EXTRACTION EFFICIENCY TESTING OF DEGRADED BONE SAMPLES: COMPARING FOUR DNA EXTRACTION METHODS FOR DOWNSTREAM MASSIVELY PARALLEL SEQUENCING APPLICATIONS

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TABLE OF CONTENTS

LIST OF TABLES v
LIST OF FIGURES vi
LIST OF ABBREVIATIONS vii
INTRODUCTION 1
DNA Profiling 1
Profiling from Skeletal Remains 2
Massively Parallel Sequencing (MPS) 4
Ion AmpliSeq™ PhenoTrivium Panel (Applied Biosystems 2020) 5
DNA Extraction Methods 6
Ancient DNA Extraction Methods 7
Forensic DNA Extraction Methods 9
Research Overview 11
Significance 12
MATERIALS AND METHODS 13
Sample Preparation 13
DNA Extraction 14
Rohland *et al.* (2018) 15
PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems 2012) 16
Bone DNA Extraction Kit, Custom (Promega 2019) 16
InnoXtract™ (InnoGenomics 2018) 17
Quantification 17
Statistical Analyses 18
Ion AmpliSeq™ PhenoTrivium Panel (Applied Biosystems 2020) 18
Library Preparation 18
Library Quantification 19
Sequencing and Data Analysis 19
RESULTS 20
Extraction Troubleshooting 20
Quantification 20
Statistical Analyses 26
Quantification Dilutions 27
Library Preparation and Quantification 27
Sequencing and Data Analysis 28
DISCUSSION 32
Quantification Performance 32
Rohland *et al.* (2018) 32
PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems 2012) 33
Bone DNA Extraction Kit, Custom (Promega 2019) 33
InnoXtract™ (InnoGenomics 2018) 34
Overall Performance 34
Sequencing Performance 35
Rohland *et al.* and PrepFiler® BTA Forensic DNA Extraction Kit 35

Bone DNA Extraction Kit, Custom and InnoXtract™ 36
Ancestry and Phenotypic Predictions 36
Effects of Pre-Extraction Processes on DNA Recovery 38
Intrinsic Factors 38
Extrinsic Factors 40
Processing Techniques 41
Applications: Investigative Genetic Genealogy 42
Future Directions 44
REFERENCES 45
APPENDIX A: STATISTICAL TESTS 51

LIST OF TABLES

Table 1: Comparison of extraction methods. 11
Table 2: Donor information. 13
Table 3: Quantification results for small and large autosomal target recovery for experimental samples for all extraction methods. 21
Table 4: Quantification results for small and large autosomal target recovery for experimental samples for the Promega protocols. 23
Table 5: Degradation indices for experimental samples for all extraction methods. 24
Table 6: Degradation indices for experimental samples for the Promega protocols 24
Table 7: Results of the C_T of the IPC of the experimental samples of protocols with overnight incubations 24
Table 8: Results of the C_T of the IPC of the experimental samples of the Promega protocols. ... 25
Table 9: Summary metrics for the experimental samples sequenced with the PhenoTrivium Panel. 28
Table 10: Genotype and depth of coverage of Sample FS-01 29
Table 11: Genotype and depth of coverage of Sample FS-03. 29
Table 12: Genotype and depth of coverage of Sample FS-05. 30
Table 13: Genotype and depth of coverage of Sample FS-10 30
Table 14: Eye color predictions according to the Iris Plex System 31
Table A1: Friedman test results. 51
Table A2: Wilcoxon signed-rank test results for comparison of means of small target concentrations 51
Table A3: Wilcoxon signed-rank test results for comparison of means of large target concentrations 51
Table A4: Sign rank test results 51

LIST OF FIGURES

Figure 1. Mean small autosomal target concentration 22
Figure 2. Mean large autosomal target concentration 22

LIST OF ABBREVIATIONS

µL – Microliter(s)
ADD – Accumulated Degree-Days
aDNA – Ancient Deoxyribonucleic Acid
CE – Capillary Electrophoresis
CODIS – Combined DNA Index System
C_T – Cycle Threshold
DI – Degradation Index
DNA – Deoxyribonucleic Acid
EDTA – Ethylenediaminetetraacetate
FOREST – Forensic Osteology Research Station
IGG – Investigative Genetic Genealogy
IGV – Integrative Genomics Viewer
IPC – Internal PCR Control
ISP – Ion Sphere Particle
LCN – Low Copy Number
MPS – Massively Parallel Sequencing
ng – Nanogram
NGS – Next-generation Sequencing
NTC – No-template Control
PCR – Polymerase Chain Reaction
pg – Picograms
pM – Picomolar
PMI – Post-Mortem Interval
qPCR – Quantitative Polymerase Chain Reaction
SNP – Single Nucleotide Polymorphism
STR – Short Tandem Repeat
TSS – Torrent Suite™ Software
WCHIL – Western Carolina Human Identification Laboratory
WCU – Western Carolina University

ABSTRACT

EXTRACTION EFFICIENCY TESTING OF DEGRADED BONE SAMPLES: COMPARING FOUR DNA EXTRACTION METHODS FOR USE IN DOWNSTREAM MASSIVELY PARALLEL SEQUENCING APPLICATIONS

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In recent years, investigative genetic genealogy (IGG), which involves the use of genealogical methods combined with DNA analysis to make potential familial matches, has become an important tool in solving cold and active cases. These cases can involve the identification of a perpetrator or the identification of missing persons. Estimates show that approximately 4,400 unidentified bodies are recovered each year, and up to one quarter of those individuals remain unidentified after one year. Traditionally, forensic DNA amplification methods have relied on the need to amplify 100-450 base-pair targets, specifically, short tandem repeats (STRs), for forensic profiles. With genetic genealogical approaches, smaller targets, such as single nucleotide polymorphisms (SNPs), have shown potential as tools for identification. Traditional extraction protocols for forensic DNA have focused on maximizing DNA recovery with the intent of amplifying larger STR targets. On the other hand, ancient DNA techniques have focused efforts on recovering smaller DNA fragments, like SNPs, and indeed have shown recovery of even highly degraded samples in excess of 400,000 years. This study aims to compare the extraction success and efficiency of one ancient DNA (aDNA) extraction technique from Rohland *et al.* (2018) and three forensic DNA extraction techniques, PrepFiler® BTA Forensic DNA Extraction Kit from Applied Biosystems, the Bone DNA Extraction Kit, Custom

from Promega, and the InnoXtract™ from InnoGenomics, on compromised bone samples for the purposes of massively parallel sequencing (MPS). Quantitative PCR was used to compare the extraction performance of the protocols, while an MPS-based assay, the Ion AmpliSeq™ PhenoTrivium Panel, was used to assess informative characteristics, such as phenotype and biogeographic ancestry, for an investigation. The Rohland and modified PrepFiler protocols showed the most success in terms of DNA recovery and sequencing. These results show the utility of an ancient DNA extraction method in MPS research and the success of a widely used forensic method. The results of this study may add to the process of determining the most appropriate extraction method for massively parallel sequencing applications such as IGG in forensic contexts.

INTRODUCTION

The practice of human identification can be applied to many different contexts. In criminal investigations, the identification of the perpetrator, and oftentimes the victim, is crucial to the resolution of the investigation (Butler 2012; Holobinko 2012). Additionally, questioned parentage, historical and archaeological investigations, missing persons cases and mass disasters all use a form of human identity testing (Holobinko 2012; Ambers *et al*. 2018; Kling *et al*. 2012; Leclair *et al*. 2007). Four commonly used approaches to human identification include DNA profiling, forensic anthropology, forensic radiography, and forensic odontology. Typically, multiple methods of identification are implemented during an investigation (Holobinko 2012). DNA profiling is the primary technique implemented in the field of forensic biology. The success of DNA profiling has earned it the characterization as the "model" method of identification in forensics, as its use provides critical information to investigators (Holobinko 2012).

DNA Profiling

Common sources of DNA encountered in forensic biology include skin cells, blood, saliva, bone, teeth, semen, and hair (Butler 2012; Haas *et al*. 2013; Hedman *et al.* 2008; Higgins and Austin 2013; Adhikari *et al*. 2014). DNA profiling can differentiate between individuals (with the exception of identical twins) and is therefore a useful tool to identify a suspect, victim, and human remains. DNA profiling is the process of distinguishing individuals of the same species by comparing DNA profiles, typically using length variation methods such as short-tandem repeat (STR) analysis (Butler 2012). STRs are short sections of DNA that are highly polymorphic, which allows for differentiation between individuals (Holobinko 2012). First,

DNA is extracted from the sample and separated from its cellular components. The amount of DNA present is quantified to determine the optimal amount required for subsequent analyses, generally through quantitative polymerase chain reaction (qPCR) (Butler 2012; Holobinko 2012). After quantification, specific regions of the DNA are amplified with STR markers through polymerase chain reaction (PCR). The DNA fragments can then be separated through capillary electrophoresis (CE) and analyzed using a data fragment software (Butler 2012). Currently, STR profiles that are eligible for inclusion in the Combined DNA Index System (CODIS) must include 20 carefully selected STR markers that have no known association with medical conditions or disease, known as the 20 CODIS core loci (Hares 2015).

Challenges associated with the process of obtaining sufficient DNA and amplifying an optimal DNA profile include mitigating sample degradation and maximizing quantity. In forensic contexts, the quantity of DNA available can be very low, which can restrict the amount and type of tests that can be performed. When skeletal remains are the source of DNA, the quality and quantity of the samples collected can be low (Holobinko 2012). However, in many of the previously mentioned contexts in which DNA analysis may be used, bone may be the only viable source of DNA.

Profiling from Skeletal Remains

Human skeletal remains that are subjected to extreme environments, such in cases of missing persons or mass fatalities, often contain degraded or low-copy number (LCN) amounts of DNA caused by exposure to moisture, heat, ultraviolet radiation, and microbes (Ambers *et al*. 2016; Dong *et al*. 2016; Gettings *et al*. 2015; Elwick *et al.* 2019). Specifically, the humic and fulvic acids present in soil, natural waters, and other sediments damage DNA (Alaeddini 2012). In addition, fire-related incidents cause rapid degeneration and fragmentation of DNA molecules

(Emery *et al.* 2020). Consequently, the resulting degraded DNA and environmental PCR inhibitors present challenges to traditional DNA profiling using STRs.

The ability to genotype STRs from degraded samples is limited by the minimum amplicon range of most forensically relevant STR loci, such as those included in the 20 CODIS core loci. Amplicons for forensic STR typing are generally in the 100-450 base pair range, which can prohibit optimal amplification if DNA is severely degraded. Allele and locus dropout are often seen with degraded DNA samples when the fragments are shorter than the required length for typing (Butler *et al.* 2003). As a result, low-quality DNA often produces partial profiles, which may not be sufficient to make an identification (Butler *et al.* 2003; Ballantyne *et al.* 2007; Ambers 2018).

Analysis of single nucleotide polymorphisms (SNPs), which include base substitutions, insertions, or deletions, may yield more informative results for small amplicons in degraded and LCN DNA (Greytak *et al.* 2019; Budowle and van Daal 2008). SNPs have lower mutation rates and are more abundant in the genome compared to STRs (Sobiah *et al*. 2018). Although SNPs are not as informative on a per locus basis compared to the CODIS core STR loci, they do contain a great amount of human variation that can be used in forensic contexts (Budowle and van Daal 2008). SNP genotyping has been shown to be accurate in a wide range of forensic samples, including male/female DNA mixtures, and DNA extracted from textiles and other sample types routinely found in casework (Greytak *et al.* 2019; Xavier *et al*. 2017; Silvia *et al.* 2017). Some commercial kits are able to provide discrimination with a massively parallel sequencing (MPS) SNP assay equivalent to that of a full STR profile. MPS may also be used to analyze STR and SNP markers in the same set, which shows advantages over traditional CE (Butler and Willis 2020). Commercial kits such as the ForenSeq™ DNA Signature Prep Kit, by

Verogen, operate on the MiSeq Forensic Genomics System, an MPS platform. This kit includes a combination of STRs, which includes the original 13 CODIS core loci, and SNPs associated with ancestry and phenotype. Up to 231 markers can be amplified simultaneously, which allows a wide range of information to be obtained from a sample in one workflow. (Jäger *et al.* 2017; Hussing *et al*. 2018; Ballard *et al.* 2020).

Massively Parallel Sequencing (MPS)

MPS has origins in Sanger sequencing, known as first generation DNA sequencing. MPS is a high-throughput method of DNA sequencing that has recently become a topic of interest in the forensic science field. MPS is also known as 'next generation sequencing' (NGS) due to its high-throughput nature compared to Sanger sequencing (Bruijns *et al*. 2018). MPS systems can simultaneously sequence millions of DNA molecules, without the need for CE to detect output. A workflow involving MPS, after DNA extraction, generally includes sample library preparation, cluster generation, DNA sequencing, and data analysis (Sobiah *et al*. 2018).

In forensics, MPS can overcome obstacles faced in traditional DNA profiling. For example, the limited capabilities of STR PCR and CE restrict the number of STRs with shorter amplicon lengths that can be typed in a single reaction (Bruijns *et al*. 2018). For example, the Ion AmpliSeq™ PhenoTrivium Panel includes 196 autosomal targets with a mean amplicon length of 78 base pairs that are sequenced in a single run (Diepenbroek *et al.* 2020). Comparatively, the GlobalFiler™ PCR Amplification Kit only contains 10 mini-STRs out of the 24 STRs included in the kit with an amplicon length of less than 220 base pairs (Applied Biosystems 2019). Furthermore, traditional STR typing uses the size of the resultant PCR amplicons to estimate the number of repeats for a particular allele of a marker, while MPS involves sequencing of the entire repeat and flanking regions, allowing further discrimination between individuals (Ballard

et al 2020). For samples with degraded or low-quality DNA, MPS can also be employed to recover smaller sequence fragments (e.g., SNPs) which can provide discriminatory power comparable to STRs.

MPS platforms such as the MiSeq FGx, and ThermoFisher's Ion Torrent Personal Genome Machine (PGM) and Ion S5™ have been used to explore the implementation of MPS in forensics, yet the technology is currently mostly used to assess SNP markers for identity or ancestry, or to supplement autosomal STR markers (Ballard *et al.* 2020; Brujins *et al*. 2018). Despite promising research, widespread use of MPS platforms and kits for casework and other forensic contexts has not yet been adopted. Time is a significant barrier in high-throughput laboratories; an MPS workflow can take up to several days for a single run. In addition, the lack of an organized database for MPS data, significant costs of instruments and kits, and rigorous forensic standards are important issues remaining to be addressed (Bruijns *et al*. 2018; Emery *et al.* 2020).

Ion AmpliSeq™ PhenoTrivium Panel (Applied Biosystems 2020)

The Ion AmpliSeq™ PhenoTrivium Panel, hereafter referred to as the PhenoTrivium Panel, is an assay that combines ancestry and phenotypic SNPs for a total of 320 targeted markers. The Precision ID Ancestry Panel, also from Applied Biosystems, contains 165 autosomal markers for estimating biogeographic ancestry, while the Precision ID Identity Panel uses Y-markers for estimating lineage and 90 autosomal SNP markers to aid in identification. The PhenoTrivium Panel allows for biogeographic ancestry, phenotypic characteristics, and Y-chromosomal lineage to be predicted in one targeted sequencing workflow. Although the recommended input of DNA is one nanogram, a study has shown reliable ancestry and phenotypic predictions from as little input as 125 picograms of DNA (Diepenbroek *et al*. 2020).

The low input amount, combined with the increased number of markers, makes the PhenoTrivium Panel attractive for use with degraded DNA samples, such as for the identification of skeletal remains, where predictions concerning appearance and ancestry may aid an investigation.

DNA Extraction Methods

Regardless of sequencing methods used, the DNA extraction method selected can have significant impacts on the success of downstream analyses. Highly efficient DNA extraction methods are critical, particularly with degraded and LCN DNA samples such as bone and teeth (Xavier *et al*. 2021). When DNA yields are expected to be low based on the condition of the bone or tooth, it is crucial that the implemented DNA extraction technique maximize the quality and quantity of the DNA extracted. Therefore, this thesis project was developed to inform best practices in DNA extraction for degraded bone samples intended for MPS.

Employing techniques developed in the ancient DNA community in forensic science is not a new concept; however, collaboration between the two communities has not reached its full potential. In the ancient DNA (aDNA) field, DNA extraction protocols have enabled the recovery of DNA sequences from remains over 400,000 years old (Rohland *et al*. 2018). Protocols and commercial kits are available for extraction of DNA from forensically relevant samples, including bones and teeth, however there are contexts in which an aDNA approach may be favored. Examples where aDNA techniques would be appropriate in forensic investigations include identification of victims from mass graves, historical skeletal remains, disinterred remains of soldiers, and remains from fire-related incidents where DNA is expected to be severely degraded (Hofreiter *et al*. 2021; Emery *et al.* 2020; Zavala *et al.* 2022). Ancient DNA extraction methods are optimized for recovery of shorter fragments, which may be

complementary to MPS-based applications used in certain forensic contexts, such as in the identification of human skeletal remains.

A modified protocol from Rohland *et al.* (2018), which will be known as the Rohland protocol, is currently used in the Forensic Genetics Laboratory at Western Carolina University (WCU) and was selected as the ancient DNA extraction method for this study. The protocols for PrepFiler® BTA Forensic DNA Extraction Kit from Applied Biosystems, the Bone DNA Extraction Kit, Custom from Promega, and the InnoXtract™ from InnoGenomics were chosen as the forensic extraction methods for comparison. These protocols will be known as PrepFiler, Promega, and InnoXtract, respectively. All of the selected protocols, and most current DNA extraction methods for calcified tissue, involve a digestion in ethylenediaminetetraacetate (EDTA), which demineralizes the bone and simultaneously inactivates DNAses. DNAses may impact amplification processes, so their removal is an important capability of efficient extraction methods (Loreille *et al.* 2007). In addition, the extraction methods all use a form of silica binding through particles suspended in a solution. Silica is a popular choice because it minimizes co-extraction of PCR inhibitors better than the conventional phenol/chloroform method (Rohland *et al*. 2018; Rohland and Hofreiter 2007; Alaeddini 2012). Table 1 summarizes the comparisons between DNA extraction methods in the fields of ancient and forensic DNA.

Ancient DNA Extraction Methods

The extraction of DNA from ancient samples aims to mitigate the unique characteristics that render DNA recovery difficult. Most aDNA is present in short fragments of equal to or less than 50 base pairs and in relatively low quantities. In addition, these sources of aDNA often contain inhibitors due to long-term exposure to adverse environmental conditions (Hofreiter *et al*. 2021). Similarly, exogenous DNA due to fungal and bacterial DNA from the surrounding

environment may be co-extracted in significant quantities, posing complications to downstream analyses (Rohland and Hofreiter 2007).

Several aDNA extraction methods have been developed in the past few decades for DNA sources such as bone, teeth, and sediments. Many aDNA extraction methods use silica spin columns to increase DNA yield over silica suspensions, but the columns are significantly more expensive (Rohland *et al.* 2018). With aDNA extraction methods, there is also an emphasis on low waste. Protocols specify the retention of buffers used for demineralization that may contain endogenous DNA and suggest pooling them with the subsequent digestion buffer (Hofreiter *et al*. 2021). Conversely, forensic DNA extraction protocols generally advise users to discard leftover buffers and remaining bone or tooth powder (Loreille *et al*. 2007). In addition, because of the fragile nature of the DNA that is present in ancient samples, protocols must avoid the use of high temperatures (Rohland and Hofreiter 2007).

Rohland *et al*. (2018):

Rohland, Glocke, Aximu-Petri, and Meyer released an update to a previous protocol they authored in 2007, which was widely used in ancient DNA research. The newest protocol developed by Rohland *et al.* is optimized for DNA library preparation for downstream MPS. Library preparation in this context enables recovery of short fragments that are not easily targeted by PCR amplification methods. Like many other aDNA methods, this protocol is adapted for bone, tooth, and soil samples. Rohland *et al.* also provide a thorough evaluation of the performance of a silica suspension, as compared to silica-spin columns. Other ancient DNA extraction methods are widely used, such as the Dabney method, a method developed by Yang *et al*., and a method developed by Allentoft *et al*. However, these methods include more handling steps than described by the Rohland protocol and therefore may increase the risk of

contamination (Gamba *et al*. 2016; Allentoft *et al*. 2015; Yang *et al*. 2014). Thus, the Rohland protocol was selected for this study as the ancient DNA extraction method due to its compatibility with MPS workflows, optimization for DNA recovery from bone, and use of silica for DNA binding.

Forensic DNA Extraction Methods

Forensic DNA extraction methods offer several advantages to save on time and cost that may benefit high-throughput laboratories and time-sensitive investigations. For example, the availability of commercial kits means easier access to the necessary materials and reagents. In addition, most forensic protocols are designed with shorter hands-on time in the form of reduced incubation times. Conversely, it is common for aDNA protocols to include 24–48-hour demineralization periods for skeletal remains (Rohland *et al*. 2018; Dabney *et al*. 2013; Glocke and Meyer 2017). Furthermore, while aDNA extraction methods are optimized for sample types such as bone, teeth, and sediment, forensic extraction protocols can be used on a variety of samples, including semen, hair, and materials such as denim (Alaeddini 2012; Barbaro *et al*. 2011). Finally, commercial forensic extraction kits are generally validated by multiple studies before market release, which may assure laboratories of the kit's viability before purchase.

Although modern DNA sources do not face the same levels of degradation and environmental contamination as ancient DNA sources, there are similar extraction challenges. Even in forensic samples where there are adequate levels of DNA, PCR inhibition is the most common cause of poor PCR performance. PCR inhibition is mainly due to co-extraction of PCR inhibitors that affect components of the PCR reaction such as the nucleotides, the amplification primers, and the polymerase enzyme. Additionally, forensic DNA samples face inhibitors unique to modern sources of DNA, such as the inhibitors present in clothing such as denim, hair, buccal

swabs, and dyes from latent fingerprint processing techniques (Alaeddini 2012). Therefore, forensic DNA extraction methods must still minimize the co-extraction of inhibitors, even in robust samples. The forensic methods chosen, PrepFiler, Promega, and InnoXtract, have all been shown to recover sufficient DNA for subsequent analyses without significant co-extraction of PCR inhibitors (Hasap *et al.* 2019; Calacal *et al*. 2021; Houston and Snedeker 2022).

PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems 2012):

Before the release of the PrepFiler BTA protocol, Applied Biosystems released BTA™ lysis buffer as a formulation reagent designed for use in combination with PrepFiler® Forensic DNA Extraction Kit, which is designed for common forensic sample types. The extraction kit that included BTA was released later and was developed for challenging sample types such as bone, tooth, cigarette butts, chewing gum, envelope flaps, and tape lifts. BTA lysis is known to isolate high quality DNA while effectively removing PCR inhibitors from a wide range of sample types (Barbaro 2011; Betancor *et al.* 2011; Harrel *et al*. 2018). This protocol has also been shown to perform better than conventional phenol/chloroform methods (Hasap *et al.* 2019). Advantages of this protocol includes a relatively short processing time and a limited amount of tube transfers to reduce risk of sample mix-up and contamination. In addition, the PrepFiler protocol has been shown to be successful with multiple MPS systems (Zeng *et al.* 2019).

Bone DNA Extraction Kit, Custom (Promega 2019):

Supplied by Promega, this kit and protocol were based on a demineralization buffer developed by the Armed Forces DNA Identification Lab. The Promega protocol was specifically designed for challenging bone samples. The custom bone demineralization buffer is combined with the DNA IQ™ manual and automated workflows for flexibility in a laboratory's needs. This protocol is also focused on minimal handling and transfer times, as well as reduced processing

and incubation times. Informative profiles have been produced with the DNA extracted using the Promega protocol, even with lower DNA yields and reduced sample input amounts (Duijs and Sijen 2020; Calacal *et al.* 2021). This protocol and demineralization buffer have also been shown to outperform organic-based methods and a previous bone incubation buffer from Progmega (Calacal *et al*. 2021).

InnoXtract™ (InnoGenomics 2018):

The InnoXtract protocol was designed for challenging samples such as touch DNA, rootless hair shafts, and degraded bone samples. This protocol is also compatible with manual and automated workflows. To date, no independent validations or optimizations have been published on the InnoXtract protocol for bone samples; however, a recent study reported successful DNA recovery from rootless hair shafts (Gutierrez *et al.* 2021). Unpublished data shows that the InnoXtract protocol may perform similarly to the PrepFiler protocol for bone powder, which is an established DNA extraction method (Houston and Snedeker 2022). Research is still needed to validate this protocol for challenging bone samples.

Table 1: Comparison of extraction methods from the fields of ancient and forensic DNA.

Field	Retain buffers?	Commercial kits available?	Wide sample range?	Short (<50 bp) fragments recovered?	Suitable for MPS?	Validated?	Process-ing Time
Ancient DNA	Yes	No	No	Yes	Yes	No	24-48 hours
Forensic DNA	No	Yes	Yes	No	Yes	Yes	4-6 hours

Research Overview

A total of seven bone samples were analyzed for this project. Seven humeri from Western Carolina University's John A. Williams Skeletal Collection were used. Bone samples were prepped and drilled to create bone powder for extraction. Four DNA extraction methods were

implemented for comparison, all of which are optimized for DNA recovery from degraded samples. After DNA extraction, the samples were assessed using the Quantifiler™ Trio Quantification Kit. Following assessment of the quality and quantity of DNA collected, statistical analyses were used to compare the success of each method through analysis of degradation indices, removal of PCR inhibitors, and recovery of small and large autosomal DNA targets. DNA libraries were prepared for experimental samples using the Precision ID DL8 Kit. The Ion AmpliSeq™ PhenoTrivium Panel was used to sequence 320 autosomal SNP markers for biogeographic ancestry and phenotypic predictions on the HID Ion GeneStudio™ S5 System. Results were analyzed using Torrent Suite™ Software (TSS) and Integrative Genomics Viewer (IGV).

Significance

This research seeks to aid the process of determining the most appropriate extraction method for degraded bone samples for MPS applications in forensic contexts such as investigative genetic genealogy (IGG). Several studies show the success of using ancient DNA extraction methods for forensic bone samples (Hofreiter *et al*. 2021; Emery *et al.* 2020; Zavala *et al.* 2022). Many factors must be considered in the process of implementing an extraction method for optimal recovery of short fragments of DNA while simultaneously reducing the risk of co-extracting PCR inhibitors. Suitability for MPS is also an important factor to consider when applications such as IGG are implemented. As single nucleotide polymorphisms (SNPs) become more widely used for identification, a reliable and efficient DNA extraction method is necessary for maximum DNA recovery from skeletal remains.

MATERIALS AND METHODS

This study investigated the performance of four DNA extraction protocols on degraded bone samples for the purposes of MPS applications. Seven humeri from WCU's John A. Williams Human Skeletal Collection were selected for this study. The DNA extracted from the seven bone samples were analyzed to assess the suitability of each DNA extraction method for downstream analyses in a forensic investigation. Sample preparation, DNA extraction, and preparation for quantification took place under PCR workstation hoods. Library preparation, library quantification, templating, and sequencing occurred in a separate lab from extraction.

Sample Preparation

The seven humeri used in this study each came from different donors that were placed at WCU's Forensic Osteology Research Station (FOREST) for decomposition. All donors were placed at the FOREST between 2015 and 2016. The post-mortem interval (PMI) for most of the donors is six to seven years, with time at the FOREST ranging from one to three years, represented by "deposition period" in Table 2. After the deposition period, the donors were recovered and processed into the John A. Williams Human Skeletal Collection. The age at death of the donors ranged from 57 years to 96 years, with most donors being 65 years or older at death (Table 2). The bones were destructively sampled before this study; therefore, the sampling site for this study was pre-determined by the existing sampled site on the bone. All existing sample sites for the sampled humeri were located on the diaphysis of each bone.

Table 2: Donor information for the sources of the bone samples used in this study.

Sample ID	Deposition Year	Deposition period**	PMI*	Sex	Age at death*	Bone***	Sampling site
FS-01	2015	3y	7	Female	57	Right H	Proximal
FS-02	2015	3y1m	7	Female	65	Right H	Proximal
FS-03	2016	1y4m	6	Female	74	Right H	Proximal

FS-05	2016	1y6m	6	Male	93	Right H	Distal
FS-09	2015	2y2m	7	Male	96	Left H	Proximal
FS-10	2016	1y7m	6	Female	57	Right H	Proximal
FS-11	2015	2y5m	7	Male	73	Left H	Proximal

* In years.
** 'y' represents the number of years, while 'm' represents the number of months.
*** H = humerus.

DNA extraction of bones and teeth requires extra steps to ensure that the DNA is released from the bone matrix. Drilling bone material to create a powder increases the ability of extraction reagents to access the DNA. Prior to drilling, bone surfaces must be decontaminated to remove exogenous DNA (Duijs and Sijen 2020; Colón *et al.* 2018). For all samples, an approximately one-by-two-inch area was cleaned near the proximal or distal end of the bone with several cotton-tipped swabs of 6% sodium hypochlorite, then several FLOQ swabs of ultrapure water were used to remove the bleach from the area. The cleaned areas were dried under a PCR workstation hood blower for approximately five minutes before drilling. A Dremel hand tool with a rotary end was used to drill four aliquots of 50-65 milligrams of bone powder from each bone element. The drill was set to its lowest speed and was turned off often to prevent heat generation, which may damage DNA (Colón *et al.* 2018). All tools were cleaned with bleach or DNA Away and ethanol and UV-eradiated along with other consumables and reagents for 15-30 minutes. The powdered samples were held at room temperature until extraction.

DNA Extraction

After drilling to create a bone powder, samples must also be decalcified to release DNA from the mineral bone matrix. At death, DNA fuses with inorganic hydroxyapatite, collagen, and other minerals, allowing DNA to persist in skeletonized tissues long after the soft tissue sources of DNA have decomposed (Duijs and Sijen 2020; Colón *et al.* 2018; Corrêa *et al.* 2021). This

creates challenges for extraction methods that must remove DNA from the calcified matrix while maintaining the integrity of the DNA present.

The Rohland, PrepFiler, Promega, and InnoXtract protocols were selected for comparison of extraction performance with the seven humeri samples. The forensic protocols were modified to make them more comparable to the Rohland protocol. Specifically, the incubation times, bone powder input, and elution volume output were standardized as described below. An overnight demineralization soak in 0.5 M EDTA was added to the PrepFiler and Promega protocols, as per Pajnič *et al*. (2016). Total demineralization of skeletal samples is the current recommendation for extraction methods to improve DNA recovery (Calacal *et al.* 2021). For a pilot run with the Promega protocol, samples from the seven humeri were extracted with the Promega protocol, excluding an overnight demineralization soak. In addition, 50-60 milligrams of bone powder were used per sample in all protocols with a final extracted DNA eluate volume of 30 microliters, following the Rohland protocol. Negative controls, in the form of reagent blanks containing no sample, were included in all extraction protocols to assess the presence of contamination. After extraction, all samples containing eluted DNA were stored at two degrees Celsius prior to quantification.

Rohland *et al.* (2018)

In this modified protocol adapted for the Forensic Genetics Laboratory at WCU, an overnight incubation of 20 hours in a 0.5 M EDTA and proteinase K solution allows for total demineralization of the samples. A maximum temperature of 37 degrees Celsius during sample lysis reduces further damage to the sample DNA due to high heat. No more than 50 milligrams of bone powder is recommended for this protocol. Buffer D, primarily composed of guanidine hydrochloride and Tween-20, is used in combination with a silica suspension as described in this

protocol for DNA binding. In addition, a single wash buffer composed of buffer PE and ethanol is used for a total of three washes. An elution buffer made of 1 M Tris-HCl, 0.5 M EDTA, and Tween-20 is used for a resulting 30 microliters of eluted DNA.

PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems 2012)

This study followed the protocol for bone or tooth samples. Up to 50 milligrams of powdered bone is specified for this protocol. A 20-hours incubation in 0.5 M EDTA at 56 degrees Celsius was added to the protocol (Pajnič *et al*. 2016). After the overnight incubation, samples were washed with ultrapure water to remove the EDTA. A second incubation took place with a lysis buffer composed of PrepFiler® BTA Lysis Buffer, 1.0 M DTT, and proteinase K for two hours at 56 degrees Celsius. PrepFiler® Magnetic Particles were used for binding DNA in this protocol. Two included wash buffers reduce the risk of detergent carryover, which can contain PCR inhibitors if co-extracted. Three washes were performed, and the specified amount of 50 microliters of elution buffer was reduced to 30 microliters.

Bone DNA Extraction Kit, Custom (Promega 2019)

For this study, the procedure for "Preprocessing Using Demineralization Buffer and Purification Using DNA IQ™ Protocol" was followed. The protocol specifies 100 milligrams of bone powder to be inputted per sample; this was reduced to 50 milligrams for this study. A 20-hour incubation in 0.5 M EDTA at 56 degrees Celsius was added to the protocol (Pajnič *et al*. 2016). An initial test of the Promega protocol was performed omitting the overnight demineralization soak. This protocol also uses two bone lysis cocktails to release any DNA that was not accessed during the overnight incubation. The first bone lysis cocktail consists of a proprietary lysis buffer, proteinase K, and 1-Thioglycerol, while the second cocktail omits the proteinase K. The samples are further demineralization with the first bone lysis cocktail for 2.5

hours at 56 degrees Celsius. Magnetic beads, in the form of DNA IQ™ Resin, were used for binding. Three washes were performed with a single wash buffer comprised of some proprietary elements, isopropyl alcohol, and 95% ethanol. The specified 50 microliters of elution buffer was reduced to 30 microliters for this study.

InnoXtract™ (InnoGenomics 2018)

For this study, the user guide for "Bone DNA Extraction and Purification Kit" was followed. This kit includes a bone DNA binding buffer and a bone digest buffer for 40 milligrams of bone powder per sample. The sample input was increased to 50 milligrams. After a 20-hour incubation in the bone digest buffer and proteinase K at 56 degrees Celsius, the bone DNA binding buffer and magnetic bead suspension were added to all samples in two stages. A proprietary wash buffer was used in three stages, followed by two washes in 80% ethanol. The protocol specifies 40 microliters of elution buffer to be added to the dried magnetic particles containing the extracted DNA, but this was reduced to 30 microliters for this study.

Quantification

Real-time PCR, also known as quantitative PCR (qPCR), was used to assess the extracted DNA from the bone samples from all methods. The Quantifiler™ Trio Quantification Kit from Applied Biosystems was used in this study. This kit uses multiple copies of three human-specific target loci, including a Small Autosomal, Large Autosomal, and Y-chromosome targets. The Small Autosomal and Large Autosomal targets can be used to assess degradation of the DNA samples. The Y-chromosome targets are used in biological sex-determination and can also give a male-to-female ratio. In addition, this kit includes an internal PCR control (IPC) to confirm that all reaction components are functioning properly. The IPC also shows the presence of, and level of, PCR inhibition in each sample. All samples, including standards, were run in duplicate.

Samples were diluted to a concentration of 0.067 nanograms per microliter prior to library preparation for the PhenoTrivium Panel.

Statistical Analyses

Following quantification, the results for small autosomal target DNA, large autosomal target DNA, and degradation indices were analyzed. In addition, the ability of each extraction method to remove PCR inhibitors was analyzed through by comparing sample internal PCR control (IPC) cycle thresholds (C_T) to IPC C_T values of the no-template control (NTC). For this study, R (version 4.0.4) and RStudio were used to perform statistical analyses. Non-parametric repeated measures tests were performed to compare small and large target DNA recovery across methods. Finally, IPC deviations and degradation indices were qualitatively analyzed.

Ion AmpliSeq™ PhenoTrivium Panel (Applied Biosystems 2020)

The PhenoTrivium Panel consists of 320 total markers, which include 200 autosomal SNPs and 120 Y-chromosomal SNPs. These markers were selected for predictions of biogeographic ancestry, appearance, and Y-chromosomal lineage. A single primer pool, at a 2X primer pool concentration, contained all 320 markers. The Precision ID SNP Panels protocol was followed for compatibility with the HID Ion GeneStudio™ S5 System, hereafter referred to as the Ion S5 (Applied Biosystems 2019). This study followed the workflow for the "Custom Ion AmpliSeq™ SNP Panel" for a 1-pool panel.

Library Preparation

The Precision ID DL8 Kit and protocol for the Ion Chef System was selected for an automated library preparation workflow (Applied Biosystems 2019). The advantage of automated workflows include reduced hands-on time and fewer transfer steps to reduce the possibility of contamination. Once primer pools were loaded, the extracted samples, including

reagent blanks and NTCs, were added Precision ID DL8 IonCode™ Barcode Adapter Plates for barcode tagging during library preparation. The barcodes give each sample a unique identifier for data analysis. A maximum of eight samples can be loaded per run on the Ion Chef System; therefore, the libraries were prepared in three separate runs. Each run took seven hours to complete. After library preparation, the libraries for all samples are combined into a single tube. Prior to library quantification, the prepared sample libraries were stored at two degrees Celsius.

Library Quantification

For most of the samples, the input was less than one nanogram, which may result in library concentrations of less than the expected 100 picomolar. Therefore, library quantification was required before proceeding with sequencing. The libraries were quantified with the Ion Library TaqMan® Quantitation Kit on the 7500 Real-Time PCR Instrument with HID Real-Time PCR Analysis Software v1.2, both from Applied Biosystems. Libraries were diluted to 30 picomolar and stored at two degrees Celsius until sequencing.

Sequencing and Data Analysis

Prior to sequencing, templating was performed on the Ion Chef Instrument with the Ion S5™ Precision ID Chef & Sequencing Kit on an Ion 530™ Chip. This study followed the protocol for "single chip loading workflow" (Applied Biosystems 2019). Following templating, the loaded 530 Chip was sequenced on the HID Ion GeneStudio™ S5 System. The SNP panel run summary metrics, which include metrics for ion sphere particle (ISP) data and individual sample results, were analyzed in the Torrent Suite™ Software (TSS). The SNP data was analyzed with the Integrative Genomics Viewer (IGV) (Thorvaldsdóttir *et al.* 2013). Within the 320 targeted SNPs included in the PhenoTrivium Panel, six phenotypic-related SNPs for eye color were analyzed using the HIrisPlex-S Webtool (https://hirisplex.erasmusmc.nl/).

RESULTS

Extraction Troubleshooting

During the InnoXtract extraction procedure, no issues were seen prior to overnight incubation and after removing the samples from the thermomixer the next morning. Following a 10-minute shaking period, a gelatinous solid formed in most of the samples, excluding the reagent blank. After attempts to bring the samples back into a solution by incubation at 56 degrees Celsius, the extraction protocol was aborted. After recommendations from customer support, the cause of the failure still could not be determined. Therefore, the sample input was reduced to the recommended 40 milligrams of bone powder for a second attempt. The InnoXtract protocol was repeated with all seven bone samples and a reagent blank. Slight evaporation was observed in some samples after the overnight incubation, and the lysates were supplemented with lysis buffer. Three samples again failed in the second attempt due to solidification, but four experimental samples were successfully extracted. The manufacturer recommended adding ethanol to the solidified samples and heating at 56 degrees Celsius; however, the failed samples did not return to solution after 150 microliters of 95% ethanol were added and ten minutes of heating at 56 degrees Celsius. The IDs for the failed and successful samples can be seen in Table 3.

Quantification

Quantification of extracted DNA via Quantifiler™ Trio Quantification Kit was used to initially examine the success of the four extraction methods compared in this study. The recovery of small and large DNA fragments for each experimental sample across methods can be seen in Table 3. Some samples from the Promega and InnoXtract extractions were flagged for either

undetected amounts of DNA or the duplicate reactions showed significantly different concentrations of DNA, and those samples were quantified in a second reaction. Negative control and positive controls performed as expected in all quantification reactions. The results for quantification of samples extracted through protocols with overnight demineralization soaks can be found in Table 3. Figure representations of the data from Table 3 can be found in Figures 1 and 2. The concentration data were square-root transformed for visualization. A comparison of the results obtained from the Promega protocols with and without demineralization soaks can be found in Table 4.

Table 3: Quantification results for small and large autosomal target recovery for experimental samples for all extraction methods in nanograms per microliter.

Sample ID	Rohland		PrepFiler		Promega		InnoXtract	
	Small Target (ng/µL)	Large Target (ng/µL)	Small Target (ng/µL)	Large Target (ng/µL)	Small Target (ng/µL)	Large Target (ng/µL)	Small Target (ng/µL)	Large Target (ng/µL)
FS-01	0.1041	0.0033	0.1897	0.0150	0.0008	0.0001	0.1106	0.0028
FS-02	0.0048	0.0009	0.0035	0.0056	Undet	Undet	Failed	Failed
FS-03	0.0037	0.0020	0.0039	0.0029	Undet	Undet	0.0073	0.0031
FS-05	0.8595	0.2960	2.3210	1.1351	0.0162	0.0283	1.9465	0.0286
FS-09	0.0045	0.0016	0.0112	0.0041	0.0031	0.0096	Failed	Failed
FS-10	0.0133	0.0150	0.0132	0.0169	0.0001	0.0001	0.0012	0.0009
FS-11	0.0324	0.0148	0.0522	0.0290	0.0009	0.0004	Failed	Failed

*Undet = The recovery of DNA was undetermined.

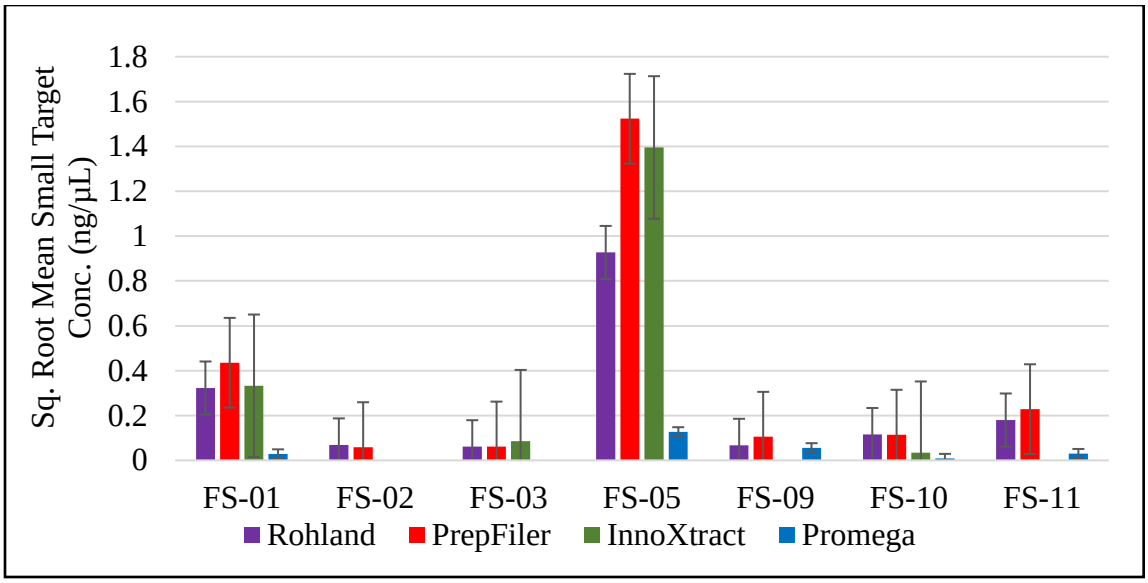

Figure 1. Mean small autosomal target concentration with a square-root transformation.

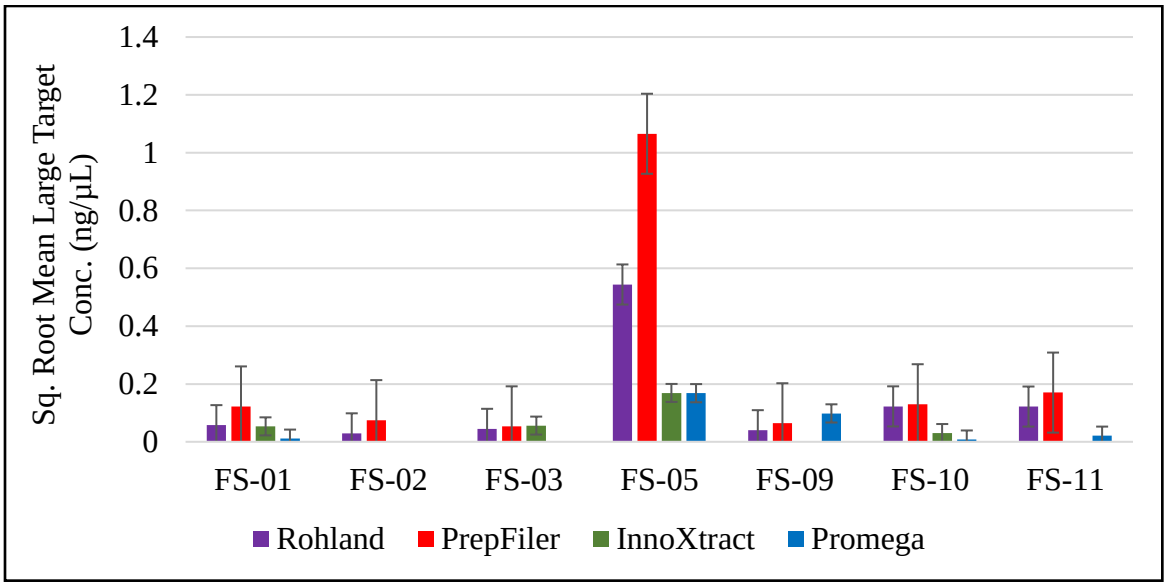

Figure 2. Mean large autosomal target concentration with a square-root transformation.

For small autosomal target DNA recovery, concentrations ranged from undetermined to 2.3210 nanograms per microliter. For large autosomal target DNA recovery, concentrations ranged from undetermined to 1.1351 nanograms per microliters. Samples with undetermined recovery amounts indicate that amplification was completely inhibited due to the presence of

PCR inhibitors (Applied Biosystems 2017). The sample from which the most small autosomal target DNA and large autosomal target DNA was recovered within each extraction method was sample FS-05. For the InnoXtract protocol, samples FS-02, FS-09, and FS-11 failed during the extraction process and were not quantified (Table 3). Compared to the Promega protocol with the overnight soak, the Promega protocol without the overnight soak recovered DNA from more samples. In addition, the small and large DNA targets were recovered in higher concentrations on average by the Promega protocol without the overnight demineralization soak (Table 4).

Table 4: Quantification results for small and large autosomal target recovery for experimental samples for the Promega protocols with and without the overnight demineralization soak.

Sample ID	Promega with overnight soak		Promega without overnight soak	
	Small Target (ng/µL)	Large Target (ng/µL)	Small Target (ng/µL)	Large Target (ng/µL)
FS-01	0.0008	0.0001	0.0175	0.0042
FS-02	Undet	Undet	0.0015	0.0016
FS-03	Undet	Undet	0.0092	0.0073
FS-05	0.0162	0.0283	1.6598	0.9779
FS-09	0.0031	0.0096	0.0134	0.0085
FS-10	0.0001	0.0001	0.0055	0.0074
FS-11	0.0009	0.0004	0.0286	0.0271

*Undet = The recovery of DNA was undetermined.

The large target concentration data is primarily used as an indication of DNA quality by comparison to the small target concentration. The ratio of small fragment to large target quantification results can be calculated by the following equation:

$$\frac{\text{Small autosomal target DNA concentration (ng/µL)}}{\text{Large autosomal target DNA concentration (ng/µL)}}$$

This ratio is known as the degradation index (DI). DI can be used as a predictor for the success of large DNA fragments as compared to small DNA fragments in STR reactions. The manufacturer interprets DIs of less than one to indicate no degradation, while a DI of between one and ten indicates the DNA is slightly to moderately degraded, and a DI of greater than ten

indicates severe DNA degradation (Applied Biosystems 2017). The results for the degradation indices are given in Table 5.

Table 5: Degradation indices for experimental samples for all extraction methods.

Sample ID	Degradation Index (DI)			
	Rohland	PrepFiler	Promega	InnoXtract
FS-01	31.54	12.69	4.95	39.40
FS-02	5.59	0.63	-	-
FS-03	1.85	1.35	-	2.72
FS-05	2.90	2.04	1.57	66.45
FS-09	2.81	2.72	3.25	-
FS-10	0.88	0.78	1.17	1.32
FS-11	2.18	1.80	2.07	-

Table 6: Degradation indices for experimental samples for the Promega protocols with and without the overnight demineralization soak.

Sample ID	Degradation Index (DI)	
	Promega with overnight incubation	Promega without overnight incubation
FS-01	4.95	4.18
FS-02	-	1.00
FS-03	-	1.26
FS-05	1.57	1.70
FS-09	3.25	1.57
FS-10	1.17	0.75
FS-11	2.07	1.05

Degradation indices for all experimental samples ranged from 0.63, indicating no DNA degradation, to 66.45, indicating severe DNA degradation. Sample FS-01 yielded the highest DI for Rohland, PrepFiler, and Promega. The InnoXtract protocol recovered DNA samples with the two highest DIs out of all experimental samples (Table 5). Overall, the DIs of the samples recovered from Promega with the overnight incubation were slightly higher than without the added incubation, sample to sample (Table 6).

Table 7: Results of the C_T of the IPC of the experimental samples of protocols with overnight incubations and their deviations from the C_T of the IPC of the NTC.

Sample ID	Rohland		PrepFiler		Promega		InnoXtract	
	C_T	Deviation	C_T	Deviation	C_T	Deviation	C_T	Deviation

FS-01	28.13	0.09	27.73	-0.31	27.43	0.20	27.52	0.29
FS-02	28.18	0.14	27.85	-0.19	-	-	-	-
FS-03	28.16	0.12	27.95	-0.09	-	-	27.43	0.20
FS-05	28.23	0.19	28.14	0.10	27.35	0.12	36.40	7.43
FS-09	27.88	-0.16	27.91	-0.13	27.33	0.10	-	-
FS-10	28.22	0.18	28.02	-0.02	29.14	0.17	27.36	0.13
FS-11	28.23	0.19	28.07	0.03	27.36	0.13	-	-

Table 8: Results of the C_T of the IPC of the experimental samples of the Promega protocols with and without overnight incubations and their deviations from the C_T of the IPC of the NTC.

Sample ID	Promega with overnight incubation		Promega without overnight incubation	
	C_T	Deviation	C_T	Deviation
FS-01	27.43	0.20	27.61	0.07
FS-02	-	-	28.00	0.46
FS-03	-	-	27.43	-0.11
FS-05	27.35	0.12	27.51	-0.03
FS-09	27.33	0.10	27.34	-0.20
FS-10	29.14	0.17	27.48	-0.06
FS-11	27.36	0.13	27.79	0.26

Table 7 shows the cycle threshold values of the internal PCR control (IPC) for each sample across the compared extraction methods. The IPC serves to confirm that assay components are functioning as expected and can be used to assess PCR inhibition. In the presence of PCR inhibitors, the IPC C_T of the sample can increase compared to the IPC C_T of the NTC. Almost all deviations for experimental samples were less than one, except for sample FS-05 for InnoXtract, indicating no significant PCR inhibition (Elwick *et al.* 2019). The average IPC deviation of the samples extracted with the Promega protocol without the overnight incubation was slightly lower than the Promega protocol without the overnight incubation (Table 8). Prior to library preparation, samples were diluted to a concentration of 0.067 nanograms per microliter for a total of one nanogram for the Precision DL8 Library Kit.

Statistical Analyses

For the purposes of statistical analyses, values for target concentrations for the failed samples during the InnoXtract protocol were inputted as zeroes. The significance threshold was place at a p-value of 0.05. Repeated measures ANOVA tests were initially used to assess the differences between the distributions of small and large autosomal target concentration results, as obtained through qPCR. However, due to small sample size, assumptions of normality in the data distribution were violated and a nonparametric alternative was used. The results of the statistical tests can be found in Appendix A. Using a nonparametric repeated measures test, known as the Friedman test, the small autosomal target concentrations were found to be statistically significantly different in the different extraction methods ($X2(3) = 11.35$, $p = 0.01$; Table A1). Pairwise Wilcoxon signed rank tests between methods revealed statistically significant differences between the small target concentrations recovered from the Promega protocol and PrepFiler protocol, and between the Promega protocol and Rohland protocol ($p = 0.016$ and $p = 0.016$; Table A2).

Friedman test was performed to compare the large target concentrations across methods. The large target concentrations were found to be significantly different in the different extraction methods ($X2(3) = 11$, $p = 0.01$; Table A1). Pairwise Wilcoxon signed rank tests between methods revealed statistically significant differences between the large target concentrations recovered from the PrepFiler and InnoXtract protocols, the PrepFiler and Promega protocols, and the PrepFiler and Rohland protocols ($p = 0.031$, $p = 0.047$, and $p = 0.016$, respectively; Table A3). In order to compare the quantification results from the Promega protocols with and without the overnight incubation, a sign test was used with a significance threshold of $p = 0.05$. The test showed significant differences between the median values of the small target concentrations ($p =$

0.0156; Table A4). No significant difference was found between the median values of large target concentrations (Table A4).

Quantification Dilutions

Due to the failure of three InnoXtract extracted samples, samples FS-01, FS-03, FS-05, and FS-10 from each extraction method were selected for MPS. The Precision DL8 Library Kit requires an input of 15 microliters of DNA at a concentration of 67 picograms per microliter for a resulting one nanogram of DNA for library preparation. Because the samples that were extracted through the Rohland and PrepFiler protocols were held in a freezer at -20 degrees Celsius for a period longer than six months, the samples were quantified with the Quantifiler™ Trio Quantification Kit before dilution calculations could proceed in order to obtain accurate concentration values. The quantification results from the second quantification were not used in comparing target recovery to the other extraction methods. Samples with small target concentrations of less than 0.067 nanograms per microliter were not diluted. Samples with concentrations greater than 0.067 nanograms per microliter were diluted with nuclease-free water with final volumes varying from 15 microliters to 60 microliters.

Library Preparation and Quantification

Three IonCode Barcode Adapter plates were used for library preparation, for a resulting 24 barcoded samples. The barcoded libraries included 16 experimental samples, four reagent blanks (from the original extraction), three NTCs, and one positive control (9947A DNA) sample. Eight sample libraries were prepared and barcoded in one Ion Chef run, with three sequential library preparations resulting in three pooled libraries. With 311 primer pairs per pool, 24 cycles of amplification for low-quality DNA at four minutes of anneal/extension time were used. The pooled libraries were stored at two degrees Celsius after library preparation. After all

libraries were prepared, library quantification was performed. Each library pool was diluted to 30 picomolar with nuclease-free water for a total of 50 microliters of solution. Twenty-five microliters from each pooled library were combined into a super-pool for 75 microliters of solution, which included all 24 barcoded sample libraries.

Sequencing and Data Analysis

Templating was performed with 25 microliters of the super-pooled library onto an Ion 530™ Chip. A custom Planned Run was created for the sequencing run with the PhenoTrivium Panel, which used the hg19 (Homo sapiens) genome assembly as a reference library. After sequencing, initial analysis took place in TSS. Summary data from the coverageAnalysis plugin is displayed in Table 9. Because review in the Converge™ Software was not possible at the time of this study, IGV and the HIrisPlex-S Webtool were used for analysis of eye color predictions. Tables 10 through 14 display the results for SNP analysis.

Table 9: Summary metrics for the experimental samples sequenced with the PhenoTrivium Panel on the Ion S5 across extraction methods.

Sample ID	Summary Metric	Rohland	PrepFiler	Promega	InnoXtract
FS-01	Mapped Reads	234,352	170,691	477	96,701
	On Target	92.65%	81.76%	58.07%	0.09%
	Mean Depth	321.5	197.7	0.607	0.016
	Uniformity	33.56%	35.00%	92.71%	99.69%
FS-03	Mapped Reads	76,976	60,871	1,302	6,033
	On Target	85.49%	80.07%	77.96%	0.81%
	Mean Depth	67.22	37.94	0.065	0.014
	Uniformity	30.75%	32.93%	98.73%	99.73%
FS-05	Mapped Reads	318,132	177,408	10,120	351,356
	On Target	95.53%	95.12%	2.27%	95.57%
	Mean Depth	555.9	311.5	0.013	661.2
	Uniformity	44.79%	48.95%	99.75%	52.84%
FS-10	Mapped Reads	68,267	3,072	1,322	1,232
	On Target	94.89%	12.66%	17.32%	8.85%
	Mean Depth	90.18	0.256	0.015	0.184
	Uniformity	31.85%	95.73%	99.70%	96.87%

A read refers to the sequence of bases in one fragment of DNA, while the mapped reads are the reads that were aligned to the reference in TSS (Bruijns *et al*. 2018). For each sample, the number of total reads was greater than the mapped reads displayed in Table 9. Of the mapped reads, a read is considered "on target" if one of the bases of the aligned sequences overlaps with a target region. The on-target reads are represented as a percentage of the mapped reads. Mean depth refers to the average depth of coverage, which is defined as the number of reads that overlap within a targeted region (Bruijns *et al*. 2018). Uniformity refers to the "percentage of bases in all targeted regions that is covered by at least 20% of the average base coverage depth reads" (Applied Biosystems 2021). For example, for sample FS-01 extracted through the Rohland protocol, uniformity means that 33.56% of the total number of bases that were targeted for this sample were covered by at least a depth of coverage of 64.3 (20% of 321.5).

Table 10: Genotype and depth of coverage of Sample FS-01 for the six IrisPlex SNPs included in the PhenoTrivium Panel across extraction methods.

IrisPlex SNP	Rohland		PrepFiler		InnoXtract		Promega	
	Genotype	Depth	Genotype	Depth	Genotype	Depth	Genotype	Depth
HERC2	A/G	70	A/G	49	-	0	G/G	1
OCA2	C/C	67	C/C	37	-	0	-	0
LOC105370627	G/G	26	G/G	9	-	0	G/G	1
SLC45A2	G/G	44	G/G	30	-	0	G/G	2
TYR	A/A	21	A/A	22	-	0	-	0
IRF4	C/C	52	C/C	50	-	0	C/C	1

Table 11: Genotype and depth of coverage of Sample FS-03 for the six IrisPlex SNPs included in the PhenoTrivium Panel across extraction methods.

IrisPlex SNP	Rohland	PrepFiler	InnoXtract	Promega

	Genotype	Depth	Genotype	Depth	Genotype	Depth	Genotype	Depth
HERC2	G/G	17	G/G	16	-	0	-	0
OCA2	C/C	16	C/C	14	-	0	-	0
LOC105370627	G/G	7	G/G	9	-	0	-	0
SLC45A2	G/G	5	G/G	4	-	0	-	0
TYR	A/A	13	A/A	11	-	0	-	0
IRF4	C/C	14	C/T	11	-	0	-	0

Table 12: Genotype and depth of coverage of Sample FS-05 for the six IrisPlex SNPs included in the PhenoTrivium Panel across extraction methods.

IrisPlex SNP	Rohland		PrepFiler		InnoXtract		Promega	
	Genotype	Depth	Genotype	Depth	Genotype	Depth	Genotype	Depth
HERC2	G/G	76	G/G	44	G/G	88	-	0
OCA2	C/C	56	C/C	39	C/C	69	-	0
LOC105370627	G/G	38	G/G	28	G/G	55	-	0
SLC45A2	G/G	41	G/G	33	G/G	55	-	0
TYR	A/G	53	A/G	33	A/G	45	-	0
IRF4	C/C	55	C/C	41	C/C	63	C/C	1

Table 13: Genotype and depth of coverage of Sample FS-10 for the six IrisPlex SNPs included in the PhenoTrivium Panel across extraction methods.

IrisPlex SNP	Rohland		PrepFiler		InnoXtract		Promega	
	Genotype	Depth	Genotype	Depth	Genotype	Depth	Genotype	Depth
HERC2	A/G	23	A/G	2	-	0	G/G	1
OCA2	C/C	19	-	0	-	0	-	0

LOC105370627	G/G	15	G/G	1	-	0	-	0
SLC45A2	G/G	7	-	0	-	0	-	0
TYR	G/G	19	-	0	-	0	-	0
IRF4	T/T	13	C/C	1	-	0	-	0

Across all six SNPs related to eye color according to the Iris Plex System in the HIrisPlex-S Webtool, Rohland and PrepFiler produced the most genotypes with the highest depth of coverage overall. The InnoXtract and Promega extraction methods did not have coverage of most Iris Plex SNPs (FS-05 for InnoXtract being the exception). For eye color, blue was consistently predicted across samples. The prediction value (p-value) is displayed in parentheses next to the eye color prediction. The highest p-value was taken to be the prediction out of blue, brown, or intermediate eye color (brown and intermediate p-values not shown). Eye color could not be predicted if there were not enough genotypes for SNPs for each sample, as seen in three InnoXtract and three Promega samples (Table 14).

Table 14: Eye color predictions according to the Iris Plex System (https://hirisplex.erasmusmc.nl/).

Sample ID	Eye Color Prediction (p-value)			
	Rohland	PrepFiler	InnoXtract	Promega
FS-01	Blue (0.848)	Blue (0.848)	-	Blue (0.867)
FS-03	Blue (0.848)	Blue (0.876)	-	-
FS-05	Blue (0.848)	Blue (0.848)	Blue (0.848)	-
FS-10	Blue (0.891)	Blue (0.856)	-	-

DISCUSSION

This study investigated the performance of four DNA extraction methods on degraded bone samples for MPS applications. Samples from seven humeri were extracted across the four selected and modified methods, which included an aDNA method from Rohland *et al.*, published in 2018, the PrepFiler® BTA Forensic DNA Extraction Kit from Applied Biosystems, the Bone DNA Extraction Kit, Custom from Promega, and InnoXtract™ from InnoGenomics. The performance of DNA recovery was assessed through qPCR, while the Ion AmpliSeq™ PhenoTrivium Panel from Applied Biosystems was used to assess the success of each extraction method with MPS technologies.

Quantification Performance

Rohland *et al.* (2018)

Small autosomal DNA target recovery from samples extracted with the Rohland protocol ranged from 0.0037 nanograms per microliter to 0.8595 nanograms per microliter (Table 3). It is important to note that the small autosomal target included in the Quantifiler™ HP and Trio assay targets amplicons of 80 bases in length, which is longer than the amplicons targeted by the Rohland protocol, which are approximately 35 bases in length (Applied Biosystems 2017; Rohland *et al*. 2018). The large target recovery ranged from 0.0009 nanograms per microliter to 0.2960 nanograms per microliter (Table 3). All but one sample, FS-01, had a degradation index less than 10, indicating that most samples were slightly to moderately degraded (Table 5). The deviations of the C_T of the IPC of the experimental samples from the C_T of the IPC of the NTC were not greater than one, indicating sufficient removal of PCR inhibitors (Table 7). For small

target concentration, the Rohland protocol recovered significantly higher concentrations of DNA than the Promega protocol (Table A2).

PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems 2012)

Small autosomal DNA target recovery from samples extracted with the PrepFiler protocol ranged from 0.0035 to 2.3210 nanograms per microliter. The large target recovery ranged from 0.0029 to 1.1351 nanograms per microliter. The small and large targets recovered with the PrepFiler protocol had the highest average concentrations out of all four methods tested (Table 3). All but one sample, FS-01, had a degradation index less than five, indicating most samples were slightly degraded. Additionally, the degradation indices for samples extracted through the PrepFiler protocol were the lowest out of all four methods tested, on average (Table 5). The IPC deviations for this method were all below one, showing no significant PCR inhibition (Table 7). The PrepFiler protocol recovered significantly higher concentrations of small autosomal target DNA than the Promega protocol and recovered significantly higher concentrations of large autosomal target DNA than all other extraction methods tested (Table A2; Table A3).

Bone DNA Extraction Kit, Custom (Promega 2019)

The Promega protocol with the overnight incubation recovered an overall lower average small and large DNA target concentration compared to the other extraction methods with overnight incubations, with two samples showing no recovered DNA through quantification (Table 3). The samples extracted from this protocol were held at two degrees Celsius prior to quantification for a period of three weeks. According to the manufacturer, this is the maximum recommended time period for storage at four degrees Celsius. Longer than three weeks, the manufacturer recommends storage at -20 degrees Celsius (Promega 2019). Degradation from

storage at two degrees Celsius is unlikely due to the small difference seen in the degradation indices between the samples extracted with the Promega protocol with and without the overnight incubation (Table 6). In addition, the average IPC deviations were comparable for both Promega extractions (Table 8).

The added overnight incubation may have had a negative effect on the performance on the Promega protocol. Conversely, the performance of the Promega protocol without the overnight incubation was comparable to the Rohland and PrepFiler protocols, both of which included overnight demineralization soaks (Table 3; Table 4). There was a significant difference in the small DNA target recovery between the Promega extractions (Table A4). The results indicate that the Promega protocol with the overnight incubation decreased overall DNA recovery compared to the Promega protocol without the overnight incubation. However, the overnight incubation did not seem to affect co-extraction of PCR inhibitors.

InnoXtract™ (InnoGenomics 2018)

Of the samples that did not fail during the extraction procedure, the small and large DNA targets were recovered in concentrations comparable to the PrepFiler and Rohland protocols. The small autosomal DNA targets ranged from concentrations of 0.0012 nanograms per microliter to 1.9465 nanograms per microliter. Additionally, the large autosomal DNA targets ranged from concentrations of 0.0009 to 0.0286 nanograms per microliter (Table 3). For large target concentration, the InnoXtract protocol recovered significantly lower concentrations of DNA than the PrepFiler protocol (Table A3).

Overall Performance

The modified protocol from Rohland *et al*. and the modified PrepFiler® BTA Forensic DNA Extraction Kit showed promising results through quantification for extraction of degraded

DNA samples. Although the degradation indices of the samples extracted through the Rohland method were comparable to the other extraction methods, higher degradation indices could be explained for the Rohland protocol's optimization for smaller targets, which would increase the degradation index. The modified protocol of the Bone DNA Extraction Kit, Custom from Promega, with the overnight incubation, and the modified protocol of InnoXtract™ from InnoGenomics presented complications that are undesirable for working with degraded DNA samples from bone. The overnight incubation added to the Promega protocol may have had a negative effect on the performance of the extraction protocol, combined with extended storage at two degrees Celsius. However, it is generally accepted that full demineralization of bone samples through increased incubation periods increases DNA recovery (Calacal *et al.* 2021). The modified protocol of the Bone DNA Extraction Kit, Custom from Promega without the overnight incubation also showed promising results, but the extracted samples from this method were not sequenced through MPS for the purpose of this study. Only samples extracted with methods including overnight incubations were sequenced for consistency.

Sequencing Performance

Rohland *et al.* and PrepFiler® BTA Forensic DNA Extraction Kit

Overall, the Rohland and PrepFiler methods performed the best in sequencing in terms of mapped reads, on-target reads, and mean depth. Between Rohland and PrepFiler, Rohland produced greater mapped and on-target reads and greater mean depths of coverage for each sample, although the results were mostly comparable. For most samples, as mean depth increased, uniformity decreased. For mean depths lower than one, uniformity was greater than 90%. When mean depths were greater than 30, the uniformity ranged from 30-50%. Therefore,

mean depth and uniformity must be interpreted together to assess sequencing output quality (Table 9).

Bone DNA Extraction Kit, Custom and InnoXtract™

The Promega and InnoXtract samples produced the lowest mapped and on-target reads on average. However, some unexpected results were seen. Although sample FS-03 extracted through the Promega protocol did not show any DNA recovery through qPCR, comparable results were produced for Promega's sample FS-10 for the metrics shown in Table 9. However, the results for FS-10 across all methods indicate a low-quantity sample. Analysis in Converge™ Software is needed to show the utility of the sequencing output for low-quantity DNA.

InnoXtract produced variable results for the sequenced samples. Although the number of mapped reads for sample FS-01 were greater than many other samples, the percentage of on-target reads was less than one percent. However, sample FS-05 extracted through InnoXtract produced the greatest number of mapped reads, on-target reads, and mean depth, and the highest percentage of uniformity across all methods. The variability in the performance of the InnoXtract method needs further investigating; however, it is likely attributed to the bone digestion step of extraction protocol, as this level of sequencing output variation was not seen in other methods.

Ancestry and Phenotypic Predictions

When available for comparison, genotypes for each SNP across the extraction methods were in agreement, except for HERC2 of samples FS-10 and FS-01. However, the genotype G/G only had a depth of one read from Promega in both samples compared to 23 reads for A/G from Rohland for sample FS-10 and 70 reads for A/G from Rohland for sample FS-01. Consensus across genotypes is important for assessing the accuracy of the extraction methods for MPS applications. The success of Rohland and PrepFiler for MPS applications is supported by the

quantification and available sequencing results. Conversely, the results from the InnoXtract and Promega modified protocols do not indicate compatibility with degraded DNA samples for MPS applications. More SNP analysis is needed for an accurate comparison of the Rohland and PrepFiler extraction methods.

Through the combined analysis of the targeted SNPs in Converge™ and using the HIrisPlex-S Webtool (https://hirisplex.erasmusmc.nl/), predictions about phenotypic characteristics and biogeographical ancestry can be made. Only the HIrisPlex-S Webtool was used for this study. For eye color, a consensus across methods indicates success, although reference information for the donors was not available for comparison. This application of SNP data in forensics, although promising, raises concerns. Incorrect interpretations of results may mislead law enforcement officials in the course of an investigation. For example, "ancestry" is a complex representation of a person's identity that may create discrepancies between genetic predictions and a cultural self-identification. Events such as adoption, misattributed parentage, and admixtures can also affect how the results are interpreted. This disconnect may complicate an investigation into the identity of an unknown individual (Diepenbroek *et al.* 2020).

With phenotypic predictions, subjectivity may affect classifications of hair color or eye color. Additionally, many aspects of a person's phenotype may be altered cosmetically. Colored contacts, dyed hair, and cosmetic surgical alterations may conflict with the genetic predictions. There are also differences in an individual's biological makeup and gender identity and expression, which may lead to some phenotypic assessments becoming less relevant, depending on the context. The concern over the interpretation of ancestry and phenotypic predictions in an investigation is recognized by scientists; however, more guidance is needed on how to effectively communicate the results of these predictions, including the limitations and cautions

that should be taken into account (Diepenbroek *et al.* 2020). Because of the current limitations of ancestry and phenotypic predictions, results should be used as investigative leads and supporting evidence.

Effects of Pre-Extraction Processes on DNA Recovery

Although there are many factors that cannot be controlled by the DNA analyst prior to receiving unidentified human remains, considerations must be made when selecting a DNA extraction method. Potential levels of degradation due to taphonomic processes or DNA yields based on bone type can be deciding factors when it comes to selecting a DNA extraction protocol.

Intrinsic Factors

Certain compositional factors of bone, known as intrinsic factors, can contribute to the preservation of DNA in extreme environments. The most relevant intrinsic factors of DNA persistence in skeletal elements are bone density, collagen content, and bone type. The inorganic phase, composed of carbonated hydroxyapatite, may provide protection of DNA when DNA is absorbed into hydroxyapatite and collagen. Denser bones, which may be more resistant to deterioration compared to other bones, are preferred for DNA analysis. These bones include the femur, tibia, and humerus. The root of tooth tissue, which is composed of dentin, pulp, and cementum, is recently becoming of interest for DNA extraction. The root is protected by the enamel of the tooth, which covers the crown. The enamel lends protection of the DNA within the root from heat, ultraviolet light, moisture, and microbes. The relationship between collagen and DNA is not well studied; however, there is evidence for binding reactions. A study by Mrevlishvili and Svintradze theorizes that the phosphate groups in DNA forms stable fibrils with collagen (2005). Collagen preservation is affected by environmental factors such as the type of

soil in which the skeletal remains are found (Raffone *et al.* 2021). The bone selected for analysis can influence the amount of extracted DNA. As mentioned previously, long bones and teeth have been the preferred bones for sampling because of their density. However, recent studies have shown evidence for increased DNA recovery for spongy bone types such as metacarpal or metatarsal bones and the petrous bones (Raffaone *et al.* 2021; Gamba *et al.* 2014).

Oftentimes, the skeletal samples available in forensic contexts leave no choice in the selection of bone type or condition. The seven humeri in this study allowed for control between bone type and DNA preservation expectation. However, sampling performed prior to this study limited the sampling site, preventing standardization of the location of sampling on each humerus. All sampling sites were classified as located on the distal or proximal diaphysis. However, some sampling sites were closer to the metaphysis than others. Studies have shown there is variation in the quantity of DNA within individual bones, especially between the epiphyses, metaphyses, and diaphyses, although the results of these studies are not in agreement. A study by Klavens *et al.* found that the mid-diaphysis produced the highest yields of DNA and the most complete STR DNA profiles in the femora the authors sampled (Klavens *et al.* 2020). Yet, a study by Antinick and Foran produced conflicting results. Although their study used bovine skeletal remains, their results showed that femoral epiphyses and metaphyses led to higher yields of DNA than the diaphyses (Antinick and Foran 2018). Many confounding variables may have contributed to the inconsistency in these studies, including post-mortem interval and other environmental factors. It is clear that more research is needed on the intra- and inter-variability of DNA in bones.

Extrinsic Factors

External factors, such as temperature, humidity, and soil and microbial composition can have significant effects on the preservation of DNA in skeletal remains. Low and high temperatures provide different mechanisms for protecting DNA. Low temperatures slow the chemical reactions responsible for organic degradation, while high temperatures slow hydrolysis, which leads to the dehydration and preservation of DNA. High humidity is detrimental to DNA in skeletal remains, leading to the penetration of organic substances that may act as PCR inhibitors. The soil in which skeletal remains are found can affect rates of DNA degradation. Low pH and permeable soils increase DNA degradation by increasing water penetration of the bone surface. Conversely, neutral or alkaline soil conditions are preferable for slowing down DNA degradation. Decomposition of organic matter in soil produces acidic compounds such as humic and fulvic acids, which can also act as PCR inhibitors during DNA extraction. Erosion by microorganisms, termed bioerosion, can affect multiple levels of DNA preservation. Microbes can damage the collagen content in the skeletal elements, as well as contribute to the presence of bacterial or fungal DNA within the bones. In addition, microbes increase the porosity of the bones, which may lead to the introduction of water and subsequently further other degradation processes (Raffone *et al.* 2021).

As mentioned previously, low temperatures slow degradation of DNA through the slowing of decomposition. Forensic anthropologists and pathologists are particularly interested in the temperatures human remains have been exposed to when it comes to accumulated degree-days (ADD). A technique for determining PMI incorporating ADD was developed by Megyesi *et al.* in 2005. ADD refer to an amount of heat or energy units as compared to a base temperature. To calculate ADD, the base temperature is subtracted from an average daily temperature, which

may be performed for the days from death to the discovery of the remains. Megyesi *et al.* suggest using zero degrees Celsius as a base temperature for the decomposition of humans, since freezing temperatures inhibit processes associated with decomposition, such as bacterial growth (Megyesi *et al.* 2005).

At the FOREST, all donors are placed above the surface for decomposition. The donors sampled in this study were placed at different times throughout the year, which may contribute to varying ADD for each sample due to seasonal temperature changes. Although it is not the responsibility of a DNA analyst to determine PMI for an individual through skeletal remains, ADD may be helpful for assessing potential DNA degradation. If there is a correlation between ADD and DNA recovery, then forensic anthropological assessments of human remains may supplement the choice of a DNA extraction method. More research is needed to explore this possible correlation.

Processing Techniques

In addition to the effects that taphonomic processes may have on the preservation of DNA in skeletal remains, there are also processes that may further impact the available DNA upon recovery and initial analysis. Prior to DNA analysis, human skeletal remains may be processed to remove soft tissue for other types of analyses, such as by forensic anthropologists. There is a variety of processing techniques implemented by forensic anthropology laboratories which may include boiling or simmering the bones in water, sometimes with the addition of degreasers. Various cleaning procedures have been criticized for their effects on the structural integrity of the bone. A study performed on the effects of processing techniques on subsequent DNA analyses indicated that methods using prolonged heating may have a negative effect on amplification reactions (Arismendi *et al.* 2004).

At the FOREST, donor remains are recovered and processed in the Western Carolina Human Identification Laboratory (WCHIL) when little soft tissue remains. Long bones are rinsed with water and dry brushed to remove dirt and remaining tissue. Bones that contain high amounts of ligaments and tendons, such as the bones of the hands and feet, are simmered on low heat until the tissue can be removed (Zejdlik-Passalacqua 2022, personal communication). Whenever possible, the handling of skeletal remains prior to DNA analysis should be taken into consideration when selecting a DNA extraction method. When the skeletal elements have been exposed to prolonged heat, the DNA extraction method should be concerned with balancing additional heat exposure with maximum DNA recovery.

Applications: Investigative Genetic Genealogy

The quality, quantity, and type of DNA recovered from samples can limit downstream applications. Especially when dealing with degraded samples that may contain highly fragmented DNA, the selected extraction method can greatly impact what sequencing and bioinformatic analysis can be generated from the extracted DNA. As discussed previously, when challenging samples such as bone and teeth are the only viable source of DNA, SNP data is becoming preferable as an alternative to STR DNA profiling. Investigative genetic genealogy (IGG) is a recently developed method of investigation that combines genealogical methods with DNA data to make inferences about genetic relationships between individuals (Kennett 2019). Forensic use of IGG has progressed rapidly in recent years due to the growing popularity of direct-to-consumer (DTC) genetic testing and open-source genealogy sites (e.g., GEDmatch and FamilyTreeDNA), which has enabled law enforcement to identify suspected perpetrators and missing persons through familial searching (Harding *et al.* 2020; Greytak *et al.* 2019; Kennett 2019). Most DTC companies use microarrays to genotype 500,000 to 1 million SNPs (Greytak *et*

al. 2019; Butler and Willis 2020; Kennett 2019). Genealogists can use these SNPs to determine genetic relatedness due to their inheritance patterns, taking into account the occurrence of recombination.

Genealogy algorithms can account for genetic relatedness and find relatives up to a seventh-degree relationship, which denotes a third cousin once-removed or other distant cousins (Butler and Willis 2020; McDermott 2020; Greytak *et al.* 2019). The next step to finding potential candidates to match the unidentified person is to form clusters of related people and work backwards to build family trees. Common ancestors are identified and a descendancy search is performed to work forward to find a group of candidates that could be the target person. Law enforcement takes this list of candidates and filters to identify the target person (McDermott 2020). IGG has played a prominent role in generating investigative leads over 200 cold cases, and it is clear that the technique will continue to be explored (Kling *et al.* 2021). However, in addition to IGG's growing use in the forensic community, there are some concerns and drawbacks associated with using genetic databases. There is overrepresentation of Northern European genetic ancestry in databases (Butler and Willis 2020). Genetic privacy and other ethical concerns are important topics that show a need for ethical considerations, regulation, and law enforcement transparency when using genetic databases (Court 2018; Kennett 2019).

DTC companies can genotype SNPs from fresh buccal samples obtained from consumers, which are abundant in DNA. IGG is limited by the SNP profile obtained from evidentiary DNA sources, which may be decades old and severely degraded. When skeletal remains are the source of DNA in a cold case, obtaining enough DNA to genotype sufficient SNPs for investigative leads is especially difficult. In order to genotype enough SNPs, the amount of DNA obtained

from the sample must be maximized. Therefore, the extraction method selected is crucial for the success of the application of IGG on degraded bone samples.

Future Directions

One of the common features of ancient DNA extraction methods is the increased processing time in the form of longer demineralization or incubation periods at lower temperatures than forensic DNA extraction methods. More research is needed on the effects of longer demineralization periods at lower temperatures with degraded bone samples of forensic age. This study showed the success of a modified PrepFiler® BTA Forensic DNA Extraction Kit that included an overnight incubation in 0.5 M EDTA. Future studies should compare the performance of the PrepFiler kit and protocol with and without the overnight soak. Processing time is an important factor in selecting DNA extraction methods, especially in high-throughput laboratories. In addition, future studies may also compare the performance of extraction methods with overnight incubation periods at higher temperatures (e.g., 56 degrees Celsius) and at lower temperatures (e.g., 37 degrees Celsius) to assess the level of heat degradation of DNA.

Although analyzing the general performance of the sequencing output through number of reads and depth of coverage provides valuable information about the compatibility of the extraction methods with MPS technologies, more detailed information is needed. Through software such as Converge™ and the HIrisPlex-S Webtool, the SNPs targeted in the PhenoTrivium Panel can be assessed for phenotypic and ancestry results. The results for each sample can be compared across methods for discrepancies and compared to reference data, when available. This would allow the accuracy and the success of the extraction methods to be more thoroughly assessed.

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APPENDIX A: STATISTICAL TESTS

Table A1: Friedman test results for the comparison of means of DNA autosomal target concentrations between the four DNA extraction methods with overnight incubations.

DNA Autosomal Target	Friedman Statistic	Degrees of Freedom	P-value
Small Target	11.35	3	0.01**
Large Target	11	3	0.01**

Table A2: Wilcoxon signed-rank test results for comparison of means of small target concentrations between pairs of extraction methods with overnight incubations.

Interaction	Wilcoxon Statistic	P-value
InnoXtract x PrepFiler	6	0.219
InnoXtract x Promega	21	0.297
InnoXtract x Rohland	14	1.000
PrepFiler x Promega	28	0.016**
PrepFiler x Rohland	24	0.109
Promega x Rohland	0	0.016**

Table A3: Wilcoxon signed-rank test results for comparison of means of large target concentrations between pairs of extraction methods with overnight incubations.

Interaction	Wilcoxon Statistic	P-value
InnoXtract x PrepFiler	1	0.031**
InnoXtract x Promega	13	0.675
InnoXtract x Rohland	3	0.078
PrepFiler x Promega	26	0.047**
PrepFiler x Rohland	28	0.016**
Promega x Rohland	4	0.109

Table A4: Sign rank test results for the comparison of median values of the DNA targets recovered by Promega protocols with and without overnight incubations.

DNA Autosomal Target	Sign Test Statistic	Degrees of Freedom	P-value
Small Target	0	7	0.0156**
Large Target	1	7	0.125