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# From Ancient DNA to Forensic Genetics: Validation Studies in Degraded DNA

Frankie Lane West
*University of Tennessee*, flwest@vols.utk.edu

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R. Alexander Bentley, Major Professor

We have read this dissertation and recommend its acceptance:

David G. Anderson, Bridget Algee-Hewitt, Dawnie Wolfe Steadman, Benjamin Auerbach, James Fordyce

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

# From Ancient DNA to Forensic Genetics: Validation Studies in Degraded DNA

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**Doctor of Philosophy**
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## DEDICATION

To my mom, Jill, and to John “Buckey” Davis, for making this possible.

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# Abstract

In forensic and archaeological applications, degraded DNA presents challenges during extraction, amplification, and analysis. Many of these issues can be addressed through the application of next-generation sequencing (NGS) techniques, including maximizing yields of fragmented DNA and identifying contaminant DNA. NGS is prompting a convergence between ancient and forensic genetic methods along several avenues, including DNA extraction. This dissertation discusses the convergence of extraction techniques contextualized within validation studies of ancient and modern DNA research.

Two validation studies are presented. The first study validates and explores the impact of a non-destructive DNA extraction technique developed by Bolnick and colleagues (2012). The "non-destructive" (Bolnick) DNA extraction technique yields both mitochondrial and nuclear DNA. While the teeth tested remained macroscopically intact, there was loss of tooth microstructure in the tooth root and enamel, shown through treated vs. untreated weights and histological analysis. Scanning electron microscopy (SEM) results showed minimal staining of the tooth. There were no significant carbon or oxygen isotopic difference between treated and untreated teeth. The damage characterized shows that the protocol is minimally destructive but may still be of interest to stakeholders desiring maintenance of macroscopic, but not microscopic, integrity.

The second study focuses on the quantity and quality of DNA extracted from post-mortem blood samples stored on untreated blood cards. Short-tandem repeats

(STRs) typed from the cards are used for ancestry-based analysis. The study validated two different hypotheses: 1) cadaveric blood stored on untreated blood cards yields enough DNA for typing of STRs and 2) STRs typed from blood cards yield geographic ancestry information. Results of the second case study indicate that post-mortem interval impacts the DNA quality of samples extracted from untreated blood cards. Tri-hybrid ancestry and admixture analysis indicate that the original thirteen CODIS loci have utility in estimating geographic ancestry.

These validation studies show the complications of working with degraded DNA in both ancient and forensic contexts. NGS approaches provide an opportunity in both fields to move beyond traditional markers to type expansive regions of the genome for both subfields and provides a way of addressing many issues of degraded DNA facing ancient and forensic researchers.

# PREFACE

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# CHAPTER 1: INTRODUCTION

The process of DNA extraction from human cellular material is well into its second century. Today, most human DNA analysis relevant to clinical settings as well as to direct-to-consumer genetic tests is readily isolated from fresh, recently collected sample material composed of blood, saliva, or other tissues (Brown et al. 2015; Yang et al. 2018). The high quality of clinically-sourced DNA stands in stark contrast to the kinds of samples that are recovered in forensic and archaeological contexts. Unlike DNA collected from living organisms, DNA from these contexts is typically highly degraded, characterized by a low starting template, high levels of fragmentation, and miscoding lesions (Butler 2012; Shapiro and Hofreiter 2014; Burrell et al. 2015; Hansen et al. 2017).

While both forensic and ancient DNA present challenges to the researcher, extending from the initial stages of sample preparation through the analysis, these are unique. Furthermore, while the objectives of forensic and ancient DNA laboratory work – including the recovery, amplification, and analysis of nuclear and mitochondrial DNA from skeletal remains and hair – are shared, a disconnect exist in the approaches applied to these two forms of degraded DNA. This critical disconnect in the theory, methods, and practice used in ancient DNA and forensic laboratories can be attributed to several fundamental reasons.

The end goals of ancient and forensic DNA differ, prompting an emphasis on different markers and analytical techniques. In forensic genetics, the primary focus of most DNA-based applications is individual identification, most commonly involving the comparison of a target (Q) profile against a known (K) reference sample for direct ("K to Q") and kinship-based matching (Butler 2012; 2015; Murphy 2017). In contrast, ancient

DNA applications rarely, with few exceptions (Coble et al. 2009; King et al. 2014), deal with individual identifications. Rather, ancient DNA research questions, as they apply to humans, are largely driven by questions of human history and prehistory, including evolutionary relationships (Green et al. 2010; Meyer et al. 2012; Meyer et al. 2014; Rogers et al. 2017), human migrations (Haak et al. 2005; Skoglund et al. 2012; Llamas et al. 2016), pathogens (Warinner et al. 2014; Margaryen et al. 2018; Søe et al. 2018), and diet (Seersholm et al. 2016; Weyrich et al. 2017).

These divergent research avenues have traditionally required different sets of DNA markers to achieve their respective ends. Forensic genetic approaches have been almost solely dependent on the typing of nuclear DNA short tandem repeats (STRs) and their smaller counterparts, miniSTRs (Butler et al. 2003; Alaeddini et al. 2010). While STRs have shown some limited utility for ancestry estimation (Algee-Hewitt et al. 2016), the primary focus has remained on identification by profile matching. In contrast, early studies in ancient DNA focused almost solely on mitochondrial DNA, which is present in higher copy number than nuclear DNA and provides the most common avenue for ancient population genetics prior to the advent of next-generation sequencing (NGS) (Merriweather et al. 1994; Stone and Stoneking 1993; Schurr et al. 1999). While in recent years, improved methods have tremendously expanded the scope of studies in ancient DNA analysis to encompass sequencing of whole mitochondrial genomes (Llamas et al. 2016; Lindo et al. 2017), nuclear genomes (Rasmussen et al. 2010; Rasmussen et al. 2014; Ávila-Arcos et al. 2017), and large-scale panels of nuclear single nucleotide polymorphisms (SNPs) (Lazaridis et al. 2014; Olade et al. 2018).

Unlike ancient DNA laboratories, forensic DNA analysis requires the validation of all laboratory procedures and analyses following Quality Assurance Standards (QAS) as issued by the DNA Advisory Board of the FBI beginning in 1998 (Butler 2012). Such validation practices are structured typically according to three different assessment levels: developmental validation, internal validation, and periodic performance checks. Developmental validation occurs external to the lab in question and is performed by manufacturers, typically focusing on kit and technological platforms. Internal validation is performed in the lab by testing new methods and reagent kits. Finally, periodic performance checks are also made internally in the lab and are designed to ensure that current protocols and reagents are working as expected. The necessity for validation of methods used in medico-legal contexts is understandable, due to the requirements set forth in the *Daubert* (1993) and *Frye* (1923) rulings, allowing for discovery requests of laboratory procedures to be admissible in court (Butler 2012). In contrast, ancient DNA laboratories are not subject to such constraints and have more leeway in regard to methods development and modifications to laboratory protocols. This contrast in research practice, along with the different markers targeted, has influenced the protocols used for and the analyses applied to these two forms of degraded DNA.

However, in spite of the traditional differences between methodologies, technological advances in DNA sequencing may now prompt a convergence between ancient and forensic DNA approaches. NGS, or massively parallel sequencing (MPS) methods are increasingly applied in forensic DNA contexts. Platforms such as the Illumina MiSeq FGx and Applied Biosystems GeneStudio S5 system with Precision ID

combine traditional STR markers with modern sets of SNPs. Like these platforms, new directions in forensic genetics now incorporate library preparation methods, which are commonly used in NGS for ancient DNA, into their workflows. Library preparation readies extracted DNA for sequencing on NGS platforms by sizing DNA fragments to specified lengths and attaching platform-specific chemistry oligonucleotides adapters to each fragment (Head et al. 2014). Integration of library preparation and targeted captures allow for sequencing of smaller fragment sizes, appropriate for degraded forensic DNA, while allowing for increased coverage of the genome, providing more statistical robusticity for analyses. While the constraints on forensic genetics laboratories regarding validation remain, the overlap between methods used in ancient DNA applications and forensic genetics will increase as forensic methods integrate more NGS-based practices.

This dissertation draws connections between current standard practices in ancient DNA research and forensic genetics. Methods of extraction in ancient DNA studies are explored in detail in Chapter 2, providing a review of past and present protocols for isolating degraded DNA. While forensic genetic applications currently require relatively large fragments for STR analysis (approximately 100-400 basepairs, or bp), new approaches harnessing the capabilities of NGS may require the adoption of ancient DNA approaches to DNA isolation. Extraction protocols in forensic genetics tend to focus on automation and reduction of inhibiting substances (Davoren et al. 2007, Rucinski et al. 2011, Pajnič et al. 2016); however, ancient DNA protocols have been adapted to purify and retain more and, increasingly, smaller fragments (Dabney et al. 2013, Rohland et al. 2018). The third chapter validates the effectiveness of a non-destructive extraction

method developed by Bolnick and colleagues (2012), here using ancient and forensically-significant human teeth and testing its applicability to a forensically-targeted next-generation sequencing kit. While one of the primary limitations of DNA analysis is the requirement for destruction of sample material, some alternatives exist, including the non-destructive extraction method explored in the aforementioned chapter. Additionally, Chapter 3 features a microscopic-level characterization of the impact of this non-destructive extraction protocol and explores the potential applications of this method. The fourth chapter explores traditional forensic DNA methods in a case study assessing the quality and quantity of DNA for typing forensic short tandem repeat (STR) markers from cadaveric blood samples. This chapter uses a model-based clustering program, STRUCTURE (Pritchard et al. 2000) to conduct ancestry and admixture estimation using STR markers typed from the untreated blood cards.

The following discussion highlights the importance of effective extraction techniques for forensic and ancient DNA research contexts. Traditional markers of ancient and forensic DNA research, including mitochondrial DNA and STRs, are the focus of analyses in the subsequent chapters, along with the application of NGS for modern samples. Through exploring the role of and technical aspects of ancient DNA extraction, critically validating and assessing a non-destructive DNA extraction protocol, and offering insights into untreated blood cards for long-term DNA storage and STR typing, this work offers a view of the congruencies between degraded DNA approaches in both ancient DNA and forensic genetics research. Overviews of each chapter are provided, contextualized within the scope of degraded DNA research.

## A Short History of Research on Degraded DNA

Two separate technological breakthroughs have revolutionized work with degraded DNA over the last three decades: the invention of polymerase chain reaction (PCR) in 1985 and the development of next-generation sequencing (NGS) by 454 Life Sciences in 2005 (Kulski 2016). These innovations dramatically altered the landscape for both forensic and ancient DNA research by significantly increasing the potential for data retrieval using degraded DNA. The quality of downstream analyses like traditional PCR, library preparation, capture methods, and sequencing nevertheless remain both highly dependent on the quality of extracted DNA and the availability biological material for DNA sampling. In contrast to PCR and high-throughput sequencing as watershed innovations, DNA extraction methods have seen gradual, but marked, improvement over the last thirty years. While these methods have improved, they, nevertheless, require the destruction of sample material which can be problematic as well as prohibitive in some situations involving human remains, sensitive museum collections, or forensic cases.

The saying, "garbage in, garbage out" applies for DNA extraction and subsequent analyses. If DNA extraction methods fail to recover any or at least adequate amounts of endogenous DNA, even the most advanced, downstream technologies are practically useless. Failure to remove inhibitors, or the adoption of extraction protocols that introduce additional inhibitors, can compromise chances of sample amplification, regardless of target markers. When working with degraded samples in both ancient and forensic contexts, choosing the most appropriate DNA extraction protocol is critical to successful amplification, sequencing, and analysis. While most of the emphasis over the

past decade has focused on novel approaches, such as next-generation or high-throughput sequencing (Hofreiter et al. 2015, Sarkissian et al. 2015) and bioinformatics-based analyses (Jonsson et al. 2013, Skoglund et al. 2014), it is critical to state how these new innovations are always constrained by the initial quality and quantity of DNA isolated during extraction. Additionally, many projects focused on ancient DNA are limited due to the destructive nature of the extraction process, thus leaving room for methodological improvement in both sampling techniques as well as DNA extraction methods.

The importance of choosing the most appropriate DNA extraction and the downstream applications cannot be overstated. These decisions should be based on a careful consideration of the sample type, sample preparation, recovery site context, and ultimate goal of the analysis or desired outcome. In spite of great technological advances in sequencing technologies over the past three decades, research on degraded DNA remains unavoidably dependent on the suitability of sampling techniques and efficacy of extraction methods, given the nature of the target samples. In addition to providing a thorough validation and assessment of a proposed non-destructive DNA extraction protocol, this research also emphasizes the importance of DNA extraction in research design for both ancient DNA and forensic genetics and provides a comprehensive overview of both past and present methodologies.

The first documented extraction of cellular DNA occurred by happenstance, during attempts by Friedrich Miescher to isolate proteins from human cellular material in 1869 (Dahm 2005). While endeavoring to separate proteins from human pus gathered from hospital bandages, Miescher isolated another material which he dubbed “nuclein,”

since it was clearly from the nucleus, a substance which we now recognize as deoxyribonucleic acid, or DNA (Dahm 2005).

More than one hundred years later, the field of DNA research witnessed another leap forward with the first successful extraction of ancient DNA from a museum specimen, the extinct quagga (Higuchi et al. 1984). Soon after, Svante Pääbo (1985) published his account of successful extraction of DNA from an ancient human, drawn from the tissue of an Egyptian mummy. These discoveries launched the field of ancient DNA, and many publications touting successful extraction of DNA from ancient materials, even from dinosaurs, soon followed. Researchers soon realized, however, that much of the DNA extracted was in fact contamination and not endogenous DNA (Lindahl 1997, Pääbo et al. 2004). The term endogenous simply means that the DNA is that of the individual being sampled for the analysis, in contrast to exogenous DNA, which comes from outside sources and can include the human researcher and other sources of contamination.

Further complicating matters, degraded forms of DNA, both materials from ancient and forensic contexts, share distinctive characteristics that separate these forms from DNA extracted from living organisms. Unlike DNA from living individuals, "ancient" and "forensic" DNA is typically extracted from deceased individuals, for which the organism's DNA repair-mechanisms are no longer functioning. These two types of degraded DNA are defined for the purpose of this dissertation's discussions as "forensic" and "ancient." Forensic samples are those samples up to 100 years in age and/or of medico-legal significance which exhibit degraded DNA characteristics, such as high

fragmentation, and which may also be low copy number (LCN), or DNA recovered from small number of cells (Gill 2001).

Similarly, "ancient" samples are almost always highly degraded, always exceeding 50 years of age, and typically much older, and are not of medico-legal significance. "Modern" DNA is simply defined as DNA from contemporary, living organisms that is expected to be well preserved. Accordingly, it is used in discussions of good quality, high or ultra-high molecular weight, DNA that is not classified as ancient or forensic. In ancient samples, DNA is typically highly fragmented and characterized by deaminated bases, caused by hydrolytic damage and microbial action over tens or hundreds or thousands of years (Hofreiter et al. 2001, Pääbo 2004). Based on these characteristics, ancient DNA is more difficult to amplify and sequence than modern and forensic DNA. Moreover, the superior quality of modern DNA will often be preferentially selected over degraded DNA in chemical reactions designed to copy or amplify DNA fragments, such as polymerase chain reaction (PCR).

This dissertation provides a comparison between DNA types, using case studies to illustrate the issues relating to extraction and analysis of ancient DNA versus modern DNA samples. While ancient samples are often more fragmented and express an increased number of deaminated bases than forensic samples, the concerns related to dealing with highly degraded samples are similar between these two subfields of human genetics. Sample preparation, contamination control, and removal of inhibitors are key factors required of the extraction protocols used in both ancient and forensic lab contexts, and these are thoroughly explored through the lens of ancient DNA in Chapter 2.

Limitations on sample material access for both ancient and forensic DNA analysts also is a shared concern, for which a potential solution is presented and tested in Chapter 3. Issues associated with degradation of post-mortem blood from untreated blood cards offer insights for forensic body donation programs but also show the limitations of small marker sets for ancestry and admixture analysis as shown by a comprehensive assessment of the archival value and research potential of these kinds of samples in Chapter 4.

### *Ancient DNA*

The realization that much of the previously published ancient DNA research was contamination led to caution regarding ancient DNA and authentication (Lindahl 1997, Cooper and Poinar 2001). Best practices for traditional lab-based methods, including PCR and Sanger sequencing were set forth by Cooper and Poinar (2001), who set forth several principles for quality ancient DNA research and confirmation of authenticity of endogenous results. The most important principles include: 1) placement of ancient DNA work must occur in dedicated "clean" lab facilities, in which PCR is performed in a separate area (often a separate building) from sample preparation, extraction, and PCR preparation; 2) independent extractions from separate sample materials should be performed; if possible, these should be independently verified in a separate lab facility; 3) replicability – the results should be replicable with additional sample material from the same individual; and 4) results should show characteristics of ancient DNA behavior, including fragmentation and deamination of bases (Cooper and Poinar 2001). These

recommendations set the standard for judging the quality of experiments, remain the gold standard for traditional Sanger sequencing methods, and have even been extended with some modifications to NGS analyses (see Skoglund et al. 2014).

Over the last ten years, Sanger sequencing has been largely replaced by next-generation sequencing technology (NGS), also known as high-throughput sequencing. The majority of NGS sequencing for ancient and forensic DNA, and most laboratory-based sequencing, is performed on the Illumina platform, with small percentages of projects performed on platforms such as Applied Biosystems' Ion Torrent (Tackney et al. 2015), Ion GeneStudio S5 (Bochtler et al. 2018) and SNaPshot machines, as well as Affymetrix platforms and restriction-site associated DNA sequencing (RAD-seq) or RAD-tagging (Tin et al. 2014).

NGS technology provides an ideal platform for ancient DNA in that it favors small fragment sizes and requires library preparation techniques that allow for capture of degraded DNA (Hofreiter et al. 2014, Marciniak et al. 2015). Traditional Sanger sequencing requires flanking primers of at least 15 base pairs on each end of a target fragment to sequence, with the shortest possible fragment sizes around 60-100 base pairs, whereas NGS allows for sequencing of fragment sizes between 30-400 base pairs, which enhances the capabilities for working with degraded samples.

While some researchers in traditional lab sciences prefer longer fragments, the so-called "limitations" of NGS actually benefit the degraded DNA research where shorter fragment sizes (30 bp and above) tend to be the norm (Dabney et al. 2013). NGS methods also allow for rapid typing of large portions of the genome, whether through random

assortment using shotgun sequencing methods or targeted capture using hybridization capture methods, in contrast to the limitations of small regions of the genome specifically targeted in Sanger sequencing.

NGS has enabled multiple breakthroughs in the analysis of degraded DNA. Researchers became able to sequence samples that were too degraded for traditional Sanger technology, including the Kennewick Man or the Ancient One (Rasmussen et al. 2015), the Anzick child (Rasmussen et al. 2014), and Cheddar Man (Brace et al. 2018). NGS also allows for sequence DNA from specimens of especially great antiquity, including the whole mitochondrial genome of an ancient hominid ancestor from Sima de los Huesos dating to over 400,000 years (Meyer et al. 2014) and the genome from a Pleistocene horse dating to over 700,000 years (Orlando et al. 2013). Despite these advances, however, ancient DNA research is still constrained by the limitations presented by DNA extraction, as explored in Chapter 2.

### *Forensic Genetics*

Forensic samples range from high quality samples that require dilution prior to amplification to extremely degraded samples and/or those containing high levels of inhibitors that impede PCR reactions (Pineda et al. 2014). While the CODIS core loci set of twenty short tandem repeats (STRs) remains the gold standard in forensic genetics, recent research has expanded beyond sole dependence on STRs. While mini-STRs (those under 125 bp) were once the most promising option for working with degraded samples

(Coble and Butler 2005), their application in forensic contexts now has limited utility (Nieuwerburgh et al. 2014). Instead, the use of NGS, including library preparation and targeted captures, allows for an enhanced marker set recovery and improved discrimination criteria, both with reduced total input DNA.

For example, NGS target enrichment probe captures allow for sequencing of both STRs and single-nucleotide polymorphisms (SNPs) with deep coverage for robust variant calling (Shih et al. 2018). To compare methods, in a validation of the GlobalFiler™ Express PCR Amplification Kit which includes typing for 21 autosomal STRs including the CODIS core loci, 1 Y-STR, 1 Y indel, and an Amelogenin marker, minimal input was 0.05 ng, though optimal input was much higher – between 2.5-10 ng (Flores et al. 2014). In contrast, in validations of the Illumina FGx ForenSeq™ DNA Signature Prep Kit, input DNA amounts as low as 0.065 ng produced full profiles with 100% call rates of expanded marker sets, including 27 autosomal STRs, 24 Y-STRs, 7 X-STRs, and 94 identity-informative SNPs (Jäger et al. 2017). Input amounts as low as 0.00782 ng produced partial profiles of 50% concordance (Jäger et al. 2017). Traditional PCR based chemistries, like the GlobalFiler™ Express PCR Amplification Kit require intact primer binding sites to sequence STRs and other markers (Shih et al. 2018). NGS methods are not bound by primer binding sites, allow for deep coverage of captured regions providing more discriminatory power, and can also be used to distinguish between mixtures (Shih et al. 2018).

With improvements in sequencing technology, library preparation, and targeted captures, forensic genetics has expanded to include ancestry informative markers (Caratti

et al. 2015), phenotypic markers (Butler 2015; Caratti et al. 2015; Parson 2018), and methylation studies for age estimation (Parson 2018). These improvements have also facilitated the development of new forensic markers with the ability to differentiate between individuals, interrogate biogeographical ancestry, and deconvolute mixtures (Bulbul et al. 2017). Kidd and colleagues have developed a panel of 65 microhaplotypes, or short DNA fragments that include two or more haplotype-distinguishing SNPs, encompassing only 198 SNPs for ancestry inference, using samples with limited input DNA (Kidd et al. 2017; Bulbul et al. 2017). NGS has expanded forensic genetic applications beyond STR profiles, incorporating ancestry-based and phenotypic markers as well as enhanced abilities to discriminate between individuals and deconvolute mixed samples. NGS platforms such as the Illumina MiSeq FGx in combination with the Verogen ForenSeq DNA Signature Prep kit may set the standard for future forensic marker sets. While other platforms, such as the Life Technologies Ion S5, allow for NGS applications for forensic samples, the ForenSeq kit is the only kit on the market that combines traditional forensic STRs with ancestry, phenotypic, and identity SNPs. Through combining 230 markers in a single reaction, the ForenSeq kit sequenced on the MiSeq FGx will allow for widespread use, thereby setting de facto protocols for NGS applications in forensic genetics.

NGS-based methods in forensic science may prompt increased convergence between forensic and ancient DNA-based methods for bone and tooth samples. Extraction methods in ancient DNA research have recovered increasingly small DNA fragments (~20-80 bp), once thought useless during the days of Sanger sequencing that

required larger sites for primer binding. With improvements in sequencing technology, those smaller fragments, which are informative, especially when targeted for SNPs. As forensic genetics continues to integrate more NGS methods, extraction methods may recover even smaller fragments (Dabney et al. 2013; Rohland et al. 2018).

Whereas forensic genetics has emphasized automated methods for high-throughput laboratory analyses (Witt et al. 2012; Kallupurackal et al. 2015), ancient DNA extractions have largely remained un-automated. Only recently has a new protocol been released that allows for automated extraction using liquid handling systems similar to those used in forensic laboratories (Rohland et al. 2018). This reflects a convergence in methods from ancient DNA to forensic genetics and vice versa.

## Non-Destructive DNA Extraction Techniques

DNA extraction methods have traditionally used destructive approaches to sample preparation, wherein a portion of the sample tissue (bone, tooth, hair, nail, exoskeleton, etc.) is pulverized to facilitate cell lysis and release of DNA from the cellular matrix (Hagelberg and Clegg 1991; Skoglund et al. 2012; Dabney et al. 2013). Sample preparation often involves manual grinding using hammers (Merriwether et al. 1994), mortar and pestles (Gondek et al. 2018), or mechanical pulverization using freezer mills (Hanni et al. 1994) or ball mills (Kistler et al. 2014). However, the destructive nature of DNA analysis may delay or prohibit access to samples (Lindahl 1997) from museums and out of respect for the views of human descendants and descendant communities.

Alternatives for destructive analysis are also needed for forensic research collections, where destructive analysis is often limited by prescribed protocols for donors or to prevent destruction of individual morphological data.

Several non-destructive DNA extraction methods have been presented. Minimally destructive techniques include those that maintain the integrity of the specimen by removing small amounts of sample material through targeted drilling, including an orthograde entrance technique for tooth samples (Alakoc and Aka 2009) and a minimally-invasive cranial base drilling method to access bony material from the petrous portion (Sirak et al. 2017).

Others suggest avoiding drilling and pulverization altogether. These approaches utilize a buffer step in which the sample is soaked and then removed, macroscopically intact (Rohland et al. 2004; Porco et al. 2010; Bolnick et al. 2012; Hofreiter 2012; Tin et al. 2014). These protocols target preserved insect specimens and use soaking buffers that are then extracted directly, maintaining specimen integrity (Gilbert et al. 2007; Thomsen et al. 2009; Porco et al. 2010; Tin et al. 2014).

The first protocol to use this approach for primate samples was developed by Rohland and colleagues for DNA extraction from museum specimens, using teeth from chimpanzees and hyena bones, coats (hides), and soft tissue dating between 47 and 164 years (2004). Rohland and colleagues compare three different extraction buffers, a sodium phosphate buffer with proteinase K, a Tris and sodium chloride buffer with proteinase K, and a guanidine thiocyanate (GuSCN) buffer without proteinase K after a two-day and seven-day soak (2004). DNA isolation by isopropanol precipitation was

used in conjunction with the sodium phosphate and Tris-sodium chloride buffers (Vigilant et al. 2001), while the GuSCN buffer utilized a silica suspension extraction protocol (Boom et al. 1990; Rohland et al. 2004). The GuSCN protocol outperformed the other methods in tests using PCR to amplify target mitochondrial DNA from 215-414 bp in length but the authors were unable to type nuclear microsatellite markers (Rohland et al. 2004).

The Rohland et al. (2004) method was validated using teeth from eight prehistoric humans from Spain (Gomes et al. 2015). Comparing traditional extraction methods (Rohland and Hofreiter 2007) with the non-destructive method, Gomes et al. (2015) showed that there was no significant difference in real-time PCR results between the two extraction protocols for a total of four samples, suggesting that the Rohland et al. 2004 non-destructive protocol performed as well as the traditional destructive method (2015).

A similar soaking buffer-based approach was used by Bolnick and colleagues (2012) for ancient bone and tooth samples and is thoroughly validated and its utility discussed in Chapter 3. Bolnick and colleagues opted for an EDTA and proteinase K soaking buffer with a limited exposure time (16-24 hours) followed by extraction using Rohland and Hofreiter's 2007 silica-suspension based protocol (2012). The method by Bolnick and colleagues produced both PCR-amplifiable mitochondrial and nuclear DNA. Both protocols reported no indications of macroscopic damage to the teeth after soaking (Rohland et al. 2004; Bolnick et al. 2012); however, this assertion is assessed more thoroughly in Chapter 3.

## Chapter Overviews

### *Chapter 2 – Next Generation Sequencing and the Convergence of Ancient and Forensic DNA Extraction Techniques*

The second chapter covers Next Generation Sequencing and the convergence of ancient and forensic DNA extraction techniques. This chapter presents a review of the literature and discussion of the parallels between DNA extraction protocols for ancient DNA research and forensic genetics. As the first comprehensive review of extraction protocols in almost twenty years (MacHugh 2000), this chapter reveals how DNA extraction methods in ancient DNA analyses are poised to influence forensic genetics protocols for next-generation sequencing (NGS) techniques. The rise of NGS of both subfields is prompting a convergence of extraction methods to exploit the strengths of NGS and yield the highest amounts of usable DNA for analysis.

Through evaluation of the most fundamental publications and widely-used extraction methods, the chapter details trends of ancient DNA extraction and compares them to forensic genetics methods. Common chemical agents used in extraction techniques are outlined and their applications explored. The three primary types of extraction techniques – phenol chloroform, silica-based, and bead-based methods – are detailed. The chapter provides an overview of DNA extraction approaches from a broad array of ancient sample materials, both human and non-human. Materials ranging from bone and tooth samples, to hair, calculus, soil, and other materials are discussed, detailing optimal sampling techniques and protocols. The limitations and capabilities of modern

extraction techniques for ancient DNA play a role in amplification as well as sequencing and analysis. This downstream effect can be seen not only in ancient samples but also in forensic genetics contexts. For example, the extraction protocol for the blood card samples used in the chapter 4 did not sufficiently remove inhibiting substances from the isolated DNA. This highlights the prime importance of optimizing effectiveness of extraction techniques, which is a need in both forensic and ancient DNA analysis.

### *Chapter 3 – Validation and Impact Assessment of a Non-destructive DNA Extraction Method on Modern and Ancient Tooth Samples*

The third chapter, Validation and Impact Assessment of a Non-destructive DNA Extraction Method on Modern and Ancient Tooth Samples, presents results from a validation study of a "non-destructive" DNA extraction method. First published by Bolnick and colleagues in 2012, this approach is tested using both ancient tooth samples, as detailed in the authors' protocol, as well as modern teeth extracted within the past five years. In addition to testing the Bolnick and colleagues' protocol, an additional extraction protocol using the Bolnick soaking buffer is tested and yields compared for modern samples to determine which is most effective. Weights, measurements, and photographs were collected before and after extraction to assess the claim of the method as non-destructive. Building on the validation study, this chapter identifies small but non-trivial damage introduced by the use of the protocol by Bolnick and colleagues on tooth samples. While the validation study confirms the potential for using the protocol to maintain the macroscopic integrity of the samples, this assessment details the effects of

the chemicals used in the protocol (bleach, EDTA, and Proteinase K) on the microstructure of teeth in modern tooth samples as well as ancient. The effect of the soaking buffer is assessed, externally, using scanning electron microscopy (SEM) and, internally, using histology. Microscopic impact is also investigated through isotope analysis to determine if this non-destructive method will impact the possibility of future, albeit destructive, analyses. Three separate analyses, tooth histology, isotope analysis, and scanning electron microscopy, are used to assess the impact of the extraction buffer on the microstructure of human tooth samples. This chapter demonstrates that the Bolnick protocol can be deemed "minimally destructive" with impacts to tooth microstructure observed through metric, histological, and SEM analyses.

***Chapter 4 – Assessing DNA Quality and Quantity from Cadaveric Blood Stored on Untreated Blood Cards: Impact on STR Quality and the Utility of Variably Amplified Markers for the Individual Estimation of Trihybrid Ancestry and Admixture Proportions***

The fourth chapter explores the quantity and quality of DNA recovered from untreated blood cards within the context of a body donation program. While extraction methods for Flinders Technology Agreement (FTA) cards have been validated for use with post-mortem blood samples, the quality and quantity of cadaveric blood stored on untreated blood cards has not been tested. Research on DNA extraction from whole blood is common place (Chacon-Cortes et al. 2012; Ghatak et al. 2013; Guha et al. 2018); however, few studies have explored the characteristics of blood extracted from cadavers. An increased understanding of the quality and quantity of DNA from post-mortem blood

samples is essential for forensic centers, enabling long-range planning of DNA-based genomic studies. The blood cards were extracted and DNA yields and quality were assessed using quantitative PCR (qPCR). Samples were amplified using the Applied Biosystems Identifiler kit and fragment analysis was carried out by capillary electrophoresis. STR profiles were assessed for relative fluorescent units (RFUs) and allele peak heights. Ancestry and admixture analysis were conducted on the data collected from the STR profiles generated through the model-based clustering program STRUCTURE (Pritchard et al. 2000, Algee-Hewitt et al. 2016). This research determined that the blood cards, when extracted between 4 months and 4 years after collection, allowed for successful amplification of a full STR profile which could then be used for preliminary ancestry estimation using the method established by Algee-Hewitt and colleagues (2016).

### *Chapter 5 – Conclusion*

This dissertation integrates current scholarship with case studies on both ancient and forensic DNA, highlighting the importance of sample storage methods, sample preparation, and DNA extraction. While some methodological differences between ancient DNA research and forensic genetics have persisted, the transition to NGS methods has encouraged convergence between these two research avenues. A deeper understanding of DNA extraction techniques allows for research planning that

incorporates sampling, decalcification, purification, and elution methods optimized for degraded samples.

Destructive DNA extraction techniques preclude access to numerous archaeological samples, as most museums and collections limit destructive analysis of human remains and other materials. By assessing the effects of Bolnick and colleagues' extraction method, the research presented in this dissertation informs future anthropological investigations of ancient DNA and provides insights into the applicability of this method for more recent, forensic-age samples. Through documenting the effects of the method on tooth structure, this research provides an assessment by which collection curators and descendant communities will be able to evaluate the potential for non-destructive or at least, minimally destructive, extraction of DNA. Provided that the protocol is deemed minimally destructive on a microstructural level, this research could be used to support research on countless museum and archaeological specimens, both human and non-human. Finally, a case study on the quality and quantity of DNA extracted from untreated blood cards provides insights into the role of sample storage materials on DNA degradation. As in ancient DNA contexts, time since death and ambient storage conditions can impact DNA preservation. Those issues, along with the utility of a limited set of STRs for ancestry and admixture analysis are assessed here.

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# BRIDGING STATEMENT

Chapter 1 introduces the three main dissertation chapters with an overview of the two fields of degraded DNA research, ancient DNA and forensic genetics, describing the similarities between the two fields while outlining the differences brought about by the sometimes divergent end goals of each. As the first comprehensive review of the history of ancient DNA extraction techniques in recent years, Chapter 2 reveals how the sometimes divergent fields of degraded DNA are poised to converge on common methods driven by the transition to next generation sequencing (NGS).

As such, Chapter 2 describes how extraction for degraded samples can be harmonized due to shared concerns over degradation, preservation, and inhibition. Outlining past examples in which the fields of ancient DNA and forensic genetics borrowed from one another regarding extraction techniques, the following chapter suggests that the newest innovation in genetic research, next-generation sequencing (NGS) is now prompting another era of exchange between the two fields. Ancient DNA researchers have advanced methods in extraction of degraded samples to exploit the benefits of NGS; forensic geneticists can utilize these improved techniques for working with problematic forensic samples.

# CHAPTER 2: NEXT GENERATION SEQUENCING AND THE CONVERGENCE OF ANCIENT AND FORENSIC DNA EXTRACTION TECHNIQUES

## Writing Statement

A version of this chapter will be submitted by F. L. West for peer-reviewed publication.

The following represents a sole-authored work written by F. L. West on the subject of DNA extraction and the convergence of these techniques in forensic genetics and ancient DNA research. The concept for and written product were developed solely by the author. Suggestions and edits were provided by all committee members.

## Abstract

In the age of next-generation sequencing (NGS) and bioinformatics-based approaches, DNA extraction remains a critical part of ancient DNA research. NGS is also driving a convergence of DNA extraction protocols used in ancient and forensic genetics, including common reagents, past and present extraction methods, and sample substrates. After a review of extraction protocols in each subfield, here we show how new techniques and sampling strategies are leading to a homogenization in forensic and ancient DNA extraction methods involving bone, teeth, calculus, soil, and other tissues. The special considerations of each degraded DNA specialization are outlined including degradation, contamination, and inhibition are discussed.

# Introduction

By detailing the extraction techniques in both forensic genetics and ancient DNA, we show that extraction protocols in both disciplines are poised to converge on more similar methods for isolating DNA. While DNA extraction poses numerous challenges, isolating DNA from degraded samples presents a unique set of concerns in both ancient and forensic contexts. However, in spite of shared concerns regarding contamination, fragmentation, deamination of bases, and low template samples, ancient DNA research and forensic genetics have differed significantly in relation to extraction techniques in recent decades. During the nascent years of research in both fields, ancient DNA drew heavily from extraction protocols in forensic genetics (O'Rourke et al. 2000). However, ancient DNA research, recent advances in extraction methods have been driven by next-generation sequencing techniques and bioinformatic approaches (Sarkissian et al. 2012; Skoglund et al. 2014; Hofreiter et al. 2014; Haber et al. 2016; Slatkin and Racimo 2016), leading to breakthrough findings involving anatomically modern humans and other hominids (Reich et al. 2010; Meyer et al. 2012; Gokhman et al. 2014), the peopling of the Americas (Rasmussen et al. 2014) and Australia (Rasmussen et al. 2011), and other ancient human migrations (Fregel et al. 2018; Olalde et al. 2018).This, in turn, has motivated proposals for improved DNA extraction protocols for recovery of endogenous DNA from ancient samples, prior to library preparation and other downstream analyses (Barta et al. 2013; Gamba et al. 2016; Nieves-Colón et al. 2017).

These developments in ancient DNA research contrast with forensic genetics, which today uses extraction protocols that are optimized for typing of traditional markers, primarily short-tandem repeats (STRs) using capillary electrophoresis (CE) rather than massively parallel sequencing (MPS). The need for developmental and internal validation of protocols, reagents, and extraction kits has largely limited the focus of forensic extractions to typing of STRs and mitochondrial DNA. However, there is increasing demand in forensic genetics to apply MPS or next-generation sequencing (NGS) approaches to DNA of medico-legal significance (Butler 2015; Jäger et al. 2017.

Here, we review the background of extraction techniques in both forensic genetics and ancient DNA. We propose that extraction protocols in both disciplines are poised to converge on more similar methods for isolating DNA, driven by NGS. In forensic genetics, especially problematic samples could be addressed using specifically optimized protocols for working with degraded samples using NGS approaches.

## Background

Ancient samples are generally defined as those exceeding 50 years in age and, more importantly, those whose sequences are characterized by high fragmentation and the presence of deaminated cytosines (Hofreiter et al. 2000; Pääbo 2004; Shapiro and Hofreiter 2014). Forensic samples are those of medico-legal significance.

The invention of polymerase chain reaction (PCR) in 1985 allowed for the advancement of both the fields of ancient and forensic DNA. PCR-based analyses of

small quantities of DNA allowed for the study of degraded DNA. The development of NGS by 454 Life Sciences in 2005 (Kulski 2016) has more recently revolutionized ancient DNA research by exponentially increasing the potential for data retrieval of fragmented and damaged DNA. These recent technological advances helped develop the field of paleogenomics, or the application of next-generation sequencing to produce whole or partial genomes from ancient samples (Shapiro and Hofreiter 2014). Since the shift to next-generation sequencing, no comprehensive review of extraction methods has been published in recent years (since MacHugh 2000), in spite of significant improvements in DNA sampling and extraction techniques.

On the other hand, forensic genetics has been slow to embrace NGS technology, restricted by validation requirements and the need for database comparisons which are predominantly based on STRs. As such, forensic extraction methods-based papers often focus more on one or more extraction techniques geared toward specific sample substrates (Adamowicz et al. 2014; Young et al. 2014; Samsuwan et al. 2018) rather than large scale overviews, but have been more broadly covered in a few exceptions (Stray et al. 2010; Lee and Shewale 2017). While extraction techniques in ancient DNA research have increasingly focused more on recovery for NGS-based projects, extraction methods in forensic genetics have largely revolved around kit-based methods, phenol-chloroform protocols, and use of automated robotics.

The importance of selecting the most appropriate DNA extraction techniques for the research design cannot be understated. Variables to consider when choosing the most appropriate method include collection site conditions, sample type and collection

methods, preservation, processing, and downstream analyses. Failure to select the most appropriate extraction methodologies can result in reduced DNA recovery, loss of time, reagent costs, and waste of precious sample material. Therefore, due consideration must be given when choosing the techniques for DNA extraction including taking important variables, such as preservation and sample type, into account.

### *DNA Preservation in Degraded Samples*

Characterizing DNA damage and understanding the mechanism behind such degradation has been of interest to ancient DNA researchers since the first investigations in the 1980s. Early on, descriptions of ancient DNA degradation opened an avenue for understanding the qualities of targeted ancient DNA and providing a means of demonstrating contaminate-free extraction and amplification (Pääbo 1989, Lindahl 1993). Soon after, these characteristics of ancient DNA degradation provided a mechanism for authenticating endogenous DNA results (Cooper and Poinar 2000; Pääbo et al. 2004) and later were exploited in combination with bioinformatic approaches to filter out contamination (Jónsson et al. 2013; Skoglund et al. 2014). DNA preservation in various substrates, from bone and teeth to environmental DNA, remains of interest with both ancient and forensic samples. Degraded DNA found in materials from both ancient and forensic contexts shares distinguishing differences from DNA extracted from living organisms.

Immediately following the death of an organism, DNA degradation begins, galvanized by both enzymatic and non-enzymatic processes (Alaeddini et al. 2010; Emmons et al. 2017). Organismal DNA is subjected to damage from enzymes created by the breakdown of the cadaver and microbially-produced enzymes, especially during the putrefaction stage of decomposition (Alaeddini et al. 2010; Emmons et al. 2017). While some evidence points to a cessation of microbial attack at around 500 years (Hedges 2002), experimentation on fragmentation has been limited. Beyond microbial action, hydrolysis and oxidation continue to damage DNA throughout the post-mortem interval producing fragmentation, miscoding lesions, and crosslinks that characterize ancient DNA sequences (Lindahl 1993; Lindahl 1997; Briggs et al. 2007; Allentoft et al. 2012). Understanding of DNA fragmentation is limited by the lack of controlled studies and restraints on experimental time frames that can be assessed over the course of a human life span. One of the few studies to assess degradation intervals points to a 524-year-half life for mitochondrial DNA fragments of 242 bp in the New Zealand Moa, with a degradation rate twice as rapid for nuclear DNA (Allentoft et al. 2012).

Studies on the mechanisms of DNA preservation have also been limited. DNA molecules are preserved through binding with the apatite strictures of bones and teeth (Grunenwald 2014), through the process of adsorption in which molecules adhere to an available surface. Biomimetic studies of DNA adsorption into apatite, similar to the hydroxyapatite found in bone and tooth matrices, point to the influence of pH on the DNA preservation process. Soil pH significantly impacts DNA preservation, with more acidic conditions resulting in more adsorption and thus greater DNA preservation

(Grunenwald et al. 2014). While DNA is bound to apatite matrices, increased porosity of bone and tooth structures is caused by microbial action, marked by a reduction in collagen (Hedges 2002). Environmental variables, including temperature, humidity, and soil pH, also affect the binding of DNA to calcium phosphates such as hydroxyapatite, affecting DNA absorption and preservation. Temperature, for example, influences the adsorption of DNA into apatite material, with lower temperatures (~4 degrees C) culminating in reduced adsorption (Grunenwald et al. 2014).

DNA may also bind to soil matrices, resulting in preservation of environmental DNA (Willerslev 2003, Emmons et al. 2017). The integration of DNA into both apatite and/or soil matrices allows for persistence of DNA in both the organic and inorganic components of bone/soil. The binding of DNA to soil as well as apatite necessitates extraction techniques that target the release of DNA from both components, yielding more endogenous DNA (Sosa et al. 2013).

### *Common Components of Extraction Protocols*

Most extraction techniques employed in ancient DNA research utilize standard ingredients and protocols found in cell biology/microbiology research. The four basic steps of DNA extraction include 1) sample preparation, 2) lysis/digestion/decalcification, 3) purification, and 4) elution. Because bones and teeth are the most common sample types for ancient DNA extraction, protocols may include decalcification steps for samples

where DNA has bound to apatite matrices, which are absent from typical DNA extractions of blood, tissue, and saliva.

### *Sample Preparation*

Sample preparation begins with surface decontamination and preparation of the sample for extraction. Grinding bone and tooth samples exposes additional surface area for improved chemical lysis of cellular material: a sub-sample (from 200 milligrams (mg) up to 5 grams (g)) is typically removed from the of bone or tooth, either with a Dremel tool or by drilling, which powders the sample material during removal. Dremel tools and standard drills and bits should be used at low rpm to avoid heat damage to the sample (Sawyer et al. 2012; Sandoval-Velasco et al. 2017). Samples typically are reduced to a ground powder manually, as with a sterilized mortar and pestle (Sawyer et al. 2012; Wannajuk et al. 2013). Other mechanical approaches use liquid nitrogen-based freezer mills (Loreille et al. 2007; Pruvost et al. 2007) and laboratory ball mills (Gamba et al. 2016; Valverde et al. 2016; Sandoval-Velasco et al. 2017), which pulverize the sample into a fine powder. Extraction of DNA from soil samples often involves mechanical pulverization, such as bead beating, to release microbes from the soil and break down cell walls (Slon et al. 2017).

While early extraction protocols required large amounts of sample material exceeding one gram (Hänni et al. 1990; Hagelberg and Clegg 1991), recently more efficient protocols require far less, typically around 0.2 g (Brotherton et al. 2013;

Thomson et al. 2014; Llamas et al. 2016). Many sampling situations require minimally-destructive methods, including collections of museum specimens as well as human remains. One less-destructive option is orthograde entrance, in which the tooth sample is drilled from crown to root apex leaving the overall tooth morphology intact (Alakoç and Aka 2009). Another option is to access the petrous portion of the human cranium through a minimally invasive procedure targeting the cranial base (Sirak et al. 2017). In other protocols, the whole tooth or bone is soaked in a lysis buffer, removed, and then the buffer extracted while retaining the integrity of the sample (Rohland et al. 2004; Bolnick et al. 2012). Similarly, whole insects can be submerged in buffer that is then poured off and extracted providing non-destructive approaches for analysis of historic non-human collections (Gilbert et al. 2007; Porco et al. 2010; Tin et al. 2014).

***On Contamination***

Regardless of sample preparation method, one of the most persistent problems in ancient DNA work is that of contamination, especially relating to ancient human and hominin studies wherein contaminate DNA, from the researcher(s), archaeologist(s), or other human sources might be closely related to that of the individual(s) sampled and more difficult to separate out downstream. The transition to NGS approaches has not alleviated the need for sample decontamination but, rather, emphasized the need to remove not only DNA of closely related species but also microbial DNA in order to increase sequencing efficiency (Korlević et al. 2015). Ancient DNA protocols use various

methods for authentication of endogenous DNA, with best practices requiring extraction controls, isolated dedicated ancient DNA work areas, recognition of appropriate molecular behavior, and reproducibility (Cooper and Poinar 2000). Dedicated lab spaces for ancient DNA typically include positive pressured air flow, working in a protective cabinet such as a PCR enclosure or other such laminar flow hood, overhead UV lamps and benchtop UV crosslinkers, and practicing uni-directional workflow from the dedicated ancient lab to post-PCR laboratories. Reagents, lab consumables, lab equipment, and cross-contamination during extraction can also introduce contaminant DNA (Barta et al. 2013).

Even when strict laboratory protocols are imposed, contamination can arise prior to the sample's arrival in the laboratory. If the material was excavated under non-sterile conditions, or if bone and tooth samples were washed to remove the soil matrix, human contaminants could be introduced into the sample material. For example, exogenous DNA can be introduced during washing and can permeate both tooth enamel as well as cortical bone (Gilbert et al. 2005; Sampietro et al. 2006). While strict controls may be put in place for excavating human remains (Yang and Watt 2005), many of the individuals sampled in ancient DNA studies are part of long-standing museum collections and have been previously handled.

Both chemical and mechanical methods are used to remove contaminant DNA from surfaces. Early studies used dental drills (Haak et al. 2005; 2008) and sand paper or sanding attachments to remove portions of the surface area of bones and teeth (Matisoo-Smith et al. 1997; Yang et al. 1998; MacHugh et al. 2000; Yang 2003; Yang et al. 2004;

2008). More recent applications use sand blasting to remove the outer portion of the bone or tooth, combined with UV irradiation (Gamba et al. 2012).

Chemical decontamination is the most often used method for removing exogenous DNA, through the use of bleach (6% sodium hypochlorite), hydrochloric acid (HCl), and/or sodium hydroxide (NaOH). Yang et al. (2004) combined all three chemicals, followed by UV irradiation, while others integrate combinations of HCl and bleach (Stone and Stoneking 1993; 1998; Skoglund et al 2012). UV radiation inactivates, or damages, longer strands of DNA, such as those greater than 500 base pairs (bp) in length, especially in aqueous solutions (Sarkar and Sommer 1993). However, in analysis of damage to shorter fragments of less than 250 bp, UV radiation has been shown to be less effective in the destruction of DNA fragments (Sarkar and Sommer 1993). Sarkar and Sommer (1993) also found that dried DNA contaminants (i.e. not in aqueous solution) were less susceptible to UV damage.

Experimenting with a variety of surface decontamination methods, including sand paper, DNAaway (a common laboratory surface decontaminant), and sodium hypochlorite, Kemp and Smith (2005) found that immersion in 6% sodium hypochlorite, or regular strength bleach, for 15 minutes provides the most effective and cost efficient method for removal of exogenous DNA, while also noting that immersion in the same bleach concentration for up to 21 hours does not damage endogenous DNA in traditional PCR reactions (Kemp and Smith 2005). Additional research has demonstrated the efficacy of 6% sodium hypochlorite treatment for lower exposure times (4 minutes) (Barta et al. 2013; Kemp et al. 2017).

Even after these decontamination steps are followed, DNA contamination may persist in ancient samples (Barta et al. 2013). In these cases, bioinformatic approaches can be used separate endogenous ancient DNA from more recent contaminants based on statistical analysis of ancient DNA characteristics (Shapiro and Hofreiter 2014; Skoglund et al. 2014). Bioinformatic approaches, packages such as *PMDtools*, use degraded DNA characteristics, such as fragment length, basepair substitutions in the 5' position, and nucleotide deamination, to statistically infer whether sequences are endogenous ancient DNA or exogenous contaminant DNA (Skoglund et al. 2014).

***Lysis/Digestion/Decalcification***

Following sample preparation and decontamination, the lysis step, sometimes referred to as digestion or decalcification in protocols for bone and tooth material, involves the breaking open or "lysing" of cellular material. Lysis, or cellular disruption, can be achieved through chemical or mechanical methods, or a combination of the two. The lysis process is meant to disrupt the cellular architecture so that the DNA becomes accessible for isolation. This disruption usually involves one or a combination of chemical agents in an aqueous buffer solution that disrupt the cellular membrane, often combined with heat and/or agitation. Agents such as ethylenediaminetetraacetic acid (EDTA) and proteinase K, sourced from *Tritirachium album*, are some of the most often-used, typically with addition of detergents to aid in lysis and Tris-based buffers to maintain a stable pH.

### *Purification of DNA*

DNA purification is usually performed using one of three approaches: phenol-chloroform, silica-based methods, or bead-binding methods. The goal of the purification step is the isolation, or extraction, of DNA from other cellular materials. Some DNA extraction techniques are better suited to working with bone and tooth material than others. Other types of sample material, such as human or animal hair, may require additional chemicals for extraction.

### *Phenol-Chloroform Methods*

Phenol-chloroform (PC) extractions represent one of the most common extraction techniques used in degraded DNA research. Ancient DNA analysts adopted PC protocols from forensic genetics in the early years of research (O'Rourke et al. 2000) and they remain a standard protocol in forensic analysis (Ferreira et al. 2013; Gielda and Rigg 2017; Iyavoo et al. 2017). Phenol and chloroform are both organic solvents commonly used in DNA extraction protocols, thus these methods are often referred to as organic extraction methods. Each of these solvents denature proteins, allowing for precipitation and removal of DNA from solution. Phenol alone is unstable and oxidizes into quinones that can crosslink DNA, and thus, is often prepared with equal or almost equal volumes of chloroform to create stability (Farrell 2005). Phenol and chloroform are also usually combined with isoamyl alcohol, which reduces protein foaming during extraction (Farrell

2005). Together, often in concentration ratios of 25:24:1 (phenol:cholorform:isoamyl alcohol, or PCIA), this combination allows for denaturing and removal of proteins, while simultaneously inhibiting nuclease activity (Farrell 2005).

Both phenol and chloroform are carcinogenic and caustic agents, so work must be performed in fume hood, which are not available in all degraded DNA facilities. Moreover, both nitrile and neoprene gloves degrade within minutes of contact with PC solutions, so chemical barrier gloves such as Silvershield or 8-mil thick ChemTek gloves should be worn. PC protocols were once the preferred method of DNA extraction, but more recent trends show ancient DNA studies increasingly favor silica-based methods (Rohland and Hofreiter 2007; Dabney et al. 2013; Gamba et al. 2016; Llamas et al. 2016; Glocke and Meyer 2017; Hansen et al. 2017; Sirak et al. 2017). This is further supported by comparisons of extraction protocols on forensic age skeletal remains in which demineralization outperformed phenol chloroform (Jakubowska et al. 2012); however, PC remains a standard forensic protocol.

### *Silica-based Methods*

Silica-based methods have been used for decades in ancient DNA work (Boom et al. 1990, Yang et al. 1998, Rohland and Hofreiter et al. 2007, Gamba et al. 2016). As noted by Boom and colleagues, DNA binds to silica or glass particles with the addition of a chaotropic salt in solution (1990). Extraction protocols based on silica suspensions, in which silicon dioxide is prepared using a series of suspensions in water and HCl, have

been actively used since the 1990s (Boom et al. 1990, Baker et al. 2001) and remain relevant protocols (Rohland and Hofreiter 2007, Hansen et al. 2017). Silica is itself a PCR inhibitor, so silica in suspension must be completely removed during elution (Yang et al. 1998), which is the final step of DNA extraction (see below). More time-efficient methods use silica-based columns, such as the Qiagen Min-elute column, as the silica remains bound within the column while the sample solution passes through, reducing chances of silica carryover during elution.

*Bead-based Methods*

Extraction methods using bead-based chemistries include both carboxyl and streptavidin surface-coated magnetic and non-magnetic microspheres and silica microspheres. Carboxyl-coated, as well as silica-based, beads bind with mammalian cells without significant modification (O'Brien et al. 2009). However, both streptavidin and carboxyl-coated microspheres can be bound with oligonucleotides to capture targeted sequences. A method called solid-phase reversible immobilization, or SPRI, has allowed for efficient extraction using microspheres, combined with high levels of propylene glycol and salts, high ionic washes, and low ionic elution buffers for release of DNA from microbeads (Bangs Laboratories 2016). Used occasionally in ancient DNA applications (Zhao et al. 2017), bead-based methods are mainly used for size-selection in DNA library preparation, forensic DNA applications (Desmyter et al. 2017) and in ecological and evolutionary studies (Vo and Jedlicka 2014).

*DNA Elution*

Elution is the final step of the extraction protocol in which the DNA is released from one material, such as silica, and put into solution. Elution of DNA is often carried out in a variety of buffers, with earlier protocols favoring ultra-purified water (Bailey et al. 1996; Burger et al. 1999; Meyer et al. 2000), and more recent using TE buffer (Tris-EDTA) (Rohland and Hofreiter 2007; Rohland et al. 2010; Neparáczki et al. 2017) or TET buffer (Tris-EDTA and Triton-X) (Avila-Arcos et al. 2015; Glocke and Meyer 2017). In comparisons of long-term storage buffers, TE buffer was shown to be superior to water in tests of DNA quantity using qPCR and STR quality (Beach 2014). With the increasing popularity of kit-based extraction components and silica-spin columns, kit-based elution buffers, such as Qiagen's EB solution, are frequently used (Carpenter et al. 2013; Seguin-Orlando et al. 2013).

***On Inhibition***

Extraction of DNA from sample materials often results in the co-extraction of substances that inhibit PCR, so-called PCR inhibitors. Many substances associated with ancient remains can present as PCR inhibitors, including humic acids (Alaeddini 2012) and fulvic acids, both found commonly in soil and water, bone dust, calcium, collagen, cave sediment, materials in blood and feces, urea, peat and clay rich soils (Baar et al. 2011), and heavy metals including copper, iron, gold, and lead (Alaeddini 2012).

Furthermore, even samples lacking in environmentally-derived inhibitors can be exposed during handling and extraction, as many reagents represent inhibitory substances, including silica (Boom et al. 1990), EDTA, detergents and salts used in extraction, ethanol, isopropanol (Schrader et al. 2012), and phenol (Alaeddini 2012).

Although the mechanisms behind PCR inhibition are poorly understood, experiments demonstrate that different inhibitory substances in varying amounts affect PCR reactions in multiple ways, with most attention focused on the interaction between inhibitors and polymerase enzymes (Alaeddini 2012; Schrader et al. 2012). With the transition to more next-generation sequencing and library preparation, PCR inhibitors have received less attention; however, the co-extraction of inhibitors remains a significant issue for ancient samples regardless of post-extraction amplification strategies. Although PCR remains an integral step in library preparation and amplification, some protocols circumvent the PCR step and instead are based on high concentrations of input DNA; however, this approach is not practical for most ancient samples (Oyola et al. 2012).

A study comparing three different extraction protocols on the same sample material recognized inhibition as a significant factor in library preparation efficiency (Glocke and Meyer 2017). Various approaches can be used to circumvent the impact of inhibitors on traditional PCR and NGS library preparation. Some experiments indicate that silica-based protocols using chaotropic salts are effective in removal of inhibitors (Alaeddini 2012) and silica-based columns have been used to remove inhibitors from extracted samples (Yang et al. 1998; Kemp et al. 2006). Chimeric polymerases designed for resistance against inhibitory substances offer another solution (Baar et al. 2011). The

addition of PCR additives such as bovine serum albumin (BSA) in reactions also has been shown effective with some ancient DNA samples (Baar et al. 2011; Farell and Alexandre 2012).

## Extraction in Ancient DNA Contexts

The first extraction of DNA from cellular material was performed by Friedrich Miescher in 1869 (Dahm 2005; Tan & Yiap 2009). Using pus collected from hospital bandages, Miescher found that the addition of an acid precipitated a substance and, based on his knowledge at the time, "had to ascribe such material to the nuclei" (Dahm 2005). While Miescher's initial isolation of DNA was rudimentary, the past 150 years have witnessed numerous advances in DNA isolation methods. The extraction protocols used in the majority of ancient DNA studies today trace their origins to a few classic protocols.

A breakthrough in isolating DNA from ancient tissue utilized a phenol chloroform-based extraction method on the quagga, an extinct zebra-like species (Higuchi et al. 1984). Shortly following work on the quagga, Pääbo (1985) used another modified phenol chloroform method, following Blin and Stafford (1976), to extract DNA from the tissue of a 2,400-year-old Egyptian mummy. In the rush of early studies following these breakthroughs, however, much of the "ancient DNA" typed was later shown to be contamination from modern sources (Handt et al. 1994; Lindahl 1997) emphasizing the need for strict contamination controls during extraction (Cooper and Poinar 2000; Pääbo 2004).

Early gel-based and plasmid extractions exploited the facility of DNA to bind to silica particles in the presence of chaotropic salts for development of numerous protocols. Based on this property, Boom et al. produced a silica-based protocol for use with human serine, urine, and bacteria (1990). Creating a silica suspension of silicon dioxide suspended and resuspended with water, followed by hydrochloric acid to adjust pH to 2.0, the authors combined the silica suspension with a guanidine thiocyanate-based lysis and washing buffer to facilitate DNA binding to the silica. The Boom extraction method was modified by Hoss and Pääbo (1993) with the addition of a one to several-hour digestion soak at 60° C for ancient bone. With and without additional modification, the protocol has been used in a variety of ancient DNA applications (Spigelman et al. 1993; Taylor 1996; Austin et al. 1997; Stone and Stoneking 1998; Fricker et al. 1997; Donoghue et al. 1998; Yoder et al. 1999; Baker et al. 2001).

Several early extraction protocols used large buffer volumes (over 2 mL) for bone and tooth decalcification, incorporating Centricon-brand microcentrator columns to concentrate the buffers during elution. One such protocol, devised by Hagelberg and Clegg (1991), builds on a phenol-chloroform protocol by Maniatis et al. 1982. The authors develop an initial decalcification step for bone powder in EDTA, followed by lysis with EDTA, proteinase K, and N-lauroylsarcosine, extraction using phenol-chloroform, with concentration using a Centricon microconcentrator (1991). This protocol was subsequently used in a variety of studies (Stone and Stoneking 1993; Stone and Stoneking 1998; Adcock et al. 2001; Hervella et al. 2015).

Yang et al. (1997, 1998) proposed a protocol using a silica-spin based column to reduce inhibitors from ancient DNA for improved PCR amplification. Their protocol used Qiagen's QIAquick columns, which capture DNA fragments 100 bp and larger, producing PCR-amplifiable extracts without needing the fume hood required for phenol-chloroform-based extractions (Yang et al. 1998). The silica-spin based protocol is now used extensively for ancient DNA studies (Yang et al. 2004; Malmstrom et al. 2005; Yang and Speller 2006; Yang et al. 2008; Malmstrom et al. 2009), often with modifications (MacHugh et al. 2000; Svensson et al. 2007; Edwards et al. 2010; Skoglund et al. 2012; Gamba et al. 2014; Raghaven et al. 2014; Lazaridis et al. 2014; Teasdale et al. 2014; Pinhasi et al. 2015; Gamba et al. 2016).

Ancient DNA studies have also combined phenol-chloroform and silica-based extractions (Ausubel et al. 1995; Boom et al. 1990; Hoss and Pääbo 1993; Krings et al. 1997; Caramelli et al. 2003; Lalueza-Fox et al. 2007; Sanchez-Quinto et al. 2012). Until recently, phenol-chloroform methods were the most popular protocol for bulk of extraction in ancient DNA research (Hagelberg and Clegg 1991; Hänni et al. 1994; Scholz and Pusch 1997; Loreille et al. 2007; Orlando et al. 2002; Orlando et al. 2003; Shapiro et al. 2004; Haak et al. 2005; Salamon et al. 2005; Weinstock et al. 2005; Orlando et al. 2006; Kemp et al. 2007; Larson et al. 2007; Orlando et al. 2008; Orlando et al. 2009; Hughes et al. 2006; Deguilloux et al. 2011; Keller et al. 2012; Sarkissian et al. 2013). Another advance in extraction methods made use of a prepared silica suspension (Rohland and Hofreiter 2007). Because it provides a simple and short (2 days) means of removing PCR inhibitors, the Rohland and Hofreiter (2007, 2010) protocol and its

variants are the preferred ancient DNA extraction protocol for many publications (Green et al. 2010; Reich et al. 2010; West et al. 2017; Bolnick et al. 2012; Hansen et al. 2017). As an alternative to silica suspension, others have utilized silica-based columns, such as the Qiagen Min-elute column (Cui et al. 2013; Dabney et al. 2013; Lazaridis et al. 2014; Witt et al. 2015; Kemp et al. 2017; Sirak et al. 2017).

Prior to the development of next-generation sequencing (NGS) approaches, there was little need to recover extremely small fragments that could not be amplified during traditional PCR for Sanger sequencing. With NGS, however, sequencing of small molecules offered the possibility of retrieval of additional informative sequence data, especially in highly degraded samples. Dabney and colleagues noted a lack of very small molecules (lower than 40 bp) in downstream analyses integral for NGS sequencing, which could be due in part to size selection in double-stranded DNA library preparation as well as extraction methods (2013). To maximize recovery of these small molecules, Dabney and colleagues modified the Rohland and Hofreiter (2007) protocol, adding a guanidine-hydrochloride/sodium acetate/isopropanol binding buffer with increased binding buffer to sample ratio, accommodated through the use of reservoir extensions, and substituting silica spin columns for the silica suspension (2013). Using this method, the authors noted increased recovery of molecules 30 bp and larger from a 300,000-year-old Pleistocene cave bear from the Sima de los Huesos site in Spain (Dabney et al. 2013). The Dabney et al. protocol has been shown as effective when compared to other extraction protocols (Gamba et al. 2016; Glocke and Meyer et al. 2017) and has been

used in multiple ancient DNA studies (Lazaridis et al. 2014; Meyer et al. 2014; Sirak et al. 2017; Brace et al. 2018; Mathieson et al. 2018).

Others have shown that single-stranded library preparation, rather than library preparation methods that target only double-stranded fragments, can recover fragments as small as 17 bp, emphasizing the need for extraction methods that recover shorter fragments (Glocke and Meyer 2017). In comparison of three extraction methods, Glocke and Meyer used a new method with a 2 M guanidine HCl and 70% isopropanol binding buffer to limit the exposure of the sample to EDTA, which affects recovery of very short fragments, during the binding step. Their findings indicate that this method is more effective at recovery of short fragments (20 bp) but less effective at removing inhibitors than the silica-based protocol by Dabney et al. (2013).

Further modifications of DNA extraction techniques for ancient DNA include a pre-digestion step that reduces exogenous contamination risks and yields a nearly 3-fold increase in endogenous DNA enrichment (Damgaard et al. 2015). This one-hour pre-digestion step — considerably shorter than a 72-hour EDTA soak suggested previously by Hagelberg and Clegg (1991) — uses a buffer composed of 4.7mL 0.5M EDTA, 50μL recombinant Proteinase K, and 250μL 10% N-Laurylsarcosyl at 50 °C for varying incubation times, after which the supernatant is discarded and an identical buffer added for the digestion step (Damgaard et al. 2015).

Additional improvements are suggested by Allentoft and colleagues relating to a new buffer solution (2015). Supplementing the pre-digestion step suggested by Damgaard et al. (2015), Allentoft and others provide a new recipe for binding buffer,

combining Qiagen PB buffer, sodium acetate, and sodium chloride, resulting in a low pH (4-5) solution optimized for short fragment recovery (2015). When tested against both silica suspension (Rohland and Hofreiter 2007) and silica spin with reservoir extension (Dabney et al. 2013) recovery methods, the new binding buffer performed best with the silica suspension protocol (Rohland and Hofreiter 2007; Allentoft et al. 2015). Moreover, this combination of methods shifts the average fragment length recovered from 90 bp to 55 bp, providing an increased recovery of endogenous DNA (Allentoft et al. 2015).

Subsequent studies (Hansen et al. 2017) have used a combination of the pre-digestion (Damgaard et al. 2015) including modifications on incubation time (15 min.), improved buffer (Allentoft et al. 2015), with standard protocols (Rohland and Hofreiter 2007). In addition, some extraction protocols can also be adapted for liquid handling systems for high-throughput sample extraction, similar to those used in forensic genetics laboratories (Rohland et al. 2018).

## Extraction in Forensic DNA Contexts

The origins of ancient DNA research and forensic genetics can be traced to the 1980s. In the same year Svante Pääbo extracted DNA from the tissue of Egyptian mummies, Sir Alec Jeffreys and colleagues succeeded in typing highly polymorphic markers, so called “mini-satellites,” typed through analysis of restriction fragment length polymorphisms (RFLP) (Jeffreys et al. 1985; Weedn 2007). This produced a landmark publication on human identification (Jeffreys et al. 1985) and from there, the DNA

"fingerprinting" technique proposed by Jeffreys gained traction and was employed in court cases in the United Kingdom as well as in the United States beginning in 1986 (Weedn 2007).

Many of the earliest studies in forensics genetics used phenol-chloroform (PC) based extractions. Chelex-100, a chelating resin, was also shown to be effective in isolation of DNA by preventing further degradation of DNA by binding to cations including magnesium, used in a variety of forensic applications (Walsh et al. 1991; Sweet et al. 1996; Lorente et al. 1998; Phillips et al. 2012). After silica-based extractions debuted in the late 1990s in ancient DNA research (Yang et al. 1998), they began to be incorporated into forensic genetics workflows (Baker et al. 2001). In comparisons of PC and silica-based protocols, silica outperformed PC in recovery of DNA for forensic samples (Hoff-Olsen et al. 1999; Davoren et al. 2007) with the exception of some sample types (Jakubowska et al. 2012).

Moreover, silica-based protocols provided an effective alternative to labor-intensive PC protocols (Edson et al. 2004; Davoren et al. 2007). Column-based extractions streamlined the process of extraction (Crainic et al. 2002) and increased recovery. The transition to silica-based extractions also allowed for automated extractions, including the development of the Qiagen BioRobot EZ-1 (Montpetit et al. 2005). Automated capabilities also extended to include full demineralization protocols (Loreille et al. 2007) for robotics platforms (Amory et al. 2012). Silica protocols have further evolved to integrate magnetic particles (Nagy et al. 2005), including the PrepFiler™ Forensic DNA Extraction Kit (Brevnov et al. 2009).

Forensic DNA samples can vary widely in the amount of DNA as well as the quality of DNA; while some samples may yield DNA through simple kit-based extractions, others may be more problematic, requiring more specialized approaches (Parsons and Weedn 1996). For these more degraded samples, ancient DNA protocols for extraction and library preparation have been shown to recover whole mitochondrial genomes for human identification (Templeton et al. 2013). Additional work on multiplexed primer sets has shown recovery of the full mitochondrial genome for forensic samples, including hair, bone, and dust bunnies, allowing for massively parallel sequencing on the Illumina platform (Hickman et al. 2018). Further improvements in extraction techniques are required to move beyond traditional STR-based typing (Jäger et al. 2017) which may prompt the adoption of ancient DNA-derived protocols.

### *A Note on Bone & Tooth Sampling*

The earliest studies suggested that compact, or cortical, bone was optimal for ancient DNA analysis (MacHugh et al. 2000). Whenever available, however, teeth have almost always represented the preferred sample material in ancient contexts (Ginther et al. 1992; Merriwether et al. 1994; MacHugh et al. 2000) and have also been heavily used in forensic contexts (Higgins et al. 2013). Recently, further research on DNA yield has challenged these early assumptions regarding the preference of teeth over all other bony elements. In a study of European individuals spanning the Neolithic, Bronze Age, and Iron Age, Gamba and colleagues compare endogenous DNA yields between samples

from the human petrous bone, other skeletal elements, and teeth, including whole portions of both roots and crowns (2014). In the initial experiment involving six individuals, the authors present endogenous DNA recovery from the petrous portion 4-16-fold greater than the teeth and up to 183-fold great than other skeletal elements (Gamba et al. 2014).

Further research on the utility of the petrous bone by Pinhasi and colleagues reveals that the segment of the petrous bone containing the otic capsule, the densest portion of the bone, is the most favorable for endogenous DNA recovery, offering hope for samples from high temperature/tropical regions (2015). While it seems that the petrous portion represents one of the best sampling options for DNA recovery, the invasiveness of the procedure and destruction of cranial morphology excludes the application of this method for some research applications.

In response, Sirak and colleagues offer a minimally-invasive cranial base drilling method (CBDM) for petrous bone sampling, executed by drilling into the bony ridge between the jugular foramen and carotid canal and into the osseous inner ear (2017). In comparison to full destruction of the petrous portion, the CBDM method produces less endogenous DNA and fewer reads aligned to the human genome than the more destructive traditional petrous portion sampling, but performed far better than an unidentified postcranial bone sample (Sirak et al. 2017). This method may offer a viable option for intact crania that will also accommodate future morphological study (Sirak et al. 2017).

However, variable preservation of samples must also be considered. Sampling 34 ancient skeletons, Hansen and colleagues compared DNA yield from portions of the petrous and parietal bones as well as teeth. With the exception of poorly preserved dental remains in a group of Vikings, the authors found that tooth cementum performs equally as well as the petrous portion in situations where teeth are not poorly preserved, and that tooth samples outperform petrous samples in cases of poor petrous preservation (Hansen et al. 2017).

For analyses using dentition, improved tooth cementum isolation procedures were outlined by Damgaard and colleagues (2015). This technique demonstrates a mean increase of 5-fold higher endogenous DNA yields in cementum over dentin, accomplished through removal of the enamel with a transverse cut of the tooth and drilling out the dentin, resulting in a "root cap" which isolates the cementum (Damgaard et al. 2015). While there are some nuances depending on differential preservation, Hansen and colleagues reinforce the evidence for prioritizing the petrous portion and cementum as samples for ancient genomic studies (2017).

Although DNA yields for post-cranial elements have been thoroughly examined in forensic contexts (Mundorff and Davoren 2014), no systematic studies of post-cranial elements have been conducted in ancient skeletal remains. Whereas the general thought in forensic genetics on the preference for long bone cortical bone samples had mirrored that in ancient genetics, Mundorff and Davoren (2014) demonstrate that small cancellous bones yield more DNA than long cortical bones, even with increasing post-mortem interval. Although more research is needed, Andronowski et al. (2017) used the same

bones and DNA extracts to examine osteocyte structure and determined that the results may be due to residual soft tissue in trabeculae of cancellous bone, which may negate the applicability of cancellous bones for improved recovery in ancient contexts. More analysis is needed to determine the persistence of DNA in individual skeletal elements and bone types, both in forensic and ancient contexts.

In some cases, especially when dealing with infants with no tooth roots available, in the absence of the petrous portion, post-cranial bones are the only option for DNA extraction. Systematic examination of the preservation of DNA in post-cranial bones for ancient DNA analysis is needed and further studies are needed in forensic genetics to understand differential preservation based on diverse taphonomic conditions.

Thanks to advancements in understanding of DNA yield by skeletal element, combined with more efficient DNA extraction techniques, less overall material is needed for ancient DNA analyses. As a result, sampling strategies should be developed on a case-by-case basis. In situations where crania are intact and preservation is optimal (cold climates or cave environments), the CBDM method may offer minimally-invasive but high-yield results (Sirak et al. 2017). However, in situations wherein the crania are intact but recovered from tropical environments, tooth cementum isolation may offer the best option. Ultimately, decisions on sampling must be assessed on a situational basis, taking into account the preservation conditions as well as the opinions of archaeologists, museum curators, and descendant communities.

# Conclusion

As ancient DNA and forensic genetics studies have reached their third decade, methods for sampling and protocols that combine additional digestion steps for greater results in endogenous DNA recovery continue to improve. These advancements allow for increased DNA recovery with reduced destruction of human remains and museum specimens. Starting with decreased amounts of sample inputs, knowledge of optimal sample materials and improvements in extraction methods, ancient DNA analysis has become less destructive and exponentially more informative. Combined with advances in next-generation sequencing and bioinformatic approaches, extensive knowledge can be gained from small amounts of input material, methods that can be harnessed by forensic genetics researchers.

In both ancient and forensic contexts, extraction protocols optimized for varying sample types and down-stream analysis can be selected for increased DNA yield and data returns. Careful consideration of extraction protocols is an essential piece of all degraded DNA research. By extension, recently improved ancient DNA extraction methods may also be applied to other degraded samples, including skeletal samples in forensic genetics laboratories. The increasing use of NGS platforms in forensic DNA research is prompting the adoption of extraction protocols geared towards high-throughput sequencing (Jäger et al. 2017). This movement toward increased recovery privileges ancient DNA techniques for DNA extraction, especially those which are optimized for bone and tooth material and those which can be automated (Rohland et al. 2018) for forensic purposes for degraded skeletal samples.

Modifications including predigestion steps (Damgaard et al. 2015; Hansen et al. 2017), demineralization buffers (Loreille et al. 2007), binding buffers (Dabney et al. 2013; Allentoft et al. 2015), and silica-based columns (Dabney et al. 2013) or magnetic beads (Rohland et al. 2018) can all increase yields of DNA that can be exploited through NGS approaches. These protocols, all from ancient DNA research with the exception of the Loreille et al. (2007) method, can be applied to forensic samples. Combined with extraction techniques, library preparation techniques fore degraded samples that have been optimized for ancient samples are also transferrable to particularly degraded forensic samples where single-stranded libraries may recover more DNA.

Throughout the last three decades of ancient DNA and forensic genetics research, the two fields have influenced one another, trading protocols and techniques for working with degraded DNA. Ancient DNA researchers have pioneered the use of NGS for degraded samples, selecting prime samples for DNA yield, optimizing extraction protocols for short fragment recovery, and using bioinformatics to isolate endogenous sequences. These same practices can be applied in forensic genetics, allowing for increased DNA recovery, capture of shorter fragments for SNP and STR analysis through NGS, and deconvolution of mixed samples. Through increased understanding of sampling strategy and extraction components, operating procedures tailored to provide optimal results can be designed, reducing overall laboratory costs, time, labor, and, most importantly, limiting destruction of human remains and other important materials.

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# BRIDGING STATEMENT

Chapter 2 provides a comprehensive overview of DNA extraction techniques for degraded DNA. Tracing the history of DNA extraction methods in both the fields of ancient DNA and forensic genetics research, the chapter offers a look at a variety of techniques and protocols as well as sample substrates. Building upon the knowledge base established in Chapter 2, the following chapter provides a case study of a specific non-destructive extraction technique developed by Bolnick and colleagues in 2012.

The Bolnick non-destructive technique is validated in Chapter 3, highlighting its utility in extracting DNA from both ancient and modern (forensic) tooth samples. Situated within both fields of degraded DNA analysis, extracts produced using the Bolnick protocol were amplified using traditional polymerase chain reaction (PCR) and next-generation sequencing (NGS) library preparation. Furthermore, the non-destructive nature of the protocol was assessed using traditional metrics, weights, histology, isotopic analysis, and scanning electron microscopy (SEM).

Issues presented within Chapter 2 are of prime importance to the testing of the Bolnick protocol in Chapter 3. The reagents used, including ethylenediametetracetic acid (EDTA), proteinase K, and guanidine salts, are explained in Chapter 2, allowing for understanding of their roles within the extraction protocol used in Chapter 3. The practical aspects of challenges associated with degraded samples, including inhibition, first presented in Chapter 2, are more fully explored in Chapter 3.

# CHAPTER 3: VALIDATION AND IMPACT ASSESSMENT OF A NON-DESTRUCTIVE DNA EXTRACTION METHOD ON MODERN AND ANCIENT TOOTH SAMPLES

## Writing Statement

The design for the validation study of the Bolnick et al. (2012) protocol was developed by Algee-Hewitt, Hulsey, and West. West, Algee-Hewitt, Hulsey, and Cabana, acquired the tooth samples. Hulsey and West measured and weighed tooth samples before and after soaking. West performed lab work including sample preparation, extraction, amplification, sequencing, and analysis. Algee-Hewitt, Steadman, and Cabana provided oversight and funding for the validation portion of the project. The plan for microstructural impact portion of this project was developed by West and Auerbach. West prepared all tooth samples, including cutting and sodium chloride and extraction buffer treatments. Eleazer, Kelso, and West prepared samples for histological analysis, cut, and ground samples. Eleazer scanned images and analyzed damage patterns for histological analysis. West and Saul sampled and prepared enamel for isotopic analysis and interpreted results of isotopic analysis. Auerbach recorded and analyzed SEM images. Two opportunities grants, Steadman and Algee-Hewitt (2012) and Auerbach (2015), from The University of Tennessee, Knoxville received by Steadman and Auerbach provided funding for this project. All authors contributed to the manuscript.

## Abstract

AUTHORS: West FL, Algee-Hewitt BFB, Eleazer CB, Hulsey BI, Scopa Kelso R, Cabana GS, Auerbach BM, Steadman DW

OBJECTIVES: This research tests the application of the Bolnick et al. (2012) non-destructive DNA extraction protocol for isolating mitochondrial and nuclear DNA from both forensic-age and ancient human teeth. Any macrostructural and microstructural changes are assessed through tooth weights and measurements, histological and isotopic analyses, and scanning electron microscopy (SEM).

MATERIALS AND METHODS: Twenty modern and four ancient teeth were used to validate the Bolnick extraction method. Total extracted DNA was quantified and mitochondrial and nuclear DNA PCR primers were used to amplify samples. The Bolnick protocol was also tested on an additional sixteen modern and three ancient teeth to determine whether the protocol introduces damage to internal tooth microstructure where damage was defined as statistically significant differences between treated and untreated samples, suggesting microstructural alterations to the tooth. Tooth weights and metrics were collected; histology, isotope analysis, and SEM were used to explore the impacts of the non-destructive buffer.

RESULTS: Nineteen of the twenty modern samples and all four ancient samples yielded mitochondrial DNA. All twenty-four samples were successfully amplified using a short

nuclear target. No samples, however, yielded STR profiles using the ForenSeq DNA Signature Prep kit when sequenced on the Illumina MiSeq platform. While tooth metrics showed no significant impact of exposure to the non-destructive extraction buffer, tooth weights indicated microstructural loss. Oxygen and carbon isotopic analysis showed no significant difference in treated and untreated samples. Histological analysis revealed damage to the tooth root and enamel in samples exposed to the extraction buffer; SEM showed slight staining of the enamel surface.

DISCUSSION: We successfully extracted mitochondrial and nuclear DNA from modern and ancient tooth samples using the Bolnick protocol. However, the extracted DNA was insufficient for use with the next-generation Illumina MiSeq FGx platform. We determine that the Bolnick protocol does have a minimally destructive impact on teeth that have undergone extraction. While the protocol does yield DNA, its utility may be limited to mitochondrial DNA analyses as well as contexts in which some microstructural impact is acceptable.

# Introduction

In the past ten years, two non-destructive DNA extraction protocols have been developed specifically for use with bone and tooth material. The first, proposed by Rohland, Siedel, and Hofreiter (2004) hereafter the "Rohland protocol"—is based on three separate soaking buffers that were later modified (Hofreiter, 2012) and validated for chimpanzee museum specimens (Mohandesan et al., 2012) as well as human teeth (Gomes et al., 2015). The second, proposed by Bolnick et al. (2012) — hereafter, the "Bolnick protocol"— was for skeletal material using a demineralization soak prior to extraction, maintaining the integrity of the physical specimen while recovering amplifiable mitochondrial and nuclear DNA. The Bolnick protocol offers a non-toxic alternative to the Rohland protocol, which includes a guanidine-thiocyanate based buffer that exposes the samples to toxic substances.

While studies have validated the DNA extraction method of the Rohland protocol on archaeological and museum samples, no study to date has validated the Bolnick method nor examined the effects of the method on dental structure. In archaeological and forensic research, teeth are routinely used in a variety of analyses, including isotopic analysis (e.g. Bentley et al., 2018; Whelton et al., 2018), histological analysis (e.g. Hollund, Arts, Jans, & Kars, 2015; Eleazer & Jankauskas, 2016), and studies of enamel structure (e.g. Smith, 2008; Bocaege et al., 2010). We observe loss of mass in modern teeth after exposure to the soaking buffer, which may present a concern for subsequent analyses, including isotopic testing and histology.

The loss of mass in teeth after exposure to the Bolnick protocol reagents calls for further study into any microscopic damage to dentition. We define damages in terms of reduction in tooth mass, with statistically significant results indicating damage to the tooth microstructure. We presume that mass is lost via the non-destructive soaking buffer, which causes internal microscopic damage rather than damage to the dental structure as a whole. We examine the effect of the Bolnick protocol on dental microstructure in modern teeth and teeth from archaeological contexts. We also validate the Bolnick protocol, targeting both mtDNA and nuclear DNA. In addition, we combine a more recent extraction protocol designed for increased DNA yield in forensic samples (Kemp et al., 2012) with the nondestructive buffer (Bolnick et al., 2012) method and tested it here to determine whether downstream extraction steps can be modified for optimized DNA recovery.

## Background

Over the past three decades in forensic genetics as well as ancient DNA research, advances in sequencing technology and bioinformatic approaches have expanded the potential for working with highly degraded samples and greatly increased the data generated per sample (Shapiro & Hofreiter, 2014; Hofreiter et al., 2015; Sarkissian et al., 2017; Glocke & Mayer, 2017). In addition, improved extraction methods have increased the recovery of DNA from skeletal material from increasingly smaller samples, from larger starting quantities in excess of 1 gram (g) (Hänni et al., 1994; Yang et al., 1998;

Edson, Ross, Coble, Parsons, & Barritt, 2004; Shapiro et al., 2004) to as little as 200-300 milligrams (mg) (Rohland & Hofreiter, 2007; Dabney et al., 2013; Allentoft et al., 2105; Sirak et al., 2017).

Until quite recently, almost all advanced extraction protocols mandated the destruction (i.e., powdering, grinding, or pulverization) of bone or tooth material (Hänni et al., 1994; Yang et al., 1998; Rohland and Hofreiter, 2007; Dabney et al., 2013; Kemp et al., 2017, Mathieson et al., 2018). Destructive methods on ancient human remains raise concerns among descendant communities, museum conservation staff, and any researchers requiring the maintenance of physical integrity of remains (Rohland, Siedel, & Hofreiter, 2004; Bolnick et al., 2012; Gomes et al., 2015).

Similarly, forensic analysts may attempt to limit the amount of tissue consumed during DNA extraction due to the limited availability of sample material, high failure rates for degraded or low-copy-number templates, and return of intact remains to the investigative agency or family members of the deceased (Miloš et al., 2007; Mundorff, Bartelink, & Mar-Cash, 2009; Hickman et al., 2018). In addition, forensic analysts must reserve intact sample for future reanalysis and potentially more complex genotyping (Jäger et al., 2017; Parsons, Alonso, Muller, Roewer, & Budowle et al., 2017).

In DNA analysis of bone and tooth material, there are multiple reasons to limit the destruction of sample tissue as part of the DNA extraction process, including ethical concerns (Kaestle & Horsbaugh, 2002; Tsosie, 2007), sample availability, the need to minimize destruction of rare skeletal remains (Meyer et al., 2012; Brown et al., 2016), and the need to keep remains intact.

The Rohland and Bolnick protocols are two proposed solutions. Rohland et al. (2004) tested three different extraction protocols, with a guanidine thiocyanate (GuSCN)-silica based method yielding best results over Tris-sodium chloride (NaCl) and sodium phosphate buffers, respectively. This approach employed a five-day soak in total of 40 ml of GuSCN-based buffer [5 molar (M) GuSCN, 50 millimolar (mM) Tris, pH 8.0, 25 mM NaCl, 1.3% Triton X-100, 2.5 mM PTB, 20 mM EDTA] at 40° C. Samples tested were chimpanzee bone and tissue between 37 and 164 years old from which the authors successfully amplified mitochondrial DNA fragments (Rohland et al. 2004). While it did not yield amplifiable nuclear DNA, the authors reported no visible damage to the tooth specimens extracted, although this did not rule out potential chemical alterations to the samples. (Rohland et al., 2004). Later applications of the protocol indicated recovery of nuclear DNA of relatively small (around 250 base pairs (bp) fragment sizes (Asher and Hofreiter 2006; Fleischer et al. 2008; Hofreiter 2012).

Mohandesan et al. (2012) presented a modified version of this protocol using silica-based DNA purification was validated via extractions of 86 chimpanzee teeth from worldwide museum collections. Mohandesan et al. (2012) used the modified Rohland protocol with amplification of mitochondrial hyper-variable region-1 (HVR-1) with two overlapping primer sets of 210 bp and 130 bp. This method highlighted some drawbacks, only successfully amplifying and sequencing HVR-1 for 65% of samples, with evidence of cross-contamination between samples, likely due to surface contamination (Mohandesan et al. 2012). With both a lower success rate than Rohland et al. (2004) and introduced external contamination, Mohandesan et al. (2012) demonstrated how

inconsistency in handling of museum specimens can impact later DNA analyses. These studies did not, however, perform a pre-extraction decontamination strategy, other than to wipe down samples with HPLC-grade water (Mohandesan et al., 2012). Most ancient DNA studies, including the Bolnick protocol, employ sodium hypochlorite to reduce exogenous contaminates (Kemp and Smith 2005).

A subsequent validation of the Rohland et al. (2004) protocol performed on ancient human teeth from eight individuals used incubation in the extraction buffer for 48 hours, modified from the original 5-7 days (Gomes et al., 2015). Samples from eight individuals from archaeological sites (Neolithic and Chalcolithic periods) in Spain were analyzed, each with one tooth destructively sampled and one tooth non-destructively sampled and mitochondrial HVR-1 and HVR-2 amplified (Gomes et al. 2015). Extracts from four individuals (eight samples total) were assessed using quantitative PCR (qPCR), which determined that the non-destructively isolated extracts performed better half of the time than the destructively sampled extracts (Gomes et al., 2015).

Gomes et al. (2015) did not, however, control for variability of DNA yields between different tooth samples, which may an additional factor in DNA yields. Variation in DNA yields from teeth has been attributed to age, sex, as well as pathology. This presents difficulty in determining whether increased yields are due to improved extraction efficiency or more endogenous DNA in tooth samples without the use of large sample sizes (Higgins et al., 2011; Higgins 2013). Gomes et al. (2015) reported damage to the samples from the non-destructive treatment, but this was not further explored. Damage to the samples is also seen in the macroscopic photographs before and after

exposure to the nondestructive buffer, showing high fragmentation of one of the eight samples and increased porosity in several others Gomes et al. (2015: Table 2).

Bolnick and colleagues (2012) proposed an alternative DNA extraction protocol for human tooth and bone material using a demineralization soak prior to extraction. Like the Rohland method, this approach maintained the integrity of the physical specimen while recovering amplifiable mitochondrial and nuclear DNA. In contrast to the Rohland protocol, the Bolnick method uses ethylenediaminetetraacetic acid (EDTA), a decalcification agent, in combination with proteinase K as an alternative to guanidine thiocyanate (GuSCN)-based buffer. GuSCN can be harmful if inhaled or if it contacts skin, thus the use of GuSCN without confirmed removal after exposure would cause the tooth samples themselves to become hazardous after extraction. Bolnick and colleagues combined the alternative EDTA-based buffer with a silica-based extraction developed by Rohland and Hofreiter (2007). This extraction method uses a silica-suspension extraction which involves exposure to a GuSCN binding buffer after the tooth sample has been removed from the non-destructive soaking buffer.

Additional methods for non-destructive extraction of DNA have focused primarily on insect specimens in museum collections and may provide alternatives to both the Rohland and Bolnick protocols (Thomsen et al., 2009; Porco et al., 2010; Castalanelli et al. 2010). Details of the various non-destructive extraction protocols and their buffer compositions are outlined in Table 3: 1. Many of the methods used for insect specimens propose alternatives to EDTA-based soaks, including ammonium bicarbonate buffer used for collagen analysis (Doorn, Holland, & Collins, 2011), but these methods

Table 3: 1: Comparison of non-destructive DNA extraction protocols

| Buffer Composition | Target Sample | Soaking Volume | Incubation Time & Temp. | Downstream Analyses | Citation |
|---|---|---|---|---|---|
| 5 M GuSCN, 50 mM Tris, pH 8.0, 25 mM NaCl, 1.3% Triton X-100, 2.5 mM PTB (10), 20 mM EDTA | Chimpanzee Teeth; Hyena tissue | 40 mL | 5-7 days at 40°C | PCR (151-414 bp) | Rohland et al., 2004 |
| 3 mM CaCl2, 2% sodium dodecyl sulphate (SDS), 40 mM dithiotreitol (DTT), 250 mg/ml proteinase K, 100 mM Tris buffer pH 8 and 100 mM NaCl | Insect Specimens; Sediments | 0.5-1.5 mL | 16-20 hours at 55° C | PCR | Thomsen et al., 2009 |
| 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS (after Ivanova et al. 2006) | Insect Specimens | 50 uL | 1, 2, 4, & 12 hours | DNA barcoding for mitochondrial DNA | Porco et al., 2010 |
| 0.5 M EDTA and 0.25 mg/mL $^{-1}$ proteinase K, pH 8.0) | Ancient Human Bone and Teeth | 10 mL | Overnight at room temperature | PCR for mitochondrial and nuclear DNA | Bolnick et al., 2012 |
| After Rohland et al. 2004 | Protocol only | Extraction buffer volume dependent on sample size | 5 days in the dark with slow agitation | Mitochondrial and nuclear DNA | Hofreiter, 2012 |

Table 3: 1, continued: Comparison of non-destructive DNA extraction protocols

| Buffer Composition | Target Sample | Soaking Volume | Incubation Time & Temp. | Downstream Analyses | Citation |
|---|---|---|---|---|---|
| After Rohland et al. 2004 | Chimpanzee Teeth | 5 mL | 5-7 days in the dark with slow agitation | HVI mitochondrial DNA | Mohandesan et al., 2012 |
| 50 g guanidine isothiocyanate, 5.3 ml of 1 M Tris-HCl, pH 7.5, 5.3 ml 0.2 M EDTA, 10.6 ml 20% Sarkosyl, 1 ml b-mercaptoethanol, dissolved in 50 ml water | Insect Specimens | 200 uL | Overnight at 55° C | NGS, RAD-tagging | Tin et al., 2014 |
| After Rohland et al. 2004 | Ancient Human Teeth | 40 mL | 48 hours at 47° C | PCR & RT PCR | Gomes et al., 2015 |

have not yet been used for DNA extraction of human skeletal material. The use of EDTA in non-destructive protocols may in fact cause damage, due to its characteristic binding with calcium ions, resulting in a decalcifying effect (Hofreiter, 2012); both the Rohland and Bolnick protocols include EDTA. The alternative buffers used in these approaches may provide alternatives to EDTA-based buffers to limit the decalcification of skeletal samples.

The Rohland and Bolnick protocols both use non-destructive buffers in which the samples are immersed for 18 hours to five days, exposing the tooth to chemicals meant to decalcify (EDTA) the hydroxyapatite matrix and denature proteins (proteinase K),

notably the histones around which DNA is tightly wrapped within the cellular matrix. While traditional protocols mechanically pulverize the skeletal sample, these protocols depend on chemical lysis to release DNA trapped within the hydroxyapatite matrix of the tooth.

Relating to tooth structure, cementum has been shown to be the most DNA-enriched portion of the tooth (Damgaard et al. 2015). The pulp cavity, which contains mostly cellular material and little mineral content, has been targeted as a rich source of DNA, albeit with low recovery of nuclear DNA within even a few months after death (Higgins, Rohrlach, Kaidonis, Townsend, & Austin, 2015). Enamel and dentine have been shown to be poor reservoirs for DNA preservation, with high reliance on cementum as the primary source of DNA in tooth material (Higgins, 2013).

We suggest that the demineralizing effect of the EDTA in these protocols allows for some release of DNA from the permeable tooth matrix, but to a lesser extent than protocols that utilize mechanical pulverization and complete demineralization. Loreille and colleagues (2007) present the effectiveness of EDTA as a demineralization agent in full digestion of sample materials before extraction. If exposed to EDTA for a sufficient duration of time, the bone powder will completely demineralize into the buffer solution (Loreille et al., 2007).

# Materials and Methods

## *Materials*

A total of 36 teeth modern and seven ancient teeth were used to validate the Bolnick protocol and explore the macro- and micro-structural impacts on tooth samples (see Table 3: 2 for list). Fifteen modern and four ancient teeth were used to test the protocol as published. Five teeth were used to determine whether the soaking buffer treatment could be combined with an alternative downstream extraction protocol (Kemp 2012).

To explore the microstructural impacts of the Bolnick protocol on human tooth microstructure, we conducted histological, isotopic, and scanning electron microscopy analyses of an additional 16 modern teeth and three ancient teeth. The four ancient teeth used for the validation study were obtained from the Norris Farms #36 site (excavated in 1983), which dates to ca. 750 years BP, with permission from the Illinois State Museum. An additional three ancient teeth were used in the testing of the protocol on tooth microstructure, obtained with permission from the central Cuzco valley of Peru, dating to ~1000 CE.

Table 3: 2: List of Samples and Respective Analyses.

| Sample ID | Tooth Type | Analysis |
|---|---|---|
| Bolnick 1 | Maxillary Incisor | DNA Extraction Validation - Bolnick |
| Bolnick 2 | Mandibular 4th Premolar | DNA Extraction Validation - Bolnick |
| Bolnick 3 | Mandibular 1st Molar | DNA Extraction Validation - Bolnick |
| Bolnick 4 | Mandibular 1st Molar | DNA Extraction Validation - Bolnick |
| Bolnick 5 | Mandibular 1st Molar | DNA Extraction Validation - Bolnick |
| Bolnick 6 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 7 | Mandibular 2nd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 8 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 9 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 10 | Maxillary 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 11 | Mandibular 1st Molar | DNA Extraction Validation - Bolnick |
| Bolnick 12 | Mandibular 2nd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 13 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 14 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 15 | Maxillary 3rd Molar | DNA Extraction Validation - Bolnick |
| Bolnick 16 | Mandibular 2nd Molar | DNA Extraction Validation - Kemp |
| Bolnick 17 | Mandibular 1st Molar | DNA Extraction Validation - Kemp |
| Bolnick 18 | Mandibular 1st Molar | DNA Extraction Validation - Kemp |
| Bolnick 19 | Maxillary 2nd Molar | DNA Extraction Validation - Kemp |
| Bolnick 20 | Mandibular 3rd Molar | DNA Extraction Validation - Kemp |
| Bolnick 21 | Mandibular 1st Molar | Impact Assessment – Isotope |
| Bolnick 22 | Maxillary 2nd Molar | Impact Assessment – Isotope |
| Bolnick 23 | Mandibular 3rd Molar | Impact Assessment – Isotope |
| Bolnick 24 | Mandibular 3rd Molar | Impact Assessment – Isotope |
| Bolnick 25 | Mandibular 2nd Molar | Impact Assessment – Isotope |
| Bolnick 26 | Mandibular 3rd Molar | Impact Assessment – Isotope |
| Bolnick 27 | Maxillary 2nd Molar | Impact Assessment – Histology |
| Bolnick 28 | Maxillary 2nd Molar | Impact Assessment – Histology |
| Bolnick 29 | Mandibular 3rd Molar | Impact Assessment – Histology |
| Bolnick 30 | Mandibular 2nd Molar | Impact Assessment – Histology |
| Bolnick 31 | Mandibular 2nd Molar | Impact Assessment – Histology |
| Bolnick 32 | Mandibular 2nd Molar | Impact Assessment – SEM |
| Bolnick 33 | Mandibular 2nd Molar | Impact Assessment – SEM |
| Bolnick 34 | Mandibular 2nd Molar | Impact Assessment – SEM |
| Bolnick 35 | Mandibular 3rd Molar | Impact Assessment – SEM |
| Bolnick 36 | Mandibular 3rd Molar | Impact Assessment – SEM |
| Ancient 1 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Ancient 2 | Mandibular 3rd Molar | DNA Extraction Validation - Bolnick |
| Ancient 3 | Mandibular 2nd Molar | DNA Extraction Validation - Bolnick |
| Ancient 4 | Mandibular 2nd Molar | DNA Extraction Validation - Bolnick |
| Ancient 5 | Mandibular 3rd Molar | Impact Assessment – Histology |
| Ancient 6 | Mandibular 2nd Molar | Impact Assessment – Isotope |
| Ancient 7 | Mandibular 1st Molar | Impact Assessment – Isotope |

The 36 modern teeth were each extracted from different anonymous individuals, procured from a local dentist in East Tennessee with IRB approval through The University of Tennessee, Knoxville (UTK). Immediately after dental extraction, the teeth were placed in individual DNA-free tubes with molecular-grade water and stored at 4° C. The tooth samples were removed from the water and air dried in a biohazard hood for four months, with remaining soft tissue removed using decontaminated forceps when necessary and extracted as outlined below.

***Methods***

Teeth were cleaned and prepared for analyses at UTK. All sample preparation for the modern samples, extraction, and PCR setup occurred in the Molecular Anthropology Laboratories' (MAL) dedicated Forensic DNA Laboratory which includes a biohazard hood for working with degraded samples of a potentially biohazardous nature, including teeth with soft tissue present. All ancient samples were extracted and prepared for PCR in the MAL's dedicated Ancient DNA Laboratory. Steps following PCR amplification, including gel electrophoresis and quantification were performed in the MAL's Modern DNA Laboratory at the UTK, following best practices for ancient DNA (Cooper & Poinar, 2001) separating pre- and post-PCR activities. Next-generation sequencing on the Illumina MiSeq FGx platform took place at the Forensic Genetics Laboratory at Western Carolina University.

After preparation (see below), 19 samples were distributed to three labs for histological and isotopic analyses. The samples were prepared for isotopic analysis at the MAL and sent to IsoForensics, Inc., Salt Lake City, Utah for analysis. Histological samples were prepared in the department of Anthropology histology laboratory at UTK and analyzed at the West Virginia School of Osteopathic Medicine. All scanning electron microscope work was performed at the Advanced Microscopy and Imaging Center at UTK.

## Bolnick Protocol DNA Extraction, Quantification, Amplification, and Sequencing

A total of 24 teeth (20 modern, 4 ancient) were decontaminated by soaking in 6% sodium hypochlorite for 15 minutes, rinsed with molecular grade water, and air dried overnight (Kemp and Smith 2005; Bolnick et al. 2012). The teeth were soaked in non-destructive soaking buffer (10ml 0.5M EDTA, $0.25\ mg/mL^{-1}$ proteinase K) overnight at room temperature with gentle agitation, following the Bolnick protocol. After the buffer solution was poured off and retained for extraction, the teeth were rinsed with molecular grade water and air-dried for storage. The soaking buffer solution was extracted following the silica-based protocol by Rohland and Hofreiter (2007) as detailed by Bolnick and colleagues (2012) for 15 of the modern samples and the four ancient samples.

### *Modified Bolnick Protocol*

To determine whether the Bolnick soaking method could be used with other extraction protocols, 5 modern teeth were treated as outlined above but extracted using a protocol designed by Kemp and colleagues integrating the Promega Wizard® PCR Preps DNA Purification System designed for forensic samples (Kemp et al., 2012). This protocol was optimized to reduce the number of steps during the extraction process to minimize overall DNA loss, a consequence of extraction that is especially important when dealing with forensic and ancient samples which are often characterized by low starting template DNA (Goodwin et al., 2018). Additionally, the binding buffer for the Bolnick protocol requires 24.81g of guanidine thiocyanate (GuSCN) for each sample, a chemical which can be harmful if inhaled or comes in contact with skin and is expensive, with an average cost of $100 per 100 grams. In contrast, the Kemp protocol requires much smaller amounts (250 microliters (uL) per sample) of 6 M guanidine HCl (Teknova) which can be purchased in solution, reducing the chances of inhalation as well as the potential for production of cyanide gas, which can occur when GuSCN contacts sodium hypochlorite, a commonly-used lab-based decontaminant (Paik and Wu 2005).

The set of 24 sample extracts were quantified on the Agilent Bioanalyzer using high sensitivity chips to detect fragment size and perform DNA quantification, performed at the Molecular Biology Core lab at UTK. Samples were also quantified using AccuBlue broad range dye on the NanoDrop 3300 fluorospectrophotometer. All samples underwent polymerase chain reaction (PCR), using a short mtDNA primer (113 bp) designed for

degraded DNA (Alonso et al. 2004) as well as a slightly larger mitochondrial primer (183 bp) designed by Kemp (2006) for amplification of ancient DNA. For confirmation of nuclear DNA, samples were amplified using a 67 bp target from the c-fms proto-oncogene for the CSF-1 receptor gene (Swango et al., 2006). All 24 samples were then prepared for sequencing on the Illumina MiSeq FGx platform using the ForenSeq™ DNA Signature Prep kit with an expanded marker set using DNA Primer Mix B as follows. One nanogram (ng) of purified DNA from each sample was amplified using Primer Mix B, which includes 59 STRs and 95 identity-informative SNPs, 56 ancestry-informative SNPs, and 22 phenotypic-informative SNPs. Targets were enriched, purified, and normalized. Samples were pooled, denatured, diluted, and loaded into the reagent cartridge and sequenced on the MiSeq FGx platform.

***Methods: Dental Structural Analysis***

To assess the impact of the Bolnick protocol on tooth structure, dental metrics and weights were taken on selected samples before and after exposure to the soaking buffer. Photographs were taken before and after soaking to capture macroscopic changes in teeth due to exposure to the buffer, show in Figure 2. All 20 modern teeth were measured for mesio-distal length (MDL) and buccal-lingual width (BLW). All 24 teeth were weighed before and after soaking.

For histological analyses, tooth samples would be seen as affected by the treatment buffer through exhibiting more damage on the Oxford Histological Index

(OHI) (Hedges and Millard 1995). The OHI provides descriptions of damage observed histologically in teeth and was used as a guideline for observing damage in this study as shown in Table 3: 3.

Five contemporary tooth samples and one ancient were cut into halves, resulting in an untreated and treated sample from the same tooth. The first half of each tooth was left untreated. The modern tooth halves and one ancient half were treated with a combination of bleach and non-destructive extraction buffer. All samples were embedded in Buehler Epo-color resin epoxy with hardener. A vacuum pump and dessicator were utilized to stabilize the epoxy matrix and to remove air bubbles and ensure proper impregnation of the specimen with epoxy. In some cases, copper wire was required to position and stabilize the tooth within the epoxy resin (Marks, 1997). Thin sections were cut from the embedded wafers using an Allied low speed diamond blade saw and mounted to glass slides. The mounted sections were then ground to a uniform thickness of 100-200 micrometers with a Metaserv 2000 Polisher. Scratches produced during the grinding process were removed with fine-grained buffing paper. Digital images of the histological sections were captured with transmitted light microscopy at 1.25x and 5x using a Leica Aperio Versa microscope.

Table 3: 3: Descriptions of the Oxford Histological Index as outlined by Hedges and Millard, 1995.

| Index Value | Percentage Intact Bone | Description |
|---|---|---|
| 0 | <5 | No original features identifiable, other than Haversian canals |
| 1 | <15 | Small areas of well-preserved bone present, or some lamellar structure preserved by pattern of destructive foci |
| 2 | <33 | Clear lamellate structure preserved between destructive foci |
| 3 | <67 | Clear preservation of some osteocyte lacunae |
| 4 | <85 | Only minor amounts of destructive foci, otherwise generally well preserved |
| 5 | <95 | Very well preserved, virtually indistinguishable from fresh bone |

Measurements were taken with ImageScope software (Leica Biosystems Imaging, Inc.). The total area of damaged dental tissue for both the tooth root and enamel was measured and divided by the total area occupied by the tooth. The images shown in Figure 3 illustrate the damaged areas recorded. This total area of damage does not distinguish between areas of the tooth affected by diagenesis (e.g., infiltrations by microorganisms from the burial environment) and acid corrosion from the DNA extraction process.

For isotopic analyses, damage to the tooth samples would be indicated by statistically significant differences between carbon and oxygen isotopic signatures in the treated and untreated samples. Two ancient molar teeth (~1000 years old) were sampled in the clean room laboratories at UTK. The top surface of tooth enamel was removed using a drill and conical bit and discarded. The conical bit was thoroughly cleaned with ethanol and then used to drill approximately 5 mg of pristine enamel powder from one

half of each tooth. The enamel powder from each tooth was transferred to a labeled 1.5-ml micro-centrifuge tube (comprising samples AIT-1 Untreated & AIT-2 Untreated). The tooth roots were then removed for future DNA analysis. The tooth crowns (AIT-1 Treated and AIT-2 Treated) were then soaked in bleach as per Kemp and Smith (2005) and in extraction soaking buffer as per Bolnick et al. (2012) to serve as the treated sample. A total of 5 mg of enamel was then removed from the treated enamel surface as outlined above. Five modern molars were abraded using a conical drill bit to remove surface impurities, after which 5 mg of enamel was removed from one half of the tooth using a diamond wheel bit and transferred to a 2 ml polypropylene tube (comprising samples IT-1 Untreated – IT-5 Untreated). For samples IT-1 Treated – IT-3 Treated, the tooth was then soaked in bleach as per Kemp and Smith (2005) and in extraction soaking buffer as per Bolnick et al. (2012) to serve as the treated sample. For samples IT-4 Treated & IT-5 Treated, only the bleach step was performed and then enamel removed as with previous samples.

In preparation for isotope analysis, all samples were treated with 3% hydrogen peroxide ($H_2O_2$) for 15 minutes. Oxidized samples were rinsed three times with deionized water, then treated with 0.1 M acetic acid ($CH_3COOH$) for 15 minutes. Acid-treated samples were rinsed three times with deionized water before being dried in an oven at 60°C overnight. A total of 2 mg per sample was submitted to IsoForensics, Inc., in Salt Lake City, Utah for analysis.

We examined the effects of the treatment on microwear and dental enamel perikymata in six contemporary molars. Perikymata are microscopic grooves found along

the tooth's enamel surface, which can provide information regarding dental growth and development (Smith, 2008; Bocaege et al., 2010). Obscuration or destruction of the perikymata would indicate that the protocol is microstructurally destructive. The teeth acquired from archaeological (ancient) samples had significant enamel damage that impeded analysis and were excluded from the study. The six modern teeth were imaged before exposure to the non-destructive buffer solution (untreated) and after exposure (treated). Dentition was cleaned with HPLC water for pre-treatment tooth imaging, and the same protocol was used for application of the extraction solution as other samples in post-treatment teeth before imaging using a Zeiss Auriga scanning electron microscope (SEM). Samples were oriented visually, with imaging focused on the buccal surface of each tooth. A focus on the buccal surface allowed for comparisons of effects on both microwear and perikymata.

Lower magnification images (around 50x) were taken to establish consistent orientation between pre- and post-treatment teeth, as well as for finding the same fields of view for higher magnification (between 80 and 110x). These resulted in regions of interest (ROIs) measuring approximately 2700 micrometer (μm) by 1800 μm. Images were taken consistently at an extra high tension (EHT) voltage level of 4kV and beam current at 30kV/50pA. Because teeth were remounted for each imaging, working distances and magnifications varied slightly between pre- and post-treatment analyses.

*Statistical Analysis*

Paired, or matched sample, *t*-tests were conducted to assess the before and after metrics and weights to determine if significant changes were caused by exposure to the non-destructive soaking buffer. Paired *t*-tests were also used to compare carbon and oxygen isotope results between untreated and treated samples. All statistical tests were analyzed using R version 3.5. 1.

# Results

## *Validation of DNA Extraction*

The majority of samples (19 of 20 modern teeth and 4 out of 4 ancient teeth) were successfully amplified using the mtDNA primers designed for degraded DNA of 113 and 185 bp, respectively (Alonso et al., 2004; Kemp, 2006), with the exception of Sample ID - Bolnick 8. All 24 samples were successfully amplified using the short (67 bp) nuclear DNA primer (Swango et al., 2006). Sample concentrations were analyzed using the Agilent Bioanalyzer to detect fragment size and perform DNA quantification for total DNA. The high sensitivity chip, which detects fragments between 35-10,380 bp and quantities between 5 picograms/microliter (pg/uL) and 5,000 pg/uL was employed. While fragment sizes are presented as an average, most samples included a broad range of

fragment sizes from 35 bp and higher, as can be seen in Figure 3: 1, depicting results from typical bioanalyzer sample run. Bioanalyzer totals are shown in Table 3: 4.

Samples extracted using the Bolnick protocol yielded results from 10 pg/uL to 1353 pg/uL. The samples extracted using the Kemp protocol yielded the highest amounts of DNA, with an average of 685 pg/uL, the lowest yield of 120 pg/uL, and the highest at 1472 pg/uL. The ancient samples yielded between 10 pg/uL and up to 233 pg/uL of DNA. For comparison, most library preparation kits, such as the Illumina Nextera XT Library Prep kit, require 1 ng of total input DNA.

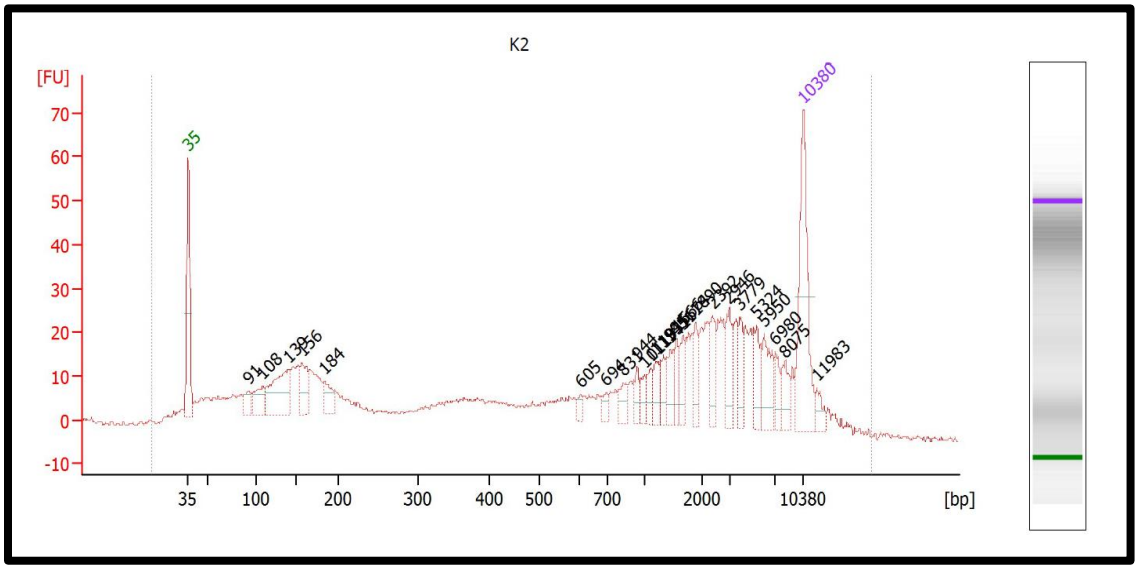



Figure 3: 1: Bioanalyzer results for sample Kemp 2. The y axis depicts fluorescence units and the x axis basepair lengths. Standard ladders of known basepair length and fluorescence are used to determine the size and quantity of DNA fragments in each sample. Note the range of fragment sizes from 91 bp to 8,075 as shown on the x-axis, showing that there are multiple fragment sizes of various concentrations present in this sample.

Table 3: 4: Bioanalyzer results for samples, including total DNA quantity in pg/uL and average fragment size. An. denotes an ancient sample.

| Sample ID | Total DNA (pg/uL) | Average Fragment Size (bp) |
|---|---|---|
| Bolnick 1 | 115 | 815 |
| Bolnick 2 | 2,900 | 4,798 |
| Bolnick 3 | 135 | 2,378 |
| Bolnick 4 | 7,530 | 142 |
| Bolnick 5 | 1.05 | 314 |
| Bolnick 6 | 6,765 | 3,485 |
| Bolnick 7 | 295 | 9,346 |
| Bolnick 8 | 105 | 3,915 |
| Bolnick 9 | 1,525 | 7,434 |
| Bolnick 10 | 8,755 | 108 |
| Bolnick 11 | 825 | 157 |
| Bolnick 12 | 5,575 | 166 |
| Bolnick 13 | 5,785 | 358 |
| Bolnick 14 | 5,285 | 143 |
| Bolnick 15 | 2,895 | 4,798 |
| Bolnick – An. 1 | 175 | 9,520 |
| Bolnick – An. 2 | 50 | 274 |
| Bolnick – An. 3 | 415 | 2,072 |
| Bolnick – An. 4 | 1,165 | 231 |
| Kemp 1 | 4,780 | 3,906 |
| Kemp 2 | 7,360 | 3,003 |
| Kemp 3 | 2,695 | 5,001 |
| Kemp 4 | 600 | 136 |
| Kemp 5 | 1,635 | 4,866 |

All 24 samples were run on the Illumina MiSeq platform, along with positive and negative controls. None of the samples extracted using the Bolnick protocol yielded positive results using the ForenSeq kit, although the positive control and three additional low template samples that were run with the Bolnick validation samples produced positive results, thus demonstrating that the lack of results was due to problems with the samples and not the kit or sequencing run. There are two possibilities for the lack of amplification from the Bolnick extraction. The first is that ForenSeq kit targets only nuclear DNA. The DNA from the samples may have been too fragmentary, since the STRs in the kit range from approximately 80 bp to almost 400 bp. The kit also includes forensic single nucleotide polymorphisms (SNPs), many of which are <125 bp in length; however, these were not successfully amplified even though some nuclear DNA was present in the samples, based on amplification of the 67 bp nuclear target using traditional PCR.

The second possible reason for lack of amplification of the Bolnick samples is presence of inhibitors which prevented target amplification; this may be a weakness of the ForenSeq kit compared with traditional PCR. For the traditional PCR, platinum taq was used in the reactions which may provide more resistance to inhibitors than the proprietary polymerase used by the ForenSeq kit. EDTA and silica, both used in the Bolnick protocol, are common PCR inhibitors, as is calcium, which is present in teeth. Incomplete removal of these substances may have resulted in lack of amplification using this next-generation sequencing approach.

*Macroscopic results*

A total of 24 forensic-age teeth were weighed before and after soaking, with weights reported in Table 3: 5. Thirteen forensic-age teeth were measured for mesio-distal length (MDL) and buccal-lingual width (BLW), with measurements reported in Table 3: 6. Paired, or matched sample, *t*-tests were conducted to assess the before and after metrics and weights.

Pre- and post-soak dental measurements and weights were analyzed using paired *t*-tests to determine whether significant differences could be found after samples were exposed to the soaking buffer. Measurements of the mesio-distal length and buccal-lingual width indicated no significant differences before and after soaking. For mesio-distal (MD) measurements, a paired *t*-test returned a *p*-value of 0.2747, $\alpha = 0.05$, with t = 1.98 and for buccal-lingual (BL) measurements, a *p*-value of 0.216, with t = 1.30. Effect sizes for MD were calculated using Cohen's d as 0.23 and for BL as 0.18. These effect sizes would be seen as small, indicating that significant results may be seen with a larger sample sizes.

Visual assessment of the tooth samples before and after indicate only minimal differences following extraction. As noted by previous studies (Rohland et al. 2004, Mohandesan et al. 2012, Bolnick et al. 2012, Gomes et al. 2015), tooth samples extracted appear cleaner and lighter in color than their original state after exposure to the soaking buffer, as can be observed in Figure 3: 2. This alteration in color is an observed change due to buffer treatment.

Table 3: 5: Sample weights before and after soaking, with total difference reported.

| Sample ID | Weight Before (grams) | Weight After (grams) | Total Difference |
|---|---|---|---|
| **1** | 1.96 | 1.950 | 0.010 |
| **2** | 1.65 | 1.550 | 0.100 |
| **3** | 1.99 | 1.940 | 0.050 |
| **4** | 1.93 | 1.830 | 0.100 |
| **5** | 1.69 | 1.650 | 0.040 |
| **6** | 1.87 | 1.780 | 0.090 |
| **7** | 1.82 | 1.800 | 0.020 |
| **8** | 2.12 | 2.040 | 0.080 |
| **9** | 1.58 | 1.550 | 0.030 |
| **10** | 1.71 | 1.650 | 0.060 |
| **11** | 2.48 | 2.410 | 0.070 |
| **12** | 2.43 | 2.390 | 0.040 |
| **13** | 2.55 | 2.490 | 0.060 |
| **14** | 2.08 | 1.970 | 0.110 |
| **15** | 2.11 | 2.000 | 0.110 |
| **16** | 2.12 | 2.050 | 0.070 |
| **17** | 1.67 | 1.650 | 0.020 |
| **18** | 1.67 | 1.630 | 0.040 |
| **19** | 2.46 | 2.350 | 0.110 |
| **20** | 1.87 | 1.760 | 0.110 |
| **21** | 1.61 | 1.610 | 0.000 |
| **22** | 1.67 | 1.670 | 0.000 |
| **23** | 2.46 | 2.420 | 0.040 |
| **24** | 1.53 | 1.520 | 0.010 |

Table 3: 6: Dental metrics, before and after soaking. Measurements are for mesio-distal length (MDL) and buccal-lingual width (BLW).

| | **MDL (Before)** | **BLW (Before)** | **MDL (After)** | **BLW (After)** |
|---|---|---|---|---|
| **1** | 11.01 | 9.72 | 10.90 | 9.78 |
| **2** | 11.98 | 10.37 | 10.58 | 9.66 |
| **3** | 11.97 | 10.16 | 11.07 | 10.26 |
| **4** | 11.44 | 10.02 | 11.16 | 10.07 |
| **5** | 10.74 | 10.19 | 10.80 | 10.18 |
| **6** | 11.53 | 9.75 | 11.55 | 9.65 |
| **7** | 10.54 | 9.88 | 10.58 | 10.02 |
| **8** | 11.45 | 11.98 | 11.70 | 8.98 |
| **9** | 12.17 | 10.75 | 11.45 | 10.09 |
| **10** | 11.37 | 9.41 | 11.19 | 10.57 |
| **11** | 12.39 | 9.73 | 11.30 | 8.90 |
| **12** | 11.47 | 10.07 | 11.11 | 9.32 |
| **13** | 11.43 | 10.54 | 11.82 | 9.46 |
| **14** | 11.42 | 10.72 | 10.27 | 10.92 |

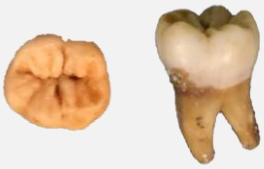
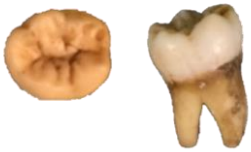
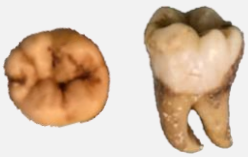
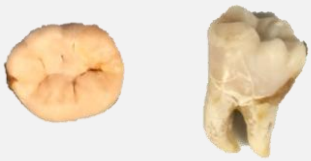
| Sample | Before Treatment | After Treatment |
|---|---|---|
| **1** |  |  |
| **2** |  |  |
| **3** |  |  |
| **4** |  |  |
| **5** |  |  |

Figure 3:  2: Photographs of teeth before and after exposure to the Bolnick non-destructive buffer.

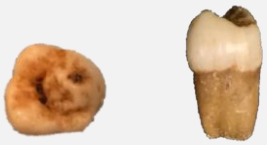
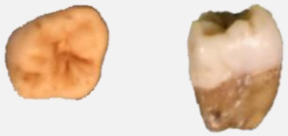
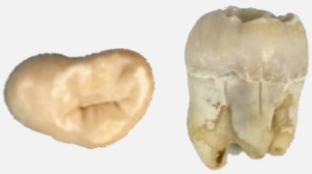
| Sample | Before Treatment | After Treatment |
|---|---|---|
| **6** |  |  |
| **7** |  |  |
| **8** |  |  |
| **9** |  |  |
| **10** |  |  |

Figure 3: 2, continued: Photographs of teeth before and after exposure to the Bolnick non-destructive buffer.

In regard to the weights of the teeth before and after soaking, results were significant. Damage was defined as a significant reduction in tooth mass suggesting microstructural impacts to the teeth. Paired t-tests observed a reduction in tooth weights after exposure to the buffer, (*p*-value <0.0009, t = 4.29). The effect size of 0.12 was also calculated using Cohen's d, showing that even with a small effect size, the impact of the soaking buffer produced significant results.

### *Histology Results*

Comparisons of root damage versus enamel damage were used to determine if one type of tissue was more affected than the other by the soaking buffer. Damage was defined as per the Oxford Histological Index (OHI) (Hedges and Millard 1995) (see descriptions in Table 3: 3 as depicted in the original publication).

Across all samples, the teeth that were treated had a higher percentage of damage, including destruction of lamellar structure and osteocyte lacunae. On average, the untreated samples displayed 24.33% root damage and 6.58% enamel damage, shown in gray in Table 3: 7. The treated samples exhibited 46.59% root damage and 11.38% enamel damage. For the ancient tooth, the enamel surface and roots were so damaged (OHI index of 0) that the percent damaged could not be calculated before or after exposure to the buffer treatment.

Table 3: 7: Damage percentages for tooth roots and enamel, untreated and treated samples.

| Sample ID | Root Damage % | Enamel Damage % |
|---|---|---|
| MT-1 Untreated | 8.91 | 4.82 |
| MT-1 Treated | 41.25 | 9.22 |
| | | |
| MT-2 Untreated | 14.73 | 7.89 |
| MT-2 Treated | 58.40 | 16.68 |
| | | |
| MT-3 Untreated | 28.26 | 1.93 |
| MT-3 Treated | 58.04 | 5.36 |
| | | |
| MT-4 Untreated | 1.79 | 4.35 |
| MT-4 Treated | 4.83 | 0.43 |
| | | |
| MT-5 Untreated | 16.63 | 7.31 |
| MT-5 Treated | 37.01 | 13.81 |
| | | |
| AT-1 Untreated | 75.66 | NA |
| AT-1 Treated | 80.00 | NA |

These damage assessments do not distinguish between damage due to diagenesis and damage caused by exposure to the buffer (see Figure 3: 3 for areas of tooth damage outlined). That said, the treated teeth show an increased percentage of both root and enamel damage and overall, twice the damage to the root and enamel as in the untreated samples, suggesting that the soaking treatment introduces increased damage to both regions of the teeth. Thus, the histological assessment indicates that the protocol cannot be deemed non-destructive in this respect.

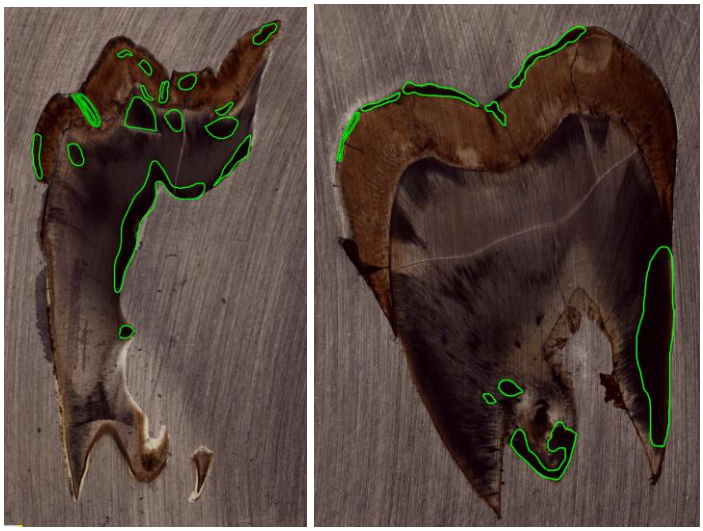


Figure 3: 3: Areas of tooth damage shown bordered by green lines.

Cutting the tooth prior to exposure to treatment may have an impact on buffer exposure as a whole; however, the same tooth was used to control for differential diagenesis between tooth samples. Even intact teeth are permeable to water and other liquids such as the extraction buffer, as demonstrated by the presence of DNA in dental pulp cavities derived from sample washing (Gilbert et al. 2005, Sampietro et al. 2006). Thus, it can be concluded that both the surface of the tooth and interior of the root and pulp cavity would be exposed to buffer regardless of sectioning.

***Isotopic Analysis Results***

Carbon and oxygen stable isotope results from treated and untreated tooth enamel samples are reported in Table 3: 8. All samples passed quality control checks in which results from replicate samples were compared. Stable isotope contents are expressed in

"delta" ($\delta$) notation as values in ‰ (permil), where $\delta = R_A/(R_{Std} - 1)$ and $R_A$ and $R_{Std}$ are the ratios of the rare to abundant isotope (e.g., $^{13}C/^{12}C$) in the sample and the standard, respectively.

The average $\delta^{13}C$ difference between the non-treated and treated enamel samples was 0.01‰, and 0.11‰ for $\delta^{18}O$. Neither $\delta^{13}C$ values (*p*-value = 0.888, paired *t*-test) nor $\delta^{18}O$ values (*p*-value = 0.675, paired *t*-test) were significantly different between pre-and post-treatment groups.

For the purposes of defining damage, it is important to remember that within stable isotope analyses, statistical differences and interpretational differences are not the same. Chesson, Berg, Kenyhercz, and Regan (2018) defined real interpretive differences (RIDs) for tooth enamel bioapatite carbonate analyses as 0.6‰ for $\delta^{13}C$ values and 1.6‰ for $\delta^{18}O$ values. The greatest differences observed in pre- and post-treatment values within the current study were 0.43‰ for $\delta^{13}C$ and 1.05‰ for $\delta^{18}O$. These differences are not statistically significant and are less than the real interpretive differences defined by Chesson and colleagues (2018). Based on these results it is reasonable to infer that neither the bleach method nor the combination of bleach and non-destructive buffer impacted results obtained during isotopic analysis, therefore the method was non-destructive in this respect.

Table 3: 8:Results of carbon and oxygen isotopic analysis on untreated and treated tooth samples.

| Sample ID | $\delta^{13}C$ | $\delta^{18}O$ |
|---|---|---|
| **IT-1 Untreated** | -7.45 | -5.79 |
| **IT-1 Treated** | -7.51 | -5.02 |
| | | |
| **IT-2 Untreated** | -10.89 | -5.38 |
| **IT-2 Treated** | -10.90 | -5.39 |
| | | |
| **IT-3 Untreated** | -9.19 | -4.58 |
| **IT-3 Treated** | -9.37 | -4.47 |
| | | |
| **IT-4 Untreated** | -8.83 | -4.55 |
| **IT-4 Treated** | -9.09 | -5.50 |
| | | |
| **IT-5 Untreated** | -10.77 | -5.98 |
| **IT-5 Treated** | -10.65 | -5.92 |
| | | |
| **AIT-1 Untreated** | -11.92 | -8.69 |
| **AIT-2 Treated** | -11.49 | -8.39 |
| | | |
| **AIT-1 Untreated** | -11.50 | -11.10 |
| **AIT-2 Treated** | -11.63 | -12.15 |

### *Enamel Structure Results*

Given the small sample size and the subtle changes observed between pre- and post-treatment teeth, we elected to restrict analyses to qualitative comparisons. Damage was defined as anything that would prevent the observation of perikymata for future analyses. High magnification images of the same region of interest on each tooth were compared for pre- and post-treatment. Staining, structure (perikymata) obliteration due to chemical exposure, the presence of electron charging, and mechanical damage were noted on each image. Approximate areas of staining were measured by superimposing ellipses over images in ImageJ 1.8.0 for Windows, measuring the pixel area of these ellipses, and calculating them as a percentage of the area of regions of interest, ROIs, occupied by enamel.

Figures 3: 4 and 3: 5 represent the untreated (A) and treated (B) comparisons between the same ROIs on two of the modern molars. The green lines in Figure 3: 4 illustrate the same locations on both SEM images for reference; orientations are identical in both figures. Both teeth are representative of the results obtained from all five molars examined for effects on microwear and microscopic surface features. In both the untreated and treated ROIs, perikymata remain identifiable in most cases, as are major features of the dentition, including the cementoenamel junction and micro-cracks.

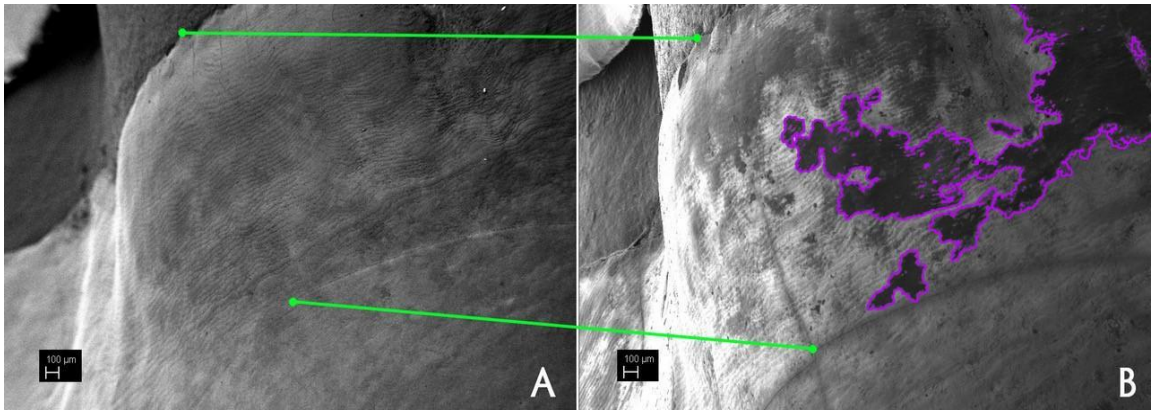



Figure 3:  4: Modern tooth before (A) and after (B) exposure to non-destructive buffer. Green lines indicate landmarks for orientation.

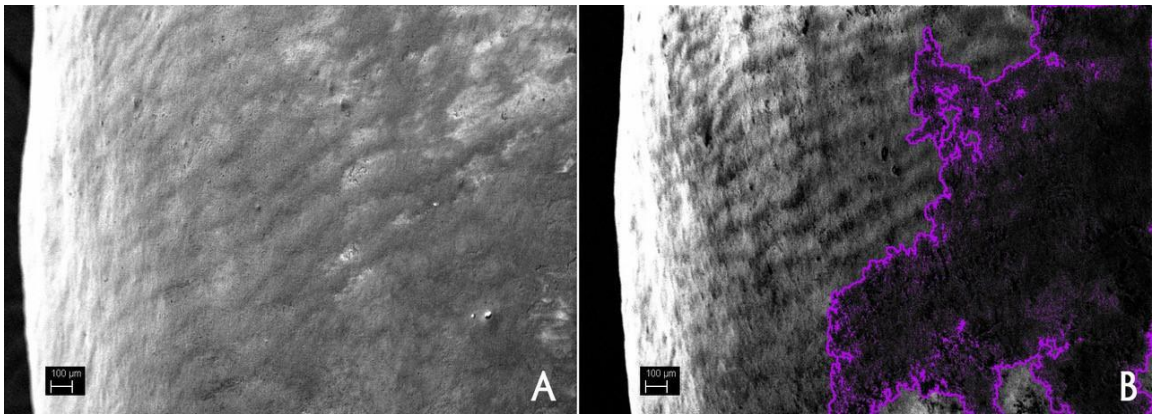



Figure 3:  5: Modern tooth before (A) and after (B) exposure to non-destructive buffer; image alignment is identical.

However, acquired dark areas are present on the buccal surfaces of post-treatment molars (outlined in purple in Figures 3: 4B and 3: 5B). This dark coloration appears to be due to staining rather than chemical etching or erosion of the enamel surface and does not cover the entirety of the treated enamel surface; it ranges from approximately 16% of the enamel surface to 50% of the enamel surface. At its most severe, staining obscures perikymata and other microscopic features of the enamel. It is worth noting that staining is the most visible effect of exposure to the extraction solution. Staining was especially evident in regions where there was existing damage including small enamel micro-cracks or porosity. Enamel micro-cracks did not propagate or expand due to the treatment. Etching and other chemical damage to the enamel was not evident and the electrical conductivity of the enamel was unaffected. However, due to the staining and obscuring of perikymata, the assessment using SEM shows that the protocol cannot be deemed non-destructive in this respect.

## Discussion

Here, we validate the use of the Bolnick non-destructive DNA extraction protocol for modern and ancient tooth samples. Our findings demonstrate that this current "non-destructive" DNA technique does in fact result in alterations to the structure of the tooth. While the macroscopic integrity was maintained in the samples utilized in this study, it is important to note that this technique causes loss and damage at a microstructural level as shown through loss of mass and histological analysis as well as staining of the enamel surface. The maintenance of macroscopic dimensions may be acceptable for museum

collections and forensic applications, however, working with ancient human remains also presents special cultural considerations.

In the case of ancient human individuals from the Americas, cultural considerations of the importance of bodily tissues must be considered. Many Native groups in North America place great importance on the maintenance of the integrity of the body (Tsosie 2007), which may be compromised with even microstructural loss in teeth. This loss of integrity would have implications for not only the deceased individual but that individual's descendants and potentially the community as a whole (Tsosie 2007). In these cases, methods such as the Bolnick protocol may not provide an acceptable alternative to traditional destructive methods.

Our results validate the utility of the Bolnick protocol for successful extraction of degraded tooth samples of both mitochondrial and nuclear DNA. Similarly, our research demonstrates that other protocols may be modified using the steps of the Bolnick protocol including the soaking buffer with intact sample in place of more destructive preparations. Protocols such as the Kemp and colleagues' method may provide results of equal quality using a faster and less-toxic extraction process and that adaptations may be used with other protocols more specialized for small fragment recovery (Dabney et al. 2013) or single step modifications including improved binding buffer (Allentoft et al. 2015, Hansen et al. 2017).

However, the failure to successfully amplify and sequence samples extracted using the non-destructive with the Illumina ForenSeq kit on the MiSeq FGx platform may point to limitations in the method as a whole for the purposes of nuclear DNA analysis.

The lack of success may indicate presence of inhibitors that prevent successful amplification during library preparation and amplification of nuclear targets. Another reason for the lack of success could be that the method produces highly fragmented nuclear DNA that falls under the threshold of the STRs targeted in the kit but allows for amplification of the 67 bp nuclear target. This could represent a trade-off between sacrificing the morphological integrity of the sample to produce higher yields with potentially larger fragment sizes over maintaining the macroscopic tooth structure with more limited yields and perhaps more fragmentary and inhibited DNA.

These methods offer options which will allow investigators to maintain sample integrity while obtaining mitochondrial DNA and short nuclear DNA fragments. However, the research presented here indicates that purportedly "non-destructive" protocols do in fact impact tooth microstructure and should be thoroughly considered before use, based on the needs of the stakeholders involved in the proposed research. By assessing the effects of Bolnick et al.'s (2012) extraction method, we demonstrate that the protocol cannot be supported as fully non-destructive due to damage to the tooth surface microscopically and the internal root structure. Rather, we suggest that the protocol be more appropriately deemed minimally destructive. Through documenting the effects of the method on dental structure and morphology, we provide a guide for understanding the impacts of this option for sensitive samples and collections. Researchers, collection curators, and descendant communities must determine whether or not the minimally-destructive protocol will meet their expectations and needs for extraction of DNA

through a macroscopically non-destructive and microscopically minimally destructive method.

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# BRIDGING STATEMENT

Chapter 3 represents a validation study of a non-destructive extraction technique for ancient and forensic tooth samples. Building on information provided on extraction techniques for degraded DNA presented in Chapter 2, Chapter 3 provides a case study example of some of the challenges associated with DNA extraction, including inhibition and issues with STR typing.

Chapter 4 draws from Chapter 2 in regard to sample storage conditions and both Chapters 2 and 3 in regard to DNA extraction and issues with degraded DNA. Chapter 4 provides a validation study of the STR-typing capabilities using post-mortem collected blood stored on untreated blood cards. Issues associated with the extracts from the blood cards include low template samples (discussed in Chapter 2), need for repeated sampling (discussed in Chapters 2 and 3), and the presence of PCR inhibitors (discussed in Chapters 2 and 3). Chapter 4 further builds upon the information presented in Chapters 2 and 3 in successful typing of the original set of CODIS short tandem repeat (STR) markers. Quality and quantity of DNA extracted from the cadaveric blood samples are assessed and predictive models for the impact of sampling time intervals and storage are explored. Furthermore, these marker sets are used to validate the use of STRs to predict geographic ancestry using the unsupervised clustering program STRUCTURE (Pritchard et al. 2000).

# CHAPTER 4: ASSESSING DNA QUALITY AND QUANTITY FROM CADAVERIC BLOOD STORED ON UNTREATED BLOOD CARDS: IMPACT ON STR QUALITY AND THE UTILITY OF VARIABLY AMPLIFIED MARKERS FOR THE INDIVIDUAL ESTIMATION OF TRIHYBRID ANCESTRY AND ADMIXTURE PROPORTIONS

## Writing Statement

The concept for this paper was conceived of by F. West and B. Algee-Hewitt. D. Steadman provided data from the William M. Bass donated collection at the Forensic Anthropology Center at The University of Tennessee, Knoxville. F. West generated the statistical analyses and writing with advice from B. Algee-Hewitt. All authors contributed to the manuscript. The paper will be submitted for publication to the journal *Forensic Science International*, *Forensic Science International: Genetics*, or *Forensic Anthropology*.

# Abstract

AUTHORS: West FL, Steadman DW, Algee-Hewitt BFB

OBJECTIVES: Blood cards are widely used for long-term body fluid (e.g. blood, saliva, etc.) storage for DNA analysis. They are especially useful for DNA sample collection at forensic anthropology research centers, given their straightforward handling and sampling protocols, stability in ambient temperatures, small storage footprint, and minimal financial investment. Little is known, however, about the long-term value of blood cards in forensic anthropological research and forensic genetic casework. Here, we investigate the quantity and quality of DNA extracted from post-mortem blood samples, in terms of DNA preservation and typing success. As part of a body donation program, these samples were collected upon donor intake and stored on FITZCO untreated (non-FTA) blood cards. We evaluate these cards in terms of DNA preservation and typing success and test the effect of age of the blood card versus their STR yields, the gold standard in forensic genetics, are used for forensic identification and as potential markers for global ancestry and admixture estimation. Degraded samples, including those stored on blood cards, can result in reduced STR markers sets and, in turn, compromised analyses. We assess these blood cards, therefore, with special consideration given to profile matching for positive identification and ancestry estimation for biological profile estimation.

METHODS: We quantify the degree of DNA degradation in terms of both the amount and fragment sizes of the individual templates, test for disagreement in genetically determined and reported sex, and evaluate the forensic genetic typing potential of the DNA by evaluating CODIS profiles generated for each case using evidence of allelic drop/in out, degradation curves, and relative fluorescent units as assessment criteria for 20 blood card samples. We analyze the impact of the DNA template on ancestry and admixture estimation, offering insights into the impact of degradation on population identifiability.

RESULTS: While STR profiles were successfully generated for most samples, our results indicate length of storage and time interval between date of death and sample collection have an impact on DNA quantity and quality of DNA, in terms of typing success. There is a statistically significant decrease in relative fluorescent unit (RFU) values with increasing time interval between date of death and collection, indicating degradation in the blood card samples related to the post-mortem interval prior to sample collection. The STR profiles generated were used to estimate ancestry and admixture using the software program STRUCTURE, demonstrating utility of the markers beyond individual identification purposes.

# Introduction

While next generation sequencing (NGS) methods have dramatically altered the fields of medical genetics and paleogenomics, short tandem repeats (STRs) identified through traditional capillary electrophoresis remain the gold standard for forensic identification. Although new technologies integrating NGS approaches hold promise for ancestry estimation (Bulbul et al. 2018; King et al. 2018), phenotyping (Walsh et al. 2017), and fluid identification (Bartling et al. 2014), STRs remain the primary genotyping method due to extensive validation as a marker set and the availability of large databases of typed individuals. Since the 1990s, forensic analysts have focused most attention on a set of core STR loci, composing the Federal Bureau of Investigation's (FBI) Combined DNA Index System (CODIS), consisting of a set of 13 traditional markers, plus Amelogenin, and recently enlarged to include seven additional markers (Hares 2015). While new approaches apply NGS to STR typing as an alternative to traditional capillary electrophoresis (CE) and offer opportunities to expand beyond the core markers (Steffen et al. 2017), the set of CODIS loci remain the primary means of genetic identification in forensic contexts (Gettings 2018). Moreover, recent work demonstrates the utility of this marker set beyond identification for population inference, demonstrating a capacity for revealing biogeographic ancestry and patterns of admixture (Algee-Hewitt et al. 2016).

Here, we analyze DNA quantities and STR profile results from post mortem blood drawn from 20 body donors and stored between 4 months and 4 years in ambient

conditions on FITZCO untreated blood cards. While FTA cards have been validated for DNA preservation for a variety of sample materials, including blood (Tredoux et al. 2015; Rahikainen et al. 2016), tissue (Santos 2018), and saliva (Hall and Roy 2014), the quality of DNA extracted from post-mortem blood samples stored on untreated cards is unknown. While FTA cards use proprietary technology to protect DNA from further degradation after samples are applied to the cards, the FITZCO FP705$^{TM}$ card is untreated and, so, does not lyse cells, denature proteins, or prevent microbial activity after sample deposition.

We focus on the quality of DNA from blood cards for long-term storage in forensic anthropology centers. Today, there are eight forensic anthropology decomposition facilities in the United States. Collection of biological samples from donors, including blood and saliva, is common practice for body donation programs. Long-term storage solutions are necessary in situations where DNA extraction and typing cannot be conducted immediately after sample collection. Here, we test the applicability of the FITZCO FP705$^{TM}$ card for long-term storage of blood samples collected post-mortem.

STRs, or short-tandem repeats, have been the standard DNA profiling method for forensic identification since the 1990s (Butler 2012). STRs lend themselves to identification based on the large number of alleles at each locus, high discriminatory power provided by the combination of STR loci, suitability for multiplexing, and relatively small size (approximately 100-400 base pairs, or bp) which allow for use with degraded samples (Butler 2012). The core STR loci which make up the standardized

CODIS set were primarily selected for their highly polymorphic qualities, enabling discrimination between unrelated individuals, with some overlap with the European Standard Set (ESS) (Butler 2012).

In addition to autosomal STRs, STRs associated with sex chromosomes can also reveal important data for forensic identification, familial relationship determination, and deconvoluting mixtures. More broadly, preestablished panels of STRs, like those used in forensic profile matching, also serve as ideal markers for management of biological sample collections. Guidelines have been established by the American National Standards Institute for the authentication of human cell lines using STRs (Barallon et al. 2010). As a marker set, the CODIS set of STRs provides a cost effective and straightforward method for matching individual cell lines with their source individuals (Nims et al. 2010). This approach could likewise be applied to skeletal collections and body donation programs, especially for elements that may become disassociated during decomposition and processing.

### *Limitations of STRs*

While STRs are well-suited to identification based on their high heterogeneity between individuals and large number of alleles per locus, the large DNA fragments required for typing can present issues when dealing with degraded DNA. The set of original 13 CODIS core loci range in size between 100-400 bp (Butler et al. 2003). Primers must be able to anneal on each side of the target amplicon during polymerase

chain reaction (PCR) in order to amplify the target region. If the DNA sample is too degraded or if PCR inhibitors are present (including indigo dyes, humic acid from soil, heme from blood, to name a few), the reaction can fail to amplify the target loci, creating situations in which one allele at the target locus drops out or both alleles, resulting in locus drop out (Butler 2012).

Allele and locus drop out are commonly seen with larger STR loci, resulting in electropherogram results that resemble a ski-slope pattern, in which smaller loci amplify in contrast to a reduction in amplification in larger loci, common in degraded samples (McCord et al. 2011). Alternatives to traditional STR marker kits have been proposed to reduce the amplicon length of larger loci, suggesting "mini-STRs" to reduce chances of allelic/locus drop out (Butler et al. 2003). While many mini-STRs have been included on expanded commercial kits, such as the Applied Biosystems AmpFℓSTR® MiniFiler™ PCR Amplification kit, they have not replaced traditional STRs as the typing method of choice (Nieuwerburgh et al. 2014).

The Amelogenin gene is present on both the X and Y chromosomes, with a distinguishing 6-bp deletion on the X chromosome not present on the Y chromosome. When typing the Amelogenin locus, a female profile will exhibit one large peak, whereas the male profile exhibits a separate peak for each chromosome. One of the issues complicating analysis of the Amelogenin locus is the phenomenon of Y-allele and X-allele dropout. In situations with degraded or inhibited DNA, Y-chromosomal specific DNA fragments can fail to amplify resulting in allelic drop-out, wherein the signal only amplifies the shorter fragment from the X-chromosome, or conversely there is dropout of

the X-chromosomal Amelogenin marker. Dropout of the Y-chromosomal marker is much more common (Kim et al. 2013). In cases of Y-allele dropout, an incorrect sex estimation can be made wherein the profile reads as female, X,X, rather than the true profile of X,Y.

Various biological sample types, including bone and tooth, blood, buccal cells/saliva, hair, and tissue present different challenges in DNA extraction and typing, resulting in differential yields and varying levels of potential PCR inhibitors. Expectations for DNA yields differ by sample type, with highest yields expected from blood (Butler 2012). Bones, teeth, blood, and hair all contain potential inhibitors that could interfere with PCR reactions, including calcium, heme, and melanin, respectively. Bone and tooth samples require extra demineralization steps to break down the hydroxyapatite matrix for DNA extraction (Loreille et al. 2007; Amory et al. 2012; Lee and Shewale 2017; Correa et al. 2018). Hair samples also require additional steps using DTT to lyse the keratin of the hair shaft (Butler 2012; Grisedale et al. 2018). As a substrate for sample storage, FTA (Flinders Technology Agreement) cards are a popular option for a variety of sample types, including blood and buccal cells. Extraction from FTA cards can be largely automated (Stangegaard et al. 2013) and can be used for direct PCR when dealing with robust samples (Hall and Roy 2014).

In contrast to robust samples, biological samples collected post-mortem may present difficulties in extraction and amplification, based on time since death and sample type, and are represented in far fewer studies regarding these sample types (Tredoux et al. 2015; Rahikainen et al. 2016). Tredoux et al. (2015) determine that both post-mortem femoral blood and buccal cell samples transferred to FTA paper produce successful

profiles at 16 STR loci with the caveat that each produced low-quality DNA when evaluated by UV absorbance. Rahikainen et al. (2016) assess DNA quality and quantity from blood collected post-mortem from autopsy samples and stored on FTA cards. The authors show that post-mortem interval and storage time both have a significant impact on DNA quantity and quality as assessed by relative fluorescence units (RFUs). FTA cards are a commonly used substrate for long-term sample storage.

Despite the importance of understanding the constraints placed upon DNA results, given the potential for technological and sample issues, no studies have assessed quantity and quality of DNA from blood samples stored on untreated blood cards for analyses of interest to forensic and anthropological geneticists, especially in the context of pursuing research using bio-banked blood samples from deceased individuals. The inability to produce complete CODIS profiles places limitations on individual identification and increases random match probabilities. When conducting ancestry estimation using STR marker sets, a reduced number of markers limits the resolution of ancestry inference as shown by Algee-Hewitt et al. (2016).

In this study, we test 20 untreated blood cards to assess the quality and quantity of DNA extracted. DNA quantity and the presence of inhibitors are assessed through qPCR. The relationship between time intervals between date of death and sample collection (IDDC) and sample collection and STR analysis (CST) and DNA quantity are evaluated. DNA quality is measured through a variety of methods, including peak height ratios and RFUs. Microvariants and off-ladder alleles are identified for each individual. We also assess the utility of these typed loci for generating ancestry and admixture proportions

using the unsupervised clustering methods implemented via the program STRUCTURE (Pritchard et al. 2000).

## Materials and Methods

Here we analyzed DNA quantity and STR quality from blood samples collected post-mortem and stored on FITZCO FP705$^{TM}$ untreated cards. STR data collected from the blood cards were then used to evaluate sample quality and make ancestry estimations using STRUCTURE.

The FITZCO FP705$^{TM}$ blood card used here was originally designed for use by the U.S. military, beginning in 1991 (FITZCO). The collection area of the card is made of biological grade cotton linter paper which prevents sample diffusion off of the substrate surface. The card design consists of four circles with a "fold-over" flap to reduce contamination risk following collection. Unlike FTA blood cards which are treated to lyse cells, deactivate nucleases, and deter microbial activity (Ahmed et al. 2011), the FITZCO FP705$^{TM}$ card is untreated.

Blood cards (FITZCO FP705$^{TM}$) were collected, postmortem, from donors of the William M. Bass Body Donation Program at the Forensic Anthropology Center (FAC) at The University of Tennessee, Knoxville (UTK). Blood was drawn from the aorta or subclavian artery of each cadaver using a syringe and placed on blood cards as part of the standard intake process, which involves documentation of the individual donor and sample (blood, hair, nails) collection for future research. Sample IDs and time interval

between date of death (DoD) and sample collection as well as interval between collection and DNA analysis are shown in Table 4: 1. Information on individual donor demographics, including geographic ancestry, or identity, was collected prior to or during the donation process. The blood card donors included individuals designated as pre-donors, individuals donated by family members, and one individual donated by a medical examiner's office. A total of nine individuals were pre-donors, individuals who planned donation and provided self-identified demographic data, including identity. Ten individuals were donated by family members and their identities offered by next-of-kin. One individual was donated by the office of a medical examiner, thus the identity provided was done so based on the assessment of the medical examiner rather than self or familial identification.

The blood cards were stored in a dessicator until sealed in plastic FoodSaver bags with a silica-based dessicant. One half-inch circle (outlined by the manufacturer) of the blood card was removed using sterilized scissors and placed in a DNA-free 50-mililiter (ml) conical tube. All samples were sent to Bode Cellmark Forensics for DNA extraction and fragment analysis.

Table 4: 1: Demographic data and time interval information for post-mortem blood donors.

| Sample ID | Sex | Age (in years) | Interval DoD/Collection (in days) - IDDC | Interval Collection/Storage Time (in days) - CST |
|---|---|---|---|---|
| **1** | M | 70 | 1 | 1568 |
| **2** | F | 75 | 1 | 1551 |
| **3** | M | 64 | 1 | 1548 |
| **4** | F | 65 | 0 | 1537 |
| **5** | M | 79 | 0 | 1519 |
| **6** | M | 64 | 3 | 1513 |
| **7** | F | 29 | 17 | 1492 |
| **8** | M | 50 | 74 | 1448 |
| **9** | F | 75 | 3 | 1443 |
| **10** | F | 58 | 1 | 1436 |
| **11** | F | 71 | 12 | 911 |
| **12** | M | 60 | 2 | 1206 |
| **13** | F | 94 | 1 | 1184 |
| **14** | M | 74 | 16 | 1181 |
| **15** | F | 79 | 3 | 1180 |
| **16** | F | 62 | 3 | 782 |
| **17** | M | 58 | 4 | 782 |
| **18** | M | 51 | 1 | 154 |
| **19** | F | 66 | 0 | 490 |
| **20** | M | 81 | 3 | 133 |

***Sample Treatment***

All samples were extracted at Bode Cellmark Forensics laboratories using the automated Qiagen EZ-1 Investigator Kit with an initial incubation, storage at 4° C overnight and extraction on the following day. Samples were quantified using the proprietary BodeQuant quantitative PCR (qPCR) for low-copy number samples. This qPCR method includes a nuclear DNA target to assess quantity of nuclear DNA as well as an Internal Positive Control (IPC) to assess presence of inhibitors within the sample extract. Following quantification, samples were amplified using the Applied Biosystems Identifiler kit. This multiplex PCR kit included the thirteen original CODIS loci plus the D2S1338 locus, the D19S433 locus, and Amelogenin. STR typing through kit-based approaches, including the Applied Biosystems Identifiler kit, uses fluorescent dyes attached to primers for each of the multiplexed loci. Samples were sequenced on the Applied Biosystems 3130 capillary electrophoresis machine in which fluorescently labeled primer fragments and lengths were detected using a charge-coupled device. The 3130 detects fluorescence of the labeled fragments and reports this output as relative fluorescence units (RFUs) which are used to interpret fragment lengths as well as quality thresholds when compared against an allelic ladder with size standard. Positive and negative controls were used throughout the entire process.

### *STR Quantity and Quality Assessment*

Sample quantities were compared to the IDDC and CST using a linear regression model, followed by the Spearman's $\rho$ (rho) test to assess the relationship between time intervals and DNA quantity. We suggest that an increased time interval between donor death/sample collection as well as increased time between collection and STR typing will result in lower average DNA yield.

To assess STR quality and impact of time intervals between DoD, collection, and extraction, RFUs were averaged across sample and locus size class and compared to IDDC and CST using a linear regression model, followed by the Spearman's $\rho$ test. Locus sizes classes were grouped on the basis of size as per Rahikainen et al. (2016) with Class 1 (<130bp), Class 2 (130-200 bp), Class 3 (200-300 bp), and Class 4 (>300 bp) as seen in Table 4: 4. As with DNA quantity, we suggest that an increased time interval between donor death/sample collection as well as increased time since collection/STR typing will result in a reduction in DNA quality. Through assessing degradation from a decrease in RFUs across locus size, we determined whether patterns of differential amplification are present in the profiles generated using the blood cards.

Peak height ratios were calculated for each individual and locus by dividing the lower peak (Peak A) RFU by the higher (Peak B) RFU as outlined by the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines. Peak height ratios of below 70% were designated as severe imbalance, a threshold indicative of multiple contributors or other issues (Glider et al. 2009). From single source samples (i.e. not

mixtures), peak height ratio imbalance can be attributed to several issues, including low starting DNA template, preferential amplification, DNA degradation, the presence of inhibitors, or a combination of these factors (Word 2013). Profiles were also checked for the presence of stutter and off-ladder alleles.

### *STR Analysis for Sex Determination*

The Amelogenin marker was typed and compared to self-reported biological sex and skeletal estimations. As a smaller marker, we expected that the Amelogenin markers would successfully amplify and match recorded biological sex. We also identified off ladder alleles, i.e., those not found within the allelic ladder for each STR kit. Off-ladder alleles can include full repeats, which are uncommon within known typed populations. Microvariants, a form of off-ladder allele with incomplete repeat units, were also noted for each individual profile. An example of a microvariant would include a simple tetranucleotide (4 bp) locus with 14 repeats, but with the addition of a partial repeat of 2 bases, making the allele call 14.2. For each microvariant and other off-ladder alleles, the frequency relative to the U.S. population was also assessed.

### *Population Inference from Amplified STRs*

Trihybrid ancestry estimation was conducted using the unsupervised clustering program STRUCTURE, v. 2.3.4 (Pritchard et al. 2000). Thirteen CODIS loci were

compiled for 332 individuals from the Human Genome Diversity Panel (HGDP-CEPH) H1048 subset (Rosenberg 2006; Algee-Hewitt et al. 2016), including 94 individuals from Sub-Saharan Africa, 83 individuals from the Americas, and 155 individuals from Europe, who together served as the parental reference sample. An initial STRUCTURE run was used to determine the optimal range of *K*, or number of clusters, with parameters set at 10,000 for burn-in and 10,000 Markov Chain Monte Carlo (MCMC) repetitions (reps). The next run set parameters between 1 and 3 for number of *K* clusters, limiting the analysis to the maximum number of populations under a trihybrid ancestry model, with 10,000 reps for burn-in and 10,000 MCMC reps with 10 iterations. We used the No Admixture model which assumes origin of individuals from only one population and is appropriate for discrete populations (Falush 2003). We assumed that allele frequencies were independent among populations with parameters of alpha ($\alpha$) and lambda ($\lambda$) set at 1. Post-processing was performed using Structure Selector (Li and Liu 2018) which integrates several approaches for data interpretation, including the Puechmaille (2016) method and Clumpak (Kopelman et al. 2015).

To evaluate admixture, we performed a second analysis with STRUCTURE. For this analysis, a subset of the National Institute of Standards and Technology (NIST) dataset (Steffen et al. 2017; Gettings et al. 2018) were used in place of the HGDP-CEPH parental populations and included 149 self-identified African Americans, 151 European Americans, and 101 Hispanics. We used the Admixture model, operating under the assumption that that each of the individuals shares genetic ancestry with one or more of the clusters included (Pritchard et al. 2010), and that allele frequencies were independent

between populations, with the $\alpha$ and $\lambda$ set at 1. The Admixture model assumes run parameters were set for *K* between 1 and 3, with 10,000 reps for burn-in and 10,000 MCMC reps with 10 iterations.

# Results

### *Extraction and Quantification*

Of the 20 samples, five had to be re-extracted twice and one sample three times to obtain sufficient quantities of DNA to produce a complete STR profile. Quantities ranged from 15.72 ng/uL to 153.81 ng/uL (Table 4: 2). Five samples exceeded the average internal positive control (IPC) threshold of 20.64 by more than 2 cycles for the standards, indicating the presence of inhibitors in those samples.

Table 4: 2: DNA quantities, internal positive control cycle threshold (IPC CT), and average peak height ratios and RFUs across all loci. Those that exceed the IPC CT and indicate the presence of inhibitors are in bold.

| Sample ID | Sample ID | DNA Quantity (ng/uL) | IPC CT | Average Peak Height Ratios | Average RFUs Across all Loci |
|---|---|---|---|---|---|
| UT09-08D | 1 | 33.65 | 20.77 | 81% | 837 |
| UT13-08D | 2 | 87.22 | **22.52** | 86% | 1369 |
| UT14-08D | 3 | 106.59 | **22.98** | 86% | 1455 |
| UT21-08D | 4 | 42.56 | 20.26 | 90% | 1074 |
| UT28-08D | 5 | 131.02 | **23.24** | 91% | 1861 |
| UT29-08D | 6 | 82.66 | 20.38 | 82% | 262 |
| UT33-08D | 7 | 90.91 | 20.33 | 79% | 409 |
| UT49-08D | 8 | 69.19 | 19.91 | 85% | 1896 |
| UT55-08D | 9 | 84.18 | 20.70 | 90% | 1087 |
| UT57-08D | 10 | 96.70 | 20.22 | 84% | 645 |
| UT117-09D | 11 | 75.25 | 19.43 | 82% | 434 |
| UT17-09D | 12 | 15.72 | 19.60 | 85% | 1033 |
| UT23-09D | 13 | 64.56 | 19.68 | 82% | 359 |
| UT26-09D | 14 | 153.81 | **22.77** | 84% | 416 |
| UT27-09D | 15 | 68.48 | 19.65 | 88% | 1194 |
| UT36-09D | 16 | 146.88 | **22.88** | 85% | 683 |
| UT37-09D | 17 | 118.96 | 19.96 | 83% | 597 |
| UT111-11D | 18 | 62.29 | 19.47 | 87% | 618 |
| UT20-11D | 19 | 131.18 | 20.79 | 87% | 1296 |
| UT08-12D | 20 | 19.30 | 19.70 | 87% | 1039 |

Correlations between DNA quantity in ng/uL and the time interval (in days) between a) date of death and collection (IDDC) and b) sample collection and STR testing (CST) were assessed using a linear regression model and Spearman's correlation coefficient, $\rho$. A linear regression model was used to assess whether time intervals were significant predictors of DNA quantity. Modeled with IDDC, there was no significance detected, with a *p*-value of 0.2933, F-statistic of 1.176, $R^2 = 0.065$, and 17 degrees of freedom. With CST, no significance was detected with a *p*-value of 0.8505, F-statistic of 0.0366, $R^2 = 0.00215$, and 17 degrees of freedom. Non-normal distribution of the variables representing IDDC was confirmed by a Shapiro-Wilk normality test, yielding, respectively, significant *p*-values of 1.101e-07 and 0.001574 when $\alpha = 0.01$ thus the Spearman's $\rho$ statistic. We find a small positive association with DNA quantity for IDDC with DNA quantity for both IDDC (Spearman's $\rho = 0.0823$, *p*-value = 0.7301) and a small negative association for CST ($\rho = -0.0519$, *p*-value = 0.8279), both associations being statistically insignificant. Data visualizations, plotting associations for each individual, are provided in Appendix A. These results suggest that time, when measured as IDDC and CST intervals, does not have a significant relationship to DNA quantity, which was contrary to the original expectations.

***STR Quality Assessment Results***

Peak height ratios were averaged across each sample (reported in Table 4: 2) and across each locus (reported in Table 4: 3). Several samples did not meet the 70% peak height ratio threshold, indicating that those samples were imbalanced, likely due to

degradation rather than possibility of a mixture due to lack of more than 2 alleles at multiple loci. Peak height imbalance can be attributed to sample degradation as well as potential mixed profiles, which include more than one contributor. In the profiles generated, only 1-2 alleles were present at each locus across the profile as a whole, indicating no sign of a potential second contributor (a major/minor mixture). Rather, the imbalance in peak heights can be attributed to increased degradation which is responsible for differential amplification of damaged DNA fragments, wherein one allele is replicated at a higher number than the other, producing differences in fluorescent units within the same locus.

Table 4: 3: STR loci, average peak height ratios per locus, and number of samples below peak height ratio of 70%.

| STR Locus | Average Peak Height Ratio | Number of Samples Below 70% PHR |
|---|---|---|
| **Amelogenin** | 86% | - |
| **D3S1358** | 88% | - |
| **D19S433** | 88% | - |
| **D8S1179** | 86% | - |
| **D5S818** | 88% | - |
| **TH01** | 87% | - |
| **vWA** | 87% | 1 |
| **D21S11** | 81% | 2 |
| **D13S317** | 85% | 3 |
| **TPOX** | 87% | - |
| **FGA** | 85% | 1 |
| **D7S820** | 86% | 1 |
| **D16S539** | 89% | - |
| **D18S51** | 86% | - |
| **CSF1PO** | 85% | 1 |
| **D2S1338** | 73% | 8 |

Average RFUs by locus class are reported in Table 4: 4. Results assessing the impact of IDDC and CST on DNA quality as shown through RFUs were conducted using a linear regression and the Spearman's $\rho$ test statistic. A single donor was stored frozen after date of death for a total of 74 days, creating an outlier in terms of statistical analysis. This outlier was removed prior to statistical testing. Using a linear model to assess whether IDDC was a significant predictor, significant results were found in each RFU class. For Class 1, the IDDC was a significant predictor of RFU values, with a *p*-value of 0.02043, F-statistic of 6.536, $R^2 = 0.2777$, and 17 degrees of freedom. For Class 2, the linear regression results indicate a *p*-value of 0.01407, F-statistic of 7.488, $R^2 = 0.3058$, and 17 degrees of freedom. For Class 3, results indicate a *p*-value of 0.02822, F-statistic of 5.752, $R^2 = 0.2528$, and 17 degrees of freedom. For Class 4, results indicate a *p*-value of 0.0418, F-statistic of 4.849, $R^2 = 0.2219$, and 17 degrees of freedom.

Using Spearman's $\rho$, the association between IDDC and RFUs, associations for Class 1 ($\rho$ = -0.6519, *p*-value = 0.0025), Class 2 ($\rho$ = -0.5278, *p*-value = 0.0201) and Class 3 ($\rho$ = -0.6089, *p*-value = 0.0056) were all significant, while associations for Class 4 ($\rho$ = -0.4432, *p*-value = 0.0573) were not. All associations between IDDC and RFUs for all class sizes indicate a negative correlation between time and fluorescence, demonstrating that as number of days post-mortem before sample collection increase, fluorescence values decrease across all class sizes.

Table 4: 4: Locus size classes with size range and loci included; average RFUs per locus size class across all individuals included.

| Class | Locus Size Range | Loci Included | Average RFU/Class |
|---|---|---|---|
| **1** | <130 bp | D3S1358, D19S433, D10S1248 | 1342 |
| **2** | 130-200 bp | vWA, TH01, D5S818 | 1011 |
| **3** | 200-300 bp | D21S11, D13S317, D7S820, D16S539 | 805 |
| **4** | >300 bp | CSF1PO, TPOX, D18S51, FGA, D2S1338 | 698 |

In assessing the association between CST and RFUs from each size class (1-4) using a linear model, no significant relationships were identified (Class 1 - *p*-value = 0.1701, F-statistic of 1.053, $R^2$ = 0.1077, and 17 df, Class 2 - *p*-value = 0.6523, F-statistic of 0.2104, $R^2$ = 0.0122, and 17 df, Class 3 - *p*-value = 0.1701, F-statistic of 1.053, $R^2$ = 0.1077, and 17 df, and Class 4 - *p*-value = 0.8766, F-statistic of 0.0249, $R^2$ = 0.0015, and 17 df. No significant relationships were identified using Spearman's $\rho$ (Class 1: $\rho$ = 0.4242, *p*-value = 0.0623; Class 2: $\rho$ = 0.2399, *p*-value = 0.3082, Class 3: $\rho$ = 0.2595, *p*-value = 0.2692 Class 4: $\rho$ = 0.0684, *p*-value = 0.7743).

***STR Analysis Results***

Comparisons of the Amelogenin marker returned complete agreement between the genetic sex markers, self-reported biological sex, and skeletal sex estimations. One off-ladder allele was recorded at locus D21S11 as microvariant 29.3 in individual 15 and confirmed by a second fragment analysis run. This allele is found at a frequency of 0.0005 in the combined U.S. population. Other microvariants not considered off-ladder

alleles were typed at 3 loci (D19S433, TH01, and D21S11), in three, four, and three individuals, respectively. These frequencies are reported in Table 4: 5.

STRUCTURE analysis for ancestry estimation was conducted using the HDGP-CEPH populations and the 20 blood card samples; the number of populations, *K*, was set at 3. This was also the optimal number of ancestry clusters identified by computational methods. This optimal value of *K* was determined using STRUCTURE Selector (Li and Liu 2018), implementing the MedMeaK, MaxMeaK, MedMedK, MaxMedK methods (Puechmaille et al. 2016) for choosing the best among a range of clusters numbers (See Appendix Figure 4A: 11). The MedMeaK, MaxMeaK, MedMedK, MaxMedK approaches all outperformed traditional deltaK methods for determining the true number of clusters in situations with uneven sample sizes (Puechmaille et al. 2016). Each of the 20 individuals was assigned to one of three population clusters, with membership coefficients representing the posterior probability that the individual is from selected population (shown in Table 4: 6). Results are visualized in the barplot shown in Figure 4: 1 generated by Clumpak (Kopelman et al. 2015).

Table 4: 5: Frequencies of microvariants found in surveyed STR profiles. * Denotes a lack of reported frequencies for a particular allele in the NIST database.

| Locus | Allele Variant | Number of Ind. | Frequency in U.S. Population |
|---|---|---|---|
| **D19S433** | 13.2 | 1 | * |
| **D19S433** | 15.2 | 2 | 0.0569 |
| **TH01** | 9.3 | 3 | 0.2056 |
| **D21S11** | 24.2 | 1 | 0.0005 |
| **D21S11** | 29.3 | 1 | 0.0005 |
| **D21S11** | 30.2 | 1 | 0.0217 |
| **D21S11** | 31.2 | 2 | 0.0772 |
| **D21S11** | 32.2 | 4 | 0.0912 |
| **D21S11** | 33.2 | 4 | 0.0328 |

When adopting a hard classification or single cluster approach to ancestry inference (Algee-Hewitt et al. 2016; Algee-Hewitt 2016), the documented identity of 17 of the 20 individuals matched the continental population cluster to which the individual was assigned. In one case, the individual self-identified as White but was assigned a membership coefficient of 0.844 for the African cluster and 0.156 for the European cluster. Two other individuals were documented, one self-identified and one familial identification, as Black but were grouped into the European cluster with membership coefficients of 0.906 and 0.950 respectively. The only non-self-identified or non-familial identified individual, 14, was identified by the medical examiner's office as Black and was assigned a membership coefficient of 0.965 for the African cluster and 0.014 for the European cluster.

Table 4: 6: Correspondence between the membership coefficients obtained from the trihybrid ancestry analysis using STRUCTURE and documented population identifier. Individuals were assigned to the population cluster with the highest degree of membership. Reported identity is included, with the source of the identity assignment. Abbreviations are S: self-identified, F: familial identification; ME: identity assigned by the medical examiner. *Denotes a potential disagreement between genetically inferred ancestry and self-reported population identity.

| Sample ID | European | African | Indigenous American | Reported Identity & Source |
|---|---|---|---|---|
| **1** | 0.903 | 0.097 | -- | White – S |
| **2** | 1.000 | -- | -- | White – S |
| **3** | 0.998 | 0.002 | -- | White – F |
| **4** | 1.000 | -- | -- | White – F |
| **5** | 1.000 | -- | -- | White – S |
| **6** | 0.931 | 0.069 | -- | White – S |
| **7** | 0.156 | 0.844 | -- | White * – S |
| **8** | 0.999 | 0.001 | -- | White – F |
| **9** | 0.691 | 0.011 | 0.299 | White – F |
| **10** | 0.961 | 0.001 | 0.035 | White – F |
| **11** | 0.950 | 0.050 | -- | Black * – S |
| **12** | 0.906 | 0.094 | -- | Black * – F |
| **13** | 0.856 | 0.143 | 0.001 | White – F |
| **14** | 0.014 | 0.965 | .021 | Black – ME |
| **15** | 0.978 | 0.022 | -- | White/American Indian – F |
| **16** | 0.900 | 0.092 | 0.008 | White – F |
| **17** | 1.000 | -- | -- | White – F |
| **18** | 0.988 | 0.010 | 0.002 | Hispanic – S |
| **19** | 0.161 | 0.839 | -- | Black – S |
| **20** | 0.999 | 0.001 | 0.001 | White – S |

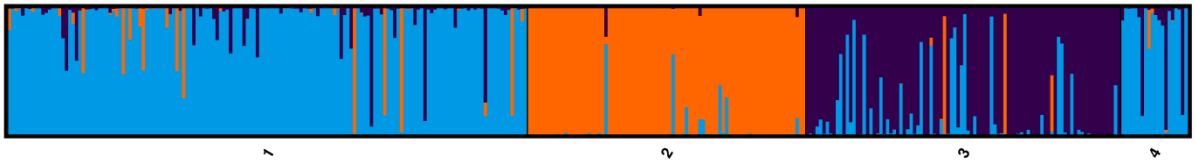


Figure 4: 1: STRUCTURE plot depicting *K*=3 ancestry clusters by population, generated in Clumpak (Kopelman et al. 2015). Each individual is represented by a single bar partitioned into *3* colored segments, which gives the individual's proportion of membership across the *3* clusters. Groups are 1) European, 2) Indigenous American, 3) African individuals, with unknown samples shown in group 4.

Results from the second STRUCTURE analysis using the Admixture model present *K=2* clusters when analyzed using STRUCTURE Selector (Li and Liu 2018). Using the Puechmaille (2016) method, the MedMeaK, MaxMeaK, MedMedK, MaxMedK preferred two distinct clusters using the NIST sub-dataset. Results are visualized in the barplot shown in Figure 4: 2 generated by Clumpak (Kopelman et al. 2015).

The best fit number of clusters was 2, with the inferred cluster assignments between two groups (Table 4: 7). Those identifying as Black had higher correlation coefficients with Cluster 1 (Table 4: 8). Those identifying as White had higher correlation coefficients on average with Cluster 2. The individual who identified as White/Native American and the individual who identified as Hispanic were split between each cluster.

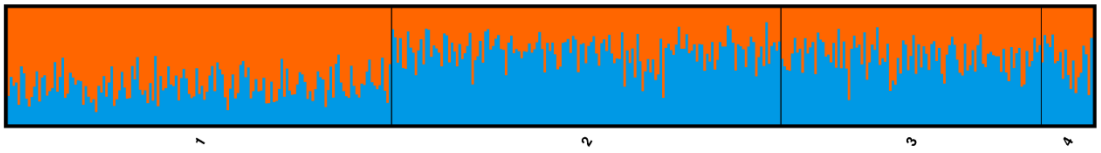


Figure 4: 2: STRUCTURE plot depicting admixture results from NIST dataset for *K = 2* cluster solution, generated using Clumpak (Kopelman et al. 2015). Each individual is represented by a single bar partitioned into *2* colored segments, which gives the individual's proportion of membership across the *2* clusters. Groups are 1) African Americans, 2) European Americans, 3) Hispanics, and 4) unknowns from blood cards.

Table 4: 7: Correspondence between the membership coefficients obtained from the admixture analysis, using STRUCTURE and the NIST reference dataset, and the documented population identifier. The optimal K=2 model was identified computationally. Individuals were assigned to one of two population clusters with the highest degree of membership.

| Sample ID | Cluster 1 | Cluster 2 | Reported Identity |
|---|---|---|---|
| **1** | 0.471 | 0.529 | White |
| **2** | 0.251 | 0.749 | White |
| **3** | 0.361 | 0.639 | White |
| **4** | 0.361 | 0.681 | White |
| **5** | 0.249 | 0.751 | White |
| **6** | 0.477 | 0.523 | White |
| **7** | 0.462 | 0.538 | White |
| **8** | 0.391 | 0.609 | White |
| **9** | 0.603 | 0.397 | White |
| **10** | 0.380 | 0.620 | White |
| **11** | 0.567 | 0.433 | Black |
| **12** | 0.682 | 0.318 | Black |
| **13** | 0.460 | 0.540 | White |
| **14** | 0.714 | 0.286 | Black |
| **15** | 0.578 | 0.422 | White /American Indian |
| **16** | 0.556 | 0.444 | White |
| **17** | 0.323 | 0.677 | White |
| **18** | 0.401 | 0.599 | Hispanic |
| **19** | 0.732 | 0.268 | Black |
| **20** | 0.282 | 0.718 | White |

Table 4: 8: Proportion of membership of each pre-defined NIST population in each of *K* = 2 clusters.

| NIST Population | Cluster 1 | Cluster 2 | Number of Individuals |
|---|---|---|---|
| **African Americans** | 0.651 | 0.349 | 149 |
| **European Americans** | 0.382 | 0.618 | 151 |
| **Hispanics** | 0.431 | 0.569 | 101 |

## Discussion and Conclusion

Here we have evaluated the effectiveness of the FITZCO FP705$^{TM}$ untreated blood card as a reliable substrate for long-term storage. Our samples were extracted after time intervals between 4 months and 4 years. We address practical laboratory concerns for the successful recovery of nuclear DNA after longer periods of time. We find that sufficient amounts of nuclear DNA can be recovered from the sampled blood cards to amplify the original 13 CODIS core loci, although several samples had to be re-extracted due to insufficient DNA recovery during the initial extraction. The 13 CODIS loci were adequate for ancestry estimation using the program STRUCTURE, for which 17 of 20 samples classified into the ancestry group that most likely corresponded with their self-reported identity. The individuals who self-identified as Hispanic and White/Native American were classified, with higher membership coefficients greater than (>0.98) into the European cluster under the trihybrid ancestry model. These individuals display opposite trends, however, when subjected to the admixture analysis using the NIST

population samples as the reference dataset. Their admixture proportions were distributed similarly to the NIST samples across the two inferred clusters. This appears to capture White and non-White variation, such that the Hispanic individual carries about 60% European admixture and the dual-identity individual about 42% European admixture.

### *Implications for Long-term Storage*

While full profiles were typed from each of the 20 sampled untreated blood cards, several issues emerged during the analysis. The presence of inhibitors in five of the 20 samples may present a concern for downstream amplification of STRs and other markers. One potential source may include heme from red blood cells, a known inhibiting substance (Butler 2012). In contrast, Rahikainen et al. (2016) reported no inhibition in DNA extracts from FTA cards. We also noted a reduction in RFUs from smaller to larger loci, as shown through the decrease in RFUs from Class 1 through Class 4 in the untreated blood cards indicating degradation. We also show that a statistically significant reduction in RFUs is associated with increased time intervals between donor death and sample collection. Increased time intervals between the date of death and collection lower the quality of STRs typed. While Rahikainen and colleagues also showed a decrease in DNA quantities over time in FTA cards, part of the reduction seen in RFUs may be indicative of DNA degradation exacerbated by nuclease activity which was not halted in the untreated blood cards. Rahikainen et al. (2016) were able to recover DNA from FTA

cards stored up to 16 years, however, this longer time interval may result in increased degradation and reduced yields in non-treated cards.

An additional aspect of the untreated blood cards to consider is the potential for pathogen exposure. Since FTA cards lyse the cells upon contact, pathogens are inactivated (Serra et al. 2018); however, pathogens can persist in the unlysed cells on the untreated substrate. While viruses such as HIV are typically undetectable within a week to a month, Hepatitis C has been identified in dried blood spots after 4 weeks and on blood in needle syringes for up to 8 months (Thompson et al. 2002). While all potentially biohazardous material should be treated with universal precautions, this aspect of blood sample storage may be a concern for forensic body donation programs, providing an additional reason to consider FTA cards over untreated cards.

Body donation programs often collect sample material for subsequent genotyping; however, DNA typing is often not the main focus of attention for decomposition facilities and budgets are limited. Based on our results, we suggest that FTA-based cards may provide a more dependable method for long-term storage in spite of the lower cost of untreated cards. If typing of large-scale marker sets may be desirable for future applications, untreated cards may not produce the high quantities and quality of DNA required for expansive SNP panels or typing combinations of multiple marker types (STRs, Y-STRs, SNPs). We recommend long-range planning for future genotyping needs when selecting sample storage substrates. For short-term preservation, untreated cards may be adequate for STR typing but for extended storage duration, FTA cards provide an

option that lyses cells, limits nuclease activity, and demonstrates DNA recovery from post-mortem collected blood samples after a decade of storage.

### *CODIS Markers for Ancestry/Admixture Estimation*

Trihybrid ancestry analysis in STRUCTURE produced membership coefficients for three ancestral groups. Out of 20 samples, 17 individuals were classified into the population which matched their reported identity. Two of the individuals, one self-identified and one familial identified as Black, had membership coefficients more closely aligned with the European cluster, whereas one individual who self-identified as White had an African membership coefficient of 0.844. Population history in the U.S. reflects admixture between groups of different continental ancestries and it has been noted that African Americans carry proportions of European ancestry (Tang et al. 2005). In a large-scale health study on Genetic Epidemiology Research on Adult Health and Aging (GERA) of over 100,000 individuals, researchers found that, of those identifying as African American, 91% had European ancestry (Banda et al. 2015). From the same study, 0.4% of self-reported Europeans had African. These discordant results between the higher population membership coefficients and reported identities may be a result of admixture, reflective of well-documented population history in the Americas.

Our STRUCTURE analyses of ancestry and admixture of the unknown individuals typed from blood cards and known-source database samples produced different results, owing to the different number of population clusters, or values of *K*,

identified computationally. One particular reason for the discrepancy between the model-based clusters in STRUCTURE can be attributed to the difference in population datasets used for each analysis. While the initial No Admixture model for ancestry estimation used the HGDP-CEPH populations, the admixture analysis used a subset of the NIST population dataset. The HGDP-CEPH populations were sampled from individuals world-wide and are routinely taken to represent parental populations – in this particular case from each of three continental regions of Africa, Europe, and the Americas. In contrast, the NIST population subset is composed of individuals from the U.S., specifically those self-identifying as African American, European Americans, and Hispanic, collected from the Interstate Blood Bank in Memphis, Tennessee or the DNA Diagnostics Center in Fairfield, Ohio.

It has been previously noted that populations in the U.S. reflect varying levels of continental admixture based on the complex population history of the country (Bryc et al. 2015; Montinaro et al. 2015). Considering that the admixture analysis used U.S. populations, all of which are known to carry on average some quantities of ancestry from each of the three major U.S. source populations (Bryc et al. 2015; Algee-Hewitt 2016; Algee-Hewitt et al. 2018), the best number of clusters was estimated at $K$=2. STRUCTURE analysis of African Americans by Lawson and colleagues (2018) demonstrated similar clustering of each into two "ancestral" population clusters based on recent admixture. Algee-Hewitt has also shown, for both genetic and proxy quantitative skeletal traits similar, 2 cluster patterns for Latinos, largely of Mexican descent, and

African Americans. The author further reported only trivial levels of admixture for European Americans, as also reflected by Banda and colleagues (2015).

While the CODIS STRs meet the recommended qualities of markers for STRUCTURE analysis in that they reflect low mutation rates, are selectively neutral, and are in linkage equilibrium (Prichard et al. 2000, Porras-Hurtado et al. 2013), alternative sets markers provide more ancestry information. Explorations of sets of forensic STRs, with different characteristics or comprising more markers, have demonstrated increased recovery of ancestry information and greater differentiation between individuals using STRUCTURE (Algee-Hewitt et al. 2016). While these particular 13 CODIS loci provide valuable insights into ancestral origin on the continental scale, the limitations must be considered when extending this panel of markers beyond its intended scope for individual identification to admixture estimation, especially for populations with complex population histories and peoples with potentially high levels of admixture.

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# Appendix A

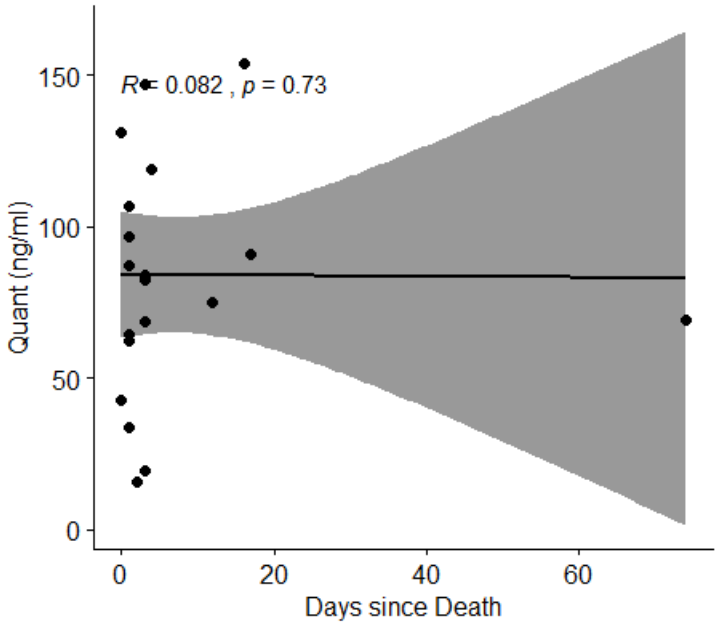



Figure 4A: 1: Spearman's ρ rank-based assessment of the relationship time since death and sample collection (IDDC) and DNA quantity

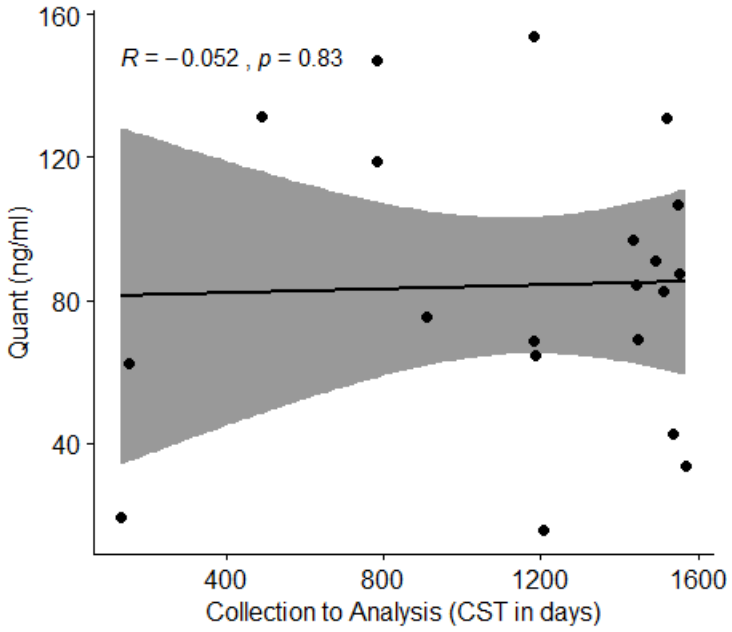



Figure 4A: 2: Spearman's $\rho$ rank-based assessment of the relationship time since sample collection and STR typing (CST) and DNA quantity

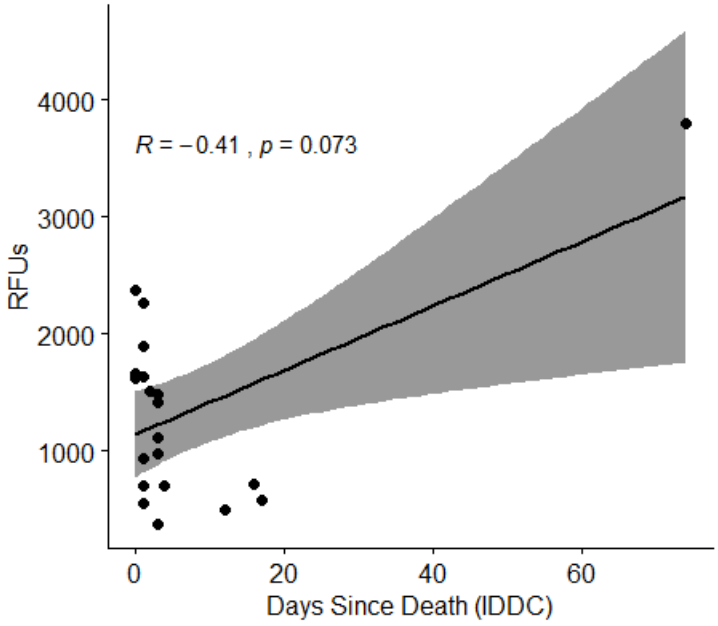



Figure 4A: 3: Spearman's $\rho$ rank-based assessment of the relationship time since death and sample collection (IDDC) and Class 1 RFUs

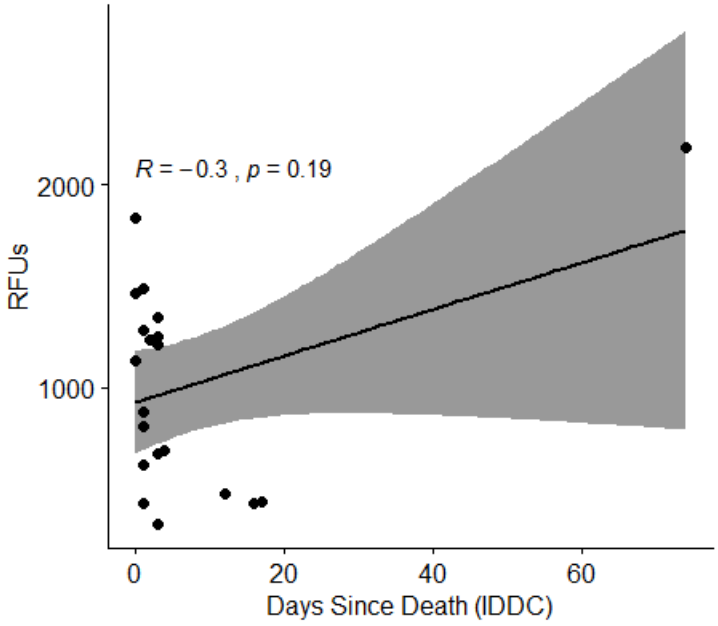



Figure 4A: 4: Spearman's $\rho$ rank-based assessment of the relationship time since death and sample collection (IDDC) and Class 2 RFUs

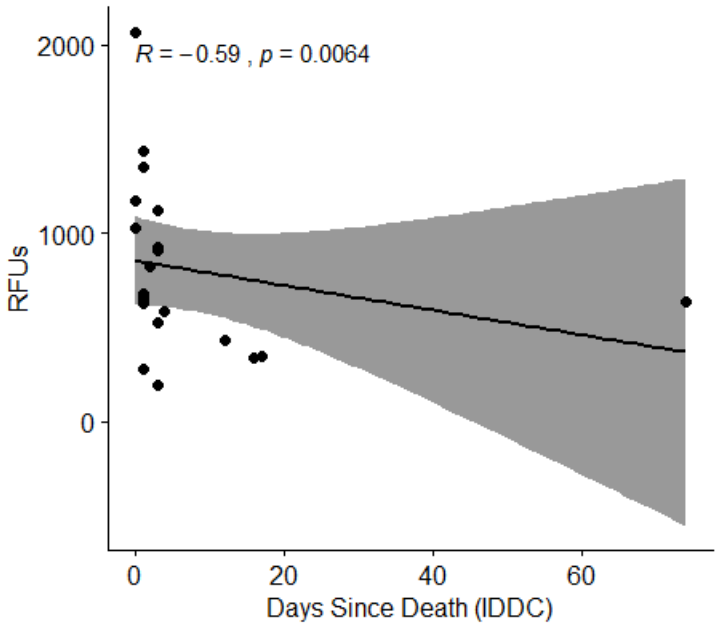



Figure 4A: 5: Spearman's $\rho$ rank-based assessment of the relationship time since death and sample collection (IDDC) and Class 3 RFUs

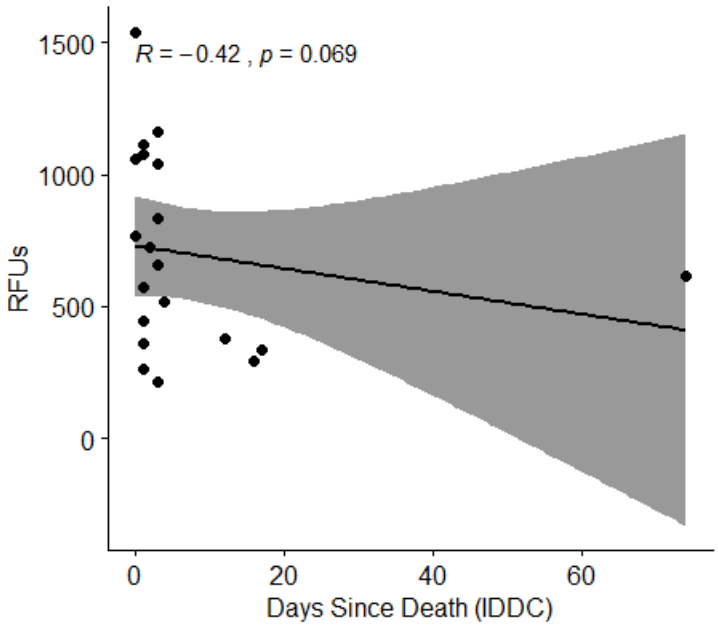



Figure 4A: 6: Spearman's $\rho$ rank-based assessment of the relationship time since death and sample collection (IDDC) and Class 4 RFUs

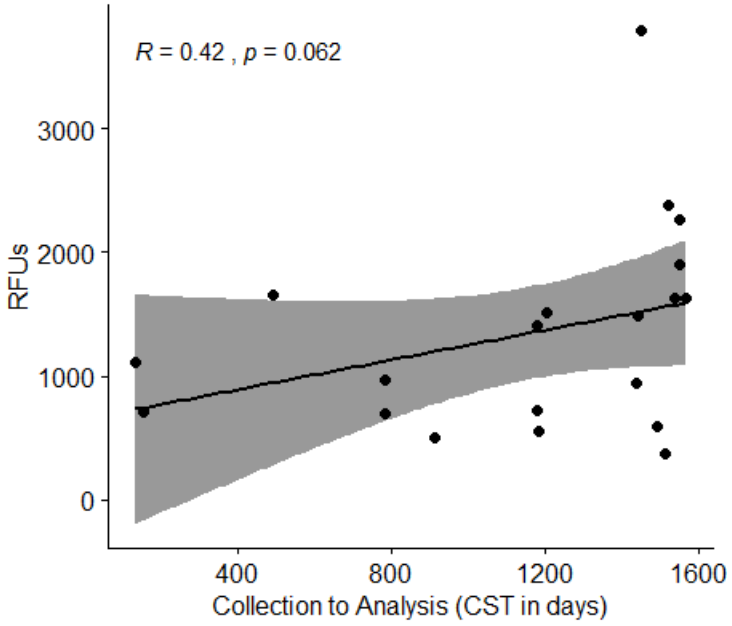



Figure 4A: 7: Spearman's $\rho$ rank-based assessment of the relationship time since sample collection and STR typing (CST) and Class 1 RFUs

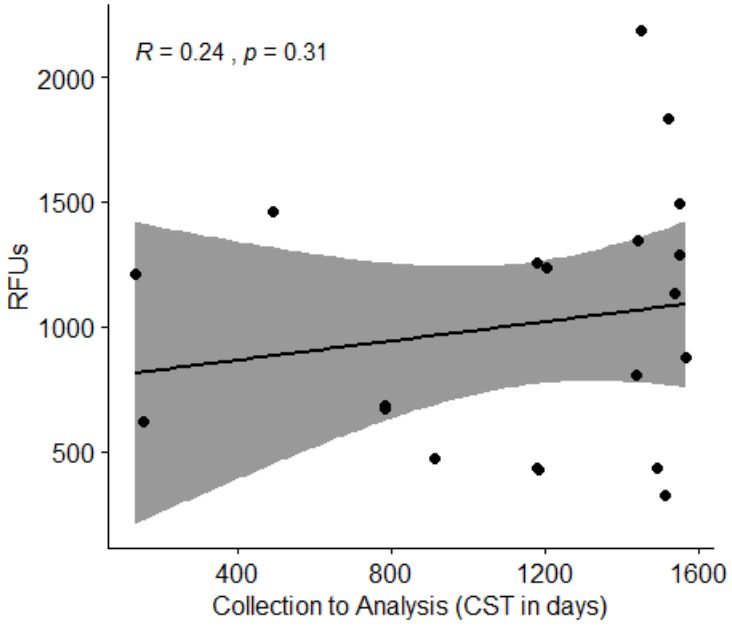



Figure 4A: 8: Spearman's $\rho$ rank-based assessment of the relationship time since sample collection and STR typing (CST) and Class 2 RFUs

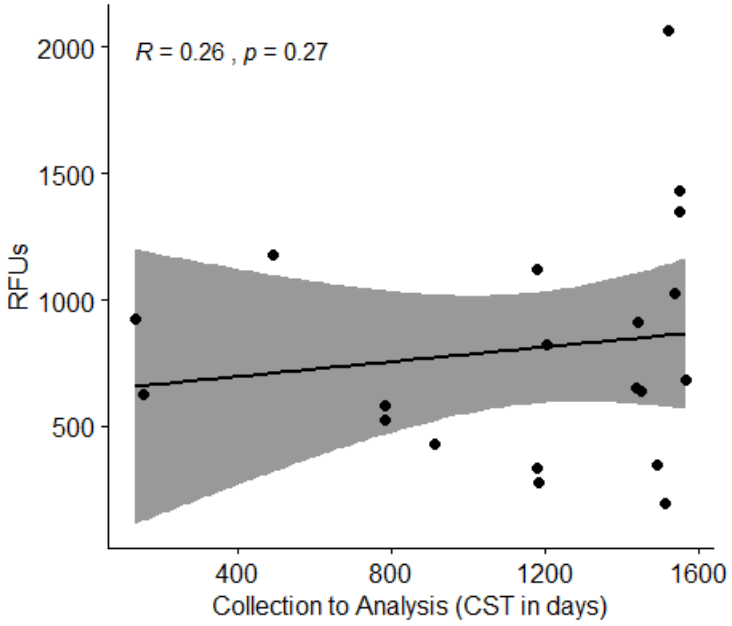



Figure 4A: 9: Spearman's $\rho$ rank-based assessment of the relationship time since sample collection and STR typing (CST) and Class 3 RFUs

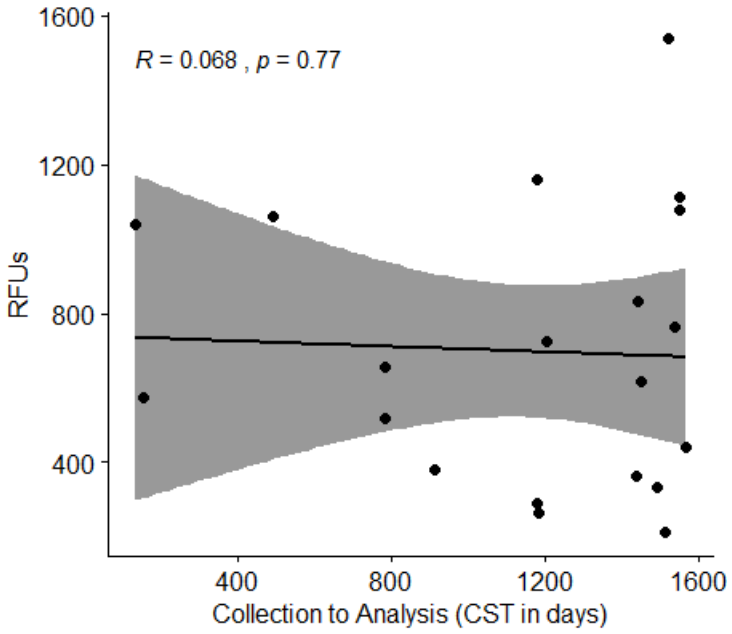



Figure 4A: 10: Spearman's $\rho$ rank-based assessment of the relationship time since sample collection and STR typing (CST) and Class 4 RFUs

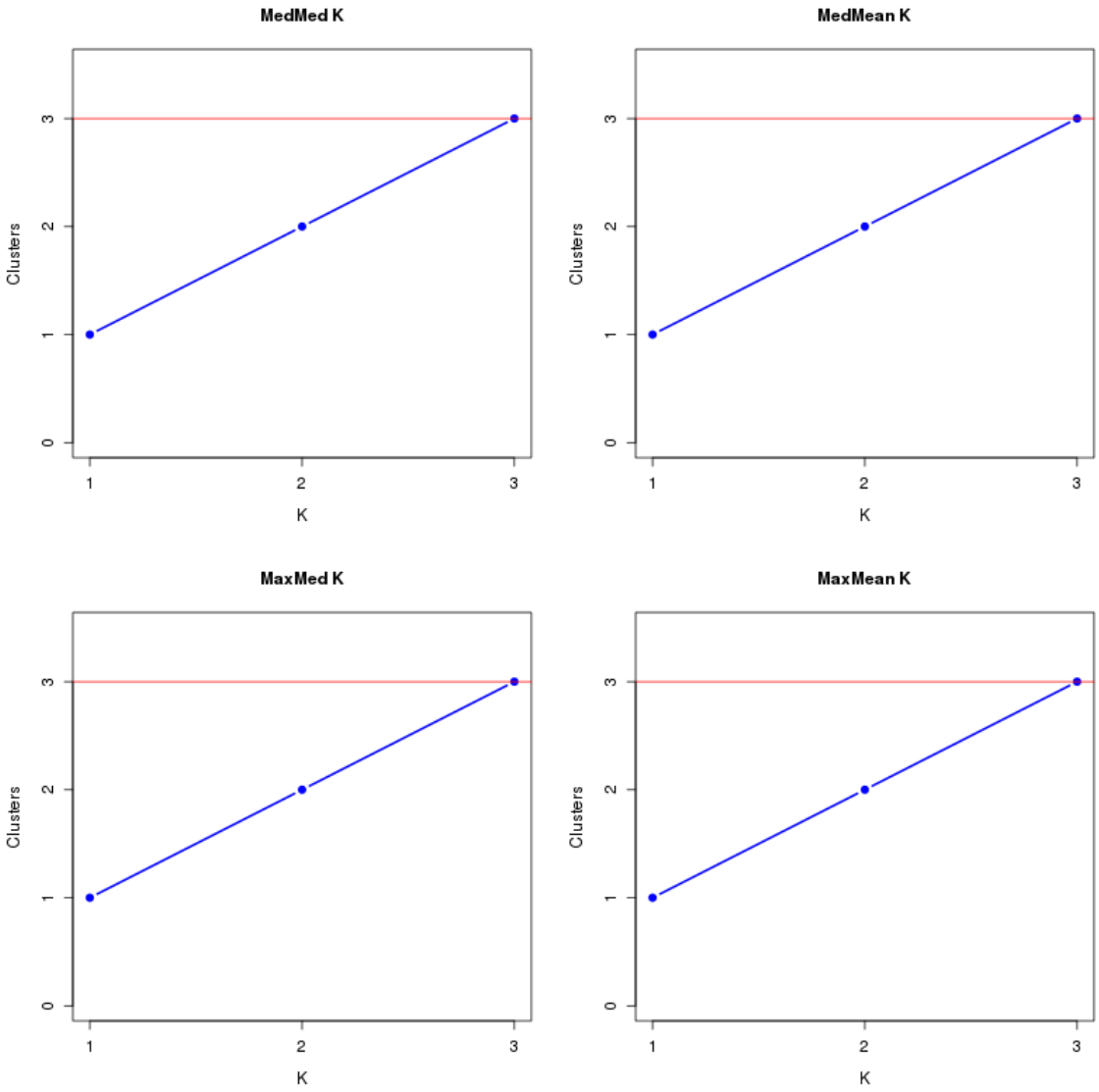
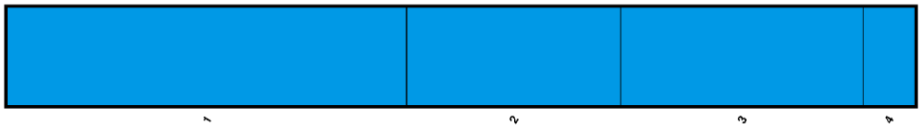
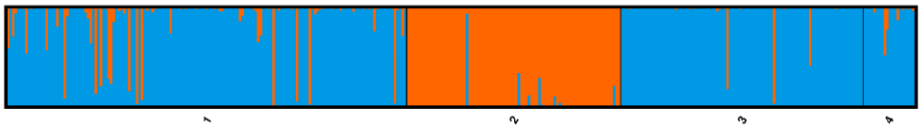



Figure 4A: 11: MedMeaK, MaxMeaK, MedMedK, MaxMedK method by Puechmaille (2016) in which the best estimate of *K* clusters is estimated to be 3.

K=110/10, Mean(LnProb) = -16608, Mean(similarity score) = 1.000



K== 210/10, Mean(LnProb) = -16045, Mean(similarity score) = 0.997



K=310/10, Mean(LnProb) = -15774 Mean(similarity score) = 0.996

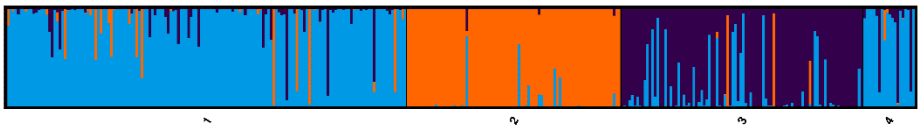


Figure 4A: 12: Clumpak-generated plots for STRUCTURE analysis using HGDP-CEPH populations assuming No Admixture model.

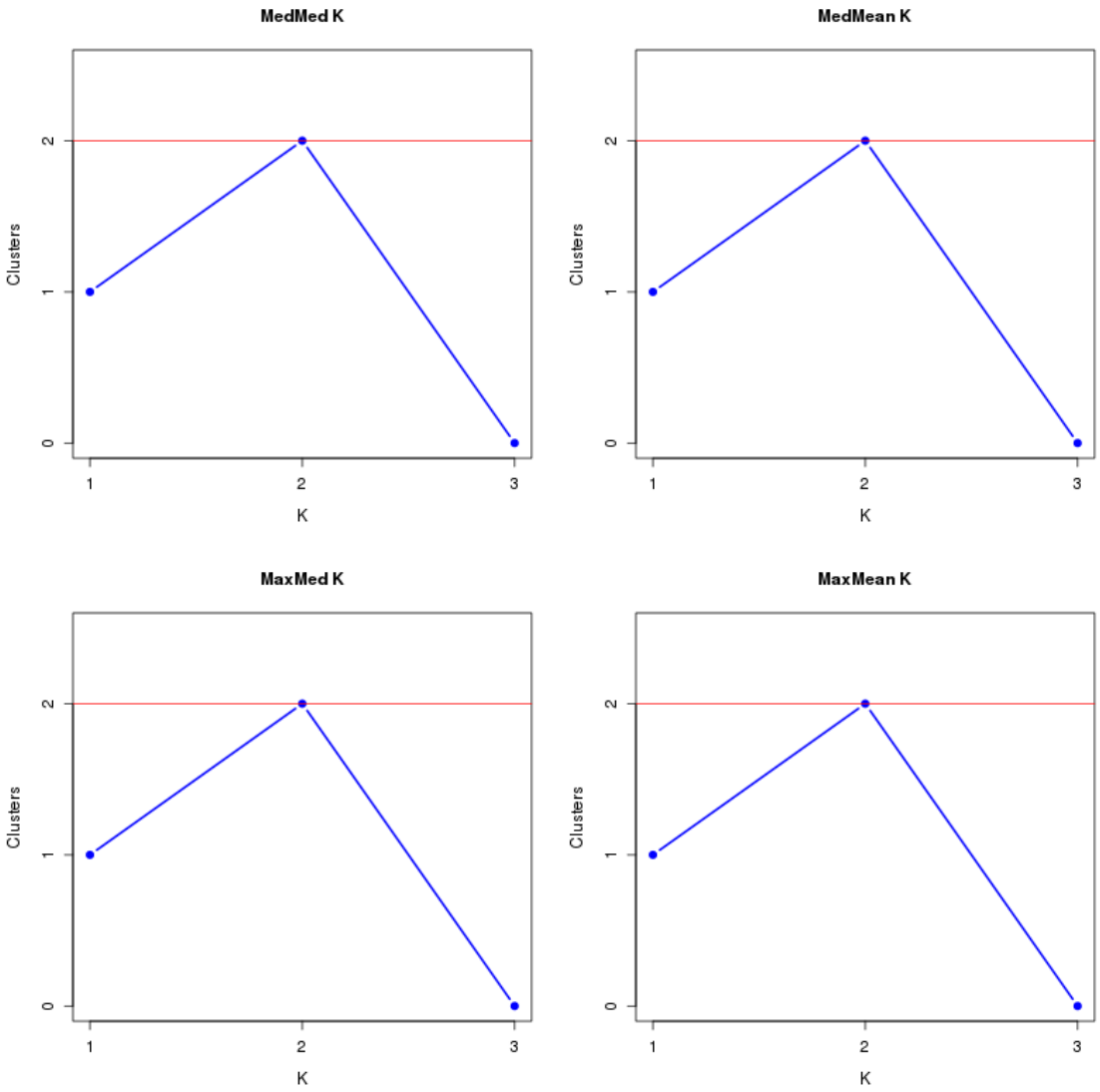
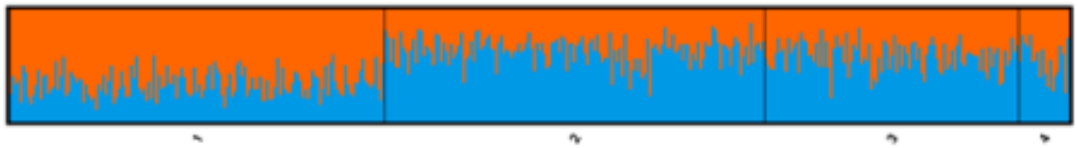



Figure 4A: 13: MedMeaK, MaxMeaK, MedMedK, MaxMedK method by Puechmaille (2016) in which the best estimate of *K* clusters is estimated as 2.

K=110/10, Mean(LnProb) = -19741, Mean(similarity score) = 1.000

K=27/10, Mean(LnProb) = -19740, Mean(similarity score) = 0.99



**K=3**10/10, Mean(LnProb) = -19946, Mean(similarity score) = 0.979

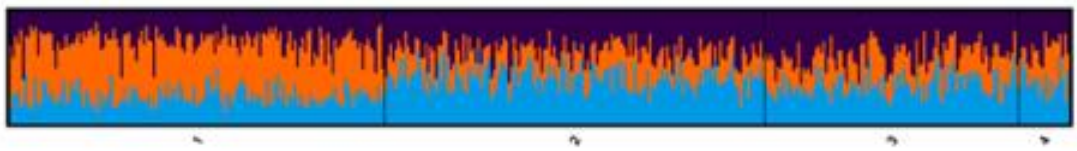


Figure 4A: 14: Clumpak-generated plots for STRUCTURE analysis using a subset of the NIST population database assuming an admixture-based model.

# BRIDGING STATEMENT

Chapter 4 provides a comprehensive view of a case study in degraded DNA, detailing sample collection, storage, DNA extraction, quantification, and short tandem repeat (STR) typing. Chapter 4 builds on the methods outlined in Chapters 1-3 and also highlights many of the challenges of degraded DNA work that were presented in the aforementioned chapters. Drawing from the conclusions of Chapter 4, the role of impact of date of death on DNA quality is emphasized, highlighting the need for attention to sample collection. Recommendations for storage of cadaveric blood samples are also presented. The ancestry prediction model used in Chapter 4 provides an additional validation study of the utility of STR data for geographic estimation as shown by Algee-Hewitt in 2016.

Chapter 5 summarizes the research presented in the previous four chapters. The chapter reiterates the similarities between the fields of ancient DNA and forensic genetics and the implications of these uniting characteristics. Building upon the validation studies outlined in Chapters 3 and 4, the shared issues of working with degraded samples are emphasized. Faced with the same issues of degradation, contamination risks, and inhibition, the prediction of a convergence between methods in ancient DNA research and forensic genetics explored in Chapter 2 is further solidified.

# CHAPTER 5: CONCLUSION

The widespread proliferation of next-generation sequencing (NGS) technologies has prompted the critical reevaluation of the prevailing methods used in these fields. While forensic geneticists still largely depend on short-tandem repeat (STR) typing for individual identifications, NGS approaches are increasing (Butler 2015). In contrast, work in ancient DNA analysis today almost exclusively uses NGS approaches, which are ideal for the small fragment sizes characteristic of archaeological samples (Shapiro and Hofreiter 2014).

While NGS has replaced traditional Sanger sequencing methods in ancient genomics, the implications of this transition to high-throughput platforms reach beyond the mere sequencing of genetic material. The move toward NGS platforms has influenced aspects of decontamination protocols, extraction methods, amplification processes, and, perhaps most significantly, analysis. The emphasis of decontamination protocols has expanded the discourse from the now standard concerns over human exogenous contamination to the need for also reducing exogenous microbial contamination (Korlević et al. 2015). NGS approaches also present the opportunity to remove sequenced contaminants bioinformatically (Skoglund et al. 2014) as well as pre-emptively even with traditional chemical-based decontamination methods (Kemp and Smith 2005).

Ancient and forensic DNA analysis depends on successful extraction of DNA. Without effective and, at times, optimal recovery of high quantity and quality DNA from characteristically difficult sample material, subsequent procedures including amplification, traditional Sanger sequencing as well next-generation sequencing cannot be fully, if at all, executed.

Today, amplification of degraded DNA involves library preparation methods being able to harness single-stranded as well as double-stranded DNA within a sample and make it available for sequencing on NGS platforms (Glocke and Meyer 2017). Prepared libraries can be sequenced directly on NGS platforms through shotgun sequencing, a method in which all fragments, both from the sampled individual and from microbial DNA, tagged with platform-specific amplicons are sequenced. With NGS kits, prepared libraries can then be subjected to targeted capture of thousands to millions of fragments, both of STRs and, more commonly, SNPs.

Analysis methods have co-evolved with new NGS methods. While traditional Sanger sequencing and capillary electrophoresis methods produce rather straightforward results that can be analyzed using individual electropherograms, NGS approaches produce multiple sequences of the same target fragment, for thousands of targets, during each run. This expanded data output requires the integration of computational methods into the wet lab, including bioinformatic analyses to parse the various sequences produced during NGS analyses. These bioinformatic approaches can also be used to authenticate ancient DNA (Skoglund et al. 2014) and make genetic sex determinations (Skoglund et al. 2013).

This dissertation has focused on several important issues affecting forensic and ancient DNA research, including sample storage, sample preparation, extraction, fragment analysis, and data analysis. Extraction techniques for ancient DNA contexts were explored in Chapter 2, including some discussion on the implications of their use in forensic genetic applications. In Chapter 2, the history of DNA extraction techniques for

ancient DNA were outlined, from the earliest methods to modern cutting-edge approaches. Beginning with the earliest approaches to DNA isolation, the evolution of DNA isolation procedures is explored, including explanations of each step in the process as well as an overview of common extraction reagents. Through understanding the various approaches to extraction, including phenol-chloroform (Hanni et al. 1994; Loreille et al. 2001), silica-based (Yang et al. 1998; Yang et al. 2004; Rohland and Hofreiter 2007; Dabney et al. 2013), and magnetic bead-based extraction (Zhao et al 2018) and the ways each use different reagents and chemistry to isolate DNA, more informed selections can be made during the project planning process (Gamba et al. 2016).

DNA extraction methods are essential to a successful research design, and Chapters 3 and 4 provide recommendations for laboratory analysis. Chapter 3 tested non-destructive DNA extraction methods on modern samples, demonstrating that DNA can be recovered from minimally-destructive extraction protocols. Chapter 3 presented a successful extraction of DNA using a technique that is minimally destructive but requires expensive and dangerous quantities of guanidine thiocyanate that also involves co-extraction of inhibitors. Chapter 4 characterized the effect of post-mortem interval on DNA quality in cadaveric blood cards. The results recommend the use of FTA-based blood cards for long-term storage.

Lastly, Chapter 4 showed that STR typing is possible on untreated cadaveric blood samples that had been stored up to four years, despite some signs of DNA degradation. The literature review in Chapter 2 provides recommendations for DNA storage and extraction strategies. For tooth samples, the survey of extraction techniques

in ancient DNA research reveals the best practice is to sample petrous portions or cementum, depending on preservation of the respective elements.

A thorough understanding of the various aspects of extraction and roles of chemical reagents is key for making informed decisions regarding extraction method choice. Various sample substrates were explored, from skeletal material to calculus, as well as more unusual substrates, including parchment and soil. In addition to the mechanics of DNA isolation and sample substrate type, the selection of extraction techniques relies on a complex set of factors, including sample source (geographic location and climate, burial context, archaeological age, individual developmental age, and taphonomic factors), downstream analyses, number of samples, research budget, as well as the needs and expectations of descendant groups, collections managers, and other stakeholders.

Other special considerations for ancient DNA analysis were also explored, including contamination and DNA authentication, both using traditional sequencing methods as well as the combination of next-generation methods with bioinformatic approaches (Skoglund et al. 2014). Attention is required when selecting skeletal material to be sampled for destructive analysis; as recommended in Chapter 2 the current literature supports sampling strategies that privilege the petrous bone (Pinhasi et al. 2014) as well as tooth cementum (Hansen et al. 2017), both of which yield the highest amounts of DNA when compared to all other elements from archaeological contexts. However, the chapter highlighted the need for a more systematic sampling of ancient skeletal remains to increase understanding of DNA yields, as has been explored previously in forensic

genetic approaches (Mundorff and Davoren 2014). Not only should multiple individuals be systematically sampled, but studies must also integrate skeletal material from differing climates and time periods. The importance of sampling strategies is also addressed in regard to destructive and non-destructive methods, to be determined by the needs of the descendant communities as well as custodial stakeholders.

Chapter 3 provided a validation study of a DNA extraction technique developed for use in contexts which require maintaining the complete integrity of the sample, a non-destructive method proposed by Bolnick and colleagues (2012). Here, the Bolnick extraction technique was shown to be successful in recovering both mitochondrial and nuclear DNA from both ancient samples as well as forensic-age samples. An additional extraction method was also validated using the Bolnick and colleagues' buffer soak, demonstrating that aspects of this protocol may be used in combination with other protocols (Kemp et al. 2012) that may be more customized to the downstream analyses and budgetary considerations of varying research questions.

Examination of the tooth samples before and after exposure to the soaking buffer revealed a lack of significant differences in tooth dimensions but also showed that the exposed teeth were reduced in weight after the buffer soak. This finding prompted the need for more intensive examination of the microstructural impact of the buffer soak on human tooth samples using multiple angles to assess damage.

Chapter 3 also offers more insight into the impact of the Bolnick non-destructive DNA extraction technique on forensically relevant and ancient teeth. Using histology, isotope analysis, and scanning electron microscopy (SEM), this research explores the

effect of this non-destructive technique to determine the extent of damage to tooth microstructure. While isotope analysis shows that there is no significant difference in isotope signatures before and after exposure to the Bolnick protocol buffer soak, differences are observed through both histological and SEM analysis.

Using a histological approach, the study demonstrates a difference between damaged areas in tooth samples that had been soaked versus not soaked. In total, the treated samples exhibit increased root and enamel damage over the untreated samples. The histology results reveal distinctive patterns associated with increased damage to microstructure by exposure to the soaking buffer and revealed almost twice as much damage in the treated teeth. SEM analysis also reveals damage to the surface of the teeth exposed to the soaking buffer, most notably staining of the enamel. Staining is especially prominent in areas with pre-existing damage and it is uncertain as to whether the staining is permanent or temporary. Based on these analyses, we determine that the Bolnick protocol should be re-categorized as a "minimally-destructive" protocol and treated accordingly.

The results from this validation research illustrate the possibilities of for non-destructive DNA extraction protocols in situations where some microstructural damage may be acceptable in exchange for genetic information obtained while maintaining macroscopic integrity of the tooth sample. While the Bolnick method produces DNA yields sufficient for amplifying mitochondrial and nuclear DNA using traditional PCR methods, researchers must weigh the costs and benefits of such protocols. Perhaps most importantly, consideration for the individuals being sampled and the descendant

communities of those individuals must be paramount. Minimally-destructive protocols hold promise for a variety of applications and may provide an alternative to destruction of human biological materials; however, this research has demonstrated that these approaches do introduce damage on a microstructural level. While this may open some samples to analysis, this is an informed decision that must be made by collections personnel and descendant communities and researchers based on the information provided in Chapter 3.

Similarly, considerations for future genotyping needs must be made when selecting long-term sample storage products for biological collections, as shown in Chapter 4. Using blood collected from 20 donors, post-mortem, and stored between 4 months and 4 years on untreated blood cards, this research shows that DNA yields and quality are sufficient for generating a full STR profiled using the Applied Biosystems Identifiler kit, with lab work performed by Bode Cellmark Forensics. Comparisons of relative fluorescent units (RFUs) between STRs separated into groups by size demonstrate a decline in fluorescence with increasing fragment length. This decline in amplification, as shown through RFUs, is indicative of DNA degradation, as fewer fragments of large size were available for amplification during PCR. In contrast to FTA (Flinders Technology Agreement) cards which lyse cells and deactivate nucleases upon sample deposition, the untreated blood cards used in the study allow nuclease activity to continue during storage. This may be the source of some DNA degradation observed in this case study.

The STR profiles generated on the 20 donors were assessed for trihybrid ancestry estimation using unsupervised clustering models in STRUCTURE (Pritchard et al. 2000). Known continental ancestry groups from the Human Diversity Genome Project (HDGP-CEPH) populations were set as parental populations assessed against unknown samples. Ancestry results were compared to self-reported ancestry and show agreement in 17 of 20 individuals. Admixture analysis was also conducted in STRUCTURE using a trihybrid subset of the NIST population database, displaying mixed levels of tri-continental ancestry, as may be expected in a U.S. population (Bryc et al. 2015). The application of ancestry/admixture analysis to the profiles generated in Chapter 4 demonstrate the utility of STR markers beyond identification, as demonstrated by Algee-Hewitt and colleagues (2016).

The selection of DNA storage materials and extraction methods requires deliberate deliberation of multiple factors, including sample type, source, research plan, budget, and subsequent analyses. Inappropriate selection of these factors can result in low extraction yields that produce no results or insufficient results. In these cases, valuable research dollars and time are spent to generate inconclusive data and, most significantly, waste precious sample material, thus attention to both sample storage substrates and extraction techniques should be a research plan and budgetary priority. Perhaps most importantly, sample preparation and extraction of DNA necessitates consideration of the cultural context of the research in order to meet the needs and expectations of the various stakeholders by using the least destructive methods to generate the highest quality data possible. Through combining responsible sample selection and processing with a

deliberate assessment of current and effective protocols that are most appropriate for target samples, researchers can obtain optimal results for downstream analyses, including next-generation sequencing.

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# VITA

Frankie Lane West was born in Waco, Texas and raised in Ben Hur, Texas, the oldest of three children. She is the daughter of Jill Johnston and Franklin Joseph West. Frankie attended Mart High School and graduated in 2000. She received a Bachelor's in History with a French minor from Baylor University in 2004. She worked as a museum professional at the Mayborn Museum Complex, Jeanes Discovery Center and Strecker Natural History Museum for five years following graduation with her undergraduate degree. Frankie moved to Knoxville, Tennessee to attend graduate school at The University of Tennessee (UTK), in Knoxville in 2009. She has taken part in a variety of research projects while at UTK, ranging from work in the Molecular Anthropology Laboratories and the Forensic Anthropology Center to working with individuals with HIV in Southern Appalachian in collaboration with the School of Nursing. She will join the faculty at Western Carolina University as an Assistant Professor of Forensic Genetics in January 2019.