McDaniel, Ethan L., A Bone and Buccal Sensitivity Study Comparison and Stability Study using the PowerPlex® Fusion 6C System. Master of Science (Biomedical Sciences), May, 2018, pp., 6 tables, 6 illustrations, bibliography, 44 titles.

A validation study, a bone sensitivity study and a stability study were performed using the PowerPlex® Fusion 6C System. These studies were performed on a 7500 Real-Time PCR System, 9700 GeneAmp Thermocycler and 3500xL Genetic Analyzer. Buccal DNA was used to develop a method to analyze the DNA profiles gathered during the bone sensitivity study and stability study. DNA profiles for specific concentrations of DNA in solution were obtained during the bone sensitivity study. The stability study showed profiles exhibiting the effects of metal PCR inhibitors being introduced to the DNA extract solutions. Full profiles were obtained for calcium concentrations less than 7.35 mM, while the instrument was fully inhibited for copper concentrations between 0mM and 7.35 mM. Based on the limited data, the PowerPlex Fusion 6C System cannot tolerate copper when present in DNA solutions; whereas, calcium may be tolerated as an inhibitor up to 7.35mM.

A BONE AND BUCCAL SENSITIVITY STUDY

COMPARISON AND STABILITY STUDY

USING THE POWERPLEX® FUSION

6C SYSTEM

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Ethan L. McDaniel, B.S.

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INTRODUCTION

Deoxyribonucleic acid, or more commonly referred to as DNA, is found within most cells of living organisms. It is composed of two categories of nucleic acids: purines and pyrimidines. Purines consist of adenine and guanine while pyrimidines consist of thymine and cytosine. These four nucleic acids make up the genetic code. Since DNA sequences have never been shown to be identical in two individuals, with the exception of identical twins, it is a unique identifier for forensic purposes. In forensics however, the whole DNA sequence is not analyzed; only sections called short tandem repeats, or STRs. [1] Short tandem repeats are highly variable, or polymorphic, sections of DNA that are found in varying length repeating sequence. [1,2] The lengths of these short tandem repeats that are analyzed during genetic analysis are called alleles.

DNA in Court

In order for any science to be accepted in court, it must first meet two standards. The standards state that the scientific evidence must be generally accepted by the scientific community and that the methodology used is sound and tested. [3] These are more commonly known as the Frye and Daubert Standards, respectively. [3]

The Frye Standard resulted from the case, *Frye v. United States*, in 1923. [4] Frye tried to get evidence admitted into the court that used a novel scientific technique at the time. The evidence got rejected due to the technique not being a "well-recognized scientific principle or discovery," resulting in the requirement that scientific evidence must be generally accepted by the scientific community. [5] Roughly 70 years later, the Frye Standard got replaced by the Daubert Standard.

This new standard was established in 1994 based on the *Daubert v. Merrell Dow Pharmaceutical* trials. These series of trials and appeals established two new rules for scientific evidence. The judge determines when scientific evidence is admissible, and the scientific techniques used must be based on scientific principles where the basis can be demonstrated. [6]

DNA was first used in court as evidence against Colin Pitchfork in England during 1986. [7] The first time DNA was used as evidence in court in the United States was against Tommie Lee Andrews in 1987. [8] DNA had been accepted by the scientific community and therefore met the Frye Standard. In both cases, DNA found in blood that was left behind at the crime scene was able to be matched to the reference sample that was obtained. In the Andrews case, DNA found in seminal fluid that was left behind was also able to be matched to Andrews' reference sample.

Sources of DNA

DNA used in forensic samples does not just come from blood and seminal fluid. DNA can be found in all different types of biological material. A few of the most commonly found biological materials containing DNA are buccal samples, blood, seminal fluid and skin cells. Buccal swab samples are used mostly as reference samples due to this type of sample being collected from known individuals and containing high quantities of DNA. [9] Blood, seminal fluid and skin cells are commonly found on evidentiary items and used when identifying an unknown individual. Items of evidence on which DNA evidence can be found on include clothing worn by the victim or perpetrator, the weapon used in homicide cases, and potentially on natural objects such as trees, bushes or grass if the crime occurred outside.

Buccal samples are collected from the inside cheek using a swab. Since this type of sample comes directly from the body, there is no question as to whom it belongs to. Buccal swab collection is also non-invasive, quick and easy to perform. [9] Blood can be collected from a number of different objects, such as a wall from a swipe or blood spatter pattern, a piece of clothing that was bled on, a weapon that punctured the skin, or any other object that might have been exposed to blood. Seminal fluid contains both skin cells and sperm cells from the male. [10] When a rape occurs, and a male is present, the skin cells from the male and the victim could produce DNA profile containing all the profiles of everyone that was involved. However, unless another male is present, the sperm cells can produce a unique single source profile, which is then used to identify the male individual. Skin cells can be found on a shirt due to perspiration, in seminal fluid when a male ejaculates, or even on an object not on the individual due to dandruff or dead skin cells falling off the body.

Sometimes these sources of DNA are found on objects that make obtaining DNA difficult. Some of these objects are bullets, explosives or bones. When loading a gun, oil and dead skin cells containing DNA transfer to the bullet and/or casing. This type of DNA is often referred to as touch DNA and can occasionally determine who loaded the gun. [11] DNA from bullets and casings can be useful in crimes that involve murder and robberies where a bullet was fired.

Another alternate source of DNA can be recovered from explosives. DNA from explosives is used when public bombings or terrorist attacks occur. Touch DNA can be present when putting together an explosive. [12,13] The amount of useful DNA present after an explosive detonates is significantly less than an undetonated bomb due to the high impact and heat that is created from

the explosion. [12,13] This can cause problems in determining who the DNA belongs to due to low-template DNA, degraded DNA or copper inhibitors being present. [12,13] Having inhibitors or degraded DNA present can cause potential partial profiles, allele drop-out or no amplification. [12,13]

Two types of evidence, bullets and explosives, can have "touch DNA" present. Touch DNA is small amounts of DNA left on an object by just touching it and is considered low copy number DNA. [11] The oil from the fingers or sweat can contain dead skin cells that still contain DNA. Touch DNA can cause alleles to drop-out and not show up in the profile, there might not be enough DNA to amplify, or there might be inhibitors that are gathered with the DNA. [14] These potential problems can all cause inconclusive results.

A third alternate source of DNA is from bone. Bone marrow contains the highest concentration of DNA in a fresh corpse. [15] However, in cases that use bone to obtain DNA, like missing person identification and identifying historical remains cases, the bones are not fresh. Bones that have been exposed to the environment have less DNA than fresh corpses due to degradation [16]. These bones are still useful though since it has been shown that bone DNA can remain stable even after being exposed to the environment for decades. [17]

Validation Guidelines

Forensic DNA testing kits take advantage of STRs and are used to obtain an individual's DNA profile. [2] These kits however, must go through validation studies before they are used on forensic evidence. There are two types of validation studies: developmental and internal.

Validation studies are a component of good quality control and quality assurance systems and are a requirement for laboratories to become and maintain accredited. Internal validation studies lead to more reliable results that allow different labs to compare results. Both types of validation studies are required to ensure reliable results that can be replicated at any lab across the nation. [18]

A developmental validation study is performed by the manufacturer of a DNA testing kit while internal validation studies are performed by an individual laboratory. [19] Developmental validation studies are more specific and look at areas that internal validations do not. Some of these are "the characterization of the genetic marker, species specificity, and population studies." [19] The characterization of genetic markers ensures that the markers meet the Hardy-Weinberg equilibrium requirements. There are five conditions that must be met to remain in Hardy-Weinberg equilibrium: a large breeding population, random mating, no allelic frequency changes due to mutation, no immigration or emigration, and no natural selection. [20] If these five conditions are met, then the marker is considered to be in equilibrium. If the markers being analyzed are all in Hardy-Weinberg equilibrium, then the product rule can be applied. The product rule states that the probability of two or more independent events occurring together is calculated by obtaining the product of probabilities of the events. [21] This then allows forensic DNA analysts to determine approximately how likely it is to see that DNA profile. Developmental validation studies look at species specificity to ensure the DNA being analyzed is actually human. Since different species, especially humans and higher primates, can share similar DNA sequences, it is important for these kits to only amplify human DNA. [22]

Internal validation studies, outlined in the FBI QAS, include some of the same studies conducted in a developmental validation study, and consist of stability studies, "sensitivity and stochastic studies, mixture studies, known and non-probative evidence samples, reproducibility and precision, and contamination assessment," along with PCR-based studies. [19] Sensitivity studies allow the laboratory to test the sensitivity levels of the tests that are performed. By testing these levels, an ideal concentration of DNA can be obtained to use during amplification. [23] Obtaining different profiles at different concentrations can help when performing stability studies. A stability study demonstrates the kit's performance when known inhibitors are present. [24] Knowing how the kit performs with inhibitors gives the analyst more information on how to reduce and prevent the issues that do arise when inhibitors are present.

Mixture studies determine the kit's ability to separate two profiles in one solution. [23] This is important especially for intimate samples to determine possible contributors. Minute traces of DNA from a second profile could be mistaken as a stutter peak, therefore, stutter studies are also performed. Stutter is what is observed in genetic analysis due to stochastic, or random, events during PCR that produce DNA strands normally one repeat unit shorter or longer than the target DNA. [25] These stochastic events typically occur in roughly 10% of the DNA found in the amplification solution. Performing a study to observe these tendencies helps eliminate these random peaks and obtain a "clean" profile.

Once all of these studies are performed and a validated analytical method made, case-type samples are analyzed. This is done to provide the analyst with practice using the validated

method. Experience with what profiles might look like when this method is used on real evidence is also obtained.

History of DNA Analysis

When DNA analysis first started, a method called restriction fragment length polymorphism, or RFLP, analysis was used. [26] This method used restriction enzymes to cut the DNA strands into smaller fragments. These fragments would then be separated by length in a gel; a technique called gel electrophoresis. The separated fragments were then transferred to a filter and fluorescently tagged. The RFLP method was very laborious, as every step was by hand. RFLP required a lot of non-degraded DNA which is hard to obtain from evidentiary samples, and it was difficult to distinguish individual alleles that were of similar length. [26]

After a few years of performing RFLP analysis, short tandem repeat, or STR, analysis was developed. This new method is what is currently used. STR analysis uses DNA analysis kits along with PCR to obtain a DNA profile. DNA analysis kits include DNA binding primers, buffers and DNA polymerases, allowing the analysis of DNA to be quicker and not reliant on large quantities of pristine DNA. [27] By using these kits, more reliable results are obtained, and DNA profiles are able to be repeatedly obtained more accurately.

In 1997, the FBI decided that there needed to be specific loci being analyzed in each kit. This led to the Combined DNA Index System 13, or CODIS 13, markers. [28] These thirteen loci were required to be in all forensic DNA analysis kits. This standardized the loci being analyzed across the country, allowing different laboratories to compare results with one another. These thirteen

loci were the minimum required loci for twenty years, until in 2017, the FBI added seven new loci to the list of required loci, bringing the required number of specific loci to twenty. [29]

Kit Comparison

Validation studies have to be done on all DNA testing materials that are used in laboratories. Two kits that are comparable to each other that are used for quantification are the QuantiFiler™ Trio DNA Quantification Kit (Applied Biosystems™, Foster City, CA) and the PowerQuant® System (Promega Corporation, Madison, WI). There are a few different amplification kits that meet the FBI requirements. Two amplification kits that are considered equivalent are the GlobalFiler™ PCR Amplification Kit (Applied Biosystems™, Foster City, CA) and the PowerPlex® Fusion 6C System (Promega Corporation, Madison, WI); however, these kits do have some slight differences from their competitor's kit.

Quantification of DNA happens after the extraction process and determines how much DNA is in the sample. This first step is necessary for the later steps of the DNA analysis process. If too much DNA is present in the amplification solution, the alleles will be blown out, resulting in peaks that do not represent the true intensity of DNA or split-peaks. If there is not enough DNA present, then the amplification primers might bind to themselves, and drop-out of some alleles may occur. Two common quantification kits that are used today are QuantiFiler™ Trio DNA Quantification Kit and the PowerQuant® System. QuantiFiler™ Trio DNA Quantification Kit uses four dyes to look at three targets (small autosomal, large autosomal and y), uses five different standard concentrations, takes approximately an hour to run and is able to quantify a DNA sample that has less than 1 pg/μL. [30] The PowerQuant® System uses five dyes to

analyze four targets (degradation, y, autosomal and the internal PCR control or IPC), uses four different standard concentrations, takes approximately an hour to complete total analysis and is able to quantify a sample with roughly 6pg of DNA or less than 1 pg/μL. [31] During a comparison of the standard curves produced by these two kits, the PowerQuant® System displayed the most consistency in amplification efficiency along with the lower coefficient of variation. [32]

The PowerPlex® Fusion 6C System, along with the GlobalFiler™ PCR Amplification Kit, are two of many kits that are used in amplification and genetic analysis. These two kits are very similar but have some slight differences. The GlobalFiler™ PCR Amplification Kit is a six-dye kit that looks at 24 loci. [33] It is optimized for database and single-source samples while having a six-month shelf-life once opened. [33] The GlobalFiler™ PCR Amplification Kit has a kit Probability of Identity (PI) value of 7.73 x 10^-28. [34] The PI is the probability that two randomly selected individuals will have the same genotype at the selected locus. The kit PI is the combined PI for all loci in that particular kit. The complementary kit, PowerPlex® Fusion 6C System, utilizes six dyes to analyze 27 loci, has a high inhibitor tolerance and requires as little as 125pg of DNA to obtain a full profile. [27,34] The PowerPlex® Fusion 6C System has a kit PI value of 2.3 x 10^-32. [34] During a comparison study, the GlobalFiler™ PCR Amplification Kit was shown to be approximately two times more sensitive than the PowerPlex® Fusion 6C System. [35] This same study showed that the PowerPlex® Fusion 6C System yielded "better intra-colour balance and preservation of mixture ratio," leading to the use of the GlobalFiler™ PCR Amplification Kit in older, degraded samples while the PowerPlex® Fusion 6C System is used in intimate samples. [35]

Inhibitors

When analyzing DNA from an evidentiary sample, pristine and copious amounts of DNA are not always available. Evidentiary samples often contain old or degraded DNA which has an increased likelihood that PCR inhibitors might be present. [16] An inhibitor has the ability to prevent optimal functionality of PCR systems and can be found throughout the environment and within the human body [16]. PCR inhibitors prevent optimal PCR functionality by interacting with the template DNA in extraction, quantification and amplification, interacting with the nucleotides, binding with the amplification primers and polymerase enzymes and by affecting the magnesium present. [16]

It is not known how these inhibitors interact with the DNA to cause reduced functionality of PCR, but there are a few mechanisms that have been proposed. Three proposed mechanisms are competitive inhibition with the polymerase, interaction with the DNA itself, and interaction with the polymerase during primer extension. [36] Possible consequences of having a sample inhibited are reduced yield of the amplicant, reaction failure, reduced detection sensitivity, and false-negative results. [16,37,38,39] These consequences can cause the analyst to come to an inconclusive, false positive or false negative result when analyzing the DNA profiles. This could potentially lead to an innocent individual being convicted of a crime or an individual who is guilty of a crime to be shown as innocent.

When performing forensic DNA analysis, there are a few possible ways for the analyst to detect whether an inhibitor is present. When increased detection threshold values or decreased

exponential phase slopes are detected during real-time PCR, an inhibitor may be present. [16] These results occur due to the decreased amount of available binding sites on the target DNA strand for the fluorescent tags. The presence of an inhibitor during the PCR amplification process is recognized when the internal standards present in each kit fail to be amplified. [16]

There are methods to prevent inhibitors from being present in the quantification, amplification, or genetic analysis process. The most common method is to dilute the extract solution to obtain more reliable quantification values or to dilute the amplification solution to reduce the amount of inhibitor present. [16] Diluting the solution does however increase the risk of potentially having a less than optimal amount of DNA present for quantification or amplification. Other methods that have been proposed and shown to have some positive effects have been purifying the DNA extract solution using silica-based methods to remove inhibitors from solution completely, using DNA-specific dyes with longer excitation wavelengths during real-time PCR, adding more polymerase enzymes and adding amplification facilitators like bovine serum albumin to bind to the inhibitors. [16]

Some examples of inhibitors that were used by the manufacturers of the PowerPlex® Fusion 6C System in the developmental stability study are hematin, humic acid, tanic acid and ethylenediaminetetraacetic acid (EDTA). [24] Using these inhibitors allowed the manufacturers to observe how the kit reacts to neutrally and negatively charged inhibitors, however, positively charged inhibitors were not tested. Since DNA is negatively charged, it is necessary to know how the kit reacts in the presence of inhibitors that are positively charged, such as metal ions.

Copper and Calcium Properties

Two known metal inhibitors are calcium and copper. Calcium is an alkaline earth metal with an oxidation state of 2+, meaning it has two valence (outer) electrons out of a possible eight in the outer shell. Calcium is the fifth most abundant element in the earth's crust. [40] The average human body contains 2% calcium, where 98% of that is found as a component of bone. [40]

Copper is a transition metal with oxidation states of 2-, 1-, 2+, 3+ and 4+. These oxidation states mean the number of free electrons change depending on the oxidation state of copper. Copper (III) and copper (IV) ions are mostly found as oxides such as Potassium cuprate and Yttrium barium copper oxide. The copper ions of interest for biological material are copper (I) and copper (II) oxidation states. Copper (I)/(II) are found in biological material such as tissue and bone. [41] Some inorganic material copper is a component of are bullets, bullet casings and electrical wiring.

Copper and calcium are occasionally encountered in forensic laboratories. It is important to know how the kit being used reacts to the presence of copper and calcium, so the analyst can be prepared to deal with drop-out, reduced peak height, or even no amplification that may occur during the PCR process or during genetic analysis.

Significance

Developmental and internal validation studies are part of kit evaluation. Internal validation studies provide stutter tendencies, stochastic and analytical threshold values, peak-height ratios and DNA concentration in the samples for amplification. Stability studies are produced during

the developmental validation study and show the state of the kit's performance under extreme conditions. The information from both types of validation studies determine whether the kit is working according to the manufacturer's recommendations and whether the laboratory's results are concordant with the manufacturer's. Conducting these studies in conjunction with the stability study will allow for an understanding of the PowerPlex® Fusion 6C System performance on inhibited samples.

The metals being tested in the inhibitory study, copper and calcium, are found in skeletal remains and other forensic samples. Calcium is encountered in forensic samples when analyzing skeletal remains from missing persons and mass disaster cases, as it is a major component of bone. Sources of copper in forensically related evidence include wires from explosive devices and in bullet cartridges. Copper and calcium were inhibitors not tested by Promega in their developmental study, therefore, additional information about the kit's performance will be assessed. [24]

Objectives

Two major goals will be achieved in this study. The first is to determine if there were any statistical differences between bone and buccal DNA for peak heights obtained in full DNA profiles. An analytical method that is used for buccal samples can be used on bone samples, but only if the sensitivity data for both types of samples look similar. This is because the quantity of DNA found in bone is much lower than that which is found in buccal swab samples. The second is to determine what effects positively charged inhibitors have on the amplified DNA samples.

Determining these effects will assist future analysis of electropherograms when positively charged ions are present in DNA evidential samples.

RESEARCH DESIGN AND METHODOLOGY

The collection and analysis of buccal samples are approved under IRB 2015-121, Internal Validation of the PowerPlex® Fusion 6C System (Promega Corporation, Madison, WI) on the Applied Biosystems® 3500xL Genetic Analyzer. The collection and analysis of blood samples are approved by IRB 2017-059, The Effects of Copper and Calcium as PCR Inhibitors were tested on the Promega Fusion 6C Human Identity DNA Testing Kit.

Sample Collection

Three types of samples were used throughout the conducted studies. Buccal swabs were collected from two volunteers by swabbing for a minimum of 15 seconds. A bone (femur) was obtained from the Willed Body Program at the University of North Texas Health Science Center. The third type of sample used was a blood sample collected from one volunteer using a purple-top blood collection tube containing 10.8 milligrams of EDTA.

Extraction

DNA from the buccal swabs and the bone sample were extracted using the DNA IQ™ Reference Sample Kit (Promega Corporation, Madison, WI) on the Promega Maxwell® 16 (Promega Corporation, Madison, WI) following the manufacturer's recommendations. DNA from the liquid blood sample was extracted using the organic extraction technique. The organic extract solution was incubated at 56°C for approximately 24 hours. Purification of the organic extract solution was performed using phenol-chloroform isoamyl alcohol (PCIA) and the Amicon®

Ultra Centrifugal Filters (Merck Millipore Corporation, Billerica, MA) following the manufacturer's recommendations.

Metal and DNA-Metal Solution Preparation

DNA solutions were made by diluting the DNA extract to a concentration of 1 nanogram (ng) DNA using TE^{-4} Buffer. Aliquots of the copper and calcium standards were made by diluting the stock solution down to a concentration of 22.04 millimolar (mM) and 20.94mM, respectively. The pH was adjusted to 4.2 for calcium and 4.9 for copper using 3M sodium hydroxide and 1M hydrochloric acid. Dilutions of each metal solution were made at concentrations of 0mM, 0.54mM, 1.64mM, 4.9mM and 14.7mM using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA). The extract and metal inhibitor solutions were made by adding 15 microliters (μL) of metal inhibitor solution to 15μL of diluted DNA extract solution, resulting in the target concentrations of 0.5ng of DNA and 0mM, 0.27mM, 0.82, 2.45mM and 7.35mM for each metal. These metal concentrations were based on research that has been performed by doctors as well as previous stability research performed in the laboratory. [8,9]

Quantification

The DNA in the extract solutions along with DNA in the extract and metal inhibitor solutions were quantified using the PowerQuant™ System (Promega Corporation, Madison, WI) following the manufacturer's protocol, "Run Setup and Thermal Cycling Using the Applied Biosystems® 7500 Real-Time PCR System and Applied Biosystems® 7500 Software, Version 2.0.6" [26]. Analysis was performed on a 7500 Real-Time PCR System (Applied Biosystems™, Foster City, CA) using the HID Real-Time PCR Analysis Software version 1.2 (Applied

Biosystems™, Foster City, CA). The threshold cycle values and estimated DNA quantity for the samples were obtained from the software. An undetermined result obtained for a DNA concentration was manually assigned a value of zero.

Amplification and Genetic Analysis

One sample of each sensitivity study concentration from the buccal and bone DNA solutions were tested with the PowerPlex Fusion 6C System. Duplicate samples of the DNA and metal inhibitor solution were tested with the PowerPlex Fusion 6C System in the same injection on the 3500xL plate. Amplification product of the DNA and metal inhibitor solution were diluted with UltraPure™ Distilled Water (Invitrogen, Carlsbad, CA) at a ratio of 3 to 1 and retested with the PowerPlex Fusion 6C System. One sample of each of the metal-DNA concentrations were tested, this time separating the controls, calcium, and copper samples into individual injections.

Short tandem repeat (STR) amplification was conducted using a GeneAmp® PCR System 9700 (Applied Biosystems™, Foster City, CA). A 3500xL Genetic Analyzer for Human Identification (Applied Biosystems™, Foster City, CA) using the 3500 Data Collection Software version 1.0 (Applied Biosystems™, Foster City, CA) was used to separate the short tandem repeat segments. The collection of STR profiles was performed on GeneMapper® ID-X version 1.4 (Thermo Fisher Scientific, Waltham, MA) using a minimum heterozygous allele detection threshold of 95 relative fluorescence units (RFU) and a minimum homozygous allele detection threshold of 205 RFU. The allele calls and peak heights were collected. Manufacturers' recommendations were followed for all instrumentation.

STR Data Analysis of the Validation Study

GeneMapper ID-X version 1.4 was used to determine the number of alleles present in each analyzed profile. Microsoft Excel 2016 for Mac was used in the different studies to determine the positive and negative limit of detection (LOD) and limit of quantification (LOQ), the analytical and stochastic thresholds, the stutter ratios, and to create the representations used to display the data collected.

The reagent blanks and negative controls were analyzed using a threshold of 1 for each dye channel to obtain the LOD and LOQ. Any off-ladder, spikes or OMR peaks were deleted, leaving just the background noise in the profile. The peak heights of each called peak were then exported to Microsoft Excel and the average along with the standard deviation (SD) were calculated. Then, using Equations 1,2,3 and 4, the positive LOD, negative LOD, positive LOQ and negative LOQ were calculated, respectively.

Equation 1: LOD = Mean + 3 X SD

Equation 2: LOD = Mean - 3 X SD

Equation 3: LOQ = Mean + 10 X SD

Equation 4: LOQ = Mean - 10 X SD

The stochastic threshold was determined by using Equation 5. The LOD value used was the largest value out of any of the dye channels between the negative and positive LOD values. The minimum peak height ratio (minPHR) used was determined by exporting the peak heights of the

true alleles of each concentration used in the sensitivity study to Microsoft Excel and calculating the peak height ratio (PHR) of each heterozygous locus. The average PHR and standard deviation were then calculated using all values obtained from each concentration. Values were then calculated for the average PHR minus 1, 2 and 3 SD. Concentrations that contained drop-out were not used in this calculation.

Equation 5: Stochastic Threshold = 3* LOD(1+minPHR)

Stutter ratio values were calculated by exporting the peak heights for each DNA concentration to Microsoft Excel. Ratios were then calculated by dividing the stutter peak height and the true allele peak height. Once each ratio had been calculated, the average of each locus along with 1, 2 and 3 standard deviations were calculated. The average plus 2 SD was used as the final stutter ratio value and each value was converted into a percentage. If the calculated stutter ratio was larger than the set manufacturer's stutter value, then it was replaced in the validated analytical method.

STR Data Analysis of the Sensitivity Study Comparison and Stability Study

GeneMapper ID-X version 1.4 was used to determine the number of alleles present in each analyzed profile. Microsoft Excel 2016 for Mac was used in the two studies to perform summary statistics and to create the representations used to display the data. The average peak height for each of the concentrations were determined for the bone, buccal and calcium-DNA samples.

RESULTS

Internal Validation Study

Multiple values are needed to produce a validated analytical method. The dye channel thresholds were obtained by analyzing the background noise peaks from the negative controls and reagent blanks used throughout the validation study. The peak heights were averaged and the standard deviation of the peak heights calculated. Table 1 shows the limit of detection and limit of quantification for when three and ten standard deviations are added to the calculated average peak height. Table 2 shows the limit of detection and limit of quantification for when three and ten standard deviations are subtracted from the calculated average peak height.

Dye Channel	Positive LOD (+3SD)	Positive LOQ (+10SD)
Blue	39.29	72.70
Green	32.03	73.64
Yellow	37.24	95.25
Red	24.73	58.56
Purple	21.39	47.28

Table 1: Calculated results for the positive limit of detection and positive limit of quantification.

Dye Channel	Negative LOD (-3SD)	Negative LOQ (-10SD)
Blue	14.63	33.61
Green	19.95	41.34
Yellow	35.19	91.99
Red	15.68	32.57
Purple	21.06	47.06

Table 2: Calculated results for the negative limit of detection and negative limit of quantification.

The positive limit of quantification results were used as the individual dye channel thresholds. The largest value of those results, 95, was used as the validated analytical threshold. The stochastic threshold was obtained by first averaging the peak height ratios of all heterozygous loci at all concentrations that contained no drop-out. The standard deviation, two standard deviations and three standard deviations using the peak heights were calculated. Figure 1 shows the log of the average peak heights at each locus vs the corresponding peak height ratio. The thresholds of the average PHR minus 1, 2 and 3 standard deviations are represented as the horizontal lines in Figure 1. The -3SD threshold contained the most values while rejecting the outliers, so a value of 0.7 was chosen as the minimum PHR. This value and the largest positive LOD value were used in Equation 5 to determine the stochastic threshold of 205. The positive LOD was used because the dye channel thresholds were determined with the positive LOQ.

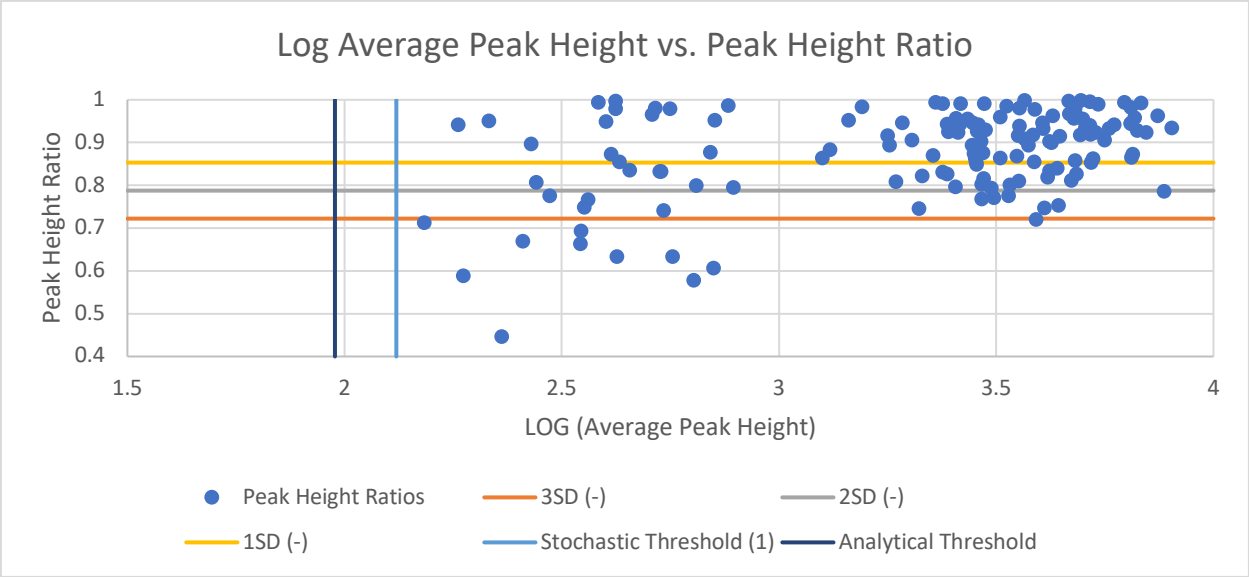


Figure 1: Peak height ratios in relative fluorescent units (RFU) for the average peak height at each locus. The four concentrations containing no drop-out are represented.

The analytical threshold of 95, stochastic threshold of 205 and minimum PHR of 0.7 were used to reanalyze the sensitivity profiles. The peak heights for the true alleles were exported and graphed with the corresponding amount of DNA in solution, as shown in Figure 2. An ideal target DNA concentration of 0.1 ng/µL, or 0.5 ng of DNA added to the amplification solution, was chosen since it produced the most consistent peak heights without containing dropped out or blown out alleles in the profile.

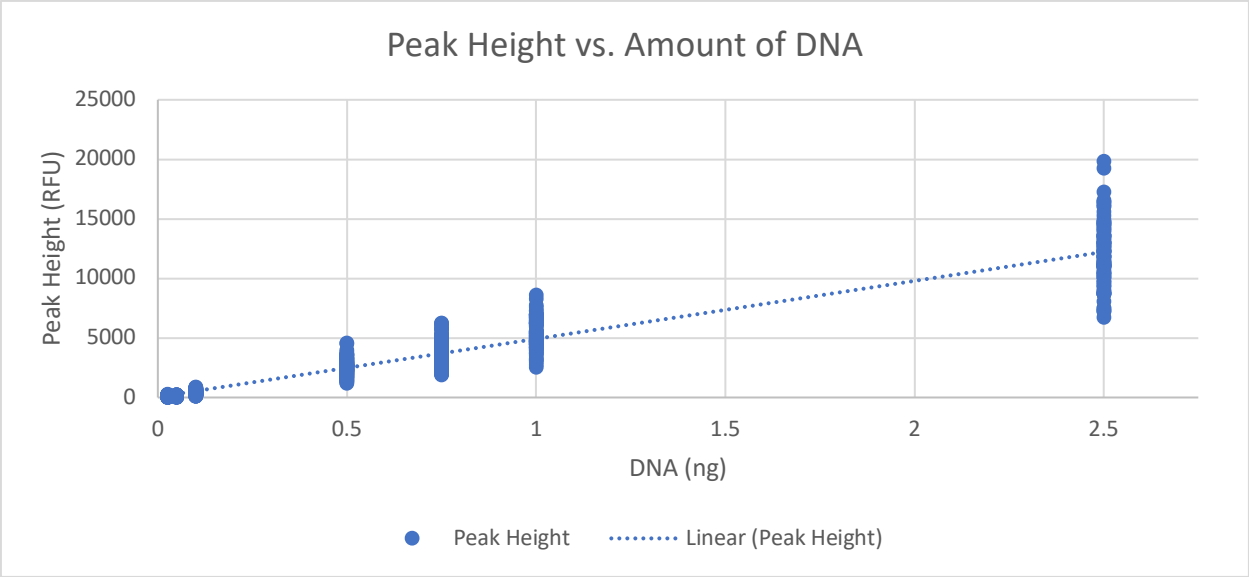


Figure 2: Peak heights in relative fluorescent units (RFU) for each allele called at each concentration for the tested buccal samples. The equation for the line of best fit is y = 4869.2x + 53.384.

The stutter study looked at the same stutter calls the manufacturer's looked at during the developmental validation study. Tables 3 and 4 show the manufacturer stutter values for plus and minus stutter in percent compared to the plus and minus stutter plus two standard deviations that were observed. Ten minus stutter values produced by the manufacturer were lower than that collected in the internal validation study. The loci that had differing stutter values were: D3S1358, D10S1248, D16S539, D18S51, D12S391, SE33, DYS391, FGA, DYS576 and DYS570. CSF1PO produced the only internal validation study value that was higher than the manufacturer's values for plus stutter. The higher observed stutter values replaced the manufacturer's stutter values in the validated analytical method. The manufacturer's stutter values that were higher than the values obtained in this study were kept as is.

Marker	Manufacturer Stutter Filter %	Mean (-) %	Mean + 2SD
AMEL	-	-	-
D3S1358	13.5	7.83	14.85
D1S1656	14.3	8.07	11.62
D1S1656(-2)	3.6	1.57	1.57
D2S441	9	4.7	6.64
D10S1248	13	7.7	15.11
D13S317	10.3	4.72	8.49
Penta E	7.2	3.14	5.78
D16S539	12	5.42	14.13
D18S51	14.6	6.89	15.51
D2S1338	13.6	7.6	10.49
CSF1PO	11.1	6.67	8.92
Penta D	4.5	2.33	3.19
TH01	4.8	3.67	4.22
VWA	14.4	7.45	9.39
D21S11	12.7	9.18	11.3
D7S820	9.7	-	-
D5S818	11	5.88	9.39
TPOX	5.4	2.22	3.03
D8S1179	11.8	13.65	11.67
D12S391	17.4	10.51	17.92
D19S433	12.1	5.72	10.4
D19S433(-2)	1.4	-	-
SE33	16.1	11.1	17.58
SE33(-2)	6.6	4.35	4.92
D22S1045(-3)	16.8	7.81	15.61
DYS391	9.4	6.9	14.51
FGA	12.4	7.68	13.33
FGA(-2)	1.2	-	-
DYS576	12.5	7.56	14.13
DYS570	13	10.61	13.36

Table 3: Manufacturer and observed values for minus stutter. The bolded values replaced the manufacturer's stutter values in the analytical method.

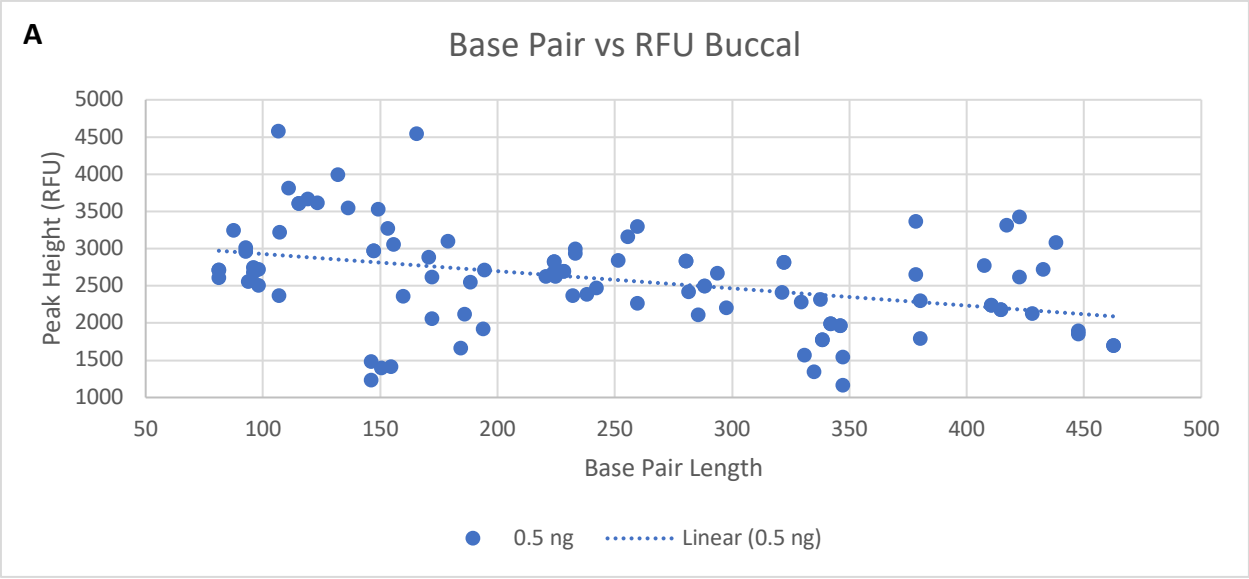
Marker	Manufacturer Stutter Filter %	Mean (+) %	Mean + 2SD
AMEL	-	-	-
D3S1358	1.7	-	-
D1S1656	2.3	-	-
D2S441	1.8	-	-
D10S1248	1.3	-	-
D13S317	2.2	-	-
Penta E	1.9	-	-
D16S539	3	1.11	1.29
D18S51	2.8	1.97	2.26
D2S1338	2.2	-	-
CSF1PO	3.7	2.83	3.96
Penta D	3.7	-	-
TH01	1.5	-	-
VWA	2.7	-	-
D21S11	2.8	-	-
D7S820	1.8	-	-
D5S818	2.3	-	-
TPOX	1.1	-	-
D8S1179	3.4	-	-
D12S391	2.7	-	-
D19S433	2.6	-	-
SE33	3.3	1.59	1.95
D22S1045(+3)	9	4.45	8.17
DYS391	2	1.77	1.77
FGA	2.8	-	-
DYS576	3.4	-	-
DYS570	2.4	-	-

Table 4: Manufacturer and observed values for plus stutter. The bolded values replaced the manufacturer's stutter values in the analytical method.

The mixture study produced two full, distinguishable profiles at the 1:1 M-F ratio. Drop-out of the minor profile started at the 1:9 M-F and the 9:1 M-F ratios. Peaks were called for the minor profile in the 1:59 M-F ratio at six loci while nine loci had alleles called for the minor profile at the 59:1 M-F ratio.

Buccal vs. Bone Sensitivity Study Comparison

Using the validated PowerPlex® Fusion 6C (PPF6C) analytical method, the buccal sensitivity study data was compared with a bone sensitivity study using the same PPF6C analytical method. Two concentrations that contained no drop out or blow out were shared between the two types of samples, 0.5ng and 1.0ng. Figure 3 shows a comparison of the buccal and bone samples at 0.5ng DNA. Figure 4 shows a comparison of the buccal and bone samples at 1.0ng DNA. The linear line of best fit for the data obtained from the buccal swabs is what is expected in a non-degraded DNA sample. The exponential line of best fit for the data obtained from the bone sample is what is expected for a degraded DNA sample.



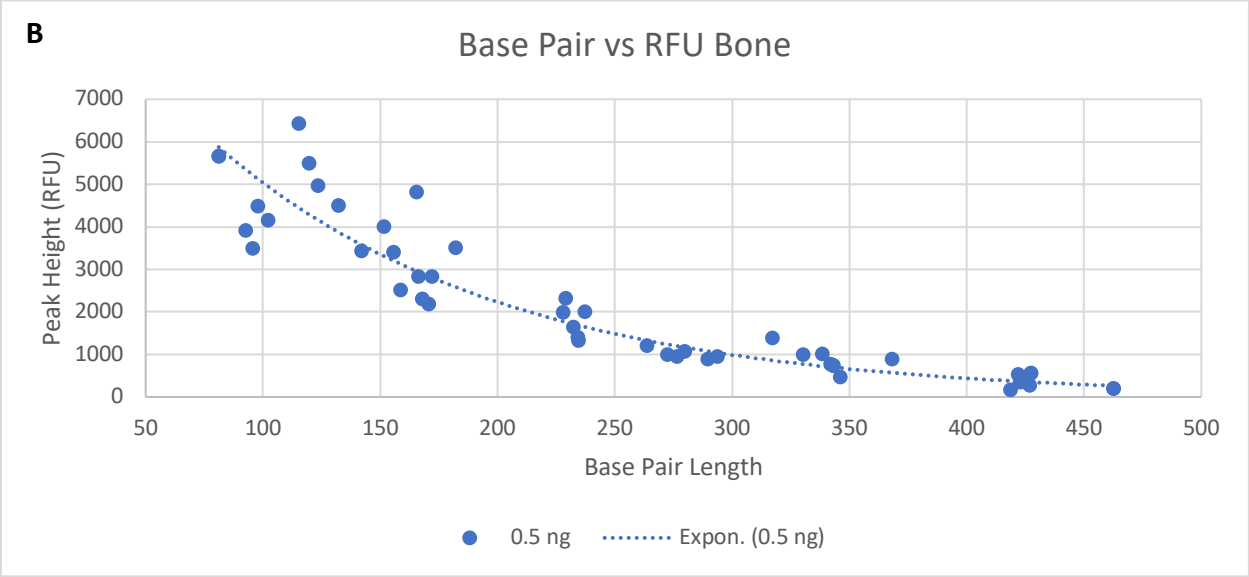
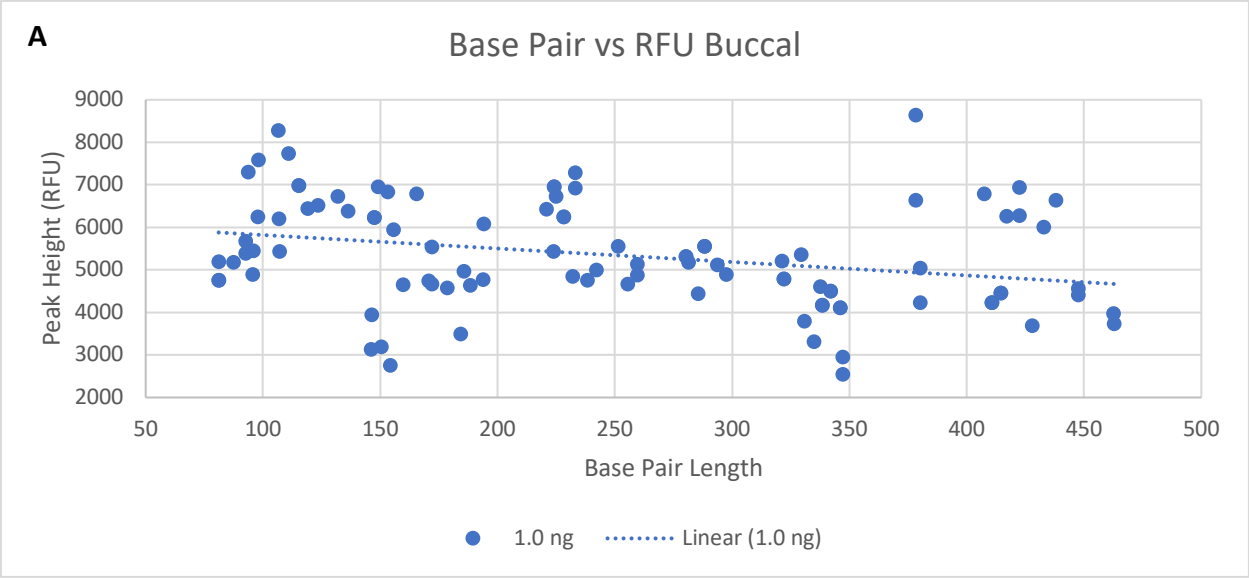


Figure 3: The allele peak heights in relative fluorescent units (RFU) for each called allele in the 0.5ng buccal (A) and bone (B) samples. The linear regression line is what is to be expected in a non-degraded DNA sample while the exponential regression line is what is to be expected in a degraded DNA sample. The equation for the line of best fit for the buccal data is $y = -2.3111x + 3159.2$ and the bone data is $y = 11373e^{-0.008x}$.



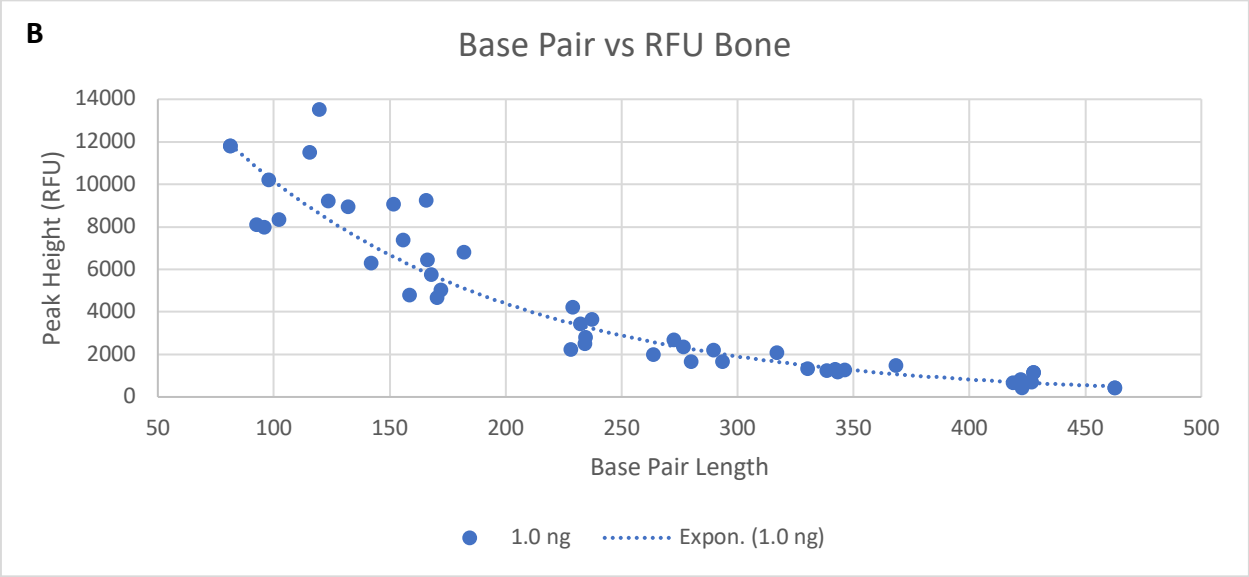


Figure 4: The allele peak heights in relative fluorescent units (RFU) for each called allele in the 1.0ng buccal (A) and bone (B) samples. The linear regression line is what is to be expected in a non-degraded DNA sample while the exponential regression line is what is to be expected in a degraded DNA sample. The equation for the line of best fit for the buccal data is $y = -3.1698x + 6137.2$ and the bone data is $y = 23461e^{-0.008x}$.

The peak heights for the true alleles in the bone samples were exported and graphed with the corresponding amount of DNA in solution, as shown in Figure 5. An ideal target DNA concentration of 0.1 ng/µL, or 0.5 ng of DNA added to the amp solution, was chosen. This is the same DNA concentration determined that was needed for the buccal samples. The averages were taken for all the peak heights contained in the six shared concentrations between the bone and buccal sensitivity studies, as shown in Table 5.

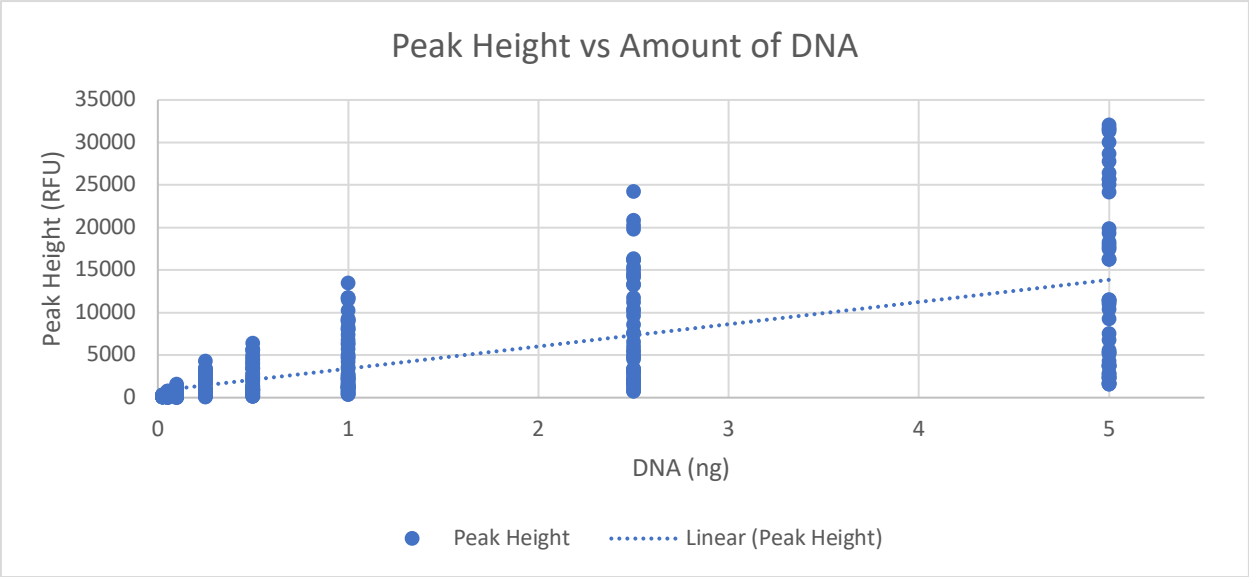


Figure 5: Peak heights in relative fluorescent units (RFU) for each allele called at each concentration for the tested bone sample. The equation for the line of best fit was y=2610.6x + 801.66.

	AVERAGES PER AMOUNT OF DNA					
	0.025 (ng)	0.050 (ng)	0.100 (ng)	0.500 (ng)	1.000 (ng)	2.500 (ng)
Bone (RFU)	158.296[1]	319.147[2]	522.750[3]	2227.313[4]	4470.729	8134.000
Buccal (RFU)	116.865[5]	121.182[5]	435.788	2574.899	5350.828	12042.390

Table 5: The average peak height of all called alleles in each shared concentration used in the buccal and bone sensitivity studies. N=48 alleles for the bone averages and N=99 alleles for the buccal averages. 1 – N=27. 2 – N=32. 3 – N=44. 4 – N=46. 5 – N=88.

Metal Stability Study

Two plates containing amplification product were run on the genetic analyzer that contained calcium or copper at 5 different concentrations. These concentrations were 0mM, 0.27mM, 0.82mM, 2.45mM and 7.35mM. The first plate, the two different metals were in the same injection, and the 3500xL produced too high of a current to produce any profiles for any of the samples.

The second plate contained the controls and reagent blank in the first injection, the samples containing calcium in the second injection and the samples containing copper in the third injection. When this plate was run on the 3500xL, profiles for the controls, reagent blank, and calcium samples were obtained while the instrument still produced too high of a voltage when the copper samples were injected. The average peak height for each concentration was calculated for the calcium samples, tabulated in Table 6.

Concentration of Calcium (mM)	Average Peak Height (RFU)
0.00	8403.49
0.27	2923.41
0.82	3905.06
2.45	4544.59
7.35[1]	3752.32

Table 6: The average peak height of all called alleles in each calcium concentration used in the stability study. N = 51 alleles. 1 – N=26.

The DNA profiles associated with each calcium concentration contained alleles that had shifted to the right, producing alleles that were either 1 or 2 base pairs smaller than what the true allele size should be for each locus. The sample that contained 7.35mM of calcium produced a partial profile, eliminating the alleles that were larger than 260 base pairs long. This allele drop-out could be caused due to the calcium interacting with the DNA, having a higher concentration in the longer DNA fragments or by binding to the free adenine, thymine, cytosine and guanine bases in solution, making longer DNA strands less likely to be copied. There were two calcium concentrations that exhibited an inhibited profile based on the line of best fit for the allele heights. These two concentrations were the 0.82mM and 7.35mM concentrations. Figure 6 shows the larger allele drop-out for the 7.35mM sample and the inhibited tendencies in the 0.82mM and 7.35mM samples.

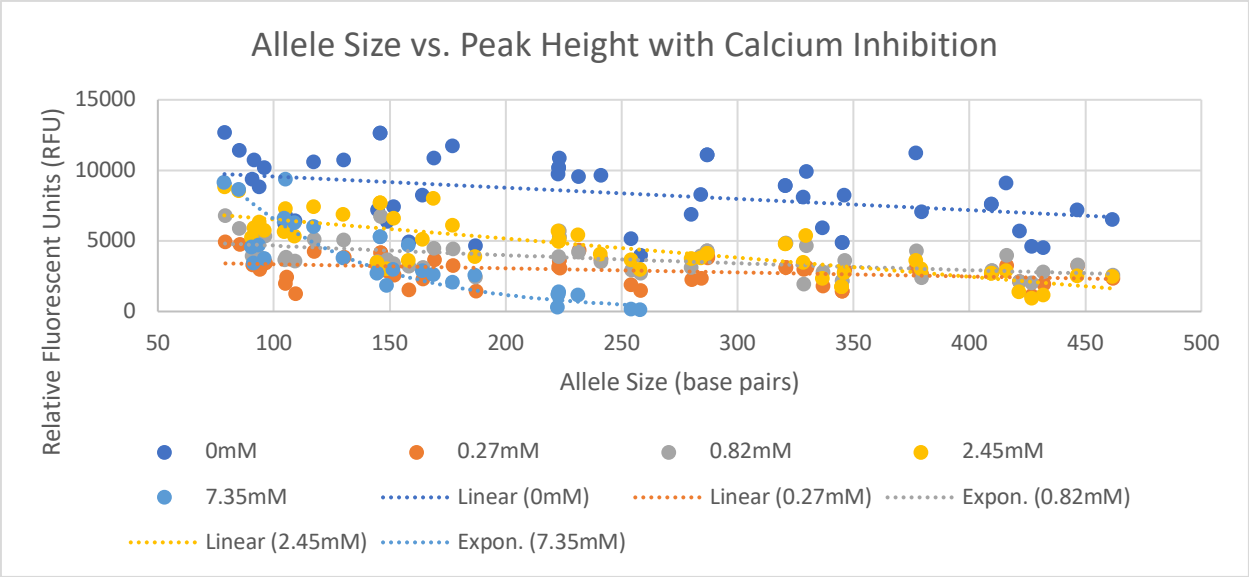


Figure 6: The allele peak heights in relative fluorescent units (RFU) for each called allele in the calcium-DNA inhibited solutions.

DISCUSSION AND CONCLUSION

Validation Study

A partial internal validation study was performed to obtain a validated analytical method to analyze case and reference samples using the PowerPlex® Fusion 6C (PPF6C) System. This particular validation study consisted of a sensitivity study, mixture study, stutter study and case samples in the form of bone. Validation studies are important to perform on the individual laboratory's instruments because every instrument creates slightly different results. The stutter ratios obtained from the performed validation study were different from the stutter ratios provided by Promega, some being higher and some being lower.

The dye channel thresholds are determined by obtaining the positive and negative limit of detection (LOD) and limit of quantification (LOQ). The positive LOQ was chosen as the dye channel thresholds. This set of values was chosen because the peaks that result from these thresholds will not show every artifact present but will also call peaks that might be minor contributors in a mixture sample or alleles that are not preferentially amplified, larger allele lengths, in a low copy DNA sample. A minimum peak height ratio (PHR) of 0.7 at three standard deviations was chosen over the standard deviation and two standard deviation values. The 0.7 ratio was low enough that it included the majority of the allele peak heights, while eliminating the extreme outliers.

Every thermocycler and genetic analyzer is slightly different, producing slightly different results and stochastic effects. When performing the stutter study, there were locus stutter ratios that were less than the manufacturer ratios, while other locus stutter ratios had a higher value than the

manufacturer's. This is to be expected due to these slightly different stochastic events that occur during amplification.

Obtaining mixture profiles can be difficult depending on the environmental conditions surrounding the DNA and the amount of time it has been exposed to the environment. When a rape occurs, the minor profile is overpowered by the major profile. It becomes even harder to obtain the minor profile if the evidence is collected a few days after the offense or if the victim washes the evidence away after the crime occurs. Observing that drop-out of the minor profile began at a ratio of 9:1 male-female and 1:9 male-female is important to know for the PowerPlex® Fusion 6C System. This allows analysts to be prepared or expect a partial profile from a mixture from a personal sample or other mixture samples that might have been exposed to harsh conditions or where one individual's DNA would be much more abundant than another.

Bone versus Buccal Sensitivity Study Comparison

The bone and buccal samples showed similar average allele peak heights. The bone profiles showed degradation though. The peak heights in the bone profiles at the longer loci are significantly less than the smaller alleles. If the bone used in the bone sensitivity study had not been degraded, then statistical testing could have been performed. The target amount of DNA in amplification for reference and evidence samples was determined to be 0.5 nanograms (ng). This value was chosen because the spread of peak heights along with the average peak height was the most ideal of the concentrations tested. The target amount of DNA in amplification for degraded bone samples was determined to be 0.5ng. The same analytical method and target amount of DNA can be used to analyze reference, evidence and degraded bone samples.

Stability Study

Copper-DNA and calcium-DNA samples were analyzed on a 3500xL Genetic Analyzer using the PPF6C System. Five concentrations of each metal were used: 0mM, 0.27mM, 0.82mM, 2.45mM and 7.35mM. Two different analyses were run on these samples. The first analysis contained too much DNA in solution, and the 3500xL produced no data due to the high voltage employed. The second analysis was performed with the correct amount of DNA to ensure the concentration of DNA was not the cause of the high voltage. This analysis showed two different tendencies for the metals.

The calcium-DNA samples had the majority of alleles show a shift to the left of the true allele by one or two base pairs. Once calcium was introduced into the DNA solution, the allele peak heights dropped. The peak heights of the samples containing calcium were approximately of similar intensity. Full profiles were obtained at the 0mM, 0.27mM, 0.82mM and 2.45mM concentrations while the 7.35mM concentration produced a partial profile. The alleles that dropped out in the 7.35mM sample occurred to all alleles that were larger than 260 base pairs long. The allele shifts, peak height drops, and allele drop outs could be caused by a few possible mechanisms.

The calcium ions in solution could bind to the Taq polymerase, nitrogen atoms and phosphate backbone of the DNA strands or primers, or to the free nucleotides in solution. When the calcium binds to the polymerase, it causes inactivation and stops the replication of the DNA strands. The DNA profile can represent this by having universal inhibition throughout the whole profile. This

can potentially cause the allele shifts and peak height drops that were observed in all concentrations. If the calcium binds to the primers or DNA strands, it would be to the phosphate backbone or to the nitrogen in the nitrogenous base. Larger alleles preferentially drop out over the smaller alleles as more calcium is able to bind to the longer DNA fragments. If the free nucleotides and calcium ions bind to each other, there would be less nucleotides available for the polymerase to make more copies of the DNA. The DNA profile would have a mixture of inhibition throughout the whole profile and also show drop out in the larger alleles when the free nucleotides are bound to the calcium ions.

The copper-DNA samples did not produce any data due to the 3500xL Genetic Analyzer producing too high of a voltage. Copper is a larger ion than calcium, containing 29 protons and neutrons in each atom. Copper also has multiple oxidation states, the number of electrons changing with each oxidation state. These different oxidation states are one reason why copper is an excellent electrical conductor. Copper (II) is an oxidation state that is commonly found in biological material and other possible evidence samples. These copper (II) ions are water soluble, making getting rid of all ions in the extract solution hard to do. Copper (II) ions also form very stable chelate complexes with amino acids. These complexes are normally removed during the purification of the extract solution. However, if the solution is not purified properly or good enough, these complexes can get through and cause possible issues in quantification, amplification and genetic analysis. The electrical properties of copper is a potential reason why the 3500xL could not obtain any data.

After performing the two sensitivity studies using buccal reference samples and a bone sample along with two stability studies using copper and calcium, the PowerPlex® Fusion 6C System has shown to be a good kit to use when dealing with degraded samples or inhibited samples. The same analytical method and target amount of DNA that is determined during the internal validation study for the PowerPlex Fusion 6C System can be used on these two different types of samples. The presence of calcium did inhibit the DNA, but full profiles were able to be obtained from concentrations of 2.45mM or less. However, the PowerPlex® Fusion 6C System used on a 3500xL Genetic Analyzer cannot tolerate the presence of copper. The presence of copper causes the instrument to produce too high of a voltage for any type of data to be obtained. Therefore, if it is suspected that there is copper present in the sample because of where the biological material was found or from indications seen in the IPC (Internal PCR Control) value during quantification of the DNA extract solution, purifying the extract solution further is a necessary step in order to obtain results.

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