	5.045	2.82	3	2	3
Cal A	5.045	2.82	3	3	1
Cal A	5.040	2.86	3	3	2
Cal A	5.038	2.88	3	3	3

### IDMS Sample Data

IDMS Assignment	Cr Recovery
0.508	0.687803
0.508	0.689788
1.805	1.680303
1.805	1.650528
4.583	3.857848
4.583	3.824103
0.508	0.680543
0.508	0.685561
1.805	1.698345
1.805	1.707379
4.583	4.036079
4.583	4.025038
0.508	0.685758
0.508	0.67371
1.805	1.679756
1.805	1.702849
4.583	3.859524
4.583	3.911734
4.583	4.566254
4.583	4.566254
1.805	1.798405
1.805	1.798405
0.508	0.506144
0.508	0.506144

### Clinical Sample Data

Data	SampleCr(mg/L)	Calc_Cr(mg/dL)
12/9/2015 c1	0.600	0.44198
12/9/2015 c1	0.599	0.44073

12/9/2015	c2	0.637	0.48822
12/9/2015	c2	0.633	0.48322
12/9/2015	c3	0.460	0.26701
12/9/2015	c3	0.455	0.26076
12/9/2015	c4	0.740	0.61695
12/9/2015	c4	0.747	0.62570
12/9/2015	c5	0.656	0.51197
12/9/2015	c5	0.671	0.53072
12/9/2015	c6	0.746	0.62445
12/9/2015	c6	0.758	0.63945
12/9/2015	c7	0.636	0.48697
12/9/2015	c7	0.652	0.50697
12/14/2015	D1	0.494	0.30950
12/14/2015	D1	0.493	0.30825
12/14/2015	D2	0.672	0.53197
12/14/2015	D2	0.698	0.56446

12/14/2015	D3	0.985	0.92315
12/14/2015	D3	0.968	0.90191
12/14/2015	D4	1.255	1.26060
12/14/2015	D4	1.232	1.23185
12/14/2015	D5	0.777	0.66319
12/14/2015	D5	0.784	0.67194
12/14/2015	D6	0.917	0.83817
12/14/2015	D6	0.916	0.83692
12/14/2015	D7	0.906	0.82442
12/14/2015	D7	0.900	0.81692
12/14/2015	D8	0.637	0.48822
12/14/2015	D8	0.666	0.52447
12/14/2015	WB	0.607	0.45073
12/14/2015	WB	0.615	0.46073
12/10/2015	Creat Lvl 1	0.686	0.54946
12/10/2015	Creat Lvl 1	0.663	0.52072

12/10/2015	Creat Lvl 2	0.777	0.66319
12/10/2015	Creat Lvl 2	0.725	0.59820
12/10/2015	Creat Lvl 3	0.647	0.50072
12/10/2015	Creat Lvl 3	0.692	0.55696
12/10/2015	Creat Lvl 4	0.692	0.55696
12/10/2015	Creat Lvl 4	0.715	0.58571
12/10/2015	Creat Lvl 5	0.715	0.58571
12/10/2015	Creat Lvl 5	0.721	0.59321
12/10/2015	Creat Lvl 6	0.769	0.65320
12/10/2015	Creat Lvl 6	0.757	0.63820
12/16/2015	X1	0.856	0.76193
12/16/2015	X1	0.873	0.78318
12/16/2015	X2	0.468	0.27701
12/16/2015	X2	*	-0.30790
12/16/2015	X3	0.469	0.27826
12/16/2015	X3	0.474	0.28451

12/16/2015	X4	0.767	0.65070
12/16/2015	X4	0.765	0.64820
12/16/2015	X5	5.216	6.21106
12/16/2015	X5	5.189	6.17731
12/16/2015	X6	4.215	4.96001
12/16/2015	X6	4.212	4.95626
12/16/2015	X7	5.352	6.38103
12/16/2015	X7	5.358	6.38853
12/16/2015	X8	4.258	5.01375
12/16/2015	X8	4.240	4.99125



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