Jeffries, Andrea Hope. <u>Estimating Human DNA Concentration in the Presence of Metal Ion</u> <u>Inhibitors: A Comparison of the Quantifiler® Human DNA Quantification Kit and the</u> <u>Investigator® Quantiplex Kit.</u> Master of Science (Biomedical Sciences, Forensic Genetics).

May, 2014. 74pp., 7 tables, 29 figures, references

Real-time quantitative PCR is the standard method used to quantify Forensic DNA. This study was conducted to determine if the Investigator® Quantiplex Kit provides more accurate estimates of DNA concentration in the presence of metal inhibitors. Single source control DNA and inhibitor solutions of aluminum, calcium, copper, iron, nickel and lead samples were prepared at a concentration range of 0.0025-18.75mM. Metal containing samples were quantified using the Investigator® Quantiplex Kit on the 7500 Real-Time PCR System, amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit, separated and detected on the 3130*xl* Genetic Analyzer and analyzed with GeneMapper® *ID-X* version 1.4. More research needs to be conducted in order to determine if the stochastic results of metal containing DNA samples can produce definite trends.

ESTIMATING HUMAN DNA CONCENTRATION IN THE PRESENCE OF METAL ION INHIBITORS: A COMPARISON OF THE QUANTIFILER® HUMAN DNA QUANTIFICATION KIT AND THE INVESTIGATOR® QUANTIPLEX KIT

Andrea Hope Jeffries, B.S.

APPROVED:

Joseph Warren, PhD, Major Professor

Rhonda Roby, PhD, MPH, Committee Member

Abe Clark, PhD, Committee Member

Laszlo Prokai, PhD, University Member

Arthur Eisenberg, PhD, Chair, Department of Molecular and Medical Genetics

Meharvan Singh, PhD, Dean, Graduate School of Biomedical Sciences

ESTIMATING HUMAN DNA CONCENTRATION IN THE PRESENCE OF METAL ION INHIBITORS: A COMPARISON OF THE QUANTIFILER® HUMAN DNA QUANTIFICATION KIT AND THE INVESTIGATOR® QUANTIPLEX KIT

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Andrea Hope Jeffries, B.S.

Fort Worth, Texas

May 2014

ACKNOWLEDGEMENTS

I would first like to thank my family and friends for providing me with an endless amount of support, encouragement and love. The faith you have in me is my constant motivation to excel. I would also like to thank my committee members, Dr. Joseph Warren, Dr. Rhonda Roby, Dr. Abe Clark and Dr. Laszlo Prokai for their guidance during this project. To Laura Combs, I sincerely thank you for your support. Additionally, I would like to thank all the professors who have pushed me academically so that I could be where I am today. My deepest gratitude extends to you all. Lastly, I would like to thank Qiagen for their financial and technical support.

TABLE OF CONTENTS

| LIST OF TABLES | iv |
|---|----|
| LIST OF FIGURES | v |
| CHAPTER 1. Introduction | 1 |
| CHAPTER 2. Hypothesis and Specific Aims | |
| CHAPTER 3. Materials and Methods | 14 |
| CHAPTER 4. Results | 19 |
| CHAPTER 5. Discussion | 52 |
| CHAPTER 6. Conclusion | 66 |
| REFERENCES | 71 |

LIST OF TABLES

| Table 1. Comparisons of the Quantifiler® Human DNA Quantification Kit and the Investigator® Quantiplex Kit |
|---|
| Table 2. Adjusted pH of metal stock solutions |
| Table 3. Metal concentrations used to experimentally treat the control DNA and the subsequent total concentrations of metal in the Quantiplex and Identifiler® Plus reactions |
| Table 4. Estimated DNA concentration and percent relative standard deviation of positive control and metal containing DNA samples using the Quantiplex |
| Table 5. Delta and mean C _T values for Al, Ca, Cu, Fe, Ni, and Pb containing samples25 |
| Table 6. Estimated DNA concentration and percent relative standard deviation of positive control and metal containing DNA samples using the Quantifiler® kit |
| Table 7. Demonstrable C_T shifts observed with the Quantifiler® and Quantiplex kits |

LIST OF FIGURES

| Figure 1. Detection of fluorescence using the TaqMan® probe in the Quantifiler® Human DNA Quantification Kit |
|---|
| Figure 2. Detection of fluorescence using the Scorpions® primers in the Investigator® Quantiplex Kit |
| Figure 3. Mean C _T values obtained from the amplification of human DNA targets in samples containing Al, Ca, and Cu ions |
| Figure 4. Mean C _T values obtained from the amplification of human DNA targets in samples containing Fe, Ni, and Pb ions |
| Figure 5. Mean C _T values obtained from the amplification of IPC DNA targets in samples containing Al, Ca, and Cu ions |
| Figure 6. Mean C _T values obtained from the amplification of IPC DNA targets in samples containing Fe, Ni, and Pb ions |
| Figure 7. Mean C _T values obtained from the amplification of human and IPC DNA targets in samples containing Al ions |
| Figure 8. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Ca ions |
| Figure 9. Mean C _T values obtained from the amplification of human and IPC DNA targets in samples containing Cu ions |
| Figure 10. Mean C _T values obtained from the amplification of human and IPC DNA targets in samples containing Fe ions |
| Figure 11. Mean C _T values obtained from the amplification of human and IPC DNA targets in samples containing Ni ions |
| Figure 12. Mean C _T values obtained from the amplification of human and IPC DNA targets in samples containing Pb ions |

| Figure 13. DNA quantification estimates (ng/µL) and heat map indicating STR profiles success |
|--|
| Figure 14. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Al ions |
| Figure 15. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Ca ions |
| Figure 16. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Cu ions |
| Figure 17. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Fe ions |
| Figure 18. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Ni ions |
| Figure 19. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Pb ions |
| Figure 20. A scatter plot of allele peak heights versus fragment sizes for samples containing Al ions at each metal ion concentration |
| Figure 21. A scatter plot of allele peak heights versus fragment sizes for samples containing Ca ions at each metal ion concentration |
| Figure 22. A scatter plot of allele peak heights versus fragment sizes for samples containing Cu ions at each metal ion concentration |
| Figure 23. A scatter plot of allele peak heights versus fragment sizes for samples containing Fe ions at each metal ion concentration |
| Figure 24. A scatter plot of allele peak heights versus fragment sizes for samples containing Ni ions at each metal ion concentration |
| Figure 25. A scatter plot of allele peak heights versus fragment sizes for samples containing Pb ions at each metal ion concentration |
| Figure 26. Mean C _T values obtained from the amplification of IPC DNA targets in samples containing Al, Ca, and Cu ions |
| Figure 27. Mean C _T values obtained from the amplification of IPC DNA targets in samples containing Fe, Ni, and Pb ions60 |

CHAPTER 1

INTRODUCTION

DNA typing has an important role within the forensic and criminal justice communities since its first described forensic use by Alec Jefferys' in 1985. This method provided a powerful approach for associating biological evidence with known or unknown individuals (1). DNA typing has been used for many types of forensic investigations such as rape, homicide, and burglary cases (2). It has also been used to exonerate individuals that have been wrongly convicted of a crime (3). Investigations such as paternity testing, missing persons, and unidentified human remains cases also utilize this approach (1, 2). While DNA typing is a powerful tool that can contribute to many types of investigations, it is dependent upon successful DNA extraction and PCR amplification of DNA (4).

Forensic DNA analysis workflow is comprised of six steps, including: DNA extraction, DNA quantification, STR amplification, separation/detection, DNA analysis and statistical interpretation (2). During the extraction process, several compounds may co-purify with the DNA, which could impact downstream PCR testing (5, 6). Casework samples may contain PCR inhibitors such as heme (7), melanin (8), indigo dye (9), humic compounds (10) and heavy metals (11). Even reagents used in DNA extraction such as detergents (12) and phenol (13) can inhibit PCR if not removed. PCR inhibitors are important to identify and understand because they have been found to prevent successful DNA amplification by PCR. The mechanisms of PCR inhibitors are not well understood, and their effects on new DNA testing chemistries need to be documented (4).

Several methods have been used for determining the DNA concentration in forensic samples. An early method used was ultraviolet (UV) absorbance, which measures the absorbance of the DNA sample at a wavelength of 260nm (14). The UV absorbance method is not very sensitive and requires a large DNA sample (15). A major limitation of this assay is that the absorbance readings are not human specific (14). A contaminated sample with other organisms' DNA or residual contaminates from the DNA extraction process will also be included in the DNA concentration estimate. This would lead to an increase in the DNA quantification estimate, which would not accurately represent the DNA concentration in the sample (15). For these reasons mentioned above this approach is generally no longer used.

The PicoGreen® assay (Molecular Probes, Eugene, OR) was also used for DNA quantification during the mid-1990s (16). This assay relies upon PicoGreen®, which is a fluorescent interchelating dye (17). The fluorescence of PicoGreen® increases when bound to double-stranded DNA molecules (16). This assay provided a higher throughput method for DNA quantification. A major limitation of the PicoGreen assay is that it is not human specific because it quantifies the total DNA concentration in the sample (17).

The QuantiBlot® Human DNA Quantitation Kit (Applied Biosystems, Foster City, CA) was more commonly used than the UV absorbance and PicoGreen® assays because it was specific for human DNA (17, 18). The DNA sample was captured on a nylon membrane and a 40 base pair probe (that binds to a target sequence on chromosome 17) would be added (18). Visual or digital capture comparisons between the DNA samples and the standards would be conducted

(19). This assay took 1.5 hours to complete and was sensitive down to approximately 150pg (18).As of 2007, the QuantiBlot® Human DNA Quantitation Kit is no longer commercially sold (20).

The AluQuant[™] Human DNA Quantitation System (Promega Corporation, Madison, WI) was introduced in the early 2000s. This assay quantified DNA concentrations by targeting *Alu* repeats. The probe-target hybridization would result in the production of light due to the oxidation of luciferin. The measured light intensity would correspond proportionally to the amount of DNA in the sample, based on a standard curve. Laboratories utilized this assay for several years because it possesses a detection range of 0.1-50ng of human DNA and is capable of being automated on robotic systems (21, 22).

Real-time quantitative polymerase chain reaction (qPCR) was first introduced in the early 1990s by Higuchi *et al* (23). This method can be referred to as "kinetic analysis" because it analyzes the change in fluorescent signaling (due to the amplification of the target sequence) from cycle to cycle (24). The fluorescent change is measured during the exponential amplification phase of PCR. The exponential phase provides a high degree of precision and efficiency surrounding the production of the newly targeted PCR products (25). Baseline fluorescent noise can be observed during the early rounds of qPCR (23). A cycle number or cycle threshold (C_T) would be set in terms of PCR amplification cycles, when the level of fluorescence exceeds the baseline noise (24). qPCR analysis utilizes a standard curve (26). The standard curve is plotted in terms of a log scale of the standard dilution DNA concentrations versus the cycle numbers (24, 26). This plot results in a linear relationship with a negative slope (26). Unknown samples can be quantified by comparing them against the standard curve to determine its initial DNA template concentration (24, 26).

The Quantifiler® Human DNA Quantification Kit (Quantifiler® kit) (Applied Biosystems) and the Investigator® Quantiplex Kit (Quantiplex kit) (Qiagen, Hilden, Germany) are two commercial qPCR kits. Downstream DNA testing relies upon qPCR kits that provide DNA quantification, which is dependent upon accurate detection of PCR inhibitors (6). These kits assume that the efficiencies between the standards and the unknown samples are equal, but this assumption does not hold in the presence of PCR inhibitors (26-28). Each kit contains an internal PCR control (IPC) that is used to detect successful amplification as well as PCR inhibitors (25, 29). The Quantiplex kit refers to the IPC as an internal control (IC). The IPC and the unknown human DNA samples are co-amplified; the IPC is used to monitor the level of PCR inhibition (6, 30, 31). In theory, when PCR inhibitors are present, the reaction will cross the detection threshold at a higher C_T when compared to uninhibited samples, indicating that a lower amount of DNA has been amplified (9). However, amplicons that vary with respect to their length, melting temperature, and primer sequences are not equally affected by inhibition (32). Quantification values based off the IPC may not be as accurate with respect to total DNA concentration since target regions are not equally susceptible to inhibition (28, 33, 34).

The Quantifiler® kit employs two nuclease assays. This kit utilizes a total reaction volume of 25µL, with a maximum template DNA input of 2µL. Each sample will undergo 40 absolute quantification PCR cycles. Furthermore, this kit utilizes a set of eight quantification standards that range from 0.023 to 50ng/µL. The sixty-two base pair target gene in the human assay is the human telomerase reverse transcriptase gene (hTERT) and is located at 5p15.33. Two primers are used to amplify this region. A Taqman® probe (Applied Biosystems) binds to a complementary sequence between the forward and reverse primers and is used to provide an estimate of the concentration of DNA in the sample. The Taqman® probe is labeled with a

4

FAM® (Applied Biosystems) reporter fluorophore on the 5' end and a non-fluorescent quencher (NFQ) and a minor groove binder (MGB) (Applied Biosystems) on the 3' end of the probe. This kit also has a 79 base pair IPC assay, which consists of a synthetic DNA strand sequence not known to be found in nature, and two primers for its amplification (25, 35). The Taqman® probe used is similar to the human assay probe, except that it is labeled with a VIC® (Applied Biosystems) reporter fluorophore (25). See Table 1.

The Quantiplex kit also utilizes a multiplex assay. This kit uses a total reaction volume of 25μ L, with a maximum template DNA input of 2μ L. Each sample will undergo 40 absolute quantification PCR cycles. A set of seven quantification standards that range from 0.0049 to $20 \text{ng}/\mu\text{L}$ are utilized in this kit. The quantification human target region (4NS1C) is proprietary to Qiagen and the patent is pending. This region is 146 base pairs in length and is located on several autosomes of the human genome. Two primers are used to amplify this region. The first primer is covalently linked to the 3' end of the probe and is designed to operate as a bi-functional molecule. A hairpin loop configuration at the 5' end of the probe includes a FAM® reporter fluorophore, NFQ and a PCR blocker. Within the hairpin loop there is a complementary sequence that will bind to a target sequence on the DNA strands. This kit also uses an IC assay that is 200 base pairs in length and consists of a synthetic DNA strand sequence not known to be found in nature. The IC requires two primers for its amplification. The first primer is identical in structure to the primer-probe previously mentioned, with the exception of being labeled with a VIC® reporter fluorophore. The second primer for both assays has no additional covalently linked molecules (29). See Table 1.

| | Quantifiler® | Quantiplex |
|----------------------------|-----------------------|------------------------------|
| Total Reaction Volume | 25uL | 25uL |
| Maximum DNA Input | 2uL | 2uL |
| Quantification PCR Cycles | | |
| | 40 | 40 |
| Number of Quantification | | |
| Standards | 8 | 7 |
| Standard Range | 0.0023-50ng/uL | 0.0049-20ng/uL |
| | Human Telomerase | |
| HumanTarget Gene (HTG) | Reverse Transcriptase | 4NS1C (Proprietary Interest) |
| | (hTERT) | |
| Number of Basepairs in the | | |
| HTG | 62 | 146 |
| Internal PCR Control | | |
| (IPC) | yes | yes |
| Number of Basepairs in the | | |
| IPC | 79 | 200 |
| Probe Technology | Taqman® | Scorpions® |

Table 1. Comparisons of the Quantifiler® Human DNA Quantification Kit and the Investigator® Quantiplex Kit

The quantification chemistry of the kits is dependent upon the reporter fluorophore signaling that is initially sequestered by a quencher molecule, when in close proximity (25, 29). The release of the reporter dye and the fluorescent signaling is the main difference between the kits. The Quantifiler® kit relies on the Taqman® probe chemistry and consists of a forward and reverse primer set as well as a linear Taqman® probe (25). The 5'-3' exonuclease activity of the Taq polymerase will cleave the probe and separate the fluorophore from the quencher, resulting in the detection of fluorescence (25) (Figure 1).



Figure 1. Detection of fluorescence using the TaqMan® probe in the Quantifiler® Human DNA Quantification Kit. A forward and reverse primer are used for the amplification of the DNA target. A linear TaqMan® probe binds to a complementary sequence in-between the two primers. As the DNA polymerase (yellow circle labeled P) synthesizes a new complementary strand, it will use its 5'-3' exonuclease activity to cleave the probe, if present. This results in the detection of fluorescent light because the reporter fluorophore (blue circle labeled R) and the non-fluorescent quencher (pink oval labeled NFQ) are separated (25).

The Quantiplex kit utilizes the Scorpions® (Qiagen) probe technology (Figure 2). This probe is designed to operate as a bi-functional molecule because the forward primer is covalently linked to the 3' end of the probe element. A hairpin loop configuration at the 5' end of the probe includes a fluorophore, quencher and a PCR stopper (29). Before the next round of amplification, the primer-probe will undergo a rapid unimolecular rearrangement, which favors binding the complementary sequence, within the hairpin loop, to a target sequence on the DNA strand (36). The unimolecular rearrangement results in the detection of fluorescent light because the quencher is no longer capable of sequestering the fluorophore (29).



Figure 2. Detection of fluorescence using the Scorpions® primers in the Investigator® Quantiplex Kit. Forward and reverse primers (red arrows) are used for the amplification of the DNA amplicon (light blue line). The forward primer is covalently linked to a hairpin probe. As a result of amplification, the hairpin probe rearranges into a linear structure. This rearrangement allows the binding of the consensus sequence (orange lines) between the probe and the amplicon, which physically separates the non-fluorescent quencher (blue circle) and the reporter fluorophore (green circle) (29).

Research is being conducted at the University of North Texas Health Science Center (UNTHSC) to investigate the impact of metal ions, as PCR inhibitors, when extracting DNA from skeletal remains. The following metal ions, aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni) and lead (Pb) were chosen to be evaluated because these metals are naturally present at high concentrations in the environment (37), previously detected in skeletal remains (38) and were speculated to inhibit PCR (11). The metal concentrations in Figure 3 were chosen to be tested because they correspond to the metal ion concentration range that is found naturally in the soil (39). The six metals and six metal ion concentrations were tested using the Quantifiler® kit. Studies conducted at UNTHSC have demonstrated that when metal ion inhibitor concentrations are increased in DNA samples, the average percent of alleles observed decreases. This was demonstrated in the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems) and the PowerPlex® 16 HS System (Promega). The percent decrease was attributed to metal ion inhibition causing a reduction in relative florescent units (RFU) values, allelic dropout, preferential amplification, electrophoretic mobility shift, and decreased electrophoretic peak heights (39).

The mean concentrations of DNA in metal containing samples and controls were also evaluated. DNA samples containing Al and Fe have shown a decrease in the mean DNA concentration estimates as the metal ion concentrations increased. Ca, Cu, Ni, and Pb containing samples demonstrated an initial increase in DNA concentration estimates when the metal concentrations were at or below 0.2mM. Cu also showed another increase in DNA concentration estimates at metal concentrations of 1.25mM and 7.5mM. Each increase in the DNA concentration estimates were subsequently followed by a decrease in the DNA concentration

10

estimates when samples were exposed to higher millimolar metal ion levels. Each metal containing sample produced a unique dose-dependent inhibitory effect (28).

A comparison of C_T values between metal containing DNA samples, IPC and DNA controls was conducted. Al, Cu, and Ni exhibited a C_t shift of at least one or more units in both the IPC and human target gene assays. Fe and Pb did not cause an IPC C_T shift but did cause shift(s) in the human DNA detector. Ca containing samples did not show a demonstrable C_T shift for either the IPC or human DNA detector. This indicates that the IPC in the Quantifiler® kit may not be providing the most accurate estimates of DNA concentrations, in relation to metal ion inhibitors (28).

Forensic DNA analysis is dependent upon successful PCR extraction and quantification. qPCR assays have helped to identify inhibited samples by comparing the C_T values of the samples to the IPC. The research conducted at UNTHSC has indicated that the Quantifiler® kit may be providing less accurate DNA concentration estimates and fails to detect lower levels of inhibitors. This is because the DNA concentration estimates given by the Quantifiler® kit do not accurately reflect the original input level of DNA. As new technology is available, it would be of interest to compare the Quantiplex kit, with its Scorpions® probe chemistry, to the results obtained with the Quantifiler® kit at UNTHSC.

11

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

Experiments using the Quantifiler® Human DNA Quantification Kit and metal containing DNA samples have yielded inaccurate DNA and IPC concentration estimates (39). The Quantifiler® Human DNA Quantification Kit produces a target amplicon and IPC of 62 and 79 base pairs, respectfully (35). The Investigator® Quantiplex Kit produces a target amplicon and IPC of 146 and 200 base pairs, respectively, which is more comparable in length to STR loci used in the AmpFLSTR® Identifiler® PCR Amplification Kit (Applied Biosystems) and PowerPlex® 16 System Kit (Promega Corporation) (29, 35, 40). Longer amplicons targeted in the Investigator® Quantiplex Kit may be useful in qPCR kits because they could indicate inhibition at a lesser concentration of metal ions when compared to smaller amplicons (35).

Hypotheses:

Null Hypothesis- The Investigator® Quantiplex Kit and the Quantifiler® Human DNA Quantification Kit will provide similar estimates of DNA and internal PCR control (IPC) concentrations in the presence of metal ion inhibitors.

Alternative Hypothesis- The Investigator® Quantiplex Kit will provide more accurate estimates of DNA and internal PCR control (IPC) concentrations in the presence of metal ion inhibitors compared to the Quantifiler® Human DNA Quantification Kit.

Specific Aims:

Aim 1- Understand the normal working conditions of the Investigator® Quantiplex Kit. This will be accomplished by running uninhibited sample sets in accordance with the manufacturers' protocols. The results obtained will be compared to the Investigator® Quantiplex Kit's developmental validation study conducted by Qiagen.

Aim 2- Determine the accuracy of the Investigator® Quantiplex Kit in the presence of metal ion inhibitors. The C_T values of the human quantification and the IPC in the Investigator® Quantiplex Kit will be collected from samples inhibited with aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni) and lead (Pb). The C_T values will be analyzed to determine if they represent an accurate estimate in terms of the sample DNA concentration that was experimentally being tested.

Aim 3-Compare and contrast the accuracy of the Investigator® Quantiplex Kit against the Quantifiler® Human DNA Quantification Kit. The C_T values obtained from the Investigator® Quantiplex Kit (in Aim 2) will be compared to the C_T values in the Quantifiler® Human DNA Quantification Kit, which were obtained previously by the same research laboratory at the University of North Texas Health Science Center. The kit which provides the most accurate DNA concentrations estimates will have C_T values that are the same or very similar to the known input level of experimental DNA concentrations.

CHAPTER 3

MATERIALS AND METHODS

Control DNA Sample Preparation

50 nanograms per micoliter (ng/µL) of single source control human DNA (Serological Research Institute, Richmond, CA) was diluted to 1ng/µL with UltraPure[™] DNase/RNase- Free Distilled Water.

Metal Solution Preparations

Al, Ca, Cu, Fe, Ni and Pb were obtained from certified analytical standards in solution (High Purity Standards, Charleston, SC). The metal stock solutions were diluted to approximately 21 millimolar (mM). The pH of each metal was adjusted to a value between 3 and 5 using 3 molar (M) ammonium hydroxide (NH₄OH) and 1M hydrochloric acid (HCl) (39) (Table 2). Serial dilutions of the metal stock solutions were prepared using UltraPureTM DNase/RNase- Free Distilled Water (InvotrogenTM, Carlsbad, CA), as previously defined (39) (Table 3). The metal ion concentration of 18.75mM was measured with a level of significance as defined in Table 3. The remaining metal ion concentrations should be evaluated as categorical values, as no significance was applied to the estimated dilution values in Table 3.

| Adjusted pH |
|-------------|
| 4.0 |
| 4.9 |
| 4.3 |
| 5.8 |
| 4.2 |
| 3.8 |
| |

Table 2. Adjusted pH of metal stock solutions.

Table 3. Metal concentrations used to experimentally treat the control DNA and the subsequent total concentrations of metal in the Quantiplex and Identifiler® Plus reactions.

| Metal | Concentrati | ion (mM) |
|-------------|-------------|-------------------------------|
| Control DNA | Quantiplex | Identifiler [®] Plus |
| 0.0025 | 0.0002 | 0.001 |
| 0.015 | 0.0012 | 0.006 |
| 0.075 | 0.006 | 0.03 |
| 0.5 | 0.04 | 0.2 |
| 3.125 | 0.25 | 1.25 |
| 18.75 | 1.50 | 7.5 |

The metal ion concentration of 18.75mM was measured with a level of significance as defined above. The remaining metal ion concentrations should be evaluated as categorical values, as no significance was applied to the above estimated dilution values.

DNA and Metal Inhibitor Solution Preparation

Samples of single source control DNA (Serological Research Institute) and the serially diluted metal solutions were prepared at various concentrations previously defined (39) (Table 3). A positive control, or 0.00mM sample, was also prepared using UltraPure[™] DNase/RNase-Free Distilled Water in place of the metal ion solution.

DNA Quantification

Absolute DNA quantification of the DNA and metal inhibitor solution samples were performed in triplicate using the Investigator® Quantiplex Kit in accordance with the manufacturer's protocol entitled, "Quantification of DNA Using the Applied Biosystems 7500 Real-Time PCR System" (29). For analysis, the 7500 Real-Time PCR System was used with Prism® 7500 Sequence Detection System (SDS) Software version 1.2.3 (Applied Biosystems). A slight adjustment was made in the "Two-step cycling" parameters. The "Combined annealing/extension" time was extended to 33 seconds, as the Prism® 7500 SDS software version 1.2.3 did not support Qiagen's recommended 32 seconds. The C_T values, standard deviation C_T, estimated DNA quantity, and the mean and standard deviation DNA quantity for the triplicate samples were obtained from the software. An undetermined result obtained for a C_T value and DNA concentration was manually assigned a value of 40 and zero, respectively.

DNA Quantification Data Analysis

Microsoft® Excel® 2008 for Mac version 12.3.6 (Microsoft Corporation, Redmond, WA) was used to calculate the average human target gene and IPC C_T values, percent relative standard deviation (RSD) and sample quantification values as a percent of the known positive control value. The average human target gene and IPC C_T values were examined by comparing the metal and positive control concentrations against the C_T values. This was also examined through the use of a linear regression. The percent relative standard deviation of each metal at each concentration was examined and compared against the other metals and the positive control. The quantification values as a percent of the known positive control. All tests where then compared against previous results (39).

STR Amplification and Detection

Duplicate samples of each DNA and metal inhibitor solution at the various concentrations were tested with the AmpFLSTR® Identifiler® Plus Kit (Applied Biosystems) (41). STR amplification was carried out on a GeneAmp® PCR System 9700 (Applied Biosystems). Short tandem repeat (STR) fragment separation was performed on a 3130*xl* Genetic Analyzer using Data Collection version 3.0 (Applied Biosystems). GeneMapper® *ID-X* Software Version 1.4 (Applied Biosystems) was used to collect STR profiles at an allele detection threshold of 50 relative fluorescence units (RFU). The allele calls, peak heights, and fragment sizes were collected. All instrumentation was used in accordance with the manufacturers' recommendations (42-44).

STR Detection Data Analysis

GeneMapper® *ID-X* version 1.4 software was used to determine the number of alleles present in each obtained profile. The number of alleles present in the duplicate samples for each concentration were combined and designated into four defined profile categories (full profile, partial profile, poor profile and no profile). A full profile consists of 52 alleles since the positive control used produces a profile of 26 alleles. A partial profile has a range of 26-51 alleles and a poor profile has a range of 1-25 alleles. No profile has zero alleles present.

Microsoft® Excel® 2008 was used to calculate the average peak height values for each allele at each locus and the minimum, maximum, 25th percentile, 75th percentile, and median peak height values for each metal at each concentration. These values were plotted, which allowed for visual comparison between the metals and their concentrations. Microsoft® Excel® 2008 was also used to perform linear regression analysis with the variables of fragment size

17

(base pairs) and peak heights (RFU) for each metal at each concentration. The following three tests were then compared to previous results (28).

CHAPTER 4

RESULTS

DNA Quantification Estimates Using The Quantiplex Kit

The metals used to treat the control DNA were divided into two groups. Group 1 included the DNA metal inhibitor solutions of Al, Ca, and Cu, while Group 2 included Fe, Ni and Pb. The standard curve for both groups included all data points. The R² values were 0.9951 for Group 1 and 0.9980 for Group 2. The slope was -3.2630 and -3.3337 for Groups 1 and 2, respectively. The y-intercept was 24.6429 for Group 1 and 24.7606 for Group 2. The obtained R² and slope values are consistent with the values reported. The y-intercept indicates the expected C_T value for a sample that has a quantity of $1ng/\mu L$ (29).

The estimated DNA concentration of the positive control and metal containing DNA samples are listed in Table 4. Each metal produced a unique series of concentration values. However, a similarity among all the metals is that the lowest metal concentration of 0.0025mM always produced a DNA concentration estimate greater than the positive control. For the metal concentration of 0.015mM, all metals except Ni exceeded the positive control concentration. At 0.075mM, all DNA concentration estimate was severely affected at 0.5mM, while the remaining metals produced concentration estimates above the positive control. At a metal concentration of 3.125mM, Ca, Cu and Pb produced DNA concentration estimates that exceeded

the positive control, while Fe and Ni produced DNA concentrations that were lower than the positive control. Al did not produce a DNA concentration estimate. At 18.75mM, Ca, Cu, Fe, Ni, and Pb produced DNA concentration estimates that were less then the positive control. Once again, Al did not produce a DNA concentration estimate.

The percent RSD values did not produce a clear trend between the metal concentrations. For Al the lowest percent RSD of 0.54% occurred at 0.0025mM and the highest percent RSD of 133.48% occurred at 0.5mM. Ca produced its lowest and highest percent RSD values of 4.08% and 23.10% at metal concentrations of 18.75mM and 3.125mM, respectively. The lowest percent RSD of 2.93% occurred for Cu at 0.0015mM and the highest percent RSD of 66.06% occurred at 3.125mM. For Fe, the lowest percent RSD was 5.82% at a metal concentration of 0.015mM and the highest percent RSD was 49.01% at18.75mM. Ni produced its lowest and highest percent RSD values of 6.38% and 22.25% at 0.075mM and 3.125mM, respectively. The lowest percent RSD value of 3.44% was obtained for Pb at 0.0025mM and its highest percent RSD value of 13.53% at 0.015mM.

| | | Group 1 | | | Group 2 | |
|-------------------|---------|---------|--------|---------|---------|--------|
| Metal | Al | Ca | Cu | Fe | Ni | Pb |
| | 0.130 | 0.130 | 0.130 | 0.192 | 0.192 | 0.192 |
| Postitive control | 100% | 100% | 100% | 100% | 100% | 100% |
| | 8.69% | 8.69% | 8.69% | 3.52% | 3.52% | 3.52% |
| | 0.189 | 0.213 | 0.240 | 0.288 | 0.236 | 0.289 |
| 0.0025mM | 145% | 164% | 185% | 150% | 123% | 151% |
| | 0.54% | 17.65% | 8.42% | 11.49% | 18.60% | 3.44% |
| | 0.243 | 0.191 | 0.239 | 0.287 | 0.163 | 0.241 |
| 0.015mM | 187% | 147% | 184% | 149% | 85% | 126% |
| | 21.73% | 11.83% | 2.93% | 5.82% | 11.96% | 13.53% |
| | 0.146 | 0.301 | 0.239 | 0.207 | 0.240 | 0.282 |
| 0.075mM | 112% | 232% | 184% | 108% | 125% | 147% |
| | 20.89% | 7.01% | 9.08% | 16.18% | 6.38% | 4.96% |
| | 0.00037 | 0.225 | 0.176 | 0.226 | 0.200 | 0.224 |
| 0.5mM | 0.29% | 173% | 135% | 118% | 104% | 117% |
| | 133.48% | 13.56% | 14.43% | 7.74% | 19.35% | 10.18% |
| | 0.00* | 0.187 | 0.277 | 0.075 | 0.182 | 0.220 |
| 3.125mM | 0.00% | 144% | 213% | 39% | 95% | 115% |
| | 0.00% | 23.10% | 66.06% | 8.58% | 22.25% | 6.82% |
| | 0.00* | 0.079 | 0.099 | 0.00141 | 0.121 | 0.103 |
| 18.75mM | 0.00% | 61% | 76% | 0.73% | 63% | 54% |
| | 0.00% | 4.08% | 24.67% | 49.01% | 15.29% | 9.90% |

Table 4. Estimated DNA concentration and percent relative standard deviation of positive control and metal containing DNA samples using the Quantiplex kit.

Positive control refers to a sample that does not contain metal ions.

Reported values are the mean DNA concentration estimates ($ng/\mu L$), quantification values of DNA samples containing metal ions as a percentage of the positive control and the percent RSD. *Indicates samples that were manually assigned a DNA concentration of zero and a C_T value of 40.

Blue values indicate a greater percent value than the Quantifiler® results.

Comparison of Metal Containing DNA Samples and Positive Control C_T Values Using the Quantiplex Kit

Table 5 shows the delta and mean C_T values for Al, Ca, Cu, Fe, Ni, and Pb containing

samples. A C_T value of 40 (representing the maximum number of PCR cycles) was manually

assigned to samples that received an undetermined C_T value by the software. The mean

difference of the human target and IPC C_T values from the metal containing and the positive

control samples was used to determine if a C_T shift was occurring, which could indicate

inhibition of metal containing samples. For Al, Ca, and Cu samples, the positive control had a

mean human target C_T value of 27.54 and a mean IPC C_T value of 28.18. Fe, Ni and Pb metal containing samples had a mean human target C_T and mean IPC C_T values of 27.15 and 28.23, respectively.

The range of mean human target C_T values for Al was 26.67-40.00. The lowest Al mean human target C_T value occurred at 0.015mM, which corresponded to a delta mean of -0.87. The highest human target C_T value was obtained at the metal concentrations of 3.125 and 18.75mM. The corresponding delta mean human target C_T was 12.46. The range of the mean IPC C_T values for Al was 28.17-30.42. The lowest Al mean IPC C_T value occurred at 0.015mM, while the highest mean IPC C_T value occurred at 18.75mM. The delta mean IPC C_T values were -0.01 and 2.24, respectively.

The mean human target C_T value range for Ca was 26.35-28.24. The lowest mean human target C_T value occurred at 0.075mM, corresponding to a delta mean of -1.19. The highest mean human target C_T value was obtained at the highest metal concentration of 18.75mM. The corresponding delta mean human target C_T was 0.70. The mean IPC C_T value range for Ca was 28.22-28.62. The lowest mean IPC C_T value for Ca occurred at the highest metal concentration of 18.75mM. The delta mean IPC C_T was 0.05. The highest IPC C_T value was obtained at the second highest metal concentration of 3.125mM, with a delta mean IPC C_T of 0.44.

For Cu, the mean human target C_T range was 26.65-27.94. The mean human target C_T value was the lowest at 3.125mM. The corresponding delta mean human target C_T was -0.89. The highest C_T value for the human target was obtained at 18.75mM, with a corresponding delta mean human target C_T of 0.40. The range of the mean IPC C_T values for Cu was 28.64-29.05. The lowest mean IPC C_T value occurred at the lowest metal concentration of 0.0025mM and the

delta mean IPC C_T was 0.46. The highest IPC C_T value occurred at 3.125mM. The delta mean IPC C_T was 0.88.

The range of mean human target C_T for Fe was 26.57-34.38. The lowest mean human target C_T was obtained at the lowest metal concentration of 0.0025mM, which had a delta mean human target C_T of -0.58. The highest mean human target C_T value occurred at the highest metal concentration of 18.75mM. The corresponding delta mean human target C_T was 7.23. The range of the mean IPC C_T values for Fe was 28.23-28.91. The lowest mean IPC C_T value occurred at 0.5mM, while the highest mean IPC C_T value occurred at of 0.015mM. The delta mean IPC C_T values were 0.01 and 0.69, respectively.

For Ni, the mean human target C_T range was 26.82-27.83. The mean human target C_T value was the lowest at a metal concentration of 0.075mM. The corresponding delta mean human target C_T was -0.32. The highest C_T value for the human target was obtained at the highest metal concentration of 18.75mM, which had a corresponding delta mean human target C_T of 0.68. The range of the mean IPC C_T values for Ni was 28.50-28.82. The lowest mean IPC C_T value occurred at the highest metal concentration of 18.75mM and the delta mean IPC C_T was 0.27. The highest metal concentration of 18.75mM.

The mean human target C_T value range for Pb was 26.56-28.06. The lowest mean human target C_T occurred at the lowest metal concentration of 0.0025mM. The corresponding delta mean human target C_T was -0.59. The highest mean human target C_T value was observed at the highest metal concentration of 18.75mM and the delta mean human target C_T was 0.91. For Pb, the range of the mean IPC C_T values were 28.42-28.77. The mean IPC C_T value was the lowest at

23

0.5mM. The corresponding delta mean IPC C_T was 0.19. The highest mean IPC C_T value was observed at 0.075mM and the delta mean IPC C_T was 0.54.

| Al | Mean Human Target C_T | Delta Mean Human Target $C_{\rm T}$ | Mean IPC C_T | Delta Mean IPC C $_{\rm T}$ |
|---|---|--|---|---|
| Positive Control | 27.54 | 0 | 28.18 | 0.00 |
| 0.0025mM | 27.00 | -0.54 | 28.24 | 0.06 |
| 0.015mM | 26.67 | -0.87 | 28.17 | -0.01 |
| 0.075mM | 27.39 | -0.15 | 28.40 | 0.23 |
| 0.5mM | 37.13 | 9.59 | 28.26 | 0.09 |
| 3.125mM | 40.00 | 12.46 | 28.36 | 0.18 |
| 18.75mM | 40.00 | 12.46 | 30.42 | 2.24 |
| | | | | |
| Ca | Mean Human Target C_T | Delta Mean Human Target $C_{\scriptscriptstyle T}$ | Mean IPC C_T | Delta Mean IPC C $_{\rm T}$ |
| Positive Control | 27.54 | 0.00 | 28.18 | 0.00 |
| 0.0025mM | 26.85 | -0.69 | 28.58 | 0.41 |
| 0.015mM | 27.00 | -0.54 | 28.57 | 0.39 |
| 0.075mM | 26.35 | -1.19 | 28.50 | 0.32 |
| 0.5mM | 26.76 | -0.78 | 28.60 | 0.43 |
| 3.125mM | 27.05 | -0.49 | 28.62 | 0.44 |
| 18.75mM | 28.24 | 0.70 | 28.22 | 0.05 |
| | | | | |
| Cu | Mean Human Target C_T | Delta Mean Human Target C_{T} | Mean IPC C_T | Delta Mean IPC C $_{\rm T}$ |
| Positive Control | 27.54 | 0 | 28.18 | 0.00 |
| 0.0025mM | 26.67 | -0.87 | 28.64 | 0.46 |
| 0.015mM | 26.67 | -0.87 | 28.66 | 0.48 |
| 0.075mM | 26.68 | -0.86 | 28.90 | 0.72 |
| 0.5mM | 27.11 | -0.43 | 28.90 | 0.72 |
| 3.125mM | 26.65 | -0.89 | 29.05 | 0.88 |
| 18.75mM | 27.94 | 0.40 | 28.85 | 0.67 |
| | | | | |
| F | Maan Human Tanaat C | Dalta Maan Human Tanaat C | Maan IDC C | Dalta Maan IDC C |
| Fe | Mean Human Target C_T | Delta Mean Human Target C_T | Mean IPC C_T | Delta Mean IPC C_T |
| Fe Positive Control | Mean Human Target C _T 27.15 | Delta Mean Human Target C_T 0.00 | Mean IPC C _T 28.23 | Delta Mean IPC C_T 0.00 |
| Fe Positive Control 0.0025mM | Mean Human Target C _T 27.15 26.57 27.55 | Delta Mean Human Target C _T 0.00 -0.58 | Mean IPC C _T 28.23 28.29 | Delta Mean IPC C_T 0.00 0.06 0.66 |
| Fe Positive Control 0.0025mM 0.015mM | Mean Human Target C _T 27.15 26.57 26.57 26.57 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 | Mean IPC C _T 28.23 28.29 28.91 | Delta Mean IPC C _T 0.00 0.06 0.69 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM | Mean Human Target C _T 27.15 26.57 26.57 27.05 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 0.02 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.64 | Delta Mean IPC C _T 0.00 0.06 0.69 0.42 0.01 |
| Fe Positive Control 0.0025mM 0.015mM 0.5mM 2.125mM | Mean Human Target C _T 27.15 26.57 26.57 27.05 26.92 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 -0.23 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.23 | Delta Mean IPC C _T 0.00 0.06 0.69 0.42 0.01 0.46 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM | Mean Human Target C _T 27.15 26.57 26.57 27.05 26.92 28.51 24.28 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7 22 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.69 28.24 | Delta Mean IPC C _T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM | Mean Human Target C _T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 | Delta Mean IPC C _T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM | Mean Human Target C _T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C _T | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C _T | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _T | Delta Mean IPC C _T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C _T |
| Fe Positive Control 0.0025mM 0.015mM 0.5mM 3.125mM 18.75mM Ni Positive Control | Mean Human Target C _т 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C _т | Delta Mean Human Target C_T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C_T 0.00 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _T 28.23 | Delta Mean IPC C_T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C_T |
| Fe Positive Control 0.0025mM 0.015mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM | Mean Human Target C _т 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C _т 27.15 26.87 | Delta Mean Human Target C_T 0.00 -0.58 -0.59 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C_T 0.00 -0.28 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _T 28.23 28.23 28.82 | Delta Mean IPC C_T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C_T 0.00 0.59 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.15 26.87 27.15 34.38 | Delta Mean Human Target C_T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C_T 0.00 -0.28 0.25 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _T 28.23 28.82 28.82 28.52 | Delta Mean IPC C_T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C_T 0.00 0.59 0.29 |
| Fe Positive Control 0.0025mM 0.015mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.075mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.15 26.87 27.39 26.82 | Delta Mean Human Target C _т 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C _т 0.00 -0.28 0.25 -0.32 | Mean IPC C _T 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _T 28.23 28.82 28.82 28.52 28.79 | Delta Mean IPC C_T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C_T 0.00 0.59 0.29 0.56 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM | Mean Human Target C _т 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C _т 27.15 26.87 27.39 26.82 27.11 | Delta Mean Human Target C _T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C _T 0.00 -0.28 0.25 -0.32 -0.04 | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.82 28.52 28.79 28.75 | |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM | Mean Human Target C _т 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C _т 27.15 26.87 27.39 26.82 27.11 27.25 | Delta Mean Human Target C _т 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C _т 0.00 -0.28 0.25 -0.32 -0.32 -0.04 0.10 | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.75 28.73 | Delta Mean IPC C_T 0.00 0.06 0.69 0.42 0.01 0.46 0.11 Delta Mean IPC C_T 0.00 0.59 0.29 0.56 0.52 0.50 |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.015mM 0.5mM 0.5mM 0.5mM 0.5mM 0.5mM 3.125mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.15 26.82 27.15 26.87 27.39 26.82 27.11 27.25 27.83 | Delta Mean Human Target C_T 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C_T 0.00 -0.28 0.25 -0.32 -0.04 0.10 0.68 | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.73 28.73 28.50 | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \end{array}$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.075mM 0.5mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.15 26.82 27.15 26.87 27.39 26.82 27.11 27.25 27.83 | | $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.29 \\ 28.91 \\ 28.64 \\ 28.23 \\ 28.69 \\ 28.34 \\ \\ \text{Mean IPC } C_{T} \\ 28.23 \\ 28.82 \\ 28.52 \\ 28.79 \\ 28.75 \\ 28.73 \\ 28.50 \\ \end{array}$ | $\begin{array}{c} \mbox{Delta Mean IPC } C_T \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \mbox{Delta Mean IPC } C_T \\ \hline \mbox{0.00} \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \end{array}$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.82 27.11 27.25 27.83 Mean Human Target C_T | $ Delta Mean Human Target C_T \\ 0.00 \\ -0.58 \\ -0.58 \\ -0.09 \\ -0.23 \\ 1.36 \\ 7.23 \\ Delta Mean Human Target C_T \\ 0.00 \\ -0.28 \\ 0.25 \\ -0.32 \\ -0.04 \\ 0.10 \\ 0.68 \\ Delta Mean Human Target C_T \\ $ | $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.29 \\ 28.91 \\ 28.64 \\ 28.23 \\ 28.69 \\ 28.34 \\ \hline \\ \text{Mean IPC } C_{T} \\ 28.23 \\ 28.22 \\ 28.23 \\ 28.52 \\ 28.75 \\ 28.75 \\ 28.73 \\ 28.50 \\ \hline \\ \text{Mean IPC } C_{T} \\ \hline \\ \text{Mean IPC } C_{T} \\ \hline \end{array}$ | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ 0.00 \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \end{array}$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM 9.5mM 0.5mM 0.5mM 0.5mM 9.5mM 9.5mM< | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.82 27.11 27.25 27.83 Mean Human Target C_T | | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.73 28.70 28.73 28.50 Mean IPC C _т 28.23 | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ 0.00 \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \end{array}$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Pb Positive Control 0.0025mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.39 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.82 27.11 27.25 27.83 | Delta Mean Human Target C _т 0.00 -0.58 -0.58 -0.09 -0.23 1.36 7.23 Delta Mean Human Target C _т 0.00 -0.28 0.25 -0.32 -0.04 0.10 0.68 Delta Mean Human Target C _т 0.00 -0.59 | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.73 28.50 Mean IPC C _т 28.23 28.50 | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.06 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ 0.00 \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Pb Positive Control 0.0025mM 0.5mM 3.125mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.39 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.56 26.82 | | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.73 28.50 Mean IPC C _т 28.23 28.50 | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ 0.00 \\ 0.59 \\ 0.29 \\ 0.56 \\ 0.52 \\ 0.50 \\ 0.27 \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm T} \\ \hline \\ \mbox{Delta Mean IPC C}_{\rm J} \\ \hline \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Pb Positive Control 0.0025mM 0.5mM 3.125mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.39 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.56 26.83 26.60 | | $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.29 \\ 28.91 \\ 28.64 \\ 28.23 \\ 28.69 \\ 28.34 \\ \end{array}$ $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.82 \\ 28.52 \\ 28.79 \\ 28.75 \\ 28.73 \\ 28.50 \\ \end{array}$ $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.50 \\ \end{array}$ $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.50 \\ \end{array}$ | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \end{array} \\ \hline \begin{tabular}{lllllllllllllllllllllllllllllllllll$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Pb Positive Control 0.0025mM 0.5mM 3.125mM 0.5mM 0.5mM 0.075mM 0.0025mM 0.0025mM 0.0025mM 0.0025mM 0.015mM 0.075mM 0.075mM 0.075mM 0.5mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.39 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.56 26.83 26.82 27.11 | | Mean IPC C _т 28.23 28.29 28.91 28.64 28.23 28.69 28.34 Mean IPC C _т 28.23 28.82 28.52 28.79 28.75 28.73 28.50 Mean IPC C _т 28.23 28.50 Mean IPC C _т 28.23 28.50 | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \end{array} \\ \hline \begin{tabular}{lllllllllllllllllllllllllllllllllll$ |
| Fe Positive Control 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Ni Positive Control 0.0025mM 0.015mM 0.0025mM 0.015mM 0.075mM 0.5mM 3.125mM 18.75mM Pb Positive Control 0.0025mM 0.5mM 3.125mM 18.75mM | Mean Human Target C_T 27.15 26.57 26.57 27.05 26.92 28.51 34.38 Mean Human Target C_T 27.15 26.87 27.15 26.87 27.39 26.82 27.11 27.25 27.83 Mean Human Target C_T 27.15 26.56 26.83 26.93 26.93 26.93 | $\begin{array}{c} \mbox{Delta Mean Human Target } C_T \\ 0.00 \\ -0.58 \\ -0.58 \\ -0.09 \\ -0.23 \\ 1.36 \\ 7.23 \end{array}$ $\begin{array}{c} \mbox{Delta Mean Human Target } C_T \\ 0.00 \\ -0.28 \\ 0.25 \\ -0.32 \\ -0.04 \\ 0.10 \\ 0.68 \end{array}$ $\begin{array}{c} \mbox{Delta Mean Human Target } C_T \\ 0.00 \\ -0.58 \\ -0.32 \\ -0.04 \\ 0.10 \\ 0.68 \end{array}$ | $\begin{array}{c} \text{Mean IPC } C_{T} \\ 28.23 \\ 28.29 \\ 28.91 \\ 28.64 \\ 28.23 \\ 28.69 \\ 28.34 \\ \hline \\ \text{Mean IPC } C_{T} \\ 28.23 \\ 28.52 \\ 28.79 \\ 28.75 \\ 28.73 \\ 28.50 \\ \hline \\ \text{Mean IPC } C_{T} \\ 28.23 \\ 28.73 \\ 28.50 \\ \hline \\ \text{Mean IPC } C_{T} \\ 28.23 \\ 28.73 \\ 28.62 \\ 28.77 \\ 28.42 \\ 28.72 \\ \end{array}$ | $\begin{array}{c} \mbox{Delta Mean IPC C}_{\rm T} \\ 0.00 \\ 0.06 \\ 0.69 \\ 0.42 \\ 0.01 \\ 0.46 \\ 0.11 \\ \end{array} \\ \hline \begin{tabular}{lllllllllllllllllllllllllllllllllll$ |

Table 5. Delta and mean C_T values for Al, Ca, Cu, Fe, Ni, and Pb containing samples.

Figures 3 and 4 show the mean C_T values obtained from the amplification of human DNA targets in samples containing Al, Ca, Cu, and Fe, Ni, Pb ions.



Figure 3. Mean C_T values obtained from the amplification of human DNA targets in samples containing Al, Ca, and Cu ions. Triplicate samples containing Al, Ca, or Cu ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human DNA target and graphed against the metal ion concentration. The solid black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples.



Figure 4. Mean C_T values obtained from the amplification of human DNA targets in samples containing Fe, Ni, and Pb ions. Triplicate samples containing Fe, Ni, or Pb ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human DNA target and graphed against the metal ion concentration. The solid black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples.
Figures 5 and 6 show the mean C_T values obtained from the amplification of IPC DNA targets in samples containing Al, Ca, Cu, and Fe, Ni, Pb ions.



Figure 5. Mean C_T values obtained from the amplification of IPC DNA targets in samples containing Al, Ca, and Cu ions. Triplicate samples containing Al, Ca, or Cu ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the IPC DNA target and graphed against the metal ion concentration. The dashed black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples.



Figure 6. Mean C_T values obtained from the amplification of IPC DNA targets in samples containing Fe, Ni, and Pb ions. Triplicate samples containing Fe, Ni, or Pb ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the IPC DNA target and graphed against the metal ion concentration. The dashed black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples.

Figures 7-12 show the mean human target and IPC C_T values of metal containing and positive control samples. A logarithmic conversion of the metal concentrations was performed for ease in data comparison between previous data (28).



Figure 7. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Al ions. Triplicate samples containing Al ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Al ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Al ions to those obtained from the positive IPC control samples.



Figure 8. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Ca ions. Triplicate samples containing Ca ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Ca ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Ca ions to those obtained from the positive IPC control samples.



Figure 9. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Cu ions. Triplicate samples containing Cu ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Cu ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Cu ions to those obtained from the positive IPC control samples.



Figure 10. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Fe ions. Triplicate samples containing Fe ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Fe ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Fe ions to those obtained from the positive IPC control samples.



Figure 11. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Ni ions. Triplicate samples containing Ni ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Ni ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Ni ions to those obtained from the positive IPC control samples.



Figure 12. Mean C_T values obtained from the amplification of human and IPC DNA targets in samples containing Pb ions. Triplicate samples containing Pb ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the human and IPC DNA targets and plotted against the logarithmic metal ion concentration (mM). The solid black line is provided as a visual reference for comparison between the C_T values obtained from samples prepared with Pb ions to those obtained from the positive control samples. The dashed black line is provided as a visual reference for comparison between the C_T values obtained from IPC samples prepared with Pb ions to those obtained from the positive IPC control samples.

STR Amplification

STR amplification was performed with the Identifiler® Plus kit. Varying degrees of precipitant formed within the metal containing samples. No precipitant was observed in any metal containing samples at metal concentrations of 0.0025mM to 0.075mM. For Al, and Pb a white light precipitant formed at the highest metal concentration of 18.75mM. A similar precipitant consistency formed in Cu metal samples at a concentration of 3.125mM. A thick

white precipitant was formed in Cu, Ca, and Ni samples at the highest metal concentration. Samples containing Fe formed an orange light and orange thick precipitant at 3.125mM and 18.75mM, respectively.

STR Profiles

Figure 13 displays a heat map that shows STR profiles obtained with the Identifiler® Plus kit. Full profiles were obtained across the metal concentrations of 0.0025-18.75mM in Fe and Pb containing samples only. Al produced full profiles at 0.0025-0.075mM. Despite the zero quantification values determined by the Quantiplex kit, a poor profile was obtained at 0.05mM and 3.125mM. In all metal concentrations full profiles were observed for Ca containing samples, except at 18.75mM where a partial profile was produced. Cu and Ni produced full profiles at metal concentrations of 0.0025-3.125mM. No profiles were obtained for Al, Cu and Ni at 18.75mM, even though Cu and Ni had DNA concentration estimates greater than zero.

| Metal Ion | | Group 1 | | | | Group 2 | | | |
|--------------------|------------|---------|-------|-------|------------|---------|--------|-------|--|
| Concentration (mM) | Metal Ions | | | | Metal Ions | | | | |
| | PC | Al | Ca | Cu | PC | Fe | Ni | Pb | |
| 0.0025 | | 0.189 | 0.213 | 0.240 | 0.192 | 0.288 | 0.236 | 0.289 | |
| 0.015 | 0.130 | 0.243 | 0.191 | 0.239 | | 0.287 | 0.163* | 0.241 | |
| 0.075 | | 0.146 | 0.301 | 0.239 | | 0.207 | 0.24* | 0.282 | |
| 0.500 | | 0.000 | 0.225 | 0.176 | | 0.226 | 0.200* | 0.224 | |
| 3.125 | | 0.000 | 0.187 | 0.277 | | 0.0753 | 0.182 | 0.220 | |
| 18.750 | | 0.000 | 0.079 | 0.099 | | 0.00141 | 0.121* | 0.103 | |

| Legend | | | | | | | |
|-----------------|-------------------------|--------|--|--|--|--|--|
| No Profile | 0 out of 26 alleles | 0% | | | | | |
| Poor Profile | 1-12 out of 26 alleles | 1-49% | | | | | |
| Partial Profile | 13-15 out of 26 alleles | 50-99% | | | | | |
| Full Profile | 26 out of 26 alleles | 100% | | | | | |

Figure 13. DNA quantification estimates (ng/µL) and heat map indicating STR profiles success. Duplicate samples prepared with 0.0025 to 18.75mM Al, Ca, Cu, Fe, Ni, and Pb were quantified using the Investigator® Quantiplex Kit. The samples were subsequently amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. Human DNA quantification estimates and number of STR alleles were collected and displayed for each metal ion at each concentration investigated.

PC denotes the positive control.

*Only one profile was evaluated due to low sizing quality of the internal size standard.

Profile Peak Heights

Figures 14-19 represent box and whisker plots of the allele peak height fluorescence

(RFU) obtained from the positive control samples and samples containing 0.0025 to 18.75mM

Al, Ca, Cu, Fe, Ni, or Pb ions. For each concentration the bars represent the minimum and

maximum values. The 25th percentile and 75th percentile are represented by green and purple

boxes, respectively. The median is where the two boxes meet.

The 75th percentile box for all metals at all concentrations were larger generally larger in

size than the 25th percentile boxes. When this trend did not hold, it was noted that the 75th and

25th percentile boxes where nearly even in size. This was observed in the three metal concentrations of 0.015mM (Cu, Ni and Pb), 3.125mM (Al, Cu, and Ni) and 18.75mM (Ca, Fe, and Pb). There was only one instance where the 25th percentile box was greater than the 75th percentile and this occurred in Fe containing samples at 3.125mM.

When examining the 25th and 75th percentile boxes as a whole unit a general trend emerged between all metals and concentrations. At a metal concentration of 0.0025mM all metal box units (representing the peak heights) except Ni did not vary that much from the positive control. A decrease in the box units was then observed for all metals at 0.0015mM. Al was an exception, as it remained similar to the box unit of 0.0025mM. At 0.075mM all box units increased following their previous decrease except Al and Pb. Al continued to remain similar to 0.025mM, while Pb continued to decrease. The unit boxes of Al, Cu and Ni exhibited a decrease and Al, Cu, and Ni remained nearly unchanged, when evaluated at a metal concentration of 0.5mM. At 3.125mM Ca, Cu, Ni and Pb remained nearly unaltered when compared 0.5mM, while Al and Fe exhibited a decrease. No results were obtained for Al, Cu and Ni at 18.75mM. A decrease in the unit boxes was observed for Ca, Fe and Pb at this concentration.



Figure 14. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Al ions. Duplicate samples containing Al metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.



Figure 15. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Ca ions. Duplicate samples containing Ca metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.



Figure 16. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Cu ions. Duplicate samples containing Cu metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.



Figure 17. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Fe ions. Duplicate samples containing Fe metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.



Figure 18. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Ni ions. Duplicate samples containing Ni metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.



Figure 19. Box and whisker plot of the allele peak height fluorescence obtained from the positive control samples and samples containing 0.0025 to 18.75mM Pb ions. Duplicate samples containing Pb metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were graphed against the metal ion concentration. The vertical lines (whiskers) represent the minimum and maximum allele peak heights. The green and purple boxes represent the 25th and 75th percentile of all observed allele peak heights at each metal ion concentration, respectively. The line where the 15th and 75th boxes meet represent the median peak height value. A detection threshold was set at 50 RFUs.

Figures 20-25 represent a scatter plot of the peak heights versus fragment sizes for each metal at each concentration. A best-fit line was drawn to aid in the identification of trends. Each metal produced a unique scatter plot, however one similarity between them was drawn. For most metal containing samples, as the fragment size increased the observed peak heights decreased.

Al metal ion concentrations of 0.0025-0.075mM showed a slight increase in the peak

heights as the short tandem repeat (STR) size increased. The metal ion concentrations of 0.5mM

and 3.125mM remained at very low peak heights across the fragment sizes. 18.75mM did not produce any peak heights. See Figure 20.

For Ca, the metal ion concentrations of 0.0025mM, and 0.075-0.3125mM clustered together. A slight increase and sharp decrease was observed for Ca at 0.015mM and 18.75mM as the fragment sizes increased. See Figure 21.

Metal ion concentrations of 0.0025mM, 0.075mM and 0.5mM for Cu retained fairly constant peak heights across the fragment sizes, while a decrease was seen at 0.015mM and 3.125mM. No peak heights were observed for 18.75mM. See Figure 22.

Fe metal ion concentrations of 0.0025mM, 0.075mM and 0.5mM clustered together and decreased slightly as the fragment size increased. 0.015mM also showed a slight decreased, while 3.125mM exhibited a sharp decrease. At 18.75mM the peak heights remained low across the fragment range. See Figure 23.

The metal ion concentration of 0.0025mM for Ni had fairly consistent peak heights across the fragment size range. All remaining metal ion concentrations except 0.075mM exhibited a decrease in peak heights with the increased size in fragments. No peak heights were observed for Ni containing samples at 18.75mM. See Figure 24.

All metal ion concentrations for Pb showed a decrease in peak heights as the fragment size increased. The metal ion concentrations of 0.0075-3.125mM were clustered closely together with 0.0025mM and 0.015mM associated on either side in close proximity. A slight decrease was observed for Pb at 18.75mM. See Figure 25.



Figure 20. A scatter plot of allele peak heights versus fragment sizes for samples containing Al ions at each metal ion concentration. Duplicate samples containing Al metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 3.125mM. No allele peak heights were observed at 18.75mM. A detection threshold was set at 50 RFUs.



Figure 21. A scatter plot of allele peak heights versus fragment sizes for samples containing Ca ions at each metal ion concentration. Duplicate samples containing Ca metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 18.75mM. A detection threshold was set at 50 RFUs.



Figure 22. A scatter plot of allele peak heights versus fragment sizes for samples containing Cu ions at each metal ion concentration. Duplicate samples containing Cu metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 3.125mM. No allele peak heights were observed at 18.75mM. A detection threshold was set at 50 RFUs.



Figure 23. A scatter plot of allele peak heights versus fragment sizes for samples containing Fe ions at each metal ion concentration. Duplicate samples containing Fe metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 18.75mM. A detection threshold was set at 50 RFUs.



Figure 24. A scatter plot of allele peak heights versus fragment sizes for samples containing Ni ions at each metal ion concentration. Duplicate samples containing Ni metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 3.125mM. No allele peak heights were observed at 18.75mM. A detection threshold was set at 50 RFUs.



Figure 25. A scatter plot of allele peak heights versus fragment sizes for samples containing Pb ions at each metal ion concentration. Duplicate samples containing Pb metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights were plotted against the fragment size. A best-fit line for each metal ion concentration was provided as a visual reference for comparisons between 0.0025 to 18.75mM. A detection threshold was set at 50 RFUs.

CHAPTER 5

DISCUSSION

DNA Quantification Estimates: A Comparison Between the Quantiplex and Quantifiler® Kits

The estimated DNA concentration of the positive control and metal containing DNA samples produced by the Quantifiler® kit are listed in Table 6. This table was created based on previous research for ease of comparison (28). Unlike the DNA concentration estimates obtained with the Quantiplex kit, only Ca, Cu, Ni and Pb produced a DNA concentration greater than the positive control at the lowest metal concentration of 0.0025mM. At 0.015mM, Ca, Ni and Pb exceeded the positive control concentration. Ni did not produce a DNA estimate above the positive control with the Quantiplex kit at this metal concentration. Only Ni and Pb produced DNA concentration estimates greater than the positive control at 0.075mM. Ca and Pb exceeded the positive control DNA concentration estimate at a metal concentration of 0.5mM. Only Cu produced a DNA concentration estimate greater than the positive control at 3.125mM and 18.75mM. The remaining metals where either below the positive control DNA concentration estimate or were manually assigned a DNA concentration of zero. Even though the Quantifiler® kit had samples that exceeded the positive control DNA concentration estimate, the Quantiplex kit often had DNA concentration estimates that were greater than the results obtained by the Quantifiler® kit. This can be observed in Figures 4 and 5 represented by blue and red values, respectively.

There are a few similarities between the Quantiplex and Quantifiler® kit in terms of DNA concentration estimates. No obvious trend was observed between the concentration estimates obtained from either kit, as each metal produced a unique series of concentration values. Al was an exception, in which a trend was observed. The DNA concentration estimate decreased as the metal concentration increased. Based on both kits, Al had the biggest impact on the DNA concentration estimate. The Quantiplex kit was unable to produce a DNA concentration estimate at 3.125mM or 18.75mM, while the Quantifiler® kit was unable to produce estimates at 0.5mM-18.75mM. Fe had the next biggest impact on DNA concentration estimates. At a metal concentration of 18.75mM, the Quantiplex kit was able to provide a DNA concentration estimate, however the estimate was below 1% of the positive control. The Quantifiler® kit was not able to provide a DNA concentration estimate for Fe at 3.125mM and 18.75mM. The remaining metals did not produce as drastic results as Al or Fe.

64% and 36% of the metal containing samples for the Quantiplex and Quantifiler® kits, respectively, had DNA quantification estimates above the positive control. Previous research has suggested that heavy metal ions may be fluorescing at a similar wavelength to that of the FAM reporter fluorophore used in both kits (28). This would lead to an increase in the in the overall detected fluorescence and therefore an increase in the DNA concentration estimates. However, no trend was observed between the Quantiplex and Quantifiler® kits in terms of the observed increase fluorescence. This indicates that the heavy metal ions are affecting the quantification chemistry of the kits differently. Future research should be conducted in order to determine which component of the Quantiplex chemistry the metal ions are interacting with. This would help determine if the metal ions are increasing the overall fluorescence by emitting a similar wavelength to that of the reporter fluorophore or if the metal ions are somehow enhancing the

fluorescence by acting as a reflective surface. Additionally, each fluorophore exhibits specific quantum yields, which is defined by the efficiency of a fluorophore to convert light that has been absorbed to emitted light (45). A higher quantum yield results in higher fluorescent intensities (45). Quantum yields are affected by temperature and pH (45). Future research should be conducted to determine if the temperature and pH of the Quantiplex and the Identifiler® Plus kits are affected by the addition of metal ions, as this may directly affect the fluorophore's quantum yield.

Like the Quantiplex kit, the percent RSD values obtained from Quantifiler® did not produce a clear trend between the metal concentrations. The lowest percent RSD for Al was 7.03%, which occurred at 0.015mM. The highest percent RSD of 51.05% for Al was observed at 0.0075mM. For Ca, the lowest percent RSD of 0.93% occurred at 0.0015mM and the highest percent RSD of 30.59% occurred at 0.0025mM. Cu produced its lowest and highest percent RSD values of 1.04% and 68.63% at 0.075mM and 0.5mM, respectively. The lowest percent RSD for Fe was 6.61%, which was observed at 0.015mM. Fe obtained its highest percent RSD of 51.49% at 0.5mM. For Ni, the lowest percent RSD of 16.05% occurred at 0.0025mM and the highest percent RSD of 50.11% was observed at 3.125mM. Pb produced its lowest and highest percent RSD values of 1.99% and 31.69% at 0.0015mM and 0.075mM, respectively.

A definite trend for the percent RSD values between the Quantiplex and Quantifiler® kits did not emerge. It was expected that as the metal concentration increased that the percent RSD would decrease. This was not seen. Most samples exhibited an increased percent RSD value over the positive control. This indicates that the metal ions are acting in a stochastic manner thereby creating variability between the triplicate samples. However, two similarities were observed regarding the lowest and highest percent RSD. The lowest percent RSD for Fe

occurred in both kits at a metal concentration of 0.015mM. Ni produced its highest percent RSD in both kits at a metal concentration of 3.125mM. Running additional replicates of the experiment would help to determine if trends could be deciphered or if stochastic results are inherit to metal containing samples.

Table 6. Estimated DNA concentration and percent relative standard deviation of positive control and metal containing DNA samples using the Quantifiler® kit (28).

| | | Group 1 | | Group 2 | | | | | |
|------------------|--------|---------|--------|---------|--------|--------|--|--|--|
| Metal | Al | Ca | Cu | Fe | Ni | Pb | | | |
| | 0.0891 | 0.0891 | 0.0891 | 0.0813 | 0.0813 | 0.111 | | | |
| Positive Control | 100% | 100% | 100% | 100% | 100% | 100% | | | |
| | 8.44% | 8.44% | 8.44% | 7.90% | 7.90% | 23.15% | | | |
| | 0.0838 | 0.119 | 0.124 | 0.0759 | 0.0891 | 0.151 | | | |
| | 94% | 134% | 139% | 93% | 110% | 136% | | | |
| 0.0025 mM | 16.95% | 30.59% | 3.18% | 24.24% | 16.05% | 6.27% | | | |
| | 0.0613 | 0.135 | 0.0569 | 0.0732 | 0.092 | 0.145 | | | |
| | 69% | 152% | 64% | 90% | 113% | 131% | | | |
| 0.0150 mM | 7.03% | 0.93% | 13.37% | 6.61% | 38.59% | 1.99% | | | |
| | 0.0239 | 0.0792 | 0.0864 | 0.0562 | 0.0933 | 0.16 | | | |
| | 27% | 89% | 97% | 69% | 115% | 144% | | | |
| 0.0750 mM | 51.05% | 12.63% | 1.04% | 44.48% | 32.80% | 31.69% | | | |
| | 0.00* | 0.115 | 0.0593 | 0.0606 | 0.0788 | 0.143 | | | |
| | 0.00% | 129% | 67% | 75% | 97% | 129% | | | |
| 0.5000 mM | 0.00% | 1.81% | 68.63% | 51.49% | 30.96% | 9.09% | | | |
| | 0.00* | 0.0761 | 0.112 | 0.00* | 0.0183 | 0.0946 | | | |
| | 0.00% | 85% | 126% | 0.00% | 23% | 85% | | | |
| 3.125 mM | 0.00% | 29.57% | 8.93% | 0.00% | 50.11% | 11.63% | | | |
| | 0.00* | 0.0659 | 10.9 | 0.00* | 0.00* | 0.00* | | | |
| | 0.00% | 74% | 12267% | 0.00% | 0.00% | 0.00% | | | |
| 18.75 mM | 0.00% | 27.62% | 39.43% | 0.00% | 0.00% | 0.00% | | | |

Positive control refers to a sample that does not contain metal ions.

Reported values are the mean DNA concentration estimates (ng/ μ L), quantification values of DNA samples containing metal ions as a percentage of the positive control and the percent RSD. *Indicates samples that were manually assigned a DNA concentration of zero and a C_T value of 40.

Red values indicate a greater percent value than the Quantiplex results.

Comparison of DNA Samples Containing Metal Ions and Positive Control C_T Values: A Comparison Between the Quantiplex and Quantifiler® Kits

Previous research evaluating metal containing and positive control C_T values with the Quantifiler® kit has indicated that a C_T shift of plus or minus one in relation to the positive control is demonstrably affected by the heavy metal ions (28). This is shown in Table 7.

The most demonstrable C_T shifts were observed in Al containing samples for both quantification kits. This indicates that Al had the biggest impact on the DNA concentration estimates out of all the metals. The Quantiplex kit exhibited human target C_T shifts at metal concentrations of 0.5-18.75mM. A C_T shift was observed at an earlier metal concentration of 0.075mM and through 0.5-18.75mM with the Quantifiler® kit. Both quantification kits exhibited IPC C_T shifts at 18.75mM, while the Quantifiler® also showed IPC C_T shifts at 0.5mM and 3.125mM.

Only one human target C_T shift was observed for Ca. A C_T shift was observed for the Quantiplex kit at 0.075mM. No IPC C_T shifts were exhibited for either quantification kit.

The Quantiplex kit did not show a human target or IPC C_T shift for Cu and Pb. At a metal concentration of 18.75mM, the Quantifiler® kit exhibited a human target and IPC C_T shift in both Cu and Pb metals.

For Fe, a human target C_T shift was observed for Quantiplex at 3.125mM and 18.75mM, while a C_T shift was only observed at 18.75mM in the Quantifiler® kit. Neither kit produced an IPC C_T shift.

No human target or IPC C_T shift was noted for Ni when using the Quantiplex kit. The Quantifiler® kit produced C_T shifts in both the human target and IPC at 3.125mM and 18.75mM.

| | Al | | С | a | Cu | | F | Fe | | Ni | | Pb | |
|-------------------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--|
| | Quantifiler® | Quantiplex | |
| 0.0025mM | - | - | - | - | - | - | - | - | - | - | - | - | |
| 0.015mM | - | - | - | - | - | - | - | - | - | - | - | - | |
| 0.075mM | 1.88 | - | - | -1.19 | - | - | - | - | - | - | - | - | |
| 0.5mM | >8.19 | 9.59 | - | - | - | - | - | - | - | - | - | - | |
| 3.125mM | >8.19 | 12.46 | - | - | - | - | - | 1.36 | 2.18 | - | - | - | |
| 18.75mM | >8.19 | 12.46 | - | - | -6.49 | - | >7.97 | 7.23 | >7.97 | - | >6.77 | - | |
| Mean IPC C _T | | | | | | | | | | | | | |
| | Al | | C | a | Cu | | Fe | | Ni | | Pb | | |
| | Quantifiler® | Quantiplex | |
| 0.0025mM | - | - | - | - | - | - | - | - | - | - | - | - | |
| 0.015mM | - | - | - | - | - | - | - | - | - | - | - | - | |
| 0.075mM | - | - | - | - | - | - | - | - | - | - | - | - | |
| 0.5mM | 2.31 | - | - | - | - | - | - | - | - | - | - | - | |
| 3.125mM | 4.89 | - | - | - | - | - | - | - | 2.32 | - | - | - | |
| 18.75mM | >12.01 | 2.24 | | | 4 74 | | | | >13.04 | | 12.66 | - | |

Table 7. Demonstrable C_T shifts observed with the Quantifiler® and Quantiplex kits. Mean Human Target C_T

The values listed indicate the delta mean between the metal containing samples and the positive control C_T values (28).

More demonstrable human target than IPC C_T shifts were observed when using the Quantiplex kit. This indicates the possibility that the IPC is not as sensitive as the manufacturers have stated or that the metal ions are not affecting the IPC as much as the human target. In most samples, if a demonstrable human target C_T shift was observed with Quantifiler® kit, then an IPC C_T shift was also observed. These results could imply that the metal ions are having a greater affect on the shorter amplicons. At higher metal concentrations, there is a higher likelihood that there are free metal ions in the solution that are not interacting with the control DNA. For this reason, it is plausible that the limited metal ions in solution are therefore interacting with the IPC. The IPCs in both kits will have the roughly the same number of potential metal ions interacting with it in each metal concentration. However, the metal ions will saturate or interact with a smaller IPC amplicon in a denser manner than a longer IPC amplicon. This could be why demonstrable human target and IPC C_T shifts were observed in several metals with the Quantifiler® kit and not with the Quantiplex kit.

When looking at Figures 26 and 27, it becomes apparent that using a demonstrable IPC C_T shift of plus or minus one, in relation to the positive control, is not appropriate for metal ion inhibition detection by the Quantiplex kit. The earliest IPC C_T shift is at approximately 0.2,

which is represented by the red line. An IPC C_T shift at this value would indicate inhibition in most metal containing samples at all metal concentrations. The blue line represents an IPC C_T shift of 0.4. This is the maximum IPC C_T shift value that encompasses most metal containing samples at all concentrations. Therefore, a range of between 0.2-0.4 and greater IPC C_T values could be utilized as an indication of potential metal ion inhibition. However, running additional experimental replicates would help to indicate which values are most appropriate to assign for human target and IPC C_T shifts for metal containing samples. A more concrete C_T shift definition based on empirical data would aid in the true identification of inhibition by metal ions.



Figure 26. Mean C_T values obtained from the amplification of IPC DNA targets in samples containing Al, Ca, and Cu ions. Triplicate samples containing Al, CA, or Cu ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the IPC DNA target and graphed against the metal ion concentration. The dashed black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples. The red line indicates an IPC C_T shift as a difference of 0.2 from the positive control. This is the first observable C_T shift that encompasses most samples across all of the metal ion concentrations. The blue line indicates an IPC C_T shift that encompasses most samples across all of the metal ion concentrations.



Figure 27. Mean C_T values obtained from the amplification of IPC DNA targets in samples containing Fe, Ni, and Pb ions. Triplicate samples containing Fe, Ni, or Pb ions were quantified using the Investigator® Quantiplex Kit. The C_T values were obtained for the IPC DNA target and graphed against the metal ion concentration. The dashed black line is provided as a visual reference to indicate the mean C_T value obtained from the positive control samples. The red line indicates an IPC C_T shift as a difference of 0.2 from the positive control. This is the first observable C_T shift that encompasses the most samples across all of the metal ion concentrations. The blue line indicates an IPC C_T shift that encompasses the most samples across all of the metal ion concentrations.

In Figures 7-12, the logarithmic conversion allows the C_T shift trends to be observed between each kit, despite their differences in mean human target and IPC C_T shift values. For Al and Fe containing samples, both kits indicated clear inhibition at higher metal concentrations. This was not demonstrated for Ca and Cu. The Quantifiler® kit clearly showed that inhibition was occurring in Ni and Pb containing samples at higher metal concentrations. This was not evident with the Quantiplex kit. Metal containing samples often exhibit random or stochastic results, but the fact that some concordances could be drawn between two difference research studies, begins to indicate how metal containing samples behave. Future studies should be conducted to investigate the reliability of these concordant results.

STR Amplification and STR Profiles

Figure 13 shows the percentage of alleles obtained from each metal at each concentration when run with the Identifiler® Plus kit. The DNA concentration estimates for each metal at each concentration are listed in this figure. There were only three instances were a zero DNA concentration estimate was manually assigned. All occurred within the three highest metal concentrations of Al and resulted in either a poor or no profile. These results could be due to strong metal ion interactions, which are preventing the DNA from being injected into the capillaries during the electrokinetic injection. Future studies should consider concentrating samples that received a zero DNA quantification estimate, as this may yield more observed alleles. A higher injection voltage and longer injection time should also be experimentally tested because it may help to disrupt the metal ion and DNA interactions. Therefore, an undetermined or manually assigned zero DNA concentration estimate should be treated with caution. The sample may have DNA present which could be used for exclusionary purposes.

No profile was obtained in Cu and Ni containing samples at 18.75mM, even though both samples had a DNA concentration estimate of approximately 0.1ng/µL. Ca exhibited a partial profile at 18.75mM with a corresponding DNA concentration estimate of 0.079ng/µL. These results indicate that a higher DNA concentration estimate does not ensure that a profile will be obtained. Interestingly enough, these three samples formed a thick precipitate after STR amplification with the Identifiler® Plus kit. Al, Cu, Fe, and Pb containing samples formed a light

precipitate at metal concentrations of 3.125mM, 18.75mM or both. In all instances except Al, the metal containing samples produced a full profile. An observation of a thick precipitate may indicate that no profile will be obtained. At higher metal concentrations, there are more metal ions in solution which are capable of interacting with the Identifiler® Plus chemistry during PCR amplification. The repeated heating of the samples may have served as a catalyst and increased the chemical reaction between the metal ions and the Identifiler® Plus chemistry, resulting in a thick precipitate. It's plausible that in higher metal containing sample concentrations that the DNA is being sequestered by its interactions with the metal ions in the precipitate. Thus, explaining why no profile was obtained, when DNA was in fact present in the sample. Future research should be conducted to test the above hypothesis.

Similar observations in terms of percent alleles obtained were concluded by previous research (39). Full profiles or 100% of the alleles were obtained in all metal containing samples at concentrations of 0.0025-0.5mM. Al was an exception, as only a full profile was obtained at the lowest metal concentration of 0.0025mM. Pb was also an exception because samples that contained this metal produced a full profile a 3.125mM. At 18.75mM, no alleles were observed for any metal containing sample. These results are fairly consistent. Both experiments showed that Al had the greatest affect on the percent of alleles obtained and at the highest metal concentrations the percent of alleles obtained decreases.

Profile Peak Heights

One trend observed with Figures 14-19 is that the majority of peak heights for each metal at each concentration fall within the 75th percentile. This indicates that most of the alleles observed had higher rather then lower peak heights. No clear trend emerged when viewing the 25th and 75th percentiles as one unit. It was hypothesized that as the metal ion concentrations

increased that the peak heights would decrease in a gradual manner. Figure 28 is provided as an example. This however was not observed, as an increase in peak heights were seen in some higher metal concentrations. However, a general trend for all metals was observed and described previously in the results section. These trends are important to make note of because they may be able to help identify at which RFU ranges metal containing samples will produce peak heights.



Figure 28. Electropherogram of allele peak height fluorescence obtained from samples containing 0.0025, 0.075, 0.5 or 3.125mM Al ions. Duplicate samples containing Al metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights and allele number were displayed for each called allele at each locus when using the 6-FAMTM dye label. The same four loci are displayed above in samples containing 0.0025mM (A), 0.075mM (B), 0.5mM (C), or 3.125mM (D) Al ions. The solid black line in A provides a visual reference of 2000 RFUs. The long black dashed line in B provides a visual reference of 1500 RFUs. The dotted black line in C provides a visual reference of 100 RFUs. A detection threshold was set at 50 RFUs. All panels are displayed on the same scale.
When examining Figures 20-25 a definite trend was not observed between the metal concentrations, allele peak heights and fragment sizes. It was expected that as the metal concentration increases that the allele peak heights would decrease as the fragment size increased. This was seen for some of the metal containing samples at various metal concentrations. Figure 29 is provided as an example of this trend. A slight increase and nearly consistent peak heights as the fragment size increased was also noted for several metal containing samples at various concentrations. These results were fairly consistent with previous research conducted (39). However, one noticeable difference was that the peak heights obtained in the previous research were approximately 500 to 1000 RFUs smaller. This indicates that metal ions often create stochastic results that are difficult to define in terms of absolute trends. More experimental replicates and research is needed to help define these trends.



Figure 29. Electropherogram of allele peak height fluorescence obtained from samples containing 0.075mM Al ions. Duplicate samples containing Al metal ions were amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. The allele peak heights and allele number were displayed for each called allele at the sex determining region (Amelogenin) and fifteen STR loci. The solid black line in A provides a visual reference of 1500 RFUs for the 6-FAMTM dye labeled loci. The long black dashed line in B provides a visual reference of 2500 RFUs for the Vic® dye labeled loci. The small black dashed line in C provides a visual reference of 1500 RFUs for the NEDTM dye labeled loci. The dotted black line in D provides a visual reference of 1000 RFUs for the PET® dye labeled loci. A detection threshold was set at 50 RFUs. All panels are displayed on the same scale.

CHAPTER 6

CONCLUSIONS

Heavy Metal Ion Inhibition: How to Approach the Sample

If a laboratory receives a sample that may be inhibited with metal ions, they should pay close attention to the DNA concentration estimate and the C_T shifts that are occurring between the non-template control or the standards and the unknown sample. A low DNA concentration estimate or C_T shift may indicate potential inhibition across a variety of metal concentrations. Therefore, it is difficult to determine the concentration of metal ions that are present in the sample. It is important to keep in mind, however, that most metal containing samples were capable of producing a full STR profile. Even at the highest metal ion concentrations, some alleles were obtained. Based on the limited results obtained in this study, it would be recommended to run all samples suspected of metal ion inhibition for STR analysis.

The Investigator Quantiplex® Kit versus The Quantifiler® Human DNA Quantification Kit

A conclusion cannot be drawn about whether the Investigator® Quantiplex Kit or the Quantifiler® Human DNA Quantification Kit provides more accurate DNA concentration estimates in the presence of metal ions. This is because metal containing samples often produce stochastic results, which are difficult to compare. Future research needs to be conducted. The observed stochastic results can, however, be used to help determine the mechanism of inhibition by metal ions. If the metal ions were specifically binding to forensically relevant sections of the DNA, or to the DNA polymerase, it may produce results where a trend could be observed. This

is because specific binding to DNA sections or to the DNA polymerase could prevent the overall successful completion of PCR for the DNA strands or compounds affected, as each would be affected at the same point in each cycle of PCR. If the metal ions are binding in a random manner to the DNA strands, DNA polymerase or both, then it may produce stochastic results. It is also plausible that the metal ions are binding randomly to the deoxyribonucleotide triphosphates (dNTPs) before being incorporated into the complementary DNA strand, which could also result in stochastic results. It could be speculated that the random nature of the metal ions allowed for the overall successful completion of PCR in most metal containing samples because each DNA strand or compound was not affected in the same manner at each PCR cycle. Therefore, the mechanism of inhibition of metal ions could be explained by the random binding to forensically relevant DNA sections, DNA polymerases, dNTPs or a combination of these compounds.

The Quantiplex and the Quantifiler® kits can be compared in terms of potential use for laboratories. Both quantification kits are very user friendly. The time required to do the bench work and instrument setup are fairly similar and straightforward. One notable difference between the two quantification kits is the instrument run time. When running both kits on the 7500 Real-Time PCR System, the Quantiplex kit takes approximately one hour while the Quantifiler® kit takes approximately 2 hours to complete. A similar amount of time and effort was used to analyze the produced results. The volume of cases or research conducted in the laboratory will determine if a decrease in the instrument run time is beneficial.

The Quantiplex and the Quantifiler® kits can also be compared in regards to instrumentation use. The Quantiplex kit can be used on the RotoGene® Q (Qiagen) or on the 7500 Real-Time PCR System. The Quantifiler® kit can be run on the 7900HT Real-Time PCR

67

System (Applied Biosystems) and also the 7500 Real-Time PCR System. Thus a difference emerges, the Quantiplex kit is combatable with two real-time platforms (Qiagen and Applied Biosystems), while the Quantifiler® kit is only combatable with the Applied Biosystems' platform. Depending on the instruments the laboratory has or is planning on obtaining will help dictate which qPCR kit is most appropriate to use.

Furthermore, the Quanitplx and Quanifiler® kits vary in cost. Currently the Quantiplex kit costs \$450 for 200 reactions and the Quantifiler® kit costs \$1,128 for 400 reactions (20, 46). If a laboratory were to use the Quantiplex kit they would be saving approximately 57 cents for each reaction. While this number may not seem very substantial, the savings could add up. Again, the amount of samples received and research conducted by the laboratory would help to determine which kit is best for their use.

When using a real life example, the advantages of the Quantiplex kit become apparent. The UNT Center for Human Identification received 288 unidentified human remain (UHR) cases during the months of January through June in 2013 (Dixie Peters, Technical Leader of the Missing Persons Unit). If each of the UHR cases were quantified with the Quantiplex kit instead of the Quantifiler® kit, it would have roughly saved the analysts three hours or half of the required quantification time. Additionally, the Quantiplex kit would have approximately saved the laboratory \$164.16 over six months.

The Value of IPCs in Detecting Inhibition

Quantiplex and Quantifiler® both include an IPC in their quantification kits. In each kit, the IPC is supposed to indicate samples that may be inhibited so that they can be properly identified and processed before running downstream analyses. The IPC in the Quantiplex kit is 200 base pairs in length, which is similar in length to most STRs (29, 40). The longer length of the IPC found in the Quantiplex kit is very important because it is capable of giving a true representation of inhibition in DNA samples. The Quantifiler® IPC amplicon is 79 base pairs in length, which is 121 base pairs shorter than the IPC in the Quantiplex kit (25). While the shorter IPC is capable of indicating potential inhibition, it will not correspond to a true representation of inhibition occurring in the sample. This is because shorter amplicons will be either be more or less affected by the inhibitor; thereby making downstream analysis decisions more difficult.

Previous research conducted by Opel et al showed that Ca containing samples produced a minimal C_T shift. For other inhibitors like melanin, hematin and tannic acid, a C_T shift was observed for larger amplicons as the inhibitor concentration increased, while no C_T shift was observed for smaller amplicons. A C_T shift was observed in all amplicons as the inhibitor concentration increased for humic acid. Collagen containing samples exhibited a slight reduction in C_T values for all amplicons, however larger amplicons required a higher inhibitor concentration to produce a C_T a shift. A minimal or demonstrable C_T shift value was not defined and thus made comparison difficult. However, these results confirm that each inhibitor will produce unique C_T shift values. They also indicate that while small amplicons may be reliable for detecting inhibition in some samples, that it is not a consistent rule for all inhibitors (32). Future studies should be conducted in order to determine the appropriate C_T shift values for each inhibitor as well as the appropriate size amplicon used to determine the presence of inhibition. These studies are important to conduct because they would aid in the understanding of inhibition as well as in the generation of complete STR profiles.

Based on the results obtained in this study, it is recommended that a low C_T shift value be used to detect metal ion inhibition with the Quantiplex kit. However, it may be entirely plausible that relying on IPC C_T shifts is not the most dependable method for determining the presence of

69

inhibitors. Future research should thoroughly investigate if utilizing IPC C_T shifts is really the most beneficial method for detecting inhibition in qPCR.

REFERENCES

1. Jeffreys AJ, Wilson V, Thein SL. Hypervariable 'minisatellite' regions in human DNA. Biotechnology 1985;24:467-72.

2. http://www.nfstc.org/pdi/Subject00/pdi_s00.htm.

3. Hampikian G, West E, Akselrod O. The genetics of innocence: Analysis of 194 U.S. DNA exonerations. Annu Rev Genomics Hum Genet. 2011;12:97-120.

4. Alaeddini R. Forensic implications of PCR inhibition--A review. Forensic Sci Int Genet 2012 May;6(3):297-305.

5. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997 Oct;63(10):3741-51.

6. Kontanis EJ, Reed FA. Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. J Forensic Sci 2006 Jul;51(4):795-804.

7. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci 1994 Mar;39(2):362-72.

8. Eckhart L, Bach J, Ban J, Tschachler E. Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. Biochem Biophys Res Commun 2000 May;271(3):726-30.

9. Larkin A, Harbison S. An improved method for STR analysis of bloodstained denim. Int J Legal Med. 1999;112(6):388-90.

10. Tebbe CC, Vahjen W. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl Environ Microbiol 1993 Aug;59(8):2657-65.

11. Primorac D. The role of DNA technology in identification of skeletal remains discovered in mass graves. Forensic Sci Int 2004 Dec;146 Suppl:S63-4.

12. Rossen L, Norskov P, Holmstrom K, Rasmussen OF. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 1992 Sep;17(1):37-45.

13. Katcher HL, Schwartz I. A distinctive property of tth DNA polymerase: Enzymatic amplification in the presence of phenol. BioTechniques 1994 Jan;16(1):84-92.

14. Warburg O Christian W. Isolation and crystallization of enolase. Biochem Z 1942; 310:384-421.

15. Sambrook J, Russell D. Molecular Cloning A Laboratory Manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.

16. Ahn SJ, Costa J, Emanuel JR. PicoGreen quantitation of DNA: Effective evaluation of samples pre- or post-PCR. Nucleic Acids Res 1996 Jul;24(13):2623-5.

17. Hopwood A, Oldroyd N, Fellows S, Ward R, Owen SA, Sullivan K. Rapid quantification of DNA samples extracted from buccal scrapes prior to DNA profiling. BioTechniques 1997 Jul;23(1):18-20.

18. Walsh PS, Varlaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Res 1992 Oct;20(19):5061-5.

19. Budowle B, Hudlow WR, Lee SB, Klevan L. Using a CCD camera imaging system as a recording device to quantify human DNA by slot blot hybridization. BioTechniques 2001 Mar;30(3):680-5.

20. www.lifetechnologies.com.

21. Mandrekar MN, Erickson AM, Kopp K, Krenke BE, Mandrekar PV, Nelson R, et al. Development of a human DNA quantitation system. Croat Med J 2001 Jun;42(3):336-9.

22. Hayn S, Wallace MM, Prinz M, Shaler RC. Evaluation of an automated liquid hybridization method for DNA quantitation. J Forensic Sci. 2004 Jan;49(1):87-91.

23. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology 1992 Apr;10(4):413-7.

24. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. Biotechnology 1993 Sep;11(9):1026-30.

25. Applied Biosystems. Quantifiler® Kits User's Manual. Foster City, CA, March 2013.

26. Rutledge RG, Cote C. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 2003 Aug;31(16):e93.

27. Guescini M, Sisti D, Rocchi MB, Stocchi L, Stocchi V. A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics 2008 Jul;9:(1)326-38.

28. Combs LG, Warren JE, Roby RK, Golden TD. Effects of metal ion PCR inhibitors on results obtained with the Quantifiler® Human DNA quantification kit. Proceedings of the Association of Forensic DNA Analysts and Administrators; 2013 May 29-31; San Antonio, TX.

29. Qiagen. Investigator® Quantiplex Handbook. Hilden, Germany, Oct 2012.

30. Swango KL, Timken MD, Chong MD, Buoncristiani MR. A quantitative PCR assay for the assessment of DNA degradation in forensic samples. Forensic Sci Int 2006 Apr;158(1):14-26.

31. Swango KL, Hudlow WR, Timken MD, Buoncristiani MR. Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples. Forensic Sci Int 2007 Jul;170(1):35-45.

32. Opel KL, Chung D, McCord BR. A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci 2010 Jan;55(1):25-33.

33. Stahlberg A, Aman P, Ridell B, Mostad P, Kubista M. Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. Clin Chem 2003 Jan;49(1):51-9.

34. King C, Debruyne R, Kuch M, Schwarz C, Poinar H. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques 2009 Nov;47(5):941-9.

35. Thomas JT, Berlin RM, Barker JM, Dawson Cruz T. Qiagen's Investigator Quantiplex kit as a predictor of STR amplification success from low-yield DNA samples. J Forensic Sci 2013 Sep;58(5):1306-9.

36. Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 1999 Aug;17(8):804-7.

37. Kabata-Pendias A, Pendias H. Trace elements in soils and plants. 3rd ed. Boca Raton, FL: CRC Press, LLC; 2001.

38. Trueman CNG, Behrensmeyer AK, Tuross N, Weiner S. Mineralogical and compositional changes in bones exposed on soil surfaces in Amboseli National Park, Kenya: Diagenetic mechanisms and the role of sediment pore fluids. J Archaeol Sci. 2004; 31: 721-39.

39. Gaydosh LJ, Deleon VH, Golden TD, Warren JE, Roby RK. Metal Ions as Forensically-Relevant Inhibitors of PCR-based DNA Testing. Proceedings of the American Academy of Forensic Sciences; 2013 Feb 18-23; Washington DC.

40. http://www.cstl.nist.gov/biotech/strbase/multiplx.htm.

41. Applied Biosystems. AmpFlSTR® Identifiler® Plus Amplification Kit User's Guide. Foster City, CA, Mar 2012.

42. Applied Biosystems. GeneAmp® PCR System 9700 Base Model User's Manual. Foster City, CA, Jun 2010.

43. Applied Biosystems. 3130/3130xl Genetic Analyzers Getting Started Guide. Foster City, CA, Jun 2010.

44. Applied Biosystems. GeneMapper® *ID-X* Software Version 1.4 User Bulletin. Foster City, CA, Dec 2012.

45. Marras SA. Selection of fluorophore and quencher pairs for fluorescent nucleic acid hybridization probes. Methods Mol Biol 2006;335:3-16.

46. http://www.qiagen.com/products/catalog/assay-technologies/investigator-quantiplex-kit.