APPLICATION OF ENHANCEMENT STRATEGIES FOR THE IMPROVEMENT OF DISCRIMINATING FORENSIC DNA PROFILES FROM HUMAN BONES

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LIST OF ABBREVIATIONS

1/RMP – inverse random match probability

BTA – bone, teeth, adhesive

CODIS – Combined DNA Index System

CE - capillary electrophoresis

C_T – cycle threshold

DNA – deoxyribonucleic acid

dNTP - deoxyribonucleotide triphosphate

EDTA – ethylenediaminetetraacetic acid

EGTA - ethylene glycol tetraacetic acid

FOReSt – Forensic Osteology Research Station

HID – human identification

IPC – internal positive control

iSNP – identity single nucleotide polymorphism

ISP – Ion Sphere™ particles

MDA – multiple displacement amplification

MPS – massively parallel sequencing

NIST – National Institute of Standards and Technology

NTC – no-template control

PCA – principle component analysis

PCR – polymerase chain reaction

PGM – Personal Genome Machine™

qPCR – quantitative polymerase chain reaction

RFU – relative fluorescence unit

SNP – single nucleotide polymorphism

STR – short tandem repeat

Taq – polymerase isolated from *Thermus aquaticus*

UAS – Universal Analysis Software

UV – ultraviolet

WCU – Western Carolina University

WGA – whole genome amplification

ABSTRACT

APPLICATION OF ENHANCEMENT STRATEGIES FOR THE IMPROVEMENT OF DISCRIMINATING FORENSIC DNA PROFILES FROM HUMAN BONES

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Although DNA profiling techniques are considered a powerful method for identification, problems arise when low quantity and/or quality DNA is tested. Analyzing samples with low template DNA using standard genotyping techniques like STR typing can yield no or an incomplete profile, making conclusive identification nearly impossible. One challenging forensic sample material is bone. For example, DNA within bone can be degraded due to harsh environmental conditions even when the structure of the bone appears to be relatively well preserved. Consequently, the amount of usable DNA in bones can be limited which complicates downstream applications for DNA profiling. This study investigated techniques that would improve obtaining a discriminating DNA profile from human bones. First, the performances of three commercial DNA extraction kits were evaluated for the recovery of genomic DNA from human bones. The PrepFiler® BTA Forensic DNA Extraction Kit recovered the highest DNA yield according to qPCR data and was used for additional bone extractions. Next, purified DNA from a total of 12 bone samples was subject to genotyping methods using capillary electrophoresis (CE) as well as massively parallel sequencing (MPS) to determine which strategy would produce the most discriminating DNA profile. The genotyping techniques evaluated were:

CE-based STR analysis with the GlobalFiler® PCR Amplification Kit, whole genome amplification (WGA) with the REPLI-g® Mini Kit for improved CE-based STR detection, and MPS for STR/SNP analysis using the Ion PGM™ and MiSeq® FGx™ platforms. Random match probabilities were calculated to determine the discriminatory power of the resulting DNA profiles. Although the Ion PGM™ SNP profiles had the highest RMPs, the GlobalFiler® STR profiles produced similar discriminatory power. Considering the time and labor required for MPS, one could argue STR analysis using traditional CE may be better suited for DNA profiling of challenging bone samples. However, the MPS technologies provide additional information that CE-methods can't, such as Y-haplogroup and biogeographical ancestry predictions from SNP analyses.

CHAPTER ONE: INTRODUCTION

The introduction of molecular techniques for human identification, specifically DNA profiling, is a major advancement in forensic science. There is a need for the identification of deceased individuals as a result of many accidents and crimes when the identity of those involved is not known (Pan American Health Organization 2004, Jakubowsha 2012). Such incidents include traffic accidents, mass disasters, war crimes, terrorist attacks, or murders with the dismemberment of a body (Pan American Health Organization 2004, Jakubowsha 2012). Any type of biological material present at a crime scene may be collected as evidence and processed in a forensic laboratory to recover and analyze DNA. Accurate analysis depends largely on not only the quantity but also the quality of the DNA present in the sample. Many times, forensic scientists are asked to recover information from low amounts of DNA from suboptimal biological evidence. Consequently, how to properly approach and analyze samples with limited DNA has become a prominent issue in the field of forensics.

Forensic DNA Profiling

Traditional forensic DNA casework includes analyzing genetic markers called short tandem repeats (STRs). STRs are short sequences of DNA that are contiguously repeated many times throughout the human genome. These repeating sequences are widely used as a genetic marker for DNA analysis because the number of repeats can be highly variable among individuals, which make STRs very suitable for human identification purposes (Ziętkiewicz et al. 2012). STRs are classified according to the number of repeating core units; forensic analyses frequently use tetrameric repeats, which are STRs with repeating units of four base pairs; i.e. [ATCG]…[ATCG]. STR alleles from specific positions in the genome are used to evaluate the

genotype of the person whose DNA is in the sample. An individual is either homozygous (inheriting identical STR alleles from each parent, i.e. the same number of repeats) or heterozygous (inheriting a different number of repeats from each parent) at a particular locus.

Capillary electrophoresis (CE), which detects multiplexes of fluorescently labelled STRs, has been the method of choice for forensic DNA analysis for almost 20 years (Lazaruk et al. 1998). STRs are readily amplified by locus-specific polymerase chain reaction (PCR), which generates billions of copies of the particular STR fragment. For analysis, CE separates STR fragments by size in a capillary tube, excites the fluorescently tagged STRs with a laser at the end of the tube, detects the emitted wavelength with an optical device, and records the STR allele with computer software (Butler et al. 2004). The output is an electropherogram, which plots the STR alleles at each locus as peaks given the relative fluorescent units (RFUs). The genotypes from the electropherogram of a DNA sample is used to construct a genetic summary for interpretation. This process, called DNA profiling, is definitive and highly discriminating which makes it a very powerful tool for identification purposes in forensic cases. Because STRs exhibit high variation among individuals and therefore have a high power of discrimination, it is possible to identify those involved in crimes or missing person cases.

Problems with DNA Profiling

Even though forensic STR profiling techniques are considered a powerful method for identification, problems arise when low quantity and/or low quality DNA is tested. In forensic casework, it is not unusual to encounter biological samples that present challenges for DNA analysis. For instance, the sample may contain trace amounts of DNA, be compromised by sample impurities, or could be highly degraded and broken into very small fragments (Butler 2012). When analyzing challenging samples with low amounts of template DNA, standard

genotyping techniques like STR typing often yields no profile or an incomplete profile, making conclusive identification of the sample nearly impossible (Butler and Hill 2010, Butler 2012).

When examining compromised DNA by STR typing, the resulting electropherogram frequently contains increased random or stochastic effects. Stochastic effects manifest as a result of amplifying low amounts of DNA using PCR amplification (Buckleton 2009, Butler and Hill 2010). If a limited number of DNA target molecules exist in a sample, then the PCR primers used to amplify a specific region of DNA may not consistently find and hybridize to all the DNA molecules present within the amplification reaction (Buckleton 2009, Butler and Hill 2010). Due to stochastic variation, some alleles may fail to be detected and the genotype of the sample may not be interpreted correctly.

Evidence of stochastic variation in a DNA profile include artifacts such as enhanced stutter, peak height imbalance, allele drop-out, and allele drop-in. Stutter occurs when *Taq* polymerase loses its place when replicating DNA during PCR and slips either forwards or backwards four base pairs; the result is a small number of DNA fragments that are either one STR repeat larger or smaller than the true DNA fragment being amplified (Cowen et al. 2011). With low template DNA, the height of the stutter peak in the electropherogram is increased. Peak height imbalance occurs at heterozygous loci when one of the STR alleles is preferentially amplified by chance during the early cycles of PCR, resulting in a peak height that is far greater than the peak height of the other allele (Cowen et al. 2011). Typically, both allele peak heights should be equivalent in height. Lastly, allele drop-out occurs when an allele that is present in the sample fails to amplify and allele drop-in occurs when additional alleles appear at a locus that are due to sporadic contamination (Buckleton 2009, Butler and Hill 2010, Butler 2012). The presence of these stochastic effects in the DNA profile can make the interpretation of the profile

difficult and can lead the analyst to make a subjective and/or biased opinion about the identity of the sample.

A Challenging Forensic Sample

An example of a challenging forensic sample is bone material. In some cases, bones are the only biological material available that can be used to create a DNA profile for the identification of human remains (Loreille et al. 2007, Jakubowsha 2012). The main components of bone tissue are inorganic hydroxyapatite minerals (calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide and citrate), which gives bone a rigid framework for strength and support; about 70% of bone tissue consists of these minerals (Loreille et al. 2007, Clarke 2008). Since bones have this extensive mineralization in their structure, the release of DNA molecules from bone tissue is difficult through common DNA extraction procedures. Generally, only a limited amount of DNA is recovered from bones.

Furthermore, DNA that can be recovered from bones is often considered low quality due to degradation. Immediately after death, intracellular nucleases begin to break down DNA, leaving it damaged and fragmented (Didenko et al. 2003). Also, DNA within bones could be degraded if the skeleton was persistently exposed to harsh environmental conditions (e.g. direct sunlight, temperature extremes, humidity, and microbial activity). These factors decrease the number of intact DNA molecules in the sample and PCR amplification efficiency is reduced (Ye et al. 2004, Jakubowsha 2012). There is an inverse relationship between STR fragment length and successful PCR amplification; larger fragments become difficult to amplify since they are more likely to be damaged and smaller fragments are more likely to be intact and their amplification is likely to occur (Butler et al. 2003).

In addition, PCR inhibitors may also be present within the bone extract such as the calcium ions from bone content and humic acid from soil/plant material (Ye et al. 2004, Jakubowsha 2012). During PCR, calcium binds to *Taq* polymerase by competing with magnesium (a *Taq* polymerase cofactor) and reduces the enzyme's activity; the presence of calcium decreases the reaction efficiency and the total amount of PCR product (Opel et al. 2010). Humic acid inhibits the PCR reaction by directly binding to specific sequences of DNA, which limits the amount of available template to be amplified (Opel et al. 2010). Altogether, degradation and/or inhibition can alter the integrity and obtainability of DNA existing within bones, and therefore complicate the downstream applications of DNA analysis.

Enhancement Strategies

In traditional forensic DNA analysis, there are two empirically-determined thresholds that are used for normal interpretation: the analytical and stochastic thresholds. Analytical thresholds define the minimum peak height requirement above which detected peaks can be reliably distinguished from the background noise of the instrument and are usually set between 50-100 RFUs (SWGDAM 2010). In contrast, stochastic thresholds are always greater than analytical thresholds and set between 150-200 RFUs (Gill et al. 2009). It defines the peak height value above which the analyst can be confident that if one peak for a heterozygote is above this threshold, then its sister allele will be present and should be at least above the analytical threshold (SWGDAM 2010). Difficulty analyzing challenging samples like bone material arises when DNA typing results fall below the stochastic threshold. Since stochastic effects cannot be avoided when testing low amounts DNA template, it is common to see allelic peaks that fall beneath this threshold which poses a risk for the wrongful designation of a heterozygous genotype as a homozygote (Gill et al. 2009). Analysts must use caution when interpreting

profiles of low template DNA; if the stochastic threshold is set too high, then there may be some loss of information, but if it is set too low, then incorrect genotypes may be called (Butler and Hill 2010). Therefore, strategies dealing with the stochastic effects associated with low template DNA (allele drop-out, higher stutter peaks, and sporadic contamination) must be taken into consideration so that the correct DNA profile is interpreted.

To assist with analyzing compromised DNA, enhancement strategies can be used to increase the sensitivity of genetic marker detection to get as much information out of the sample as possible. There is a wide variety of techniques and emerging technologies that focus on analyzing compromised DNA; these technologies may have different chemistries and detection methods but all aim to increase the amount of reportable genetic loci to improve discriminatory power. These technologies include having larger and more sensitive multiplexes that reduce not only the amount of input DNA required but also the amount of time for analysis (Peng et al. 2015). Furthermore, high-throughput technologies such as massively parallel sequencing (MPS) can now evaluate new alternative genetic markers and can simultaneously produce large amounts of data for many samples all in one reaction.

Improved STR Typing

CE methods are limited by the minimum quantity and quality of DNA that could be reliably typed within a reaction. If a DNA sample is degraded or inhibited, the fluorescence signal associated with some STR loci, especially those corresponding to larger fragments of DNA, is reduced or may even fail to be detected, a phenomenon known as locus drop-out. However, there are now improved STR typing kits that consist of next-generation chemistries that can deliver clean and more decipherable DNA profiles, making interpretation easier. One such kit, the GlobalFiler™ PCR Amplification Kit (Life Technologies), is known to have high

sensitivity and adaptability for degraded DNA as well as high tolerance for PCR inhibitors (Gouveia et al. 2015). This kit also contains additional STR markers, some of which are significantly smaller than the original STR markers. These so called mini-STRs, which range from 75 to 220 base pairs in length (Life Technologies™ 2014), optimize performance on degraded DNA because their primers have binding sites that are designed to sit closer to the STR repeat region and amplification is more likely to occur with the smaller fragments. Altogether, this next-generation STR kit could maximize the number of alleles detected from degraded samples like bones, making successful profiling and therefore high discriminatory power more achievable.

Whole Genome Amplification

Another strategy for increasing the amount of limited DNA produced from a bone specimen is whole genome amplification (WGA), which amplifies the entire genomic DNA prior to locus-specific STR amplification steps (Maciejewska et al. 2013). WGA is a sample enrichment technique that amplifies low quantities of starting DNA template and results in large quantities of amplified product, making downstream DNA analysis more attainable for challenging samples (Ballantyne et al. 2007, Maciejewska et al 2013). Numerous WGA methods have been explored and developed, but the WGA method used in this study was called multiple displacement amplification (MDA, Figure 1).

MDA is an isothermal reaction based technique and uses random hexamer primers to theoretically amplify the entire genome of the sample (Ballantyne et al. 2007). In addition, MDA involves a different DNA polymerase (Phi29) than the commonly used *Taq* enzyme in PCR. Phi29 has high processivity and enables multiple concurrent and overlapping rounds of amplification (Ballantyne et al. 2007, Butler 2012). In this process, Phi29 polymerase uses a free

3’ end of a hexamer primer as a starting point for replication, but it does not end replication when it encounters a 5’ end of DNA. Rather, Phi29 will displace the 5’ strand and continue to replicate. New hexamer primers will bind to this displaced strand and replication will continue, leading to an almost exponential amplification reaction of the template DNA (Schneider et al. 2004). Phi29 also has a higher replication fidelity compared to the *Taq* enzyme because of its 3’ to 5’ proofreading activity (Schneider et al. 2004). The application of WGA to low quantity or degraded samples has not been thoroughly examined and may be a possible method for improved and/or successful DNA analysis of challenging bone samples.

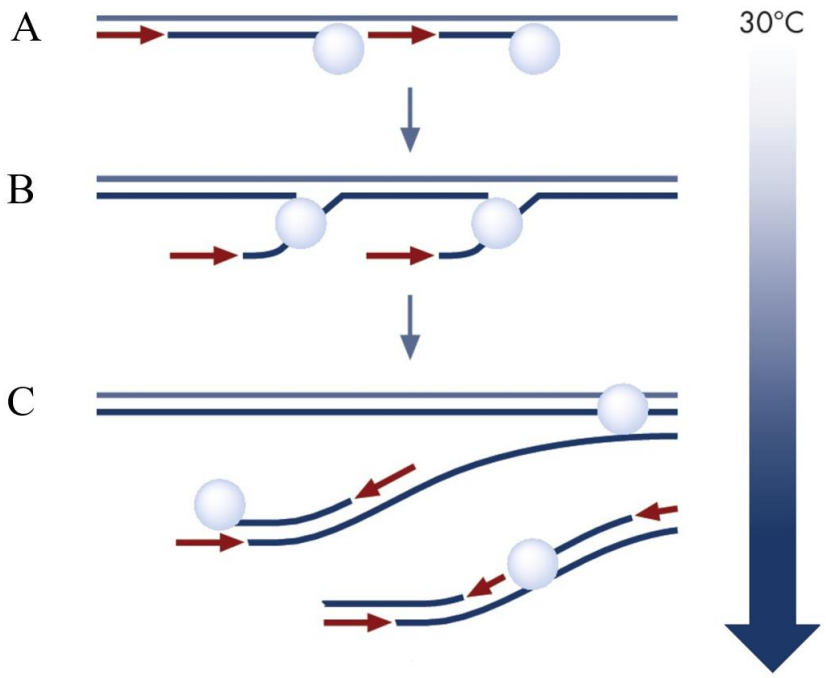

Figure 1. Schematic of whole genome amplification with isothermal MDA reaction. A) Random hexamer primers (red arrows) anneal to DNA template and Phi29 (white circle) extends at 30°C. B) Phi29 moves along the template strand and displaces the complementary strand. C) New primers bind to the displaced complementary strand and replication begins again on the new template strand. (https://www.qiagen.com/us/resources/technologies/wga/overview-on-wga/)

Massively Parallel Sequencing

Although it has revolutionized forensic DNA analysis, CE methods are limited. Not only is there a limit to the number of STR markers that can be separated by size using the same fluorescent dye color, but there are also a restricted number of dye colors that can be read by the instrumentation optics (Gettings et al. 2015). To characterize insufficient and degraded DNA from challenging samples, MPS technologies may be an alternative approach. MPS is a rapid, high-throughput technology that can produce high quality and detailed sequence information on targeted areas of the human genome; it can collect massive amounts of data from one or many individuals simultaneously (Borsting et al. 2014, Apaga et al. 2017). New MPS technologies have increased sensitivity and multiplexing capabilities compared to CE-based sequencing methods and provide a huge potential advantage for low template or degraded DNA analysis (Borsting et al. 2014, Gettings et al. 2015). MPS technologies can generate short sequence reads of typically 25 to 250 bases and can also incorporate non-traditional smaller markers called single nucleotide polymorphisms (SNPs) (Butler 2012, Gettings et al. 2015, Apaga et al. 2017).

SNPs show sequence variation, not length variation, and are classified as single positioned base substitutions, insertions, or deletions. SNPs are spread throughout the human genome and constitute almost 90% of all sequence variation in humans (Collins et al. 1998). They occur on all 22 autosomal chromosomes, both on the Y and X sex chromosomes, and on mitochondrial DNA. Their abundance provides a wide range of information and a large potential role for human identification purposes in forensics. The disadvantage of SNPs is their discriminatory power for identity testing. Because of the bi-allelic nature of SNPs, they are much less informative per locus compared to the multi-allelic nature of STR markers. The number of SNPs required to reach the discriminatory power of STRs is on average four times greater

(Sobrino et al. 2005). But if massively parallel sequencing technologies are able to successfully obtain a complete SNP profile, then the discriminatory power of the profile may be equivalent to or far exceed the discriminatory power of a traditional STR profile.

MPS technologies have improved the power of SNP markers by increasing the number of SNP loci that are analyzed in a single reaction. Also, SNP loci are small and thus the PCR amplicon size is reduced making it theoretically possible to successfully obtain sequence information about the degraded DNA from bones, which are normally too fragmented to characterize by traditional sequencing techniques. Targeting many SNPs in the genome can yield even more information from challenging samples and therefore, can increase the discriminatory power that assists with determining identity from these samples. There are several commercial kits that use MPS platforms for forensic DNA analysis, but the two MPS platforms included in this study are the MiSeq® FGx™ Forensic Genomics System (Illumina) and the Ion Torrent™ Personal Genome Machine (Ion PGM™) (Thermo Fisher Scientific). Though each platform has its own unique method for sequencing, they share two characteristics: the immobilization of DNA molecules to a medium and the clonal amplification of DNA template molecules to increase intensity of the signal.

Ion Torrent™ sequencing technology. One MPS platform that can be applied to forensic purposes is the Ion Torrent™ Personal Genome Machine (Ion PGM™) (Thermo Fisher Scientific). This MPS platform is based on semiconductor technology and is paired with a commercially available SNP panel for human identification, the HID-Ion AmpliSeq™ Identity Panel (Thermo Fisher Scientific). This MPS system targets and sequences a total of 124 SNP markers in one reaction (90 identity/autosomal SNPs and 34 upper Y-clade SNPs), having amplicon sizes ranging from 80 to 220 base pairs (Borsting and Morling 2015, Gettings et al.

2015). Before sequencing begins, PCR amplicon libraries containing SNP markers are flanked with adapters that help the library fragments attach to proprietary beads called Ion Sphere™ particles (ISPs). Clonal amplification of the libraries is accomplished by emulsion PCR so that the entire surface of the ISPs is covered with DNA template (Borsting and Morling 2015). Once library preparation is complete, sequencing is ready to be performed on the Ion PGM™ instrument.

The approach of semiconductor sequencing for the Ion PGM™ is based on the simple chemistry of DNA replication (Figure 2). When a nucleotide is incorporated into the newly synthesized strand by DNA polymerase, a hydrogen ion is released as a byproduct and there is a local change of pH. Ion Torrent™ has created a proprietary device, called a semiconductor chip, that can sense and record these subtle pH changes and translate it into digital information for DNA analysis (Rothberg et al. 2011). The semiconductor chip contains approximately one million wells, each containing millions of copies of a DNA library on a single bead. The Ion PGM™ sequencer then serially flows one nucleotide at a time over the chip. If the nucleotide is complimentary to the DNA template strands, then it gets incorporated by polymerase and the hydrogen ions are released (Liu et al. 2012). The pH of the solution changes in the well which is detected by an ion sensing layer beneath the chip and converts it into a change in voltage (Rothberg et al. 2011). The voltage change is recorded and a nucleotide base is called. This process happens simultaneously across the millions of wells in the chip. Because this is direct detection and each nucleotide incorporation is measured within seconds, the Ion PGM™ has a very short run time of only three hours. Sequencing continues until the desired number of nucleotide flows across the chip is completed.

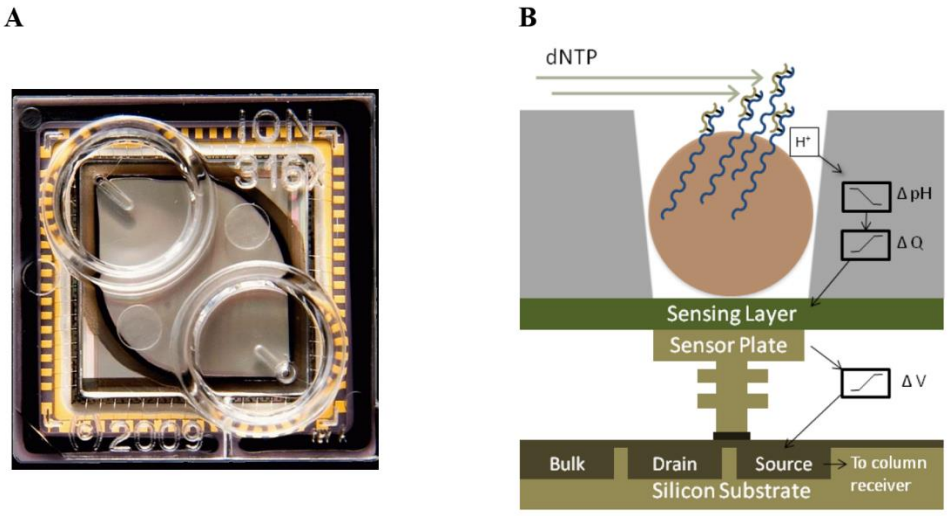

Figure 2. Ion Torrent™ semiconductor sequencing technology. A) The Ion Torrent™ 316 chip. The gray ellipse in the center contains approximately one million wells where the sequencing reaction occurs. B) Cross-section of a single well in the Ion Torrent™ chip that holds one Ion Sphere™ particle with DNA template. A hydrogen ion is released when a nucleotide (dNTP) is incorporated, which changes the pH of the well. A sensing layer beneath the well detects the pH change, coverts it to a voltage change, and translates the information into a computer (Rothberg et al. 2011, https://www.thermofisher.com/us/en/home/brands/ion-torrent.html).

Illumina® MiSeq® sequencing technology. Another MPS platform that is designed for forensic science purposes is the MiSeq® FGx™ Forensic Genomics System (Illumina). This MPS platform uses sequence-by-synthesis technology (Figure 3) and is coupled with a commercially available human identification panel, the ForenSeq™ DNA Signature Prep Kit (Illumina) which sequences a total of 172 SNPs as well as 58 STRs (Borsting and Morling 2015). The SNP loci detected in this assay include identity informative SNPs, biogeographical ancestry informative SNPs, as well as phenotypic informative SNPs; the amplicon size ranges from 63 to 231 base pairs (Illumina® 2014). The STR loci detected include globally recognized autosomal, Y-, and X-STRs; the amplicon size ranges from 61 to 467 base pairs (Illumina® 2014).

Amplicon library fragments are prepared by targeted PCR amplification and then ligated with adapters at either end of the DNA strand. These adapters allow the amplicon to hybridize to complementary oligonucleotides that are fixed to the surface of a flow cell (Liu et al. 2012). The flow cell is a proprietary, optically transparent, solid substrate that anchors the libraries in place and provides a flat surface sequencing. Clonal amplification of the libraries occurs on the instrument through bridge amplification and produces clusters that contain identical DNA fragments (Metzker 2010, Borsting and Morling 2015).

Sequencing with the MiSeq® FGx™ System requires fluorescent reversible terminator nucleotides to sequence the millions of clusters on the flow cell in parallel. Each nucleotide contains a characteristic fluorescent dye and a removable blocking group. At the beginning of each sequencing cycle, all four of the reversible terminator nucleotides are flooded across the flow cell simultaneously but only one is incorporated at a time based on the sequence of the template (Metzker 2010, Liu et al. 2012). After each nucleotide incorporation, the flow cell is washed to remove the extra unincorporated nucleotides. Then, a light source excites the fluorescent dye of the incorporated nucleotide and the resulting emittance is captured by a camera and the base is recorded as well as identified by the computer (Borsting and Morling 2015). Afterwards, the fluorophore and terminator is enzymatically cleaved which allows incorporation of the following nucleotide (Metzker 2010). This process is continued until the desired read length is achieved.

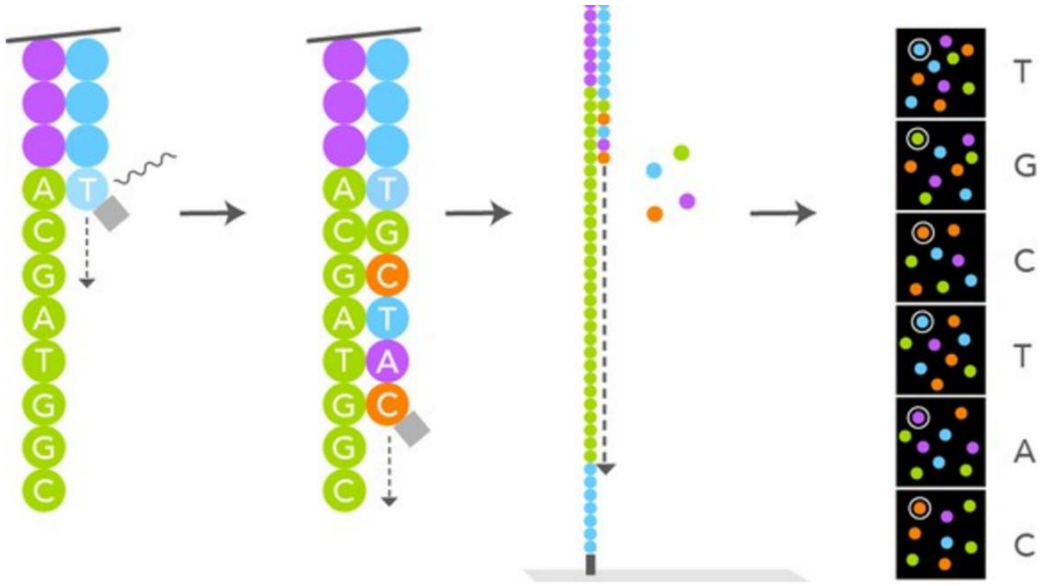

Figure 3. Illumina® sequence-by-synthesis technology. Each nucleotide is linked with a different dye and terminator group (gray square). During sequencing, all four nucleotides are simultaneously flooded across the flow cell. After incorporation of a complementary nucleotide, strand elongation is stopped due to the blocking effect of the terminator group. The fluorescent dye of the nucleotide is detected using an imaging system. Then, both the dye and the terminator group are cleaved and a new cycle starts. The circled dots on the black boxes represents a certain cluster of DNA template on the flow cell after an image is taken; the sequence is determined base by base according to fluorescent dye color.
(https://www.illumina.com/technology/next-generation-sequencing/sequencing-technology.html)

Purpose and Significance of the Study

The purpose of this study was to investigate techniques and emerging technologies that would improve the ability to obtain a discriminating DNA profile from human bones, which are considered a challenging forensic sample. Bones can be difficult to analyze with traditional STR typing methods if the amount of DNA present is limited or degraded. A variety of bones, including those that were commercially obtained and those that were more forensically relevant (environmentally degraded bones), were examined in this study. To begin, three different DNA extraction kits were tested to determine the best method for recovering the highest amount of

DNA from bones. Then, analysis of the DNA from each bone type was performed using three different genotyping/sequencing techniques: STR amplification, whole genome amplification, and massively parallel sequencing. The aim of this study was to determine which enhancement strategy produced the most discriminating DNA profile that could potentially assist with identification from these challenging forensic samples.

This study examined an area of DNA profiling that is novel to forensic investigations and crime laboratories. The improvement of current profiling techniques and emergence of MPS technologies could potentially provide a large advantage for analyzing low template and degraded DNA. This study encompassed the development of a workflow that described the best practices for all steps in the procedure for DNA profiling on bone samples, from DNA extraction procedures to enhancement strategies such as next-generation STR amplification, whole genome amplification, and MPS technologies for DNA analysis. The results of this study can be used to possibly suggest a successful enhancement strategy for actual forensic casework that deal with compromised samples such as bones.

CHAPTER TWO: METHODS

Contamination Precaution

All stages of this project were performed under sterile conditions to prevent the risk of contamination in the bone samples. All DNA extractions and amplification set-up procedures were performed in a designated laboratory, while other downstream procedures were performed in another separate post-amplification laboratory. Personal protective equipment such as a lab coat, disposable sleeves, facial mask, and nitrile gloves were worn and changed frequently throughout the laboratory procedures. Bone cutting, extraction, and PCR amplification set-up procedures were performed in dedicated laminar flow hoods. Work areas were cleaned with 10% (v/v) bleach, 70% (v/v) ethanol, and UV-irradiated for 15 minutes before and after each use. All appropriate supplies and reagents for procedures were exposed to UV light in a UV crosslinker for 15 minutes prior to use.

Bone Samples and Preparation

A total of 12 human bones were used in this study. Three bones (a femur, rib, and hand phalanx) were commercially obtained (Skulls Unlimited International, Inc. Oklahoma City, OK); information about these bones, such as origin and age, were unavailable. They are referred to as the unknown bones or "Unk Femur", "Unk Rib", and "Unk Phalanx" throughout the remainder of this paper. The remaining nine bones were obtained from Western Carolina University's Donated Skeletal Collection. This collection is composed of donors whose remains were left to decompose for varying lengths of time at WCU's Forensic Osteology Research Station (FOReSt), an outdoor human decomposition facility located in Cullowhee, NC. Bones from skeletal remains of three different individuals were used. A right femur, a right 6^{th} rib, and a right

proximal hand phalanx were selected from each set of skeletal remains. The three skeletons were documented as 11-16, 11-19, and 13-07. 11-16 was a Caucasian male that was buried in the year 2011 and was the 16th individual to be donated to FOReSt; 11-19 was a Caucasian male that was surface deposited in 2011 and was the 19th individual to be donated to FOReSt; and 13-07 was a Caucasian male that was surface deposited in 2013 and was the 7th individual to be donated to FOReSt.

Before cutting, all bones were wiped with 70% (v/v) ethanol and sanded with a Dremel® (Racine, WI) rotary tool to remove any surface contaminants and/or exogenous DNA. Bones were then cut into approximately 1 cm x 1 cm fragments with a cutting disc (Figure 4). Bone fragments were sonicated for 20 minutes in UV-irradiated 5% (w/v) Terg-a-Zyme® detergent to remove any proteinaceous contaminants. Pulverization of the bone fragments was performed in a SPEX 6770 Freezer Mill (Metuchen, NJ) to obtain bone powder. To do so, the fragments were put into separate polycarbonate vials with end caps and a stainless-steel impactor bar. The freezer mill was filled with 5 L of liquid nitrogen and set to the following parameters: 5 minutes of pre-cool time, 5 minutes of run time, 2 minutes of cool time, and a rate of 15 cycles per second. The resulting bone powder was then weighed and put into a conical tube for storage at room temperature.

Figure 4. 11-19 right femur from the WCU Donated Skeletal Collection. After the surface was cleaned, an approximately 1 cm x 1 cm fragment was cut from the bone with a cutting disc using a Dremel® rotary tool.

DNA Extraction

The performances of three commercial DNA extraction kits were evaluated for the isolation and purification of DNA from human bones. Samples used in this study were 50 mg of bone powder from the commercially obtained unknown femur, rib, and phalanx samples. The three DNA extractions kits involved in this project were: the PrepFiler® BTA Forensic DNA Extraction Kit (Life Technologies™ 2012), the QIAamp® DNA Investigator Kit (Qiagen® 2012), and the EZ1® DNA Investigator Kit (Qiagen® 2012). Each kit features different methods for extracting DNA; the PrepFiler® BTA kit uses magnetic beads, the QIAamp® kit uses a silica membrane spin column, while the EZ1® kit is fully automated and uses magnetic beads. DNA extraction procedures were performed according to the manufacturer's instructions or with a substituted total demineralization lysis step (Loreille et al. 2007) to see if DNA recovery could be improved. This protocol aimed to maximize the recovery of DNA by complete physical dissolution of the bone powder. Total demineralization included an overnight incubation of the bone powder at 56°C in a demineralization buffer (0.5 M EDTA, pH 8.0; 1% N-Lauroylsarcosine). All extractions for each bone type were performed in quintuplicate. Purified

DNA was eluted in 50 µl of elution buffer. After extraction, the amount of DNA present in the extract was quantified using a human nuclear DNA real-time quantitative PCR (qPCR) assay. A statistical t-test was calculated to determine which kit recovered the most amount of DNA, according to the results of the qPCR assay. The extraction method that consistently produced the highest yield of DNA was used for future bone extractions of this project.

DNA Quantification

DNA within the bone extracts was quantified using a real-time quantification PCR (qPCR) assay to measure the amount of amplifiable human DNA in a sample prior to PCR amplification. qPCR is a process that detects amplified product as the reaction progresses in real time which is enabled by the inclusion of fluorescently labeled probes. At the end of each qPCR cycle, the intensity of fluorescent probes is detected and the measured fluorescence is proportional to the total amount of amplicon product. One commercially available qPCR kit, the Quantifiler® Trio DNA Quantification Kit (Life Technologies™ 2015), is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample by targeting three different loci: large autosomal (214 bases), small autosomal (80 bases), and Y-chromosome (75 bases) targets. With detection of these three targets, not only can the quantity of DNA be measured but also the quality of DNA with respect to the level of DNA degradation. The level of PCR inhibition is detected by the inclusion of a 130 base pair synthetic oligonucleotide called an internal positive control that is co-amplified with the DNA sample.

The Quantifiler® Trio DNA Quantification Kit (Life Technologies™ 2015) was used in accordance with the manufacturer's protocol on an ABI 7500 Real-Time PCR System with the HID Real-Time PCR Software (Thermo Fisher Scientific). PCR conditions were as follows: 95°C hold for 2 min, 40 cycles of 95°C for 9 sec and 60°C for 30 sec. For this quantitation assay,

the amount of amplifiable DNA in the samples was normalized to a standard curve which was calculated using known DNA dilutions (50.0, 5.0, 0.5, 0.05, and 0.005 ng/µL). Inhibition in the samples was assessed by comparing the internal positive control threshold cycle (IPC C_T) values of the samples and the IPC C_T values of the no-template control (NTC). Any delay in the sample IPC C_T value indicated inhibition; if the sample IPC C_T was ±1 of the IPC C_T of the NTC, then the sample was considered contaminated with PCR inhibitors. DNA degradation was assessed by calculating a degradation index, or a ratio of the small autosomal target DNA concentration (ng/µL) to the large autosomal target DNA concentration (ng/µL). If the degradation index was greater than 1, then the DNA within the sample was degraded.

STR Amplification and Analysis

STR amplification was used to genotype the purified DNA obtained from all bone samples. The GlobalFiler™ PCR Amplification Kit (Life Technologies™ 2014) was used to amplify a total of 24 loci: 21 autosomal STR loci, one Y-STR (DYS391), one insertion/deletion polymorphic marker on the Y chromosome (Y indel), and the sex determining marker, Amelogenin. The amount of input DNA that was required for this kit was 1 ng for 29 PCR cycles or 500 pg for 30 PCR cycles. PCR amplification was carried out according to the GlobalFiler™ PCR Amplification Kit on a Veriti™ 96-Well Thermal Cycler (95°C for 1 min; 30 cycles of 94°C for 10 sec, 59°C for 90 sec; 60°C hold for 10 min). 30 PCR cycles were performed since the amount of DNA present in the extracts was less than 500 pg. The amplified products were then detected by capillary electrophoresis using a 3500*xL* Genetic Analyzer (Thermo Fisher Scientific).

The resulting data was analyzed using the GeneMapper™ ID-X Software (Thermo Fisher Scientific) at a 50 RFU detection threshold. By lowering the detection limit to 50 RFUs, more

information from low quality samples could be interpreted. The autosomal STR loci that were reported in each DNA profile was counted and calculated as a percentage. Average percent reported loci were calculated from the sample replicates. The unknown bone samples had five replicates each while the WCU Donated Skeletal Collection bones had three replicates each.

Random match probabilities (RMPs) were calculated to determine discriminatory power of the resulting STR profiles. An RMP is the probability that two randomly selected individuals from a population have identical genotypes by chance alone; it is DNA profile rarity estimate. RMPs are based on the genotypes present in profile as well as the population frequency estimates of the alleles that make up the genotypes. RMPs were calculated using STR allele frequency data from the NIST 1036 US population dataset (Caucasian, African American, Asian, Hispanic), assuming Hardy-Weinberg Equilibrium (Hill et al. 2013). The genotype frequency at each locus was calculated first using the equation $p^2 + 2pq + q^2 = 1$, where p^2 was used for a homozygote genotype and 2pq for a heterozygote genotype. If allele drop-out was suspected, then the 2p rule was used. The entire profile (multi-locus genotype) frequency was then calculated following the product rule, where each STR genotype observed was treated as an independent event. The inverse RMP (1/RMP) values were reported for easy interpretation of the DNA profile rarity in certain US population groups.

Whole Genome Amplification

The purified DNA obtained from the unknown bones samples was subject to pre-amplification using MDA WGA to determine if the amount of DNA template in the samples could be increased. MDA WGA was performed with the REPLI-g® Mini Kit (Qiagen® 2011) according to the manufacturer's instructions, with 5 µl of purified DNA. The purified DNA was denatured by adding a denaturation buffer. Denaturation was stopped by the addition of a

neutralization buffer and a MDA reaction mixture (containing reaction buffer, dNTPs, random hexamers, and Phi29 DNA polymerase) was added. Phi29 catalyzes the strand-displacing reactions that is characteristic of MDA. An isothermal amplification reaction took place on a Veriti® Thermal Cycler (Applied Biosystems) for 16 hours at 30°C and was heat inactivated at 65°C for 10 min. This method does not require the repetitive cycles of denaturing and annealing temperatures common in most PCR techniques. The resulting WGA products was quantitated using the Quantifiler™ Trio DNA Quantitation assay and diluted (if necessary) to the appropriate concentration for STR amplification with the GlobalFiler™ PCR Amplification Kit.

Massively Parallel Sequencing Using Ion PGM™

Massively parallel sequencing was conducted on the unknown bone samples using the Ion Torrent™ Personal Genome Machine, or Ion PGM™. The HID-Ion AmpliSeq™ Identity Panel was used for library amplification and preparation; this assay targeted a total of 124 SNPs (90 identity/autosomal SNPs and 34 upper Y-clade SNPs). The amount of input DNA required was 1 ng. Since the samples contained less than 1 ng of DNA, the maximum volume (6 µL) was added to the PCR reaction. Sequencing libraries were prepared using the Ion AmpliSeq™ Library Kit 2.0 according to the Ion AmpliSeq™ Library Preparation for Human Identification Applications User Guide (Thermo Fisher Scientific Inc. 2014). Library amplification was performed in the Veriti® Thermal Cycler (Applied Biosystems). Each library amplicon was barcoded using the Ion Xpress™ Barcode Adapters Kit (Thermo Fisher Scientific Inc. 2014). Barcoded libraries were purified with Agencourt® AMPure® XP Reagents (Beckman Coulter, Brea, CA) and quantified with qPCR using the Ion Library Quantitation Kit (Thermo Fisher Scientific Inc. 2014) to measure the concentration of the prepared libraries. Based on the qPCR

results, libraries were diluted to the recommended concentration and pooled together at equal concentrations for clonal amplification via emulsion PCR (emPCR).

emPCR was performed on a OneTouch™ 2 system to prepare template-positive Ion Sphere Particles (ISPs). The ISPs were enriched on a OneTouch™ ES system with the Ion PGM™ Template OT2 200 Kit according to the Ion PGM™ Template OneTouch™ 2 200 Kit User Guide (Thermo Fisher Scientific Inc. 2014). The enriched ISPs were then loaded into an Ion 316 Chip and placed in the Ion PGM™ instrument. Sequencing was performed in the Ion PGM™ following the guidelines of the Ion PGM™ Hi-Q™ Sequencing User Guide (Thermo Fisher Scientific Inc. 2015). Raw sequencing data were processed in the Ion Torrent Suite™ Software with the HID SNP Genotyper Plugin adapted for data analysis. A threshold of 20 reads was used to detect SNP genotypes. The HID SNP Genotyper Plugin also provided RMP and 1/RMP values for each sample; these were calculated from the 1000 Genomes database (American, South Asian, East Asian, European, African). An issue arose with the Ion PGM™ server instrumentation which prevented other sequencing runs to be performed. Data from the WCU Donated Skeletal Collection bone samples were not able to be collected. The results from the unknown bone samples were still included, however.

Massively Parallel Sequencing Using MiSeq® FGx™

Massively parallel sequencing was conducted on all 12 bone samples using the MiSeq® FGx™ Forensic Genomics System. The ForenSeq™ DNA Signature Prep Kit was used for library amplification and indexing following the recommended manufacturer's protocol for purified DNA samples (Illumina® 2014). DNA Primer Mix B multiplex was used for library amplification, which consisted of primer pairs that targeted 58 STRs (27 autosomal STRs, 7 X-STRs, and 24 Y-STRs), 94 identity informative SNPs, 56 biogeographical ancestry informative

SNPs, and 22 phenotypic informative SNPs. The amount of input DNA required was 1 ng. Since the samples contained less than 1 ng of DNA, the maximum volume (5 µL) was added to the PCR reaction. Prepared libraries underwent a bead-based purification and normalization process. Once normalized, the libraries were pooled together at equal volumes and concentrations. Pooled libraries were then diluted in hybridization buffer and denatured. Sequencing was performed on the MiSeq FGx™ instrument with the MiSeq ForenSeq™ Sequencing Kit (Illumina) according to the manufacturer's protocol. Sequencing data was analyzed using the Illumina® ForenSeq™ Universal Analysis Software (UAS).

The ForenSeq™ UAS provided quality and coverage data for all samples and was used to determine STR and SNP genotypes as well as estimate ancestry and phenotype. The default threshold settings were used, which were based on the number of total reads at a particular locus. The analytical threshold was set at 10 reads while the stochastic threshold (known as the interpretation threshold by the software) was set at 30 reads. Although they passed the analytical threshold, majority of the alleles were not called because the number of reads fell below the 30 read stochastic threshold. The ForenSeq™ UAS allowed for the user to modify and alter the genotype at each locus, if alleles were detected at that locus. Because increased stochastic effects were expected in these degraded DNA samples, the alleles that fell between the analytical and stochastic threshold were modified and called as true alleles. The ForenSeq™ UAS calculated 1/RMPs from the STR length-genotypes, STR sequence-genotypes, and SNP genotypes. Estimates of 1/RMPs for all bone samples were calculated using partial profiles, no complete profiles were obtained. Allele frequencies from the NIST 1036 US population datasets (Caucasian, African American, Asian, Hispanic) were used.

CHAPTER THREE: RESULTS

Performance of Three DNA Extraction Kits on Bone Material

The performances of three DNA extraction kits was determined based on DNA yield measured by a qPCR assay. The R^2 values for all standard curves was at least 0.99, showing good linearity. The average total amount of DNA isolated from the unknown bones in a final volume of 50 µl with the PrepFiler® BTA Forensic DNA Extraction Kit, the QIAamp® DNA Investigator Kit, and the EZ1® DNA Investigator Kit was 0.289, 0.021, and 0.015 ng respectively, per 50 mg of bone powder (Table 1). DNA yields were taken from the small autosomal target DNA concentrations of the qPCR assay. On average, the PrepFiler® BTA Forensic DNA Extraction Kit recovered a significantly higher concentration of DNA (M = 0.289, SE = 0.08) compared to the QIAamp® DNA Investigator Kit (M = 0.021, SE = 0.006), p = 0.003 and the EZ1® DNA Investigator Kit (M = 0.015, SE = 0.003), p = 0.0036. The PrepFiler® BTA Forensic DNA Extraction Kit was therefore used to extract DNA from the 11-16, 11-19, and 13-07 bones. DNA yields from the WCU Donated Skeletal Collection bones are reported in the appendix (Table A1).

Table 1. DNA yields from 50 mg of bone powder for the three unknown bones. Three different DNA extraction protocols were used to test their performance. The result for each bone is an average of five replicates. The average DNA yield for each extraction kit was calculated as well.

Bone	DNA yield from 50 mg of bone powder (ng)		
	PrepFiler® BTA	QIAamp®	EZ1®
Unk Femur	0.102	0.010	0.015
Unk Rib	0.062	0.002	0.006
Unk Phalanx	0.702	0.051	0.025
Average	0.289	0.021	0.015

Total Demineralization

Total demineralization of the bone powder was performed to test whether DNA yields could be increased compared to DNA yields from the PrepFiler® BTA Forensic DNA Extraction Kit according to the manufacturer's instructions. This kit's protocol was modified with a substituted total demineralization lysis step (Loreille et al. 2007). DNA extractions with total demineralization were performed on the three unknown bone samples. The results were reported with the average concentrations of the small autosomal target DNA according to qPCR data (Figure 4). Per 50 mg of bone powder, the average amount of DNA isolated from the analyzed bones with total demineralization was 0.094 ng (*SE* = 0.02), which was much lower than the average amount of DNA isolated without total demineralization (*M* = 0.289 ng, *SE* = 0.08). A statistical t-test using an alpha level of 0.05 revealed that the amount of DNA recovered with the total demineralization lysis step was significantly different from the amount of DNA recovered without total demineralization ($p = 0.004$). With this observation, the total demineralization protocol did not improve DNA yield from bone samples and was not used for later bone extractions.

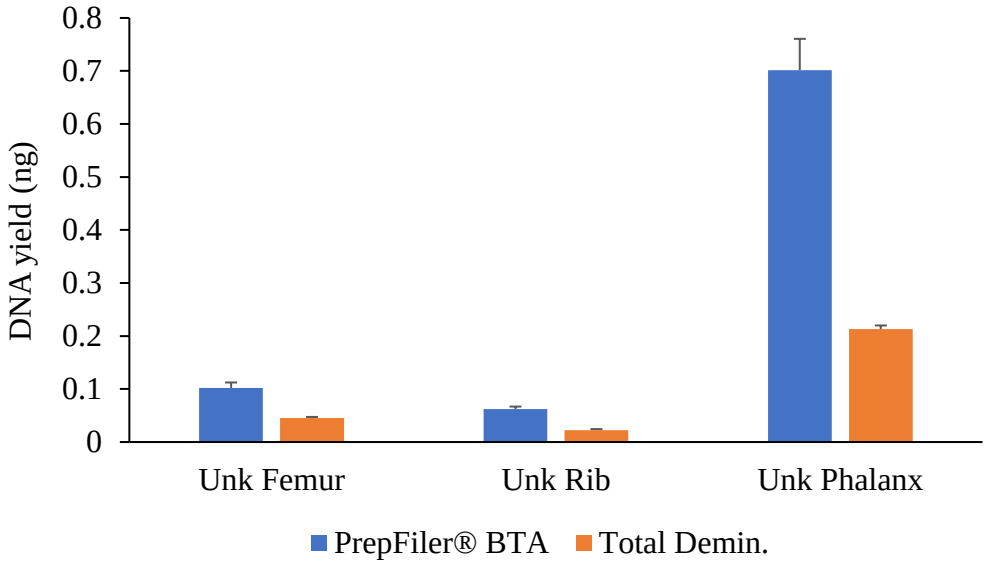

Figure 5. Average DNA yield from the unknown bone samples with total demineralization. Amounts were compared to the DNA yield obtained from the PrepFiler® BTA Forensic DNA Extraction Kit without total demineralization.

PCR Inhibition and DNA Degradation Assessment

IPC C_T values were used for the detection of PCR inhibitors in the DNA extracts. For all 12 bone samples, there was proper IPC amplification according to qPCR data which suggested that none of the extracts contained PCR inhibitors. The IPC C_T values of each sample were within ±1 of the IPC C_T of the NTC. Degradation indices were calculated for each of the bone extracts and results suggested that all extracts contained degraded DNA (Table 2). This result was characteristic of bone samples because of the decomposition process and the postmortem time period. Extreme degradation was seen in the unknown bones.

Table 2. Degradation indices for all bone samples. Ratios were calculated according to qPCR assay. An index greater than 1 indicated there was more amplification of the small autosomal target than the large autosomal target and therefore DNA degradation was possible.

Bone	Mean Large Target Quantity (ng)	Mean Small Target Quantity (ng)	Mean Degradation Index
Unk Femur	0.005	0.102	20.4
Unk Rib	0.004	0.062	17.7
Unk Phalanx	0.049	0.702	14.3
11-16 Femur	0.138	0.236	1.72
11-16 Rib	0.067	0.148	2.22
11-16 Phalanx	0.023	0.045	2.00
11-19 Femur	0.034	0.299	8.70
11-19 Rib	0.159	0.363	2.28
11-19 Phalanx	0.015	0.116	7.71
13-07 Femur	0.187	0.339	1.82
13-07 Rib	0.172	0.358	2.09
13-07 Phalanx	0.028	0.123	4.39

Whole Genome Amplification

Whole genome amplification using a multiple displacement amplification method was tested to determine its ability to increase the amount of DNA template present in the unknown bone samples. The quantity of DNA that was put into the WGA reaction was compared to quantity of DNA that was present after the WGA reaction (Figure 5); a statistical t-test using an alpha level of 0.05 was used to determine if there was a significant difference. For the unknown femur sample, results showed that the amount of DNA after the WGA reaction (M = 0.0190 ng, SE = 0.002) was significantly different than the amount of DNA present in the original sample (M = 0.0102 ng, SE = 0.001), p = 0.02505. Although the yield of amplified DNA was increased, it was still beneath the required DNA input amount for STR typing. For the unknown rib sample, results showed that the amount of DNA after WGA (M = 0.0045, SE = 0.0005) was not significantly different than the amount of DNA present in the original sample (M = 0.0062 ng, SE = 0.0005), p = 0.0745. For the unknown phalanx sample, results showed that the amount of DNA after WGA (M = 0.062 ng, SE= 0.004) was not significantly different than the amount of DNA present in the original sample (M = 0.7015 ng, SE = 0.006), p = 0.274. The yield of amplified DNA after WGA was less than the input amount for both the unknown rib and phalanx samples. Overall, these results indicated that there was no major improvement in the amount of DNA template after WGA was performed. With this observation, WGA may not be very efficient at increasing the amount of template DNA for low quantity and degraded DNA that is frequently encountered in bone samples.

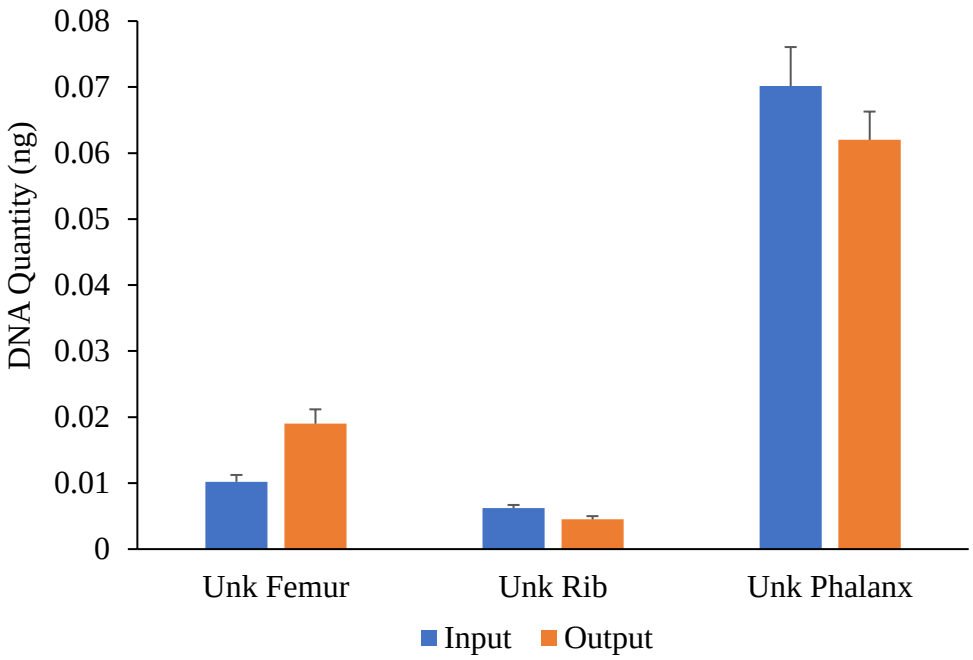

Figure 6. Comparison of DNA quantity (ng) before and after WGA reaction. The REPLI-g® Mini kit was used for WGA MDA. Bone samples are displayed along the horizontal axis. "Input" refers to the amount of DNA that was present within the bone extract, "output" refers to the amount of DNA that was amplified by WGA.

Comparison of Reported Loci from Each Genotyping Assay

The maximum volume for each genotyping assay was used for all bone samples since the amount of DNA in each extract was below the recommended 1 ng. Table 3 compares DNA input amounts for each sample amplification. The success of each genotyping assay (GlobalFiler® STRs, Ion PGM™ iSNPs, MiSeq® STRs, and MiSeq® iSNPs) was evaluated by comparing the percent of reported loci (Figure 6). The percentage of reportable loci metric normalized the assays so that no assay was penalized for containing fewer loci and conversely, no assay was at an advantage for containing more loci. It should be noted that only the genetic markers used for discriminatory purposes were reported here: autosomal STRs and identity-informative (autosomal) SNPs. The GlobalFiler® STR assay and the MiSeq® STR assay shared 20 loci and

genotype results from both assays were concordant. The Ion PGM™ iSNP assay and the MiSeq® iSNP assay shared 83 loci and genotype results from both assays were concordant.

Amplification of the degraded DNA samples resulted in partial profiles for majority of the assays tested; stochastic effects such as peak height imbalance, enhanced stutter, and allele/locus drop out were seen. However, complete profiles (100% reported loci) were achieved for all replicates of the 13-07 femur and 13-07 rib samples with the GlobalFiler® STR assay. Excluding the Ion PGM™ iSNP assay, the GlobalFiler® STR assay had the highest percentage of reported loci across all bone samples (M = 78.00%, SE = 0.063), followed by the MiSeq® STR assay (M = 43.78%, SE = 0.064), and the MiSeq® iSNP assay (M = 27.88%, SE = 0.051). By just comparing the unknown bone samples, the Ion PGM™ iSNP assay had the highest percentage of reported loci (M = 67.07%, SE = 0.098) but the GlobalFiler® STR assay was not far behind (M = 53.25%, SE = 0.108).

Table 3. Comparative input DNA amounts (ng) for each genotyping assay. The maximum volume for each assay was used for all bone samples. Maximum volumes were 15, 5, and 6 µL for the GlobalFiler® Kit, MiSeq® FGx ForenSeq™ DNA Signature Prep Kit, and Ion PGM™ Identity Panel respectively.

Sample	Input DNA amounts for each genotyping assay (ng)		
	GlobalFiler®	MiSeq® FGx ForenSeq™	Ion PGM™ Identity Panel
Unk Femur	0.0306	0.0102	0.0133
Unk Rib	0.0186	0.0062	0.0079
Unk Phalanx	0.2105	0.0702	0.0828
11-16 Femur	0.0708	0.0236	-
11-16 Rib	0.0442	0.0147	-
11-16 Phalanx	0.0135	0.0045	-
11-19 Femur	0.0897	0.0299	-
11-19 Rib	0.1089	0.0363	-
11-19 Phalanx	0.0347	0.0116	-
13-07 Femur	0.1015	0.0338	-
13-07 Rib	0.1075	0.0358	-
13-07 Phalanx	0.0369	0.0123	-

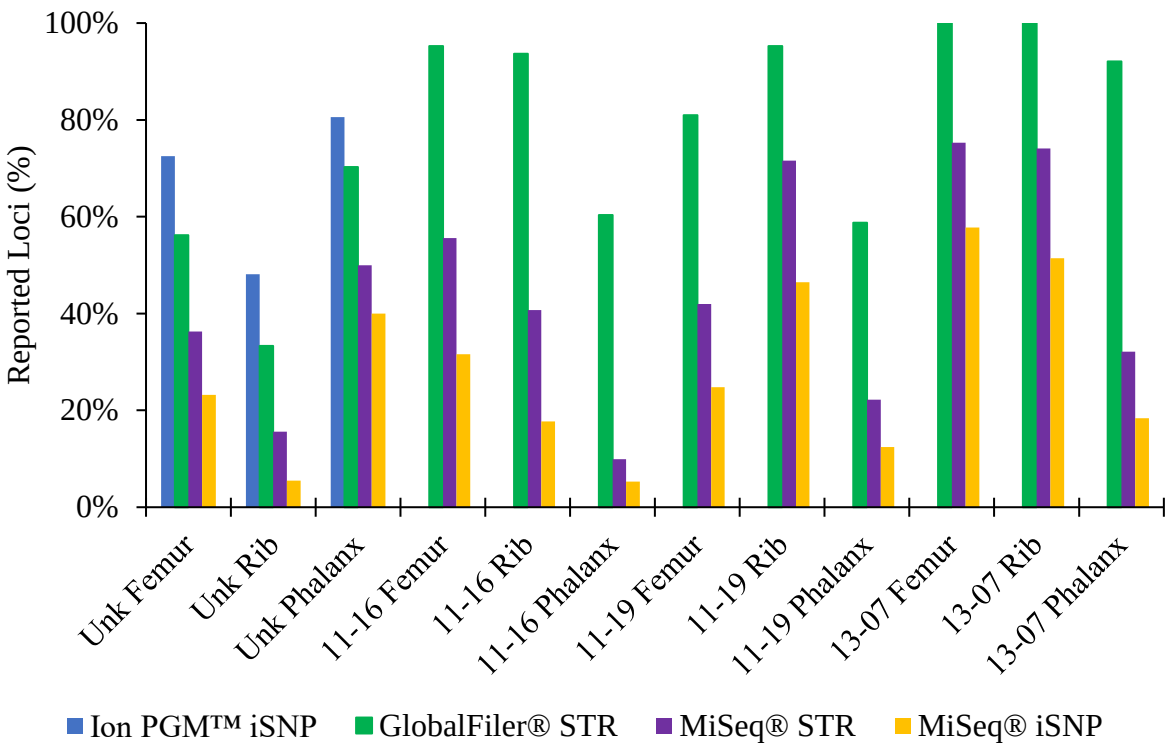

Figure 7. Comparison of reported loci (%) from each genotyping assay. All genetic markers that were used for discriminatory purposes are given (autosomal STRs or identity-informative SNPs). Most assays resulted in partial profiles, but complete profiles resulted from the GlobalFiler® STR assay for all replicates of the 13-07 femur and 13-07 rib samples.

Random Match Probabilities

To measure the discriminatory power for each analysis of degraded DNA from bones, random match probabilities were calculated. The RMP values reflected the suitability of each assay to characterize DNA; RMP values differed with each sample profile due to the varying frequencies associated with each genotype. The inverse of RMPs (1/RMPs) were reported to give the likelihood of obtaining the same DNA profile from an unrelated individual of the US Caucasian/European population group (Figure 7). Excluding the Ion PGM™ iSNP assay, the GlobalFiler® STR assay was able to consistently achieve the highest discriminating power, having an average 1/RMP value of one in one octillion (10^{27}). This was due to the high

percentage of reported loci. Lower RMP values were obtained with the MiSeq® STR and MiSeq® SNP profiles because of the small number of loci that were detected in both assays. The average 1/RMP value for the MiSeq® STR length-genotype assay was one in one quintillion (10^{18}), while the average for the MiSeq® STR sequence-genotype was one in ten sextillion (10^{22}). The STR sequence-genotype was more discriminating compared to the STR length-genotype because the underlying sequence variation of the STRs increased allelic diversity and therefore power. In addition, the small number of reported loci combined with the low discriminating power of bi-allelic SNPs suppressed the 1/RMP values for the MiSeq® iSNP assay which had an average 1/RMP value of one in ten trillion (10^{13}). Looking at the unknown bone samples, the Ion PGM™ iSNP assay generated the highest 1/RMP values, an average of one in ten undecillion (10^{37}). All other 1/RMP values for US African American, Hispanic, and Asian population groups are reported in Tables A2-A6 of the appendix.

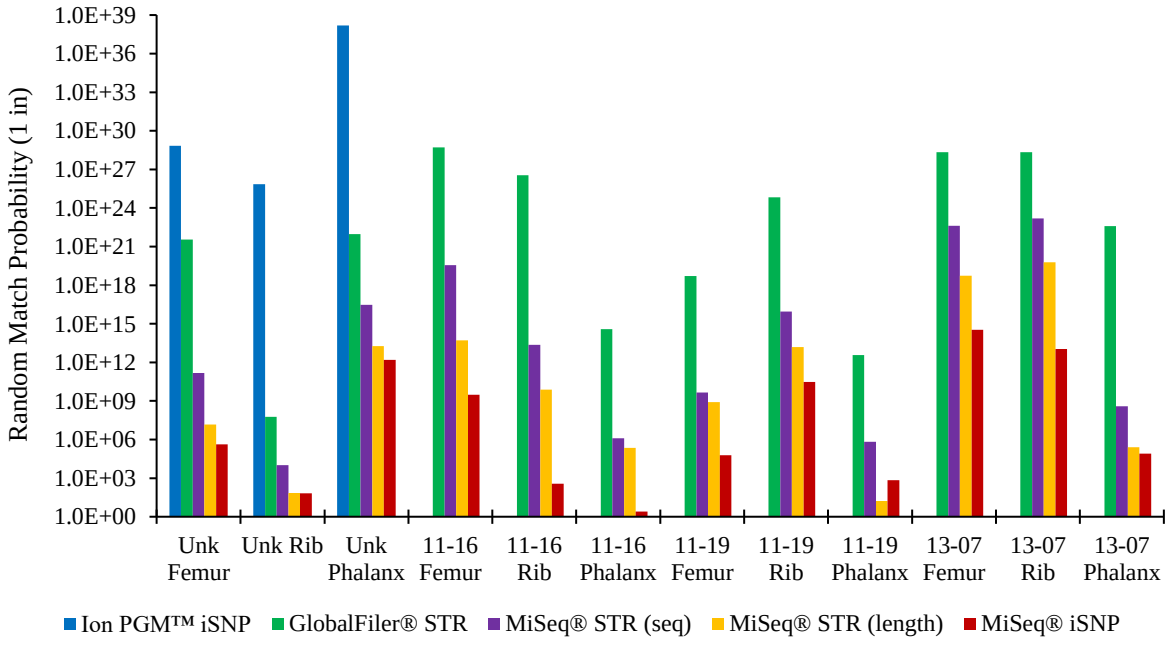

Figure 8. RMP (1 in) generated by each analysis of degraded DNA from bone samples. 1/RMP values were reported for the Caucasian/European population and displayed on a logarithmic scale.

Ancestry and Phenotype SNP Analysis

For the Ion PGM™ platform, the HID-Ion AmpliSeq™ Identity Panel examined 34 SNPs located on the upper clade of the Y chromosome that helped to predict Y haplogroups of the unknown bone samples (Table 4). Predications were made using the Ion Torrent Suite™ Software with the HID SNP Genotyper Plugin. Results predicted C, IJK/R1b, and H1 haplogroups for the unknown femur, rib, and phalanx respectively. The HID SNP Genotyper Plugin also provided descriptions about these haplogroups and all Y haplogroups had origins from South Asia and India.

For the MiSeq® FGx platform, the ForenSeq™ DNA Signature Prep Kit examined 56 SNPs that estimated biogeographical ancestry of the samples based on principle component analysis (PCA). The ForenSeq™ UAS created PCA plots that reflected the best fit population estimate of each sample's biogeographical ancestry using 1000 Genomes data: Ad Mixed American, African, East Asian, European. Results showed that the major population of biogeographical ancestry for the WCU Donated Skeletal Collection bones was European, which was consistent with antemortem donor records (Figure 9). Each of the unknown bones had ancestry that associated more with the East Asian population group. This is consistent with the vast majority of anatomical skeletal material found in the United States (Hefner et al. 2016). The MiSeq® FGx™ platform also had to the potential to estimate hair and eye color with 22 phenotype informative SNPs. The ForenSeq™ UAS could generate individual probabilities for four hair color categories (black, brown, blonde, and red) and three eye color categories (brown,

blue, and intermediate). In order to make these estimates, all phenotype SNP loci had to be detected. Unfortunately, incomplete profiles were generated from the degraded DNA of the bone samples and phenotypic estimates could not be calculated (Figure 9).

Table 4. Summary table of predicted Y haplogroups from the unknown bone samples. Predictions were made using the Ion PGM™ MPS platform with the Ion Torrent Suite™ Software and HID SNP Genotyper Plugin.

Bone	Y Haplogroup Prediction	Origin
Unk Femur	C	Indian subcontinent, Sri Lanka, parts of SE Asia
Unk Rib	IJK, R1b	Southwest Asia
Unk Phalanx	H1	Indian subcontinent

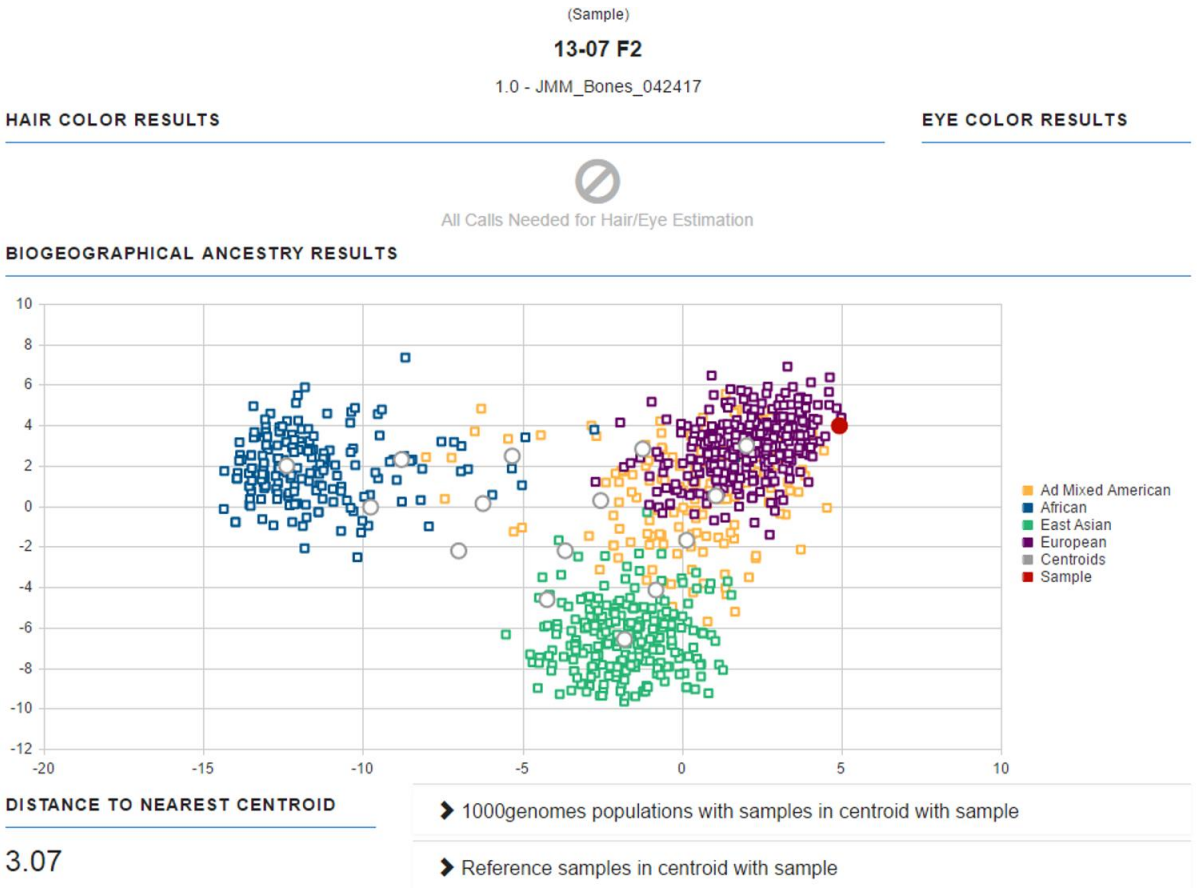

Figure 9. PCA plot of ancestry informative SNPs. Plot was generated with the Illumina® ForenSeq™ Universal Analysis Software and displayed the best fit population estimate. The population estimate for the 13-07 Femur is the red circle, which was closely associated with the European population (purple). No hair or eye color estimate could be determined.

CHAPTER FOUR: DISCUSSION

DNA Extraction

Three DNA extraction kits were evaluated in this study by their ability to recover genomic DNA from human bone samples: the PrepFiler® BTA Forensic DNA Extraction Kit, the QIAamp® DNA Investigator Kit, and the EZ1® DNA Investigator Kit. Mainly, these DNA extraction kits are solid-phase DNA extraction methods but have two different approaches: the magnetic bead technique or the silica column technique. The PrepFiler® BTA Forensic DNA Extraction Kit has a two-hour cell lysis/incubation step and uses proprietary polymer-coated magnetic beads to bind, wash, and purify DNA. The EZ1® DNA Investigator Kit also uses magnetic beads, which have a silica polymer coating, for DNA purification and is fully automated; it can process up to 14 samples in approximately 20 minutes following an overnight cell lysis/incubation step. Lastly, the QIAamp® DNA Investigator Kit requires an overnight cell lysis/incubation step and uses silica-based spin columns for DNA purification; wash steps are performed in spin columns using centrifugation.

Measurement of DNA quantity using a real-time qPCR assay showed that the PrepFiler® BTA Forensic DNA Extraction Kit recovered the highest yield of DNA from 50 mg of bone powder and far exceeded the performance of the other kits. One explanation for its improved performance is that the use of a magnetic bead technique enables fewer tube transfers during the extraction process, which prevents sample mix-ups, contamination, and/or loss of DNA. The QIAamp® spin columns require several tube transfers during the wash/centrifugation steps which increases the chance for DNA to be left behind in a tube or lost. In addition, higher yields were obtained with the PrepFiler® BTA Forensic DNA Extraction Kit because the PrepFiler®

magnetic bead size was designed to be much smaller than typical magnetic bead sizes, such as those used by EZ1® DNA Investigator Kit. A smaller bead size thus provides a higher surface area for capturing DNA molecules during the extraction process.

Another reason for the high recovery of the PrepFiler® BTA Forensic DNA Extraction Kit lies in its lysis buffer composition. Most lysis buffers are formulations of detergents, chaotropic salts, and a chelating agent such as ethylenediaminetetraacetic acid (EDTA). EDTA is used to sequester divalent metal ions in cell extracts (Mg^{2+} and Ca^{2+} for example) and inactivates cellular nucleases since metal ions are nuclease cofactors. The lysis buffers for the QIAamp® and EZ1® DNA Investigator Kit both contain EDTA. However, the PrepFiler® BTA lysis buffer does not contain EDTA, but rather ethylene glycol tetraacetic acid (EGTA). EGTA is a chelating agent like EDTA, but it preferentially binds to and has a higher affinity for calcium ions (Ca^{2+}). Because much of bone content contains calcium minerals and EGTA specifically has Ca^{2+} chelation, the PrepFiler® BTA lysis buffer worked efficiently to extract DNA from bone material and therefore resulted in high DNA yields.

In summary, the results demonstrated that the PrepFiler® BTA Forensic DNA Extraction Kit performed better than the two other commercial kits evaluated for the extraction of DNA from human bones; it yielded an almost 16-fold larger yield of DNA. With the incorporation of smaller magnetic beads and EGTA into the lysis buffer, this kit is more suitable for extracting DNA from challenging samples such as bones. An added benefit of the PrepFiler® BTA Forensic DNA Extraction Kit is that it is more time efficient because it only requires a two-hour cell lysis/incubation time, which is far less than the time required for the overnight digestion of the QIAamp® and EZ1® DNA Investigator Kits. Therefore, successful DNA extraction from

bone samples can be accomplished within a few hours using this kit, rather than the two-day time period for the QIAamp® and EZ1® DNA Investigator Kits.

Enhancement Strategies for DNA Profiling of Bone Samples

Enhancement strategies that would improve DNA profiling of low quantity and low quality DNA from bone samples were evaluated in this study. These strategies included genotyping assays that analyzed forensically informative STRs and SNPs dispersed throughout the human genome. Traditional capillary electrophoresis method using the GlobalFiler® STR assay was compared to massively parallel sequencing technologies to see which achieve the best 1/RMP values. The GlobalFiler® assay genotyped 21 autosomal STRs (including 10 mini-STRs), the Ion PGM™ assay genotyped 90 autosomal SNPs, and the MiSeq® assay genotyped 27 autosomal STRs as well as 94 autosomal SNPs. Each genotyping assay was evaluated on how discriminating the resulting DNA profile was for the bone samples examined in this study. Determining the discriminatory power of the DNA profiles reflected the evidentiary value of the samples and how the information would be used in forensic applications, such as a testimony in court.

Comparing the GlobalFiler® STR assay and the MiSeq® STR and iSNP assays, the 1/RMP values of all bone samples were greater for the GlobalFiler® STR profiles. The MiSeq® STR profiles had low discriminating power due to having fewer reported loci than in the GlobalFiler® STR profiles. For the MiSeq® iSNP profiles, low 1/RMPs were calculated because the bi-allelic SNPs are far less discriminating per locus than the STR loci examined in the GlobalFiler® assay. Additionally, the MiSeq® assay had an overwhelming number of targeted loci which may have contributed to its weakened performance compared to the other genotyping assays evaluated in this study. It has been shown that assays with a large number of markers are

more sensitive to the quantity and quality of the input DNA template (Butler et al. 2007). This was particularly true since small amounts of degraded DNA from bone samples was tested here. Issues may have arisen because the amount of input DNA was less than the recommended 1 ng and the DNA template that specific primers were targeting may not have been present or if they were, failed to amplify due to inadequate quality. Thus, a limited number of loci were detected and partial profiles were seen in the results.

Focusing on the results of the unknown bone samples, the Ion PGM™ iSNP assay achieved the highest 1/RMP values, likely due to both the small amplicon size and larger number of loci that were successfully targeted in this assay. The GlobalFiler® STR profiles produced the second highest 1/RMP values. Though the average 1/RMP of one in ten undecillion (10^{37}) from the Ion PGM™ iSNP assay is numerically much different from the average 1/RMP of one in one octillion (10^{27}) from the GlobalFiler® STR assay, both 1/RMP values are considered highly discriminating in the forensic community. The magnitude of these 1/RMP estimates has reached a point where it may be appropriate to declare source attribution, or to say that an individual is the source of an evidentiary DNA sample with a reasonable degree of scientific certainty (Budowle 2000). Forensic laboratories have established predefined thresholds for when it is appropriate to declare source attribution, like when the 1/RMP estimate is rarer than a thousand times the size of the US population, or one in 300 billion (Butler 2015). Because both these assays produced strong RMP values that exceed this source attribution threshold, a larger number of SNP markers may not offer as much discriminatory power improvement over STR markers when analyzing DNA with low template levels. Although the Ion PGM™ iSNP assay offered some improvement in 1/RMP values, the values obtained with traditional STR typing may be sufficient for discrimination purposes.

Making Sense of Discriminatory Power

For criminal investigations, two DNA profiles are typically evaluated: one from the collected biological evidence, also called the unknown sample, and one from a reference or known sample. Because DNA profiles include only a few genetic loci, it is not impossible for two entirely unrelated people to have a matching profile by chance. Random match probabilities are therefore calculated to estimate how likely it is for this chance to occur. These statistical calculations are used by forensic scientist as a part of their expert testimony, where they must measure the strength of their evidence and reason probabilistically if the DNA profile of the unknown sample confirms or excludes a match to the known DNA profile.

However, these estimates are often presented in a way that lay audiences, such as jurors in court, can't seem to comprehend. With the wide array of genetic markers available for forensic testing, it is not uncommon for these random match probabilities derived from a DNA profile to contain astronomical numbers (Ziętkiewicz 2012), i.e. the random match probabilities seen this is study. These values can often exceed the current world population of seven billion people and even the total human population that has ever lived on Earth (~110 billion) by several folds, which may be confusing for the average lay person (Thompson and Newman 2015). Such inconceivable probabilities can be difficult for jurors to assign appropriate probative value to the DNA evidence and may even dissuade their decision (Cowley 2017). Of course, the more discriminating a profile is, the more strength an observed match will have. But forensic laboratories should consider whether having MPS technologies that could potentially yield extremely discriminating numbers is worth the time and expense for them to convert from CE technologies. CE technologies have been widely accepted by the judicial system and the random

match numbers produced may be sufficient for identification purposes. Nonetheless, the tradeoffs for each technology should be thoroughly evaluated.

Implementation of MPS for Forensic Applications

Based on the results obtained from this study, analyzing STR markers using traditional CE methods may perform just as well or even better than analyzing SNP and even STR markers using massively parallel sequencing when testing low levels of degraded DNA from bones. One reason that the CE method performed so well could be because the GlobalFiler® Kit allowed for a higher volume of sample DNA input compared to the kits of the MPS platforms: 15 µL for the GlobalFiler® Kit, 6 µL for the Ion PGM™ Identity Panel, and 5 µL for the MiSeq® FGx ForenSeq™ DNA Signature Prep Kit. Though the concentration of DNA in the bone extracts was below the recommended 1 ng for each assay, the larger volume of sample DNA input may have facilitated the analysis of low template DNA of the bone samples.

Although the Ion PGM™ iSNP assay performed slightly better than the GlobalFiler® STR assay, MPS technologies may not be the best solution for DNA profiling of low template samples. Currently, there is a problem with MPS implementation into forensic laboratories for DNA analyses because capillary electrophoresis has already been fully validated and accepted into the legal system. There is a hesitation to implement high throughput instrumentation because forensic laboratories would need to put forth a dedicated effort to adopt the advanced methodology and processes of MPS (Dickens 2016). Additionally, the experimental workflow and bioinformatics required for MPS analyses are much more complex than the CE workflow currently used in forensic laboratories (Zhang et al. 2011). MPS platforms require a lot more time and labor, especially for manual preparations of DNA libraries. With so many genetic markers typed in MPS, there is an increased complexity of data to be examined and interpreted.

Because of this, data analysis becomes reliant upon advanced computer systems (Zhang et al. 2011). Though MPS platforms have user-friendly analysis interfaces for data input and output, the analyst may not be able see what happened "behind the scenes" that led the software program to produce those results. Another topic that needs to be addressed before MPS implementation is defining useful thresholds for interpretation. Analysis software programs apply default thresholds when allele calling; these default settings may have to be optimized to enhance accurate genotyping, especially of low template DNA.

Lastly, there is the question of whether SNP markers could replace STR markers for DNA profiling. Forensic laboratories primarily use standardized CE-based technology to detect length variation in STR markers for DNA profiling (Yang et al. 2014). Because CE-based STR analysis is widespread, there are well-established DNA databases that contain millions of STR profiles based on just a few core STR markers (e.g. the FBI Laboratory's Combined DNA Index System, CODIS). On the contrary, there are no nationally recognized SNP databases used for forensic investigations that are compatible with STR data due to the vast number of available SNP loci and the inconsistent use across forensic laboratories. Furthermore, multiple platforms exist for SNP typing with various methods for detection (e.g. the MPS platforms used in this study), resulting in a lack of consensus throughout the forensic community (Butler et al. 2007). Autosomal STR markers rather than autosomal SNP markers have a more dominant role in forensic DNA identification because autosomal STRs are more polymorphic and discriminating per locus. Thus, CE-based STR multiplexes can examine a smaller number of loci, have shorter generation times, and allow for more simplistic interpretation (Butler et al. 2007). For the reasons aforementioned, one could argue that SNPs may not be ready to replace STRs. Although

autosomal STRs are better suited for identification purposes, other applications of SNP assays should still be evaluated.

Other Applications of SNP Analysis Using MPS

Another application for SNP analysis using MPS are the SNP markers that predict Y haplogroups, biogeographical ancestry, and superficial phenotypic traits of the sample. Though they were not extensively analyzed in this study like the identity informative SNPs, these ancestry and phenotype informative SNPs provided more data and insight about the evaluated bones. MPS technologies can provide more information about a sample than just the simple number of repeats STR loci have.

Analyzing Y-STRs with CE methods can help to identify any males who have contributed to the sample, but analyzing Y-SNPs with MPS technology can provide deeper knowledge about the ancestral origin of the male DNA by determining Y chromosome lineage. The Ion PGM™ HID-Ion AmpliSeq™ Identity Panel coupled with the HID SNP Genotyper Plugin analysis software mapped the detected Y-SNPs to the Y-chromosome phylogenetic tree and determined Y haplogroups that originated from different geographical regions of the world. In contrast to STR markers which have high mutation rates, SNP markers are more stable by having a low mutation rate and are more suitable for phylogenetic analysis (Ochiai et al. 2016). While Y-SNPs have limited use for individualizing a sample, they may be helpful in estimating ancestral origin of the individual (Butler et al. 2007).

Since the Y haplogroup prediction of the unknown bones indicated an Indian/South Asian origin, the question of whether these bones were a part of India's bone trade comes to mind. For 200 years, India has been the world's primary source of bones used for medical or human anatomical study (Hefner et al. 2016). Since colonial times, India has had a mysterious network

of bone traders who would collect human skeletal remains from cemeteries of remote Indian villages in order to sell them to universities and hospitals around the world. In 1986 however, the export of skeletal remains was banned by the Indian government following rumors that traders were murdering people for their bones (Hefner et al. 2016). Though the bones used in this study were legally purchased and this speculation cannot be affirmed, it is still interesting that these bones could have been a part of the legal Indian bone trade. These results however provide insight as to the types of forensic cases that may require Y-SNP analysis using MPS.

By analyzing genetic markers such as ancestry-informative and phenotype-informative SNPs, MPS technology can offer new investigative leads in cases where the person's identity is not known. Information inferred from a DNA profile in regard to what part of the world a person comes from or their physical traits including hair and eye color can help law enforcement agencies decide what direction to take during criminal investigations, missing persons investigations, or mass disaster victim identification where reference samples may not be available. It may even be possible to narrow down the list of potential suspects in an investigation using these ancestry and phenotypic inferences derived from a DNA sample (Yang et al. 2014). In these cases, SNP typing could help provide investigators with information about a perpetrator or victim based solely on the biological evidence left at the crime scene (Butler et al. 2007).

Conclusions

In summary, this study evaluated DNA extraction as well as DNA profiling methods for the enhanced performance on human bones, a challenging forensic sample type. It was determined that the PrepFiler® BTA Forensic DNA Extraction Kit recovered the highest DNA yield compared to the QIAamp® DNA Investigator Kit and the EZ1® DNA Investigator Kit. Total demineralization

was not efficient for complete dissolution of bone powder, nor was whole genome amplification for improved DNA quantity for STR amplification. Random match probabilities calculated from all genotyping methods showed that the Ion PGM™ iSNP assay provided the highest discriminatory power and reported loci, followed by the GlobalFiler® autosomal STR assay. All MiSeq® assays had the poorest performance in terms of reported loci and discriminatory power.

The results of this study may help the forensic science community determine whether the use of CE-based or MPS-based genotyping methods are suitable for DNA analysis of bone samples with low quantities of degraded DNA. Though the Ion PGM™ platform produced the most discriminating SNP profiles, STR analysis with size-based CE detection had similar discriminating power which supports that CE is still a powerful and discriminating method for forensic identification applications. However, MPS technologies do have the ability to provide more information about the DNA samples such as predictions for Y-haplogroups, biogeographical ancestry, and hair and eye color phenotypes. In this aspect, MPS surpasses size-based CE detection by obtaining more information from the degraded DNA. Overall, this study provided insights as to some improvements that can be made when analyzing DNA from bone material which may help forensic scientists build discriminating DNA profiles from these challenging samples.

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APPENDIX

Table A1. DNA yields from 50 mg of bone powder for the 11-16, 11-19, and 13-07 bones. The PrepFiler® BTA Forensic DNA Extraction Kit was used. The result for each bone is an average of three replicates.

Bone	DNA yield (ng)
11-16 Femur	0.236
11-16 Rib	0.147
11-16 Phalanx	0.045
11-19 Femur	0.299
11-19 Rib	0.363
11-19 Phalanx	0.116
13-07 Femur	0.338
13-07 Rib	0.358
13-07 Phalanx	0.123

Table A2. 1/RMP values (1 in) calculated from GlobalFiler® autosomal STR assay. Values are based on alleles frequencies from four US populations (NIST 1036 Population Data).

Bone	Caucasian	African American	Hispanic	Asian
Unk Femur	3.50×10^{21}	7.46×10^{24}	3.46×10^{20}	8.87×10^{19}
Unk Rib	5.77×10^{7}	1.26×10^{10}	6.95×10^{7}	8.82×10^{8}
Unk Phalanx	9.21×10^{21}	4.43×10^{24}	4.01×10^{21}	3.98×10^{20}
11-16 Femur	5.12×10^{28}	1.95×10^{31}	1.85×10^{30}	3.59×10^{33}
11-16 Rib	3.50×10^{26}	1.05×10^{29}	9.70×10^{27}	1.43×10^{31}
11-16 Phalanx	3.85×10^{14}	2.05×10^{15}	2.44×10^{15}	1.39×10^{18}
11-19 Femur	5.01×10^{18}	1.72×10^{21}	8.66×10^{19}	3.17×10^{21}
11-19 Rib	6.87×10^{24}	6.18×10^{27}	1.93×10^{26}	6.58×10^{27}
11-19 Phalanx	3.76×10^{12}	7.54×10^{13}	1.44×10^{13}	2.67×10^{14}
13-07 Femur	2.23×10^{28}	1.98×10^{33}	3.94×10^{29}	3.97×10^{30}
13-07 Rib	2.23×10^{28}	1.98×10^{33}	3.94×10^{29}	3.97×10^{30}
13-07 Phalanx	3.83×10^{22}	7.84×10^{27}	5.93×10^{23}	2.25×10^{24}

Table A3. 1/RMP values (1 in) calculated from Ion PGM™ iSNP assay. Values are based on alleles frequencies from five world populations (1000 Genomes Data).

Bone	South Asian	American	East Asian	European	African
Unk Femur	1.36×10^{27}	4.67×10^{27}	1.07×10^{28}	6.97×10^{28}	5.48×10^{32}
Unk Rib	2.99×10^{25}	1.81×10^{27}	6.12×10^{26}	7.18×10^{25}	1.87×10^{32}
Unk Phalanx	5.25×10^{34}	4.29×10^{37}	6.70×10^{35}	1.54×10^{38}	3.36×10^{43}

Table A4. 1/RMP values (1 in) calculated from MiSeq® STR assay (length genotype). Values are based on alleles frequencies from four US populations (NIST 1036 Population Data).

Bone	Caucasian	African American	Hispanic	Asian
Unk Femur	1.51×10^{7}	7.92×10^{9}	8.60×10^{6}	2.59×10^{7}
Unk Rib	5.70×10^{1}	2.92×10^{1}	1.78×10^{1}	1.84×10^{1}
Unk Phalanx	1.86×10^{13}	6.46×10^{13}	7.11×10^{12}	5.48×10^{11}
11-16 Femur	5.12×10^{13}	1.92×10^{16}	1.01×10^{15}	5.19×10^{17}
11-16 Rib	7.56×10^{9}	5.09×10^{12}	2.53×10^{10}	9.11×10^{11}
11-16 Phalanx	2.26×10^{5}	2.43×10^{5}	9.71×10^{5}	3.93×10^{6}
11-19 Femur	7.97×10^{8}	3.05×10^{10}	3.00×10^{9}	9.19×10^{10}
11-19 Rib	1.54×10^{13}	4.74×10^{14}	1.04×10^{14}	3.55×10^{15}
11-19 Phalanx	1.66×10^{4}	2.93×10^{3}	2.72×10^{3}	3.84×10^{3}
13-07 Femur	5.54×10^{18}	6.44×10^{22}	2.41×10^{20}	1.04×10^{22}
13-07 Rib	5.94×10^{19}	8.98×10^{23}	6.23×10^{20}	5.96×10^{21}
13-07 Phalanx	2.53×10^{5}	9.65×10^{6}	5.91×10^{5}	2.22×10^{7}

Table A5. 1/RMP values (1 in) calculated from MiSeq® STR assay (sequence genotype). Values are based on alleles frequencies from four US populations (NIST 1036 Population Data).

Bone	Caucasian	African American	Hispanic	Asian
Unk Femur	1.51×10^{11}	3.28×10^{14}	8.62×10^{10}	2.70×10^{12}
Unk Rib	6.21×10^{3}	6.68×10^{2}	1.79×10^{3}	1.84×10^{1}
Unk Phalanx	3.02×10^{16}	5.60×10^{17}	2.39×10^{17}	2.46×10^{15}
11-16 Femur	3.69×10^{19}	3.54×10^{22}	1.12×10^{21}	2.22×10^{23}
11-16 Rib	2.33×10^{13}	2.30×10^{17}	1.23×10^{14}	2.99×10^{16}
11-16 Phalanx	1.29×10^{6}	3.61×10^{5}	9.80×10^{6}	3.98×10^{7}
11-19 Femur	4.43×10^{9}	2.27×10^{11}	1.10×10^{10}	2.64×10^{12}
11-19 Rib	9.02×10^{15}	1.88×10^{18}	4.53×10^{17}	5.07×10^{18}
11-19 Phalanx	6.58×10^{5}	4.02×10^{5}	2.03×10^{4}	5.15×10^{5}
13-07 Femur	4.02×10^{22}	2.89×10^{28}	8.72×10^{24}	3.28×10^{26}
13-07 Rib	1.53×10^{23}	4.46×10^{28}	8.25×10^{24}	2.19×10^{26}
13-07 Phalanx	2.84×10^{8}	5.34×10^{11}	1.61×10^{9}	1.89×10^{11}

Table A6. 1/RMP values (1 in) calculated from MiSeq® iSNP assay. Values are based on alleles frequencies from four US populations (NIST 1036 Population Data).

Bone	Caucasian	African American	Hispanic	Asian
Unk Femur	4.24×10^{5}	1.44×10^{6}	1.67×10^{5}	1.74×10^{6}
Unk Rib	6.44×10^{1}	4.08×10^{1}	5.97×10^{1}	2.30×10^{2}
Unk Phalanx	1.57×10^{12}	7.15×10^{12}	6.55×10^{11}	3.60×10^{11}
11-16 Femur	3.09×10^{9}	1.54×10^{12}	2.58×10^{10}	9.75×10^{10}
11-16 Rib	3.61×10^{2}	5.56×10^{3}	6.22×10^{2}	1.82×10^{3}
11-16 Phalanx	2.54×10^{0}	9.86×10^{0}	2.56×10^{0}	3.35×10^{0}
11-19 Femur	6.16×10^{4}	2.73×10^{6}	6.42×10^{4}	1.56×10^{8}
11-19 Rib	2.99×10^{10}	3.63×10^{13}	7.10×10^{10}	2.47×10^{15}
11-19 Phalanx	7.05×10^{2}	7.60×10^{3}	6.91×10^{2}	5.33×10^{4}
13-07 Femur	3.33×10^{14}	1.99×10^{19}	6.15×10^{14}	6.01×10^{18}
13-07 Rib	1.11×10^{13}	5.95×10^{16}	2.28×10^{13}	7.91×10^{15}
13-07 Phalanx	8.14×10^{4}	1.56×10^{6}	4.98×10^{4}	5.44×10^{4}