MITOCHONDRIAL DNA ANALYSIS OF ANCIENT HAIR SHAFTS FROM THE DAKHLEH OASIS, EGYPT:

CHALLENGES AND RECOMMENDATIONS

BY

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ABSTRACT

To date, there have been no previous reports in the literature on DNA extraction or analysis of ancient human hair. The hair shaft has an extraordinarily stable structure that enables it to remain unchanged for centuries and persist in the archaeological record. Hair shafts, in theory, should provide one of the best archives of mitochondrial DNA, since hair is well protected by the cuticle layer (anhydrotic) and is not subject to postmortem DNA destroying autolytic enzymes. In this thesis, the latest extraction and purification methodologies were tested on modern human and ancient Egyptian hair shafts. In addition, closer attention was paid to the structural components of hair, particularly keratin. PCR amplification of DNA retrieved from the ancient Egyptian hair shafts was limited in success. PCR inhibitors that co-purified with DNA extracted from the ancient hair shafts contributed to PCR failure. A variety of analytical techniques were used to identify the presence of PCR inhibitors present in the DNA extracts including Gas-chromatography-Mass spectrometry (GC-MS), Secondary Ion Mass Spectrometry (SIMS), Raman Spectrometry and Induced Coupled Plasma (ICP) Spectrometry. Real-time PCR proved to be pivotal in the analysis of DNA from ancient Egyptian hair shafts. The preliminary Real-time PCR results indicate that mtDNA is present in low yet analyzable levels in ancient human hair shafts from two Kellis burials (K2 124, G10-3). The use of Real-time PCR with the Gilbert *et al* (2004) method holds the greatest potential for future ancient DNA research.

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INTRODUCTION

PART I.

Statement of Problem

In recent years there has been much debate on the survivability of ancient DNA in human tissues from ancient Egypt, with Marota *et al* (2002) and Gilbert *et al* (2005) arguing 'against' and Zink and Nerlich (2003) arguing 'for' its preservation. The latter cite successful molecular studies that followed the stringent protocols necessary to authenticate the presence of ancient DNA (Paabo *et al,* 2004). Recent publications on mitochondrial DNA (mtDNA) extracted from human skeletal remains from the Dakhleh Oasis, Egypt (Graver *et al,* 2001; Parr, 2002; Molto, 2002), have supported the arguments of Zink and Nerlich (2003), although the results have been sporadic and often not verifiable. The interpretations are necessarily provisional because of this. A recent investigation of molecular sexing methods using skeletal remains from ancient Dakhleh has also shown that obtaining interpretable nuclear DNA in the ancient Egyptian material is problematic, though the extracts seem to contain authentic ancient human DNA (Hildebrandt, 2003). A number of recommendations have followed from these studies including the use of alternative tissue samples (Hildebrandt, 2003) since, to date, the Dakhleh research has primarily used bone samples because of their abundance and excellent histological preservation (Graver *et al,* 2001; Parr, 2002).

Following the latter recommendation, this thesis tests the efficacy of obtaining interpretable ancient mtDNA from human hair shafts from the late Roman Period sample from Kellis 2, Dakhleh, Egypt, using multiple experimental methodologies. Hair remains

are abundant in this sample (Molto, 2002) and, as noted, many of the burials have already yielded provisional mtDNA results from bone (Graver *et al,* 2001; Parr, 2002). The hypothesis investigated in this thesis is that hair should be a better archive of ancient mtDNA than bone and dentin and soft tissues. This hypothesis derives from fundamental aspects of hair biology. First, since the hair shaft is outside the body it theoretically is not impacted by the perimortem apoptosis (i.e. autolytic enzyme lysis) experienced by living tissues, like bone and dentin. Secondly, the hair shaft is nonliving, avascular and protected against moisture (anhydrotic) and other environmental DNA destroying agents by its cuticle layer, while the mtDNA is bound and thus protected by the keratin found in the cortex of the hair shaft. Part of the research design involves the use of the enzyme keratinase to determine if the yields of mtDNA can be increased. Currently, there are four main DNA hair extraction protocols available and these are tested herein in conjunction with this new enzyme methodology.

To date, no ancient (=non-historical) human DNA research has utilized hair, in part because hair is not commonly found in most mortuary samples. However, in a recent study by Bonnichsen *et al* (2001), mtDNA was successfully extracted and amplified from hair in a big horn sheep dated at 9800 years B.P. In another study by Gilbert *et al* (2004) DNA was extracted from the hair of a bison dated at 64,800 years B.P. These studies and the fact that hair is a common source of evidentiary DNA in active and cold forensic cases (Wilson *et al,* 2001) supports the possibility that ancient Dakhleh hair remains should yield interpretable DNA results.

The thesis is organized as follows. Part II provides a detailed description of the basics of hair biology that is fundamental to the hypothesis. Parts III and IV respectively

provide a historical overview and description of the methodologies used in DNA research in hair. Part V describes the material and methods (research design). Parts VI and VII provide the results and interpretation respectively, followed by conclusions and recommendations arising from the research (Part VIII).

PART II.

The Human Hair Shaft:

A POTENTIAL ANCIENT ARCHIVE OF GENETIC INFORMATION

As noted, an understanding of hair shaft biology is a necessary first step in developing appropriate DNA extraction methodologies. Even forensic laboratories recommend microscopic examination of hairs prior to DNA testing (Linch *et al*, 2001)

Hair Shaft Development

Hair is a complex biological structure that consists of both living and dead tissue, the hair follicle and the hair shaft, respectively. The hair follicle is located deep within the scalp, extending from the epidermis into the dermis. The hair shaft is an appendage that begins within the hair follicle. The average number of hair follicles present in human scalp is approximately 100,000 (Gray *et al*, 1997). This number is permanently established by 20 weeks gestation (Olsen *et al*, 1995). Each hair follicle is capable of self-regeneration, and may grow up to approximately 30 new hair shafts in an individual's lifetime (Linch *et al*, 2001).

The hair shaft is produced by mitotic activity within the hair bulb matrix that is directed by the dermal papilla (Figure 1). Pre-germinal cells within the hair bulb matrix differentiate into three types of cells, medullary, cortical and cuticular that each will migrate distally towards the epidermis. As the cortical cells migrate, they engulf the dendritic processes of melanocytes that contain melanin and mitochondria. Melanocytes are differentiated cells that exist alongside the pre-germinal cells within the hair bulb matrix. The main function of melanocytes is to produce melanin, pigment grains that

provide hair with its colouring. It is hypothesized that this event occurs because directly after cell differentiation cortical cells require a multitude of mitochondria (energy) to facilitate intense protein production (Linch *et al*, 2001). During their migration, the cuticle, cortical and medullary cells undergo the processes of dehydration and keratinization, and finally arrange into concentric layers emerging through the epidermis as the definitive hair shaft (Powell, 2002).

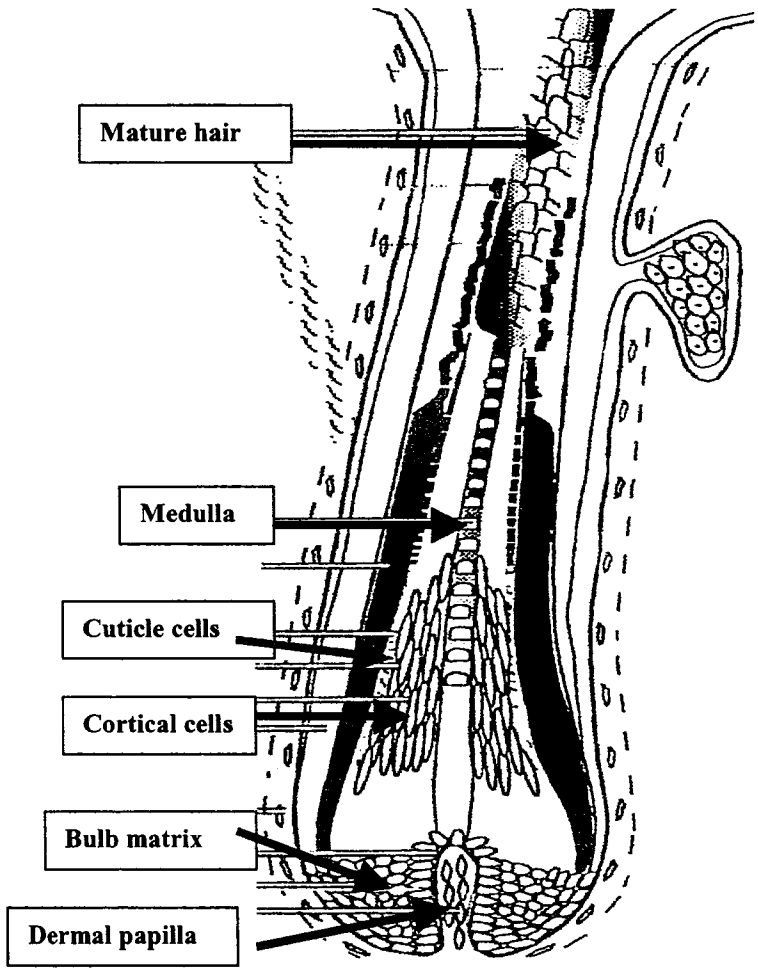

Figure 1. The histological structure of the human hair. Modified from 'Forensic Examination of Hair' edited by James Robertson, 1999.

The Mature Hair Shaft

The human hair shaft possesses three structural components, the cuticle, the cortex and the medulla. The cuticle consists of flat overlapping cells that cover and surround the entire hair shaft. The cortex forms the bulk of the shaft and contains pigment granules that determine the colour of hair. The medulla is centrally located within most hair shafts and consists of loosely packed disintegrating cells (Robertson, 1999). The cell membrane complex (CMC) is a natural adhesive that binds the cells that form the structural components together. (Robbins, 2002).

Approximately 80% of the hair shaft is comprised of keratin. Keratin is an insoluble protein present at high levels in hair, feathers, scales and the stratum corneum. Cells of the cortex and cuticle are composed almost entirely of keratin fibrils embedded in an amorphous matrix of cellular debris. (Wittig, 1982; Olsen, 1995). Unlike cortical or cuticular cells, medullary cells contain a minimal amount of structural protein. Keratin's supercoiled alpha-helical structure and intermolecular disulfide bonds provides the hair shaft with its strength and resiliency (Wittig, 1982).

The Hair Growth Cycle

The hair growth cycle consists of three stages (1) anagen phase (growth phase) (2) catagen phase (intermediate phase) and, (3) telogen phase (shedding phase). The stages of hair growth are primarily controlled by the influence of androgens (Jolles *et al*, 1997). The first phase of the hair growth cycle, the anagen phase, is characterized by high mitotic activity within the hair bulb matrix. During this phase, the hair shaft grows at a

rate of approximately 1cm per month (Gray *et al*, 1997). Mitotic activity within the hair bulb slows down significantly during the catagen phase. During the catagen phase, the hair bulb matrix begins to shrink and undergo apoptosis. In the telogen phase, hair growth terminates and the hair is eventually shed. As the telogen hair reaches maturity, a new anagen phase hair begins to grow beneath, pushing the telogen hair out, ultimately replacing it once it has been shed and thus ending the growth cycle (Jolles *et al*, 1997). Typically, at a given time approximately 10% of scalp hairs are in the telogen or shedding phase (Gray *et al*, 1997). An individual typically sheds 100 to 150 scalp hairs per day (Powell *et al*, 2002). Consequently, hair shafts are often valuable sources of biological evidence found during crime scene investigations.

DNA and the Hair Shaft

There are two types of DNA found within hair, nuclear DNA and mitochondrial DNA (mtDNA). Hairs with roots or adhering follicular tissue contain both nuclear DNA and mtDNA. Shed hairs contain a very minimal amount of nuclear DNA that is highly degraded and typically less than 100bp in size (Matsuda *et al*, 1997). The nucleus and its genome are degraded during keratinization. Conversely, the mitochondria and its genome are able to survive the keratinization process due to the protective nature of this organelle's membrane. Mitochondria can commonly be visualized amongst the keratin fibres within the cortical cells in the mature hair shaft (Linch *et al*, 2001).

Mitochondrial DNA is also more likely to be found within the hair shaft than nuclear DNA due to its multi-copy nature. MtDNA is present in as many of 1000 copies per cell whereas nuclear DNA is only available in single copy. Consequently, mtDNA is a much more suitable candidate for genetic analysis of fragmented and/or shed hairs.

The mitochondrial genome is circular in nature, maternally inherited (Hutchinson *et al*, 1974) and significantly smaller in size (16,569bp, Anderson *et al*, 1981) than the nuclear genome (Figure 2). The non-coding (control) region of the mtDNA genome is highly polymorphic with a mutation rate 5 to 10 times higher than the nuclear genome (Greenberg *et al*, 1983). Analysis of the non-coding region is useful in forensics for individuation purposes if a sample from a maternal relative is available for comparison and is also valuable in the study of past populations providing information on familial relationships and ethnicity.

A condition known as heteroplasmy is known to occasionally exist in hair shafts (Budowle *et al*, 2003). Heteroplasmy is a condition wherein a tissue contains more than one population of mitochondria. It is hypothesized that heteroplasmy may exist within the hair shaft as a result of one population of mitochondria originating from the bulb matrix and another originating from the melanocytes (Linch *et al*, 2001). Because of the developmental nature of hair, heteroplasmy is hypothesized to be more common in hair than any other tissue although it is still uncommon.

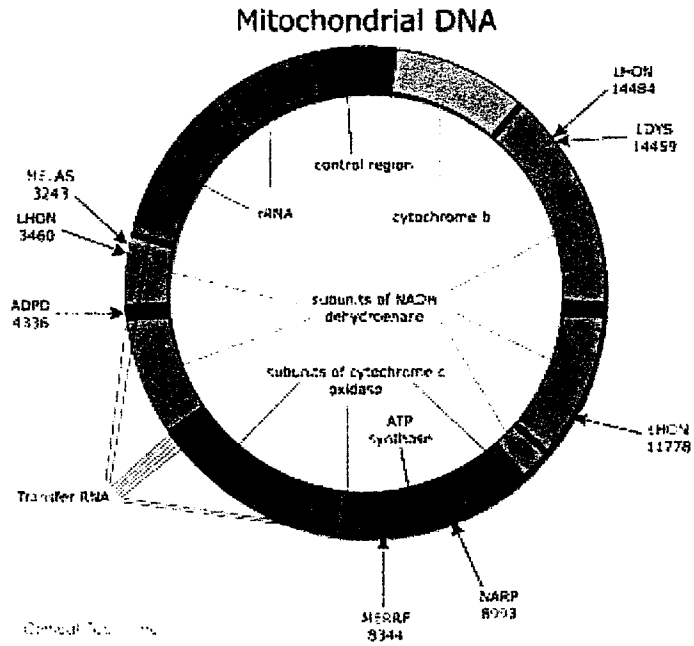

Figure 2. The mitochondrial genome

Hair Survival in the Archaeological Record

The hair shaft has an extraordinarily stable structure that enables it to remain unchanged for centuries and persist in the archaeological record (Massa *et al, 1980;* Lubec *et al,* 1987; Powell *et al,* 2002). As an example, X-ray defraction of a hair specimen from China nearly two millennia old revealed that the alpha-helical content was almost intact (Robbins *et al,* 2002). However, hair shaft survival in the archaeological record is greatly dependent on its surrounding environment. Hair is most likely to survive in hot and arid environmental conditions (e.g. Egypt, American Southwest). A burial microenvironment that is anoxic and rich in metal ions will also increase the likelihood of hair survival by retarding the growth of microorganisms (Bonnichsen *et al,* 2001).

The main constituent of the hair shaft, cysteine-rich keratin, provides hair with its resistance to degradative forces (e.g. water, UV radiation). Cysteine forms extremely stable disulphide cross-connections that provides hair with its structural resiliency (Wilson *et al*, 2000). However, if keratinolytic microorganisms and insects (e.g. fungi, beetles) are present in the microenvironment, they may alter and/or damage the hair shaft (Haglund *et al*, 1997). Fungi are ubiquitous in the environment and have the ability to damage hair. Fungal hyphae can penetrate the cuticle and tunnel through the hair shaft compromising the internal structure of the hair shaft. A histologically well-preserved cuticle does not preclude that the internal hair shaft structure has not suffered damage (Wilson *et al*, 2000).

The integrity of the hair shaft can also be affected by pre- and postmortem cultural practices, such as cosmetic treatment and mummification, respectively. These cultural practices may result in the disruption of the internal chemistry of the hair shaft (Lubec *et al,* 1997).

The Hair Shaft: An excellent archive of ancient DNA?

Hair found in the archaeological record and in forensic contexts could be valuable archives of DNA. DNA survival has been reported in bison hair that is approximately 64 800 years old (Gilbert *et al*, 2004). This date coincides with the oldest DNA retrieved from bone, teeth or soft tissue. Conditions within the hair shaft are quite favorable for DNA preservation. Due to the structure of the hair shaft, DNA is inherently protected from hydrolysis and autolysis. Hydrolysis is a major cause of DNA damage in ancient specimens. Hair cells are essentially dehydrated, thus DNA within hair escapes internal hydrolytic damage (e.g. cleavage of phosphodiester bonds and/or base linkages) (Gilbert

et al 2004, Haglund *et al* 1997). DNA within hair is protected from damage induced by exogenous water due to its hydrophobic protein structure. The hair shaft is avascular, thus, it is also not subject to the apoptotic damaging effects of post-mortem autolytic enzymes that most other DNA archives (e.g. bone, soft tissue) encounter.

Shortly after death, microorganisms proliferate and decompose bodily tissue. However, the cuticle acts as a barrier to protect DNA within the hair shaft from microbial digestion. If DNA is protected during the initial onslaught of biological and environmental activity immediately after death, it has the potential to exist indefinitely. There are several benefits for using hair as a preferred source of ancient DNA. Modern DNA contamination is a great challenge faced in the realm of ancient DNA studies. However because of the resilient hydrophobic structure of the cuticle that is in part due to the protein keratin, the hair shaft can be easily decontaminated of exogenous DNA sources. On the contrary, bone and soft tissue are not easily decontaminated due to their porous structure. Another added benefit of using hair shafts instead of other tissue types for mtDNA analysis is that hair contains minimal nuclear DNA. Consequently, the risk of unintentionally amplifying nuclear copies of mitochondrial genes is insignificant (Gilbert *et al*, 2004). An obvious benefit of using hair as a choice source of ancient DNA (i.e. instead of bone, teeth or soft tissue) is that it can easily be sampled and reduces the potential destruction of precious archaeological specimens.

Nonetheless, like other sources of DNA, hair is an excellent ion exchange system. Metallic ions and organic contaminants can absorb into hair from the burial microenvironment (Robbins *et al*, 2002). Contaminants present in the hair shaft can potentially make DNA analysis challenging. Organic and inorganic contaminants present

within the hair shaft can cause PCR inhibition and impact the genetic information that exists within the hair shaft (Wilson *et al*, 2000).

PART III.

A Historical Overview of the Extraction of DNA from Hair Shafts

Over the past two decades, a variety of methods and technologies have been successfully used to extract mtDNA from hair shafts (e.g, Scheiber *et al* 1988, Kable *et al* 1988, Higuchi *et al* 1988, Yoshii *et al* 1992, Wilson *et al* 1995, Vigilant 1999, Baker *et al* 2001, Hellmann 2001, Heywood *et al* 2003). Because of the continuing developmental status of these methodologies it is prudent to provide an historical overview and critique outlining the advantages and disadvantages of each extraction protocol. By so doing the theoretical foundation of this thesis will be established.

In the late 1980s three laboratories, independently established the presence of high molecular weight mtDNA in hair shafts (Scheiber *et al* 1988, Kalbe *et al* 1988 and Higuchi *et al*, 1988). Schreiber *et al* (1988) used the following extraction procedure: lyophilized hair was powderized using a micro-dismembrator (50 Hz) and subsequently incubated overnight in a phenol lysis buffer (sodium acetate, sodium dodecyl sulfate (SDS), ethylene-diamine-tetra-acetic acid (EDTA)). The extract was then treated with chloroform/isoamyl alcohol (25:1), and the DNA was recovered by precipitation with ethanol. Approximately 5 pg of high molecular weight DNA could be obtained from one hair shaft. However, the possibility that some of the isolated DNA was from skin flakes and/or dandruff on the hair surface could not be precluded. It has been previously reported that skin flakes and dandruff can exist in the lipid rich epidermal detritus (sebum). Kalbe *et al* (1988), digested hair shafts in an extraction buffer (EDTA, SDS, sodium chloride, Tris-HCl (pH 8.5)) with proteinase K and dithiothreitol (DTT). The extract was treated with phenol/chloroform and DNA was recovered by precipitation with

ethanol. DNA yields from single hair shafts ranged from approximately 200pg to 20ng. Kalbe *et al* (1988) concluded that skin flakes adhered to the hair shaft may have obscured results. Higuchi *et al* (1988) isolated mitochondrial DNA (mtDNA) from only a 10cm single hair shaft. The hair shaft was digested in an extraction buffer (Tris-HCl, EDTA, NaCl) with proteinase K, DTT, and SDS. DNA was extracted with phenol-chloroform followed by *n*-butanol. The extract was purified using ultrafiltration (Centricon-30, Amicon). Polymerase chain reaction (PCR) was used to detect a 9bp deletion in the mtDNA control region. This deletion is indicative of Asian ancestry and the amplicon will be either 111bp or 120bp, depending on the presence or absence of the deletion. DNA yields from 10cm hair shafts were less than 10ng. The root end of freshly plucked hairs yielded as much as 0.5μg of DNA. These early studies pointed out a number of strengths and weaknesses involved in the DNA extraction process including the possibilities of intrinsic inhibitors.

Yoshii *et al* (1992) found that water soluble-melanins are inhibitors of DNA polymerase and DNAses. Short amplicons (331bp) from the mtDNA D loop were successfully amplified from single hair extracts. Size exclusion chromatography (SEC) columns removed water soluble melanins from the hair extracts. DNA extracts not purified using size exclusion chromatography columns resulted in PCR inhibition. The use of size exclusion chromatography is significant since melanin is abundant in hair. Though not stated by Yoshii *et al* (1992), white hairs should in theory provide higher yields of DNA than pigmented hairs in the absence of SEC, an hypothesis that is entirely testable by using white hairs and pigmented hairs from the same individual.

Wilson *et al* (1995) developed a method for the extraction of mtDNA from hair shafts. This protocol, which is the standard for all forensic laboratories, involves physical crushing of a 2cm fragment of hair shaft using glass micro-tissue grinders. The ground shaft is incubated overnight in an extraction buffer (Tris-HCl (pH 8.0), EDTA) with proteinase K. Subsequently, the extract is treated with phenol chloroform isoamyl alcohol (25:24:1). The aqueous layer is then purified using microfiltration (Microcon-100, Millipore). Two small regions of the control region of the mitochondrial genome, Hypervariable Regions I (16024-16365) and II (73-340), were successfully amplified and analyzed. Clearly, Wilson *et al* (1995) were influenced by the previous methods although they did not incorporate SEC in their protocol.

Vigilant (1999) evaluated four techniques for their effectiveness for the isolation of DNA from naturally shed chimpanzee hairs. The 4 different isolation methods included (1) organic extraction subsequent to proteinase K digestion (Higuchi *et al*, 1988) (2) Qiagen tissue kit purification (protease digestion followed by DNA purification and isolation using a silica-gel based spin column) (3) Chelex ionic bead resin treatment with and without accompanying proteinase k digestion and (4) Proteinase K digestion in a PCR-compatible buffer. The most successful method was the simplest, proteinase K digestion without further purification. Though not stated, the implication of the latter is significant when dealing with degraded evidentiary or ancient DNA samples since the more steps involved carries with it the inherent problem of contamination risks.

Baker *et al* (2001) successfully retrieved mtDNA from hair shafts using a silica-based extraction method. Hair shafts were sampled from recently deceased individuals suffering from various stages of decomposition (drowned, burned, and putrefied). DNA

was isolated from a 2cm segment of hair shaft. The hair shaft was ground and subsequently incubated in an extraction buffer (GuSCN, Tris-HCl, EDTA, Triton X-100). DNA was isolated from the crude extract using a *GeneClean* DNA Purification Kit. One microlitre of the 30μL extract was sufficient for PCR amplification. Baker *et al* (2001) provided evidence that a silica-based approach may be a viable alternative to organic solvent methods.

Hellmann (2001) amplified short nuclear DNA short tandem repeat (STR) loci, (HUMFES, HUMTHO1, HUMTPOX) using 20mm of hair shaft. The hair was digested using an extraction buffer (Tris-HCl, NaCl, $CaCl_2$, SDS and DTT). The DNA was isolated using phenol/water/chloroform followed by purification with ultrafiltration (Microcon-100, Millipore). They reported that the substitution of $CaCl_2$ for EDTA significantly increased the efficiency of hair digestion (enzymatic activity of *proteinase K* is controlled by calcium). Evidence was provided that nuclear DNA does exist within the hair shaft, but due to the keratinization process it is highly fragmented. Only very small fragments less than 110bp in size can be amplified by PCR. Conventional STR loci employed by the F.B.I. for human identification range from 106bp to 350bp (mean=250bp) in size. Consequently, in forensic casework amplification of conventional STR loci from hair shaft extracts is unsuccessful with current protocols.

Pfieffer *et al* (1999) discovered that hair from different parts of the body (head, pubic, axillary) varies in terms of DNA recovery. DNA yields are highest in head hair shafts followed by pubic and axillary hair shafts, respectively. It was also reported that there was no correlation between DNA yields and gender.

Heywood *et al* (2003) assessed the effects of various hair treatments on DNA levels within the hair shaft and reported that variation in DNA levels exist between different hairs from the same head. Freshly clipped hair shafts were digested in an extraction buffer (Tris-HCl, EDTA, NaCl, DTT, SDS) with proteinase K. The extract was then treated with phenol then chloroform followed by precipitation with ethanol. The DNA extract was further purified using *Wizard DNA cleanup columns* (Promega) to remove water soluble melanins. DNA yields for single hair shafts were more than 0.4 ng. The *HLA-DQA1* locus (241bp) was amplified by PCR from the DNA extract. It was reported that in hair that was treated with permanent colour or excessively washed, DNA levels were significantly reduced.

As noted in the introduction, there have been only two previously published reports on the extraction of DNA from ancient hair shafts. Bonnichsen *et al* (2001) demonstrated that ancient DNA (aDNA) could be recovered from sheep hair. Using the Wilson *et al* (1995) protocol, mtDNA was successfully extracted from Paleo-American (7800 B.C.) Bighorn sheep (*Ovis canadensis*) hair shafts. However, only a very small amount of DNA was recovered. Also, DNA fragments (116bp) of the *cytochrome b* gene were amplified by PCR for speciation. This is the first authentication of the successful extraction of aDNA from hairs. The paper also notes the importance of the archaeological context of recovered remains. In 2004, Gilbert *et al* also reported successful extraction of DNA from ancient hair shafts. Small fragments (<234bp) of mtDNA were successfully amplified, cloned and sequenced from bison (*Bos bison*) and horse hair dating back to more than 65,000 years. Until very recently, there had been no published reports on the extraction of mtDNA from ancient or historical human hair. Gilbert *et al* (2004) reported

to have successfully extracted mtDNA from human hair approximately three centuries old using a modified version of the Wilson *et al* (1995) protocol. The hair samples were digested for 24 hours in an extraction buffer (Tris Buffer, SDS, NaCl, DTT and *N*-phenylacylthiazolium bromide (PTB)) with proteinase K. After digestion, the DNA was extracted using phenol:chloroform and then concentrated by centrifugal dialysis (Millipore, UK). A small fragment (147bp) from HVI of the mitochondrial genome was successfully amplified, cloned and sequenced. This marks the first study that illustrates the great potential of hair as an archive of ancient human DNA.

A number of interesting methodological results were reported in an unpublished MSc thesis (2003) by Ms Arlene Lahti. She reported that non-pigmented hair shafts had lower DNA yields than pigmented hair shafts. She postulated that this could be a result of the initial stage of the DNA extraction process. Grinding of the hair shaft is a critical preparatory step in the DNA extraction process. Because non-pigmented hair is difficult to visualize, it can be lost or insufficiently ground up during this step. Insufficient grinding of the hair may not fully release DNA bound in the hair shaft and may affect DNA yields. Poor DNA yields from non-pigmented hair may also be a result of solar degradation. Ultra Violet light has the ability to cross-link DNA making it unviable to PCR amplification and subsequent analysis (Riley, 1997). DNA in non-pigmented hair lacks the UV absorbent protection that melanin provides in pigmented hairs. This is of significance in forensic casework if sample selection between pigmented or non-pigmented hair is an option.

The uneven distribution of DNA within the hair shaft has also been previously reported. DNA levels are higher in the root-end of the hair shaft (Higuchi *et al* 1988,

Yoshii *et al* 1992, Heywood *et al*, 2003) than the tip-end of the shaft. Histologically, it is hypothesized that DNA resides within the cuticle of the shaft (Kalbe *et al* 1988, Heywood *et al* 2003), although, the cortex hypothetically should be the key area for the concentration of mtDNA. The cortex comprises the bulk of the hair shaft and it is protected by an external layer of resilient cuticular cells. To date there has been limited data published on the genesis of mtDNA in hair shafts, which is unfortunate since this could prove useful as our extraction methods mature.

PART IV.

Traditional and Non-Traditional DNA Isolation Methods and Technologies

The choice of a method for DNA isolation from hair shafts depends on many factors including the physical characteristics of the source, required DNA quantity, degree of purity required for downstream applications (e.g. PCR), as well as cost and time limitations. DNA extraction protocols should be designed to minimize the potential for contamination with extraneous DNA. This can be achieved by reducing the number of steps or manipulations in a DNA isolation procedure. In order to be effective, methods for the extraction of DNA from hair shafts should focus on purifying small amounts of DNA while simultaneously removing potential PCR inhibitors.

The isolation of DNA from cellular organelles is a triparate process: (1) cell disruption (2) cell lysis and (3) deproteination. The initial step in the isolation of DNA from hair shafts is to dissociate the cuticular, cortical and medullary cells. The hair shaft can be disrupted by physical maceration using a microtissue grinder (mortar and pestle), or by chemical treatment using an extraction buffer. Extraction buffers can be used to disrupt the extracellular matrix and intercellular junctions adhering the cells. Cell adhesion is highly dependent on Ca^{2+}. Consequently, extraction buffers typically contain a Ca^{2+} chelating/binding agent such as EDTA. Detergents such as sodium dodecyl sulfate (SDS) and Tween are also common components of extraction buffers. Detergents assist in the solubilization of cellular membranes. Guanidinium thiocyanate can also be included in an extraction buffer or be used on its own to disrupt cellular membranes. DTT is a reagent occasionally used in extraction buffers specific for hair. It reduces the disulphide bonds which give hair its rigid structure. In recent years, *N*-

Phenylacylthiazolium bromide (PTB), a proprietary organic compound, has recently been used in extraction buffers for DNA recovery from ancient specimens. PTB assists in the recovery of DNA from ancient materials by cleaving glucose-derived protein cross-links thereby releasing DNA trapped within sugar derived condensation products.

Traditional Methods and Technologies for the Isolation of DNA

A key step in DNA purification is protein removal. Deproteination can be accomplished using a variety of methods. Most common are the broad spectrum proteolytic enzymes namely, proteinase K and pronase. It should be noted that the lack of histones in mtDNA potentially means that deproteinization chemicals may, in fact, degrade the DNA.

Organic solvents can also isolate DNA from proteins and other cellular debris. Organic solvents such as Phenol chloroform isoamyl alcohol (24:25:1) are traditional ways of deproteinization. This process is more efficient when combining at least two organic solvents. Phenol dissociates proteins from DNA while chloroform denatures proteins and facilitates the separation of the aqueous and organic phases. Proteins and lipids will reside in the organic phase. Nucleic acids and other water soluble matter such as carbohydrates, will reside in the aqueous layer. Chloroform also acts to decrease the solubility of DNA in phenol thereby reducing losses to the organic phase. Isoamyl alcohol is combined with these two solvents to reduce foaming during the extraction process. However, methods involving organic solvents can result in poor DNA recovery and low quality DNA yields because they degrade the bonding units of the nucleotides. They require technical skill, and may not provide reproducible results. Also, DNA isolated using this methods may contain residual organic solvents, phenol and/or

chloroform, which can inhibit downstream applications, particularly PCR. Finally, organic solvents are toxic and can be hazardous if not handled properly.

Another traditional DNA isolation technique is ethanol precipitation. Precipitates form in the presence of monovalent cations such as ammonium acetate or sodium chloride. The negatively charged DNA reacts with the monovalent cations to form salts. The addition of ethanol causes the DNA to precipitate. This method is time consuming and may result in chemically damaged DNA. Residual ethanol, like organic solvents, may inhibit downstream applications such as PCR.

Non-Traditional Methods and Technologies

Size exclusion chromatography (P-30 columns Biorad, DTR columns Edge Biosystems), is effective in isolating DNA. This purification method fractionates molecules on the basis of size. Small molecules are impeded within the inert gel matrix while the DNA is excluded and passes directly through the column.

Microfilitration (Micron-100, Amicon) is also a size dependent DNA purification method. Low molecular weight molecules (e.g.DNA) are retained within the selectively permeable membrane. This can be problematic because the filter may also concentrate potential PCR inhibitors that fall above the molecular weight cut-off of the membrane. This technology is used by the F.B.I. for the purification of DNA from hair shafts (Wilson *et al*, 1995).

Many new technologies for DNA isolation are based on the negative charge of DNA. A proven alternative to traditional methods for the isolation of DNA is the use of a solid-phase support such as silica. DNA has high binding affinities for silica in the presence of high concentrations of chaotropic salts. High concentrations of chaotropic

salts, such as GuSCN, modify the structure of water allowing for the binding of DNA to the silica (Hamaguchi *et al*, 1962). DNA adsorption to silica is also highly dependent on pH. Immobilization of DNA to silica must occur at an acidic pH (pH<7.5). The bound DNA can be washed to remove cellular debris and then subsequently eluted from the silica using a low salt solution. Silica methodologies are highly selective for DNA and are unlikely to co-purify potential PCR inhibitors. However, precautionary removal of all residual silica particles is germane because they can inhibit enzymatic activity in the PCR.

Spin column technology using silica gel membranes (QIAquick, Qiagen) is an effective alternative to using silica particles. QIAquick columns have been optimized for the recovery of DNA and removal of contaminants. The filter traps DNA 100bp –10kb and excludes proteins, nucleotides and salts. QIAquick columns have a reported 95% DNA recovery rate. These columns have proven effective for removing melanin, a PCR inhibitor (Yang *et al*, 1997). In the presence of a high salt buffer and an acidic pH, DNA will bind to the silica membrane and contaminants will pass through the column. An ethanol-based buffer is used to wash the membrane bound DNA (e.g. to wash away salts, detergents, enzymes). Contrary to adsorption, elution is most efficient under basic conditions and low salt concentrations.

The latest application to DNA recovery from biological tissues is magnetic bead technology. They have been useful for cell separations, immunoassays, and the isolation of viruses, organelles and DNA (Haukanes, 1993). Magnetic beads work on an affinity or binding principal similar to silica technology. In order for magnetic beads to act uniformly in a magnetic field they must be almost identical in magnetism and size. They

must also be constructed to be superparamagnetic, that is they must exert their magnetism only in a given magnetic field. They are constructed as such by depositing colloidal magnetite (e.g. magnetic iron oxides) throughout the beads. Superparamagnetism allows for minimal settling under gravity and efficient collection in the presence of a magnetic field. The surface are of these magnetic beads is large, 50-100m^2/g, to allow for maximal DNA recovery. Sometimes the beads are coated with polymeric compounds with functional groups (Isocyanate, Epoxy, and Vinyl groups). Functional groups are applied for coupling of spacer arms with amino, hydroxyl or carboxylic end groups. DNA attaches covalently to these groups.

Several magnetic bead-based systems for the isolation of DNA have been developed. They each follow a similar mechanism adsorption of the released DNA to the magnetic beads, washing of the DNA/magnetic bead complex (removal of contaminants) followed by elution of the DNA from the magnetic beads. In principal magnetic beads are very amenable for automating the extraction process.

MATERIALS AND METHODS

Samples

Modern Hair Samples

The modern hair shaft samples analyzed in this study were collected from three individuals. Male and two related female individuals (mother-daughter) are represented in this sample set. Their ages and hair colour are shown Table 1. The hair shafts sampled from each individual had not been cosmetically altered (e.g. dyed , permed).

Table 1. Modern Hair Sample Set for MtDNA Analysis

Sample	Sex	Age	Hair colour
Lillian Murray	Female	55 yrs	Blonde
Carolyn Murray	Female	24 yrs	Light brown
Curtis Hildebrandt	Male	27 yrs	Dark brown

Ancient Hair Samples

The ancient hair shaft samples analyzed in this study were collected from a large Roman Period (*circa* 300 -400AD) cemetery, Kellis 2, associated with the ancient town of Kellis located in the Dakhlah Oasis, Egypt (Figure 3). The Dakhlah Oasis is one of five oases situated in the Sahara desert in western Egypt. Due to the extreme aridity of this region, the burials excavated from Kellis 2 are exceptionally well preserved. The burial pattern found in this cemetery consists of single interments in mudbrick tombs oriented in the head west position (Graver, 2001). Approximately 72% of the burials excavated from Kellis 2 have scalp hair present. Several hair colours are represented in this sample set (n=10) ranging from blonde to black. Both male and female individuals are represented in

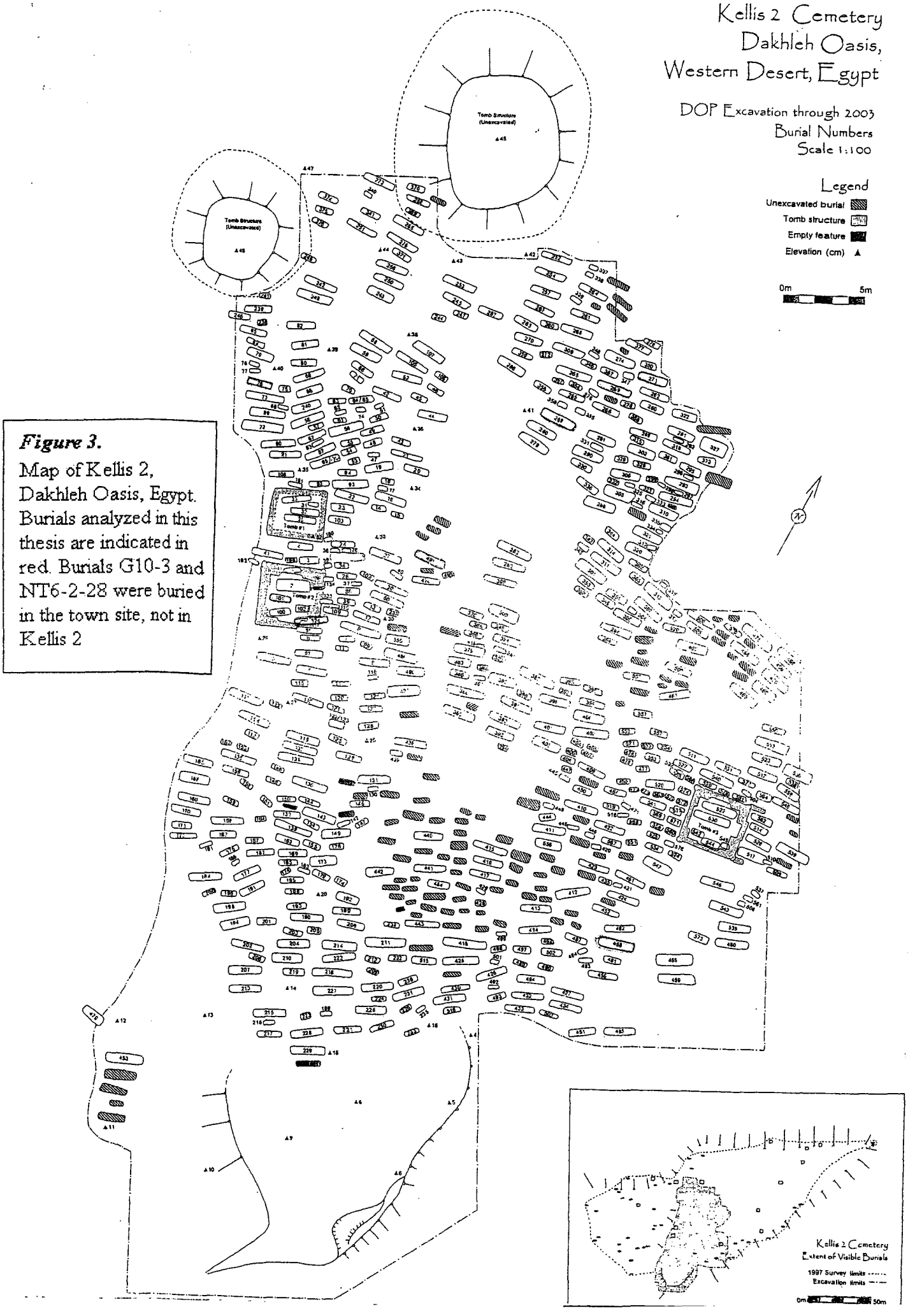

Figure 3.
Map of Kellis 2, Dakhleh Oasis, Egypt. Burials analyzed in this thesis are indicated in red. Burials G10-3 and NT6-2-28 were buried in the town site, not in Kellis 2

the sample set ranging from 6 to 55 years of age (Table 2). Prior to analysis, the hair samples were stored in sterile plastic bags at room temperature and low humidity.

Table 2. Ancient Egyptian Hair Sample Set for MtDNA Analysis

Burial number	Sex	Age	Hair colour
K2 B76*	Female	38 yrs	Red-brown
K2 B101*	N/A	6 yrs	Red-brown
K2 B124	Male	30 yrs	Brown
K2 B259*	Male	46 yrs	Light brown
K2 B269*	Female	55 yrs	Light brown
K2 B271*	Female	31 yrs	Red-brown
K2 B458	Female	50 yrs	Red-brown
K2 B491	Female	40 yrs	Brown
NT6-2-28	Female	30 yrs	Blonde
G10-3 (Nubian)	Female	24 yrs	Black

* burial has mtDNA profile from bone (Graver *et al*, 2001)

Contamination Precautions

One of the many challenges faced when conducting ancient DNA (aDNA) research is contamination from modern DNA sources (Paabo *et al*, 2004). Due to the sensitivity of the polymerase chain reaction (PCR), even minimal amount of exogenous DNA can be problematic. Stringent precautions were taken throughout this study to minimize the risk of contamination following the recommendations of Parr (2002). All research was conducted in a facility (Molecular World Inc.) with dedicated pre- and post- PCR areas. DNA extraction, purification and PCR preparation were conducted in dedicated rooms in a Clean Laboratory in which modern samples were prohibited. Post-PCR analysis was not conducted within the Clean Laboratory. Protective clothing was worn at all times to prevent the introduction of my own DNA into the ancient samples throughout all pre-PCR procedures. This clothing included a tyvek body suit, disposable sleeves, face mask, hairnet, safety glasses, and latex gloves. The experimental procedures were carried

out in laminar flow hoods with an HEPA-filtered air supply that was ultra violet (UV) irradiated before each use. UV irradiation was used to cross-link any exogenous DNA present in the hood, thereby making it unamenable to PCR. All equipment and reagents were decontaminated using an UV crosslinker (UVP CL-1000 Ultraviolet Crosslinker), bleach (10%), ethanol (70%), and/or an autoclave (VWR Accusterilizer™ ASI2). Negative controls were carried out throughout the extraction, purification and PCR procedures to ensure the authenticity of the DNA extract. The DNA profiles of all laboratory personnel including myself as well as the archaeologists who collected the hair samples were all mtDNA typed and known.

Microscopy

Modern Hair Samples

Prior to the analysis of the ancient Egyptian hair samples, the histology of the modern hair shaft was studied using Differential Interference Contrast Optics (Olympus Inverted Microscope 1X51) and Scanning Electron Microscopy (JEOL JSM-5900L V).

Ancient Hair Samples

A sample of hair from each of the twelve burials was examined using Brightfield microscopy (Micromaster ® Fischer Scientific and Reichert Microstar IV Light Microscope with Digital Photomicrographic System). Prior to microscopy, hair shafts were washed with a 1% Terg-a-zyme™ detergent (Alconox Inc., New York, NY) and rinsed with ~95% ethanol. Dry slides of each sample were viewed under 10X, 40X and 100X magnification. The histological structure and integrity of the hair shaft were observed.

DNA Extraction and Purification Protocols

The efficacy of four DNA extraction and purification protocols was evaluated for their ability to retrieve DNA from modern and ancient Egyptian hair shafts. Hair shaft segments (20mm) were treated by four different methods (1) the standard protocol currently used by the FBI (Wilson *et al*, 1995) (2) an enzymatic digestion followed by silica column purification (3) an enzymatic digestion followed by magnetic resin purification, and (4) a protocol developed by Gilbert *et al* (2004) for the extraction of DNA from ancient bison hair. Prior to assessing the efficacy of these four methods on ancient Egyptian hair shafts, their efficacy to retrieve DNA from modern hair shafts was evaluated.

Hair Sample Preparation

Prior to each DNA extraction and purification protocol, each hair sample was washed with 1% Terg-a-zyme™ detergent to remove adhering contaminants and subsequently rinsed with ~95% ethanol.

FBI Standard Protocol

The FBI standard protocol for the extraction of DNA from hair shafts was developed by Wilson *et al* in 1995. The hair shaft (~2cm) was placed in 500µL of Tris EDTA (10mM Tris HCl, 0.1µM EDTA, pH 8) buffer and ground using a sterilized 0.5mL glass micro-tissue grinder (Kontes, Vineland, NJ). The homogenate was then incubated overnight (18 to 24 hrs) at 56°C with 0.5mg/mL of proteinase K (EM Science, USA) under medium agitation (500rpm). Upon completion of the incubation, 200µL of phenyl/chloroform/isoamyl alcohol (PCIA, 25:24:1, Sigma-Aldrich, Louis, MO) was

added to the extract. The aqueous phase was purified and concentrated using microfiltration (Micron™100, Millipore, Ireland) according to the manufacturer's recommendations. The DNA extract was eluted in 40µL of 80°C double distilled sterile water.

Modification of the FBI Standard Protocol

The protocol developed by Wilson *et al* (1995) was followed with one exception, the substitution of proteinase K (0.5mg/mL) with a novel enzyme, keratinase (0.5mg/mL, BioResource International Inc, Raleigh, NC). This modified protocol was evaluated on modern hair shafts only. The modification did not result in increased DNA yields, thus was not applied to ancient hair shafts.

Proteinase K Digestion and Silica Column Purification

The hair shaft (~2cm) was physically ground with 500µL of Tris EDTA buffer (pH 8.0) using a sterilized 0.5mL glass micro-tissue grinder. The homogenate was then incubated overnight at 56°C (18 to 24 hrs) with 0.5mg/mL of proteinase K under medium agitation (500rpm). The extract was then purified using commercially available silica gel columns (QIAquick® PCR Purification Kit, QIAGEN Sciences, MD) according to the manufacturer's recommendations and eluted in 40µL of 80°C elution buffer.

Proteinase K Digestion and Magnetic Resin Purification

The hair shaft (~2cm) was physically ground with 500µL of Tris EDTA buffer (pH 8.0 using a sterilized 0.5mL glass micro-tissue grinder. The homogenate was then incubated overnight (18 to 24 hrs) at 56°C with 0.5mg/mL of proteinase K under medium agitation (500rpm). The extract was then purified using magnetic resin (DNA IQ™, Promega,

Madison, WI) according to the manufacturer's recommendations and eluted in 40μL of 80°C double distilled sterile water.

Protocol by Gilbert et al (2004) for the Retrieval of aDNA from Hair

The hair shaft (~2cm) was added to 500μL of extraction buffer (0.01M Tris, 0.01M NaCl, 1% SDS, 0.5mg/mL proteinase K, 10mg/mL DTT, 0.001M PTB) and incubated overnight (18 to 24 h) at 55°C with medium agitation (500rpm). Upon completion of the incubation, 200μL of PCIA was added to the extract. The aqueous layer was purified and concentrated using microfiltration (Microcon™100, Millipore) according to the manufacturer's recommendations. The DNA extract was eluted in 40μL of 80°C double distilled sterile water.

DNA Quantification

DNA extracts were quantified using a fluorometer (TBS-380 Mini-fluorometer, Turner Biosystems, Sunnyvale, CA). 10.0μL of purified DNA extract was re-suspended in 89.5μL Tris EDTA buffer with 0.5μL of Pico Green reagent (Molecular Probes, Eugene, OR) to bring to a total sample volume of 100.0μL (1:10 dilution). Pico Green reagent is a fluorescent nucleic acid stain for specifically quantitating double-stranded DNA. The solution was protected from light, vortexed, transferred into a cuvette and placed into the fluorometer chamber for immediate DNA quantification according to the manufacturer's recommendations. It should be noted that the detection limit of the fluorometer is 15pg of DNA.

DNA Amplification

DNA extracts were routinely used for PCR immediately, but if necessary were stored at –20°C. Amplifications were carried out in 25μL reactions containing 10X PCR reaction buffer (Invitrogen, Carlsbad, CA), 50mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 10mM dNTPs, 5U/μL Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) 10μM mtDNA primers (Integrated DNA Technologies, Coralville, IA), Omni Pur® DEPC water (EM Science, USA) and various amounts of template (5μL, 10μL, 15μL). The template was also diluted to varying degrees ranging from 1:10 to 1:100. The reactions were vortexed and immediately placed in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems) for PCR amplification. Positive and negative controls for each primer set were run in parallel to each PCR.

PCR Primers

Ancient DNA is usually quite fragmented. Consequently, I attempted to amplify short sections (100bp-250bp) from the mtDNA genome. These amplicons are Hypervariable regions I and 2 (HV-1 and HV-2) and a small variable region from the *28s rRNA* gene (Table 3). HV-1 and HV-2 are useful for determining maternal relationships between individuals. The *28s rRNA* gene is highly conserved amongst all organisms and is quite useful for distinguishing both prokaryotic and eukaryotic species.

Table 3. Primers used for PCR Amplification of MtDNA from Modern and Ancient Extracts

mtDNA Region	Primer Set	Amplicon Size
HV-1	*15977* 5'-CCACCATTAGCACCCAAAG-3' *16223* 5'-GGGTTGATTGCTGTACTTGC-3'	246bp
	16193 5'-CATGCTTACAAGCAAGTACAGC-3' *16425* 5'-GATATTGATTTCACGGAGGATGG-3'	232bp
HV-2	*162* 5'-CGCACCTACGTTCAATATTACAG-3' *258* 5'-TCTGTGTGGAAAGTGGCTG-3'	96bp
28S rRNA gene	*A* 5'-ATCTAGTAGCTGGTTCCCTC-3' *B* 5'-CCTCTAATCATTCGCTTTAC-3'	100bp

Thermocycling conditions were as follows: 35 cycles of amplification with denaturation at 94 °C (30 s), annealing at 55 °C (30 s), and extension at 72 °C (1min).

Gel Electrophoresis

Aliquots (5μL) of the PCR product were analyzed by polyacrylamide gel electrophoresis (PAGE). 5μL of PCR product along with 2μL of 6x loading buffer (Promega, Madison, WI) was loaded on to a 6% polyacrylamide gel and run at 150volts for 30 min. DNA fragments were visualized by staining the gel with ethidium bromide and viewing on an UV transilluminator (UVP, Upland, CA). Photographs of each gel were taken using a Polaroid Gel Cam. A size maker, 100bp DNA ladder (Promega, Madison, WI), was run in parallel with each set of PCR products.

Sequencing Analysis

PCR products were purified for sequencing by using Performa DTR gel filtration cartridges (Edge Biosystems, Gaithersberg, MD). Sequencing was performed using ABI

PRISM BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations. Electrophoresis and sequence analysis were performed using a 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing primers used were identical to those used in the amplification.

Identification of PCR Inhibitors

Gas-Chromatography-Mass Spectrometry (GC-MS)

An analysis of the organic composition of crude DNA extract (200.0μL) from two Kellis 2 hair samples (K2 B259 and K2 B269) was performed using GC-MS (Varian 1200, Walnut Creek, CA). The organic composition of intact hair shafts belonging to these two burials were also analyzed using GC-MS. Prior to GC-MS analysis, the hair shafts (2cm) were ground with the assistance of liquid nitrogen and a ceramic mortar/pestle and subsequently dissolved in an alkaline solution.

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Elemental and molecular analysis of hair samples from two Kellis 2 individuals (K2 B76 and K2 B124) was performed using SIMS (Trift II ToF-SIMS, Physical Electronics Inc) as per Kempson *et al* (2003).

Raman Spectroscopy

Elemental and molecular analysis of hair samples from two Kellis 2 individuals (K2 B76 and K2 B124) was performed using Raman spectroscopy as per standard protocol at the Ian Wark Research Institute, University of South Australia.

Inductively Coupled Argon Plasma Spectrometry (ICAP/ICP)

An elemental analysis of 60.0µL of purified DNA extract from two representative Kellis 2 hair samples, NT6-2-28 and G10-3, was performed using ICP spectrometry (Varian Vista Pro ICAP Radial with a Cetac Autosampler). An acid (10% HNO_3) digested soil sample (~600mg dry weight) from the tombs of NT6-2-28 and K2 B269 was also analyzed using ICP spectrometry.

Real-time PCR analysis of DNA Extracts from Ancient Egyptian Hair Shafts

Sample preparation

DNA was extracted from burials G10-3 (Nubian) and K2 B124 using the Gilbert *et al* (2004) protocol. The DNA extracts were quantified using a fluorometer and Pico Green reagent.

Quantification and Melting Curve Profiles

Amplifications were carried out using the Bio-Rad iQ Sybr Green qPCR kit. Each 25µL reaction contained: 2X qPCR reaction mix, mtDNA primers (250nm)(Table 4) PCR grade water and 5µL of template (modern Asian, modern Caucasian, G10-3, K2 B124). The reactions were placed in a thermocycler (DNA Engine Opticon ® 2 Continuous Fluorescence Detection System) for PCR amplification.

Thermocycling conditions were as follows: 60 cycles of amplification with denaturation at 95 °C (30 s), annealing at 57 °C (30 s), and extension at 72 °C (30s). After 60 cycles samples were incubated at 72°C for 10 min. The plate was read after every cycle. The melting curve was generated from 50°C to 105°C and read every 1°C.

A standard curve was created using modern Caucasian template. The template was quantified using a NanoDrop® spectrophotometer. The quantified value was

normalized to create a standard curve. The ancient (G10-3 and K2 B124) and control (modern Asian and Caucasian) templates were subsequently amplified. Negative controls were processed in parallel. The data was analyzed using MJ Opticon Monitor® (version 3) Analysis Software.

Table 4. Primers used for Quantitative PCR and Melting Curve Analysis of Ancient Egyptian Extracts and Modern Human Controls

mtDNA Region	Primer Set**	Amplicon Size
*12S rRNA**	708 5'-cgttccagtgagttcaccctc-3' 946 5'-cactctttacgccggcttctattcac-3'	238bp

**The 12S rRNA region of the mtDNA genome is variable yet conserved across populations*
** Primers designed by Genesis Genomics Inc. They are guaranteed not to amplify nuclear mitochondrial pseudogenes (Pseudofree™)

RESULTS

Modern Hair Microscopy

Differential Interference Contrast Microscopy and Scanning Electron Microscopy revealed the complexity of the histological structure of the human hair shaft (Figures 4 to 8).

Figure 4. Differential Interference Contrast micrograph of isolated cuticular cells. It is speculated that *N* is the cell nucleolus. Cuticular cells are 50-60μm in length.

Figure 5. Differential Interference Contrast micrograph of frayed hair shaft. Many cuticular cells have separated from the cortex. Bundles of cortical cells (~100 μm in length) are visible. The hair shaft is ~87μm wide.

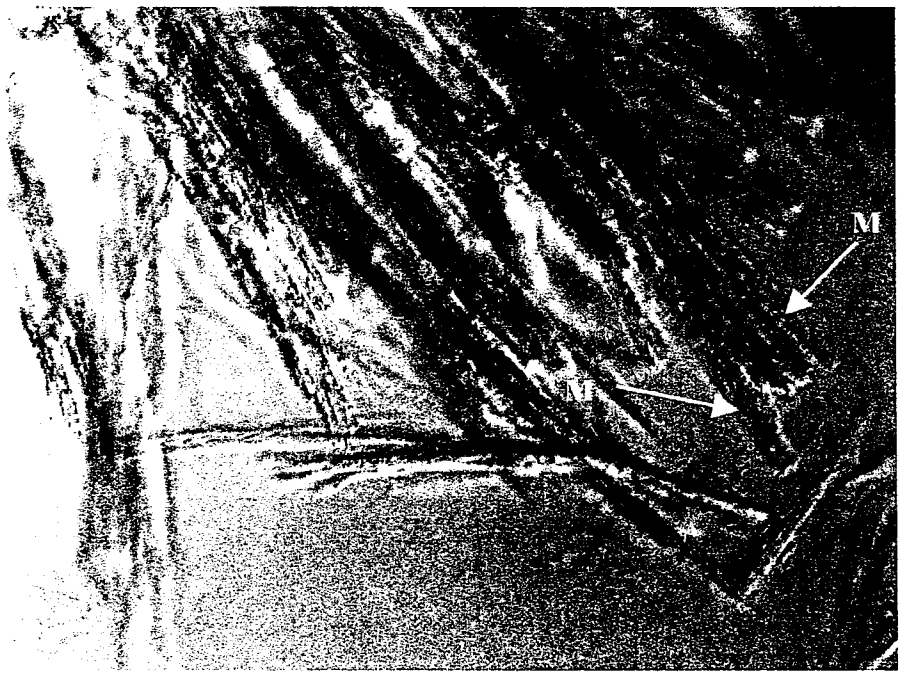

Figure 6. Differential Interference Contrast micrograph of frayed hair shaft. Melanin granules (M) within cortical cells are visible.

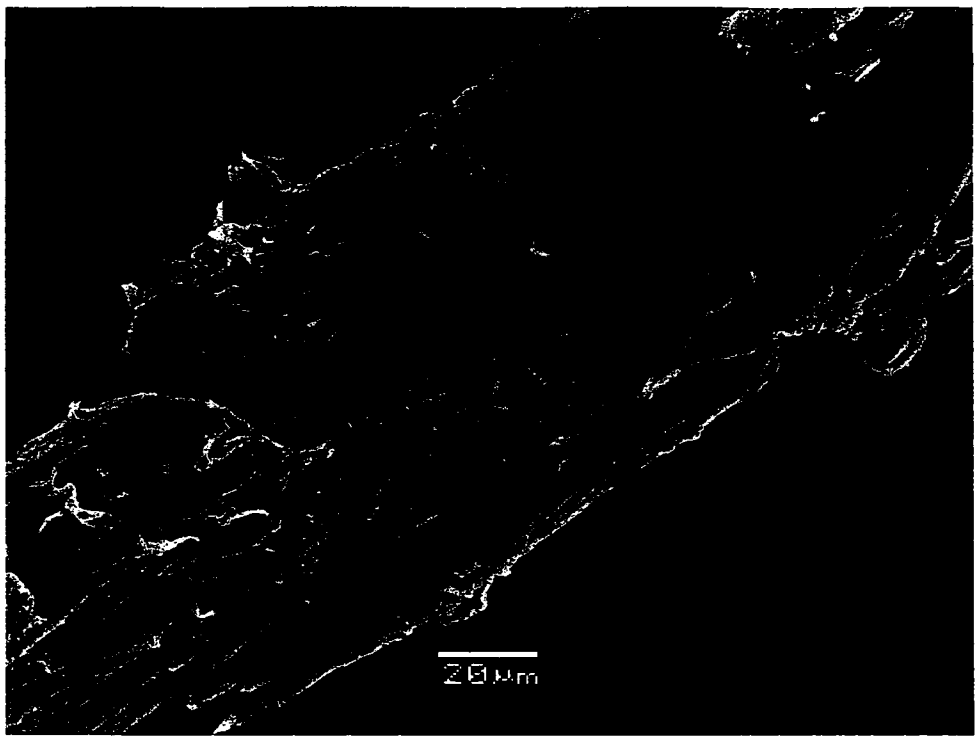

Figure 7. SEM of a fractured hair shaft. Cuticular cells appear as overlapping scales.

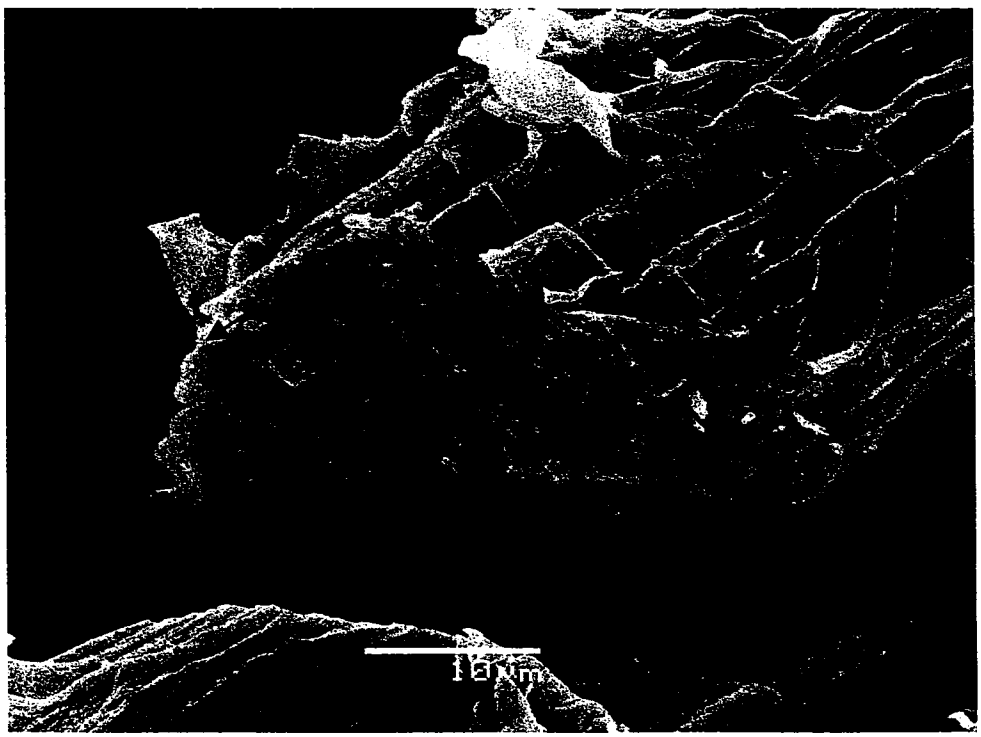

Figure 8. SEM of fractured hair shaft. Cortical cells are visible at the fracture site but outlines are indistinct.

In general, the ancient Egyptian hair from Kellis appears to be very well preserved. The cuticle scale pattern appears relatively intact in most specimens (Figure 9). However, there is evidence of damage or contamination as shown in Figures 10 to 15.

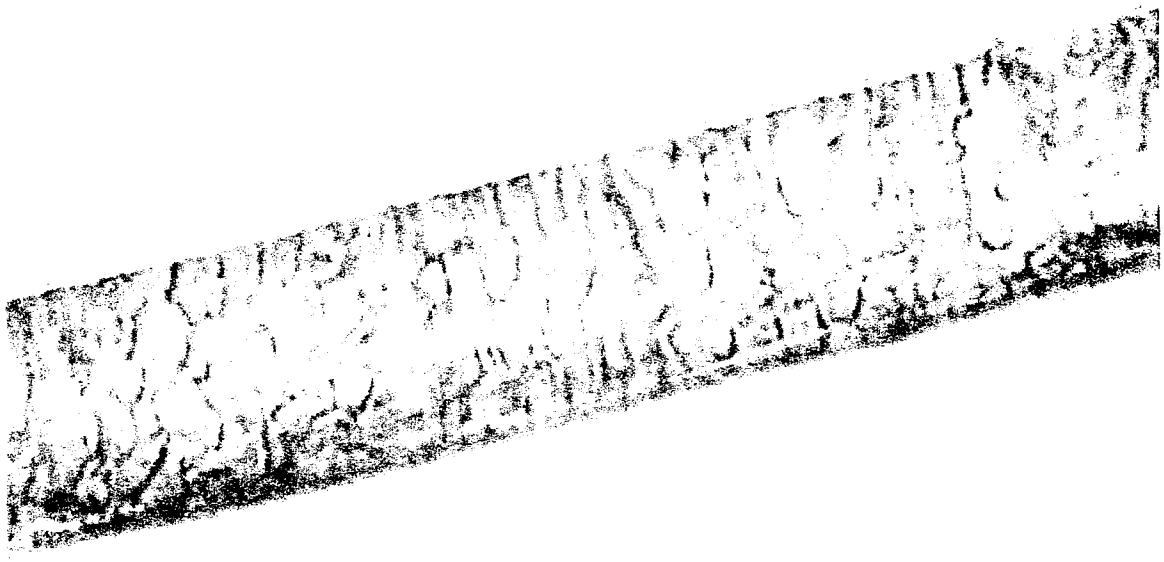

Figure 9. Micrograph of hair shaft from burial NT6-2-28. The cuticular scale pattern is relatively well preserved. 400X magnification.

Figure 10. Micrograph of hair shaft from burial K2 458. Hair shaft fracture. 400X magnification.

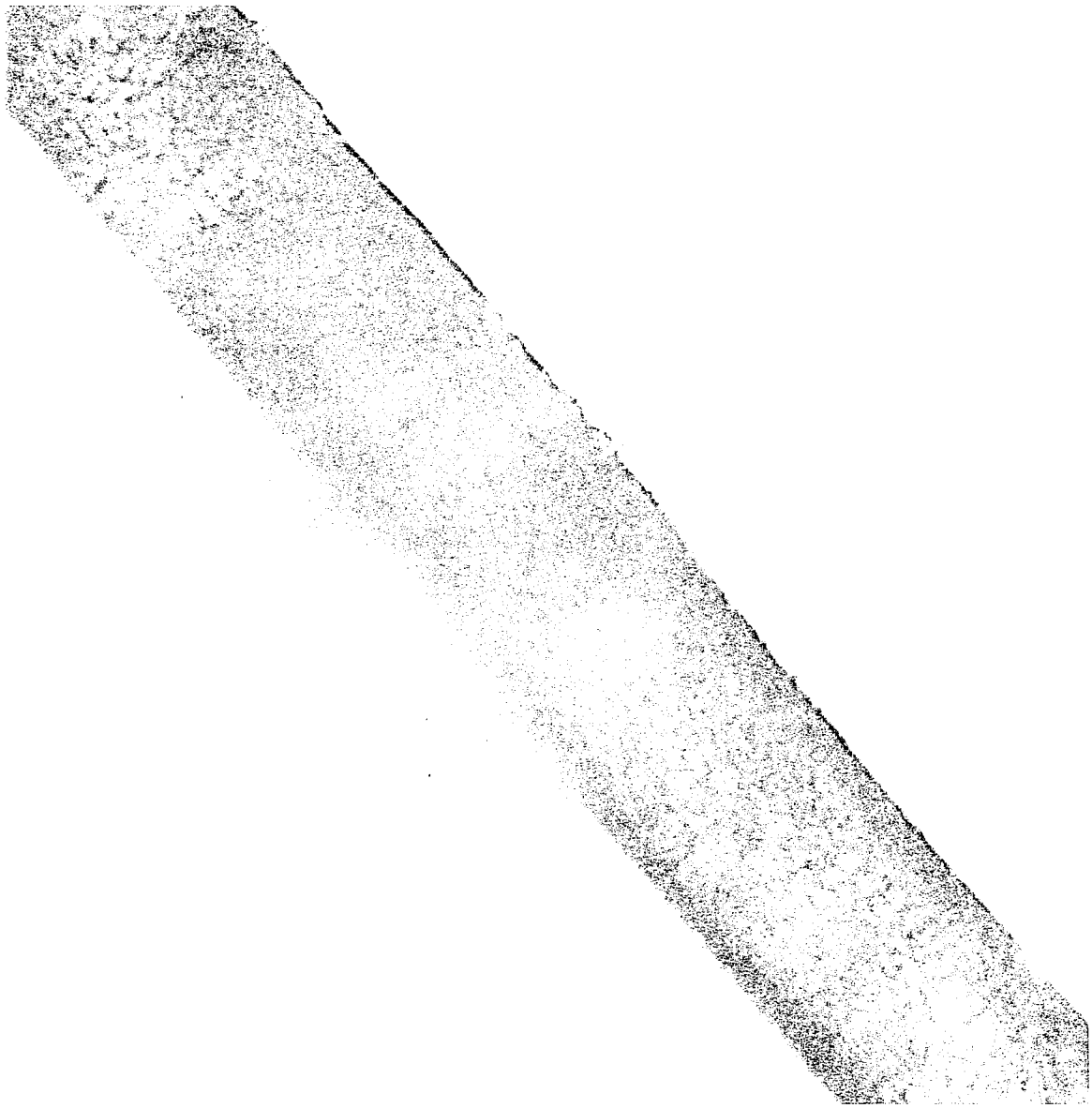

Figure 11. Micrograph of hair shaft from burial K2 458. Possible fungal contamination on hair shaft exterior. 400X magnification.

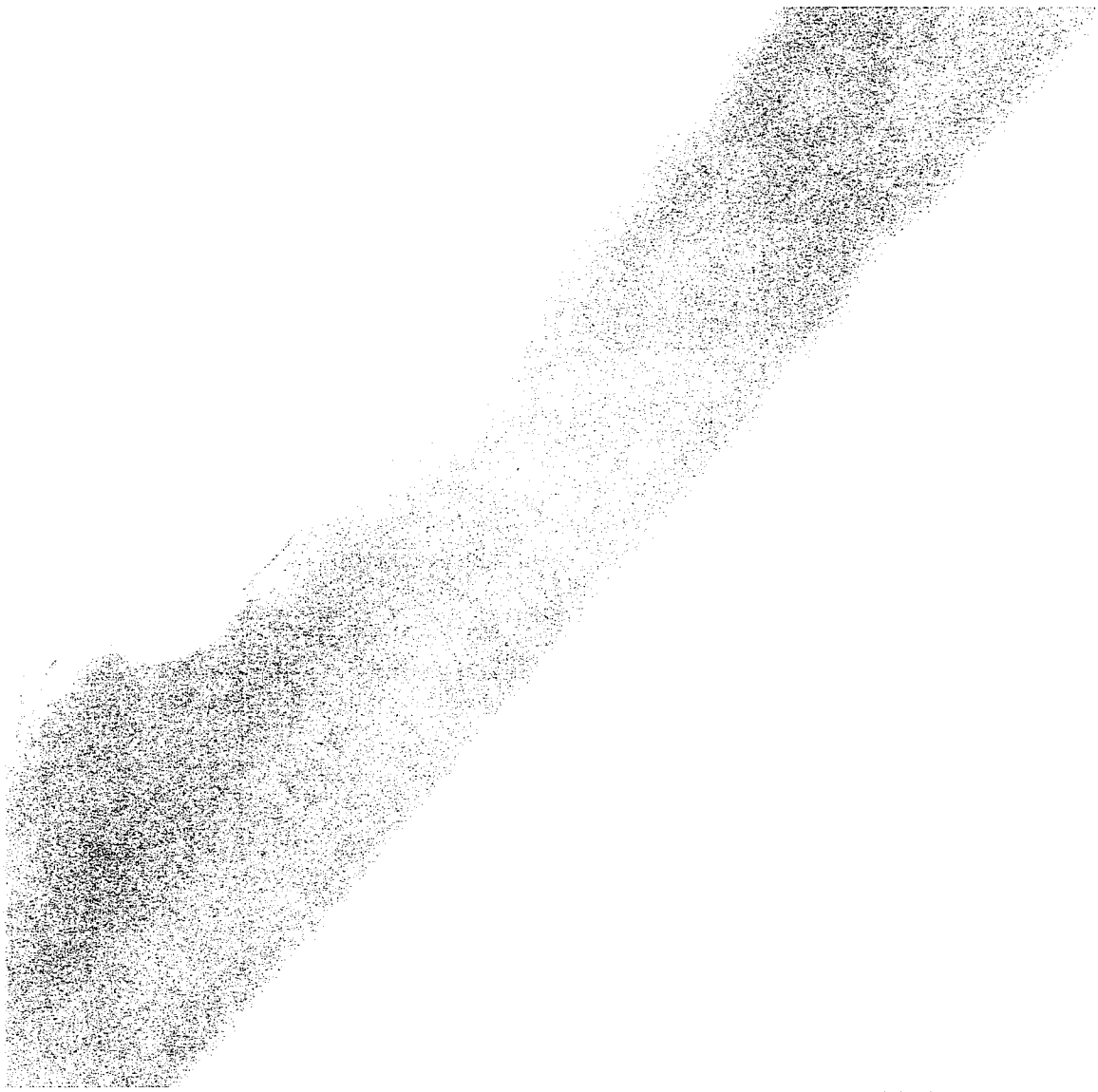

Figure 12. Micrograph of hair shaft from burial G10-3 (Nubian). Possible insect damage. 400X magnification.

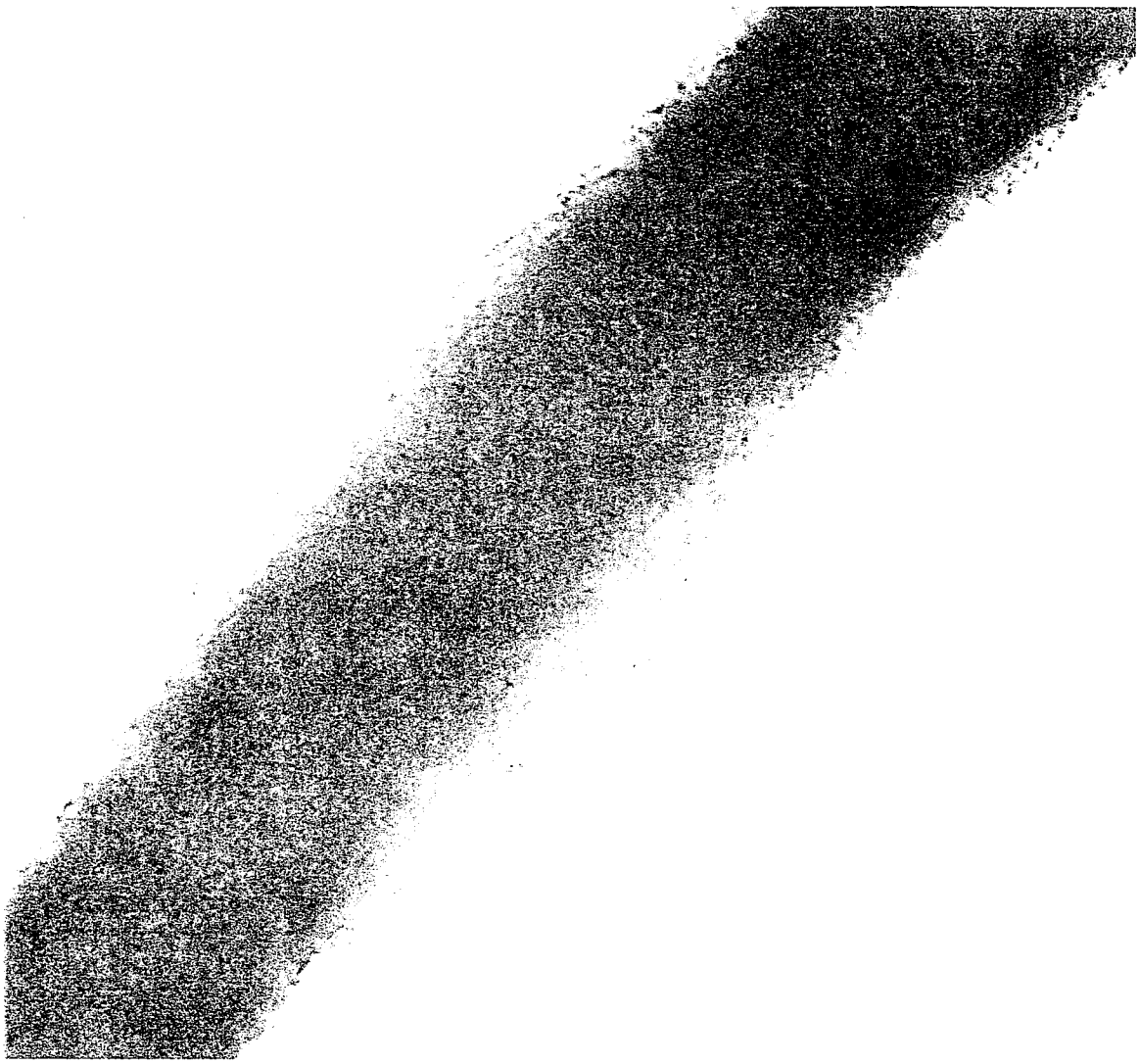

Figure 13. Micrograph of hair shaft from burial G10-3. Evidence of cuticular scale ruffling. 400X magnification.

Figure 14. Micrograph of hair shaft from burial K2 124. Fragmented medulla. 400X magnification.

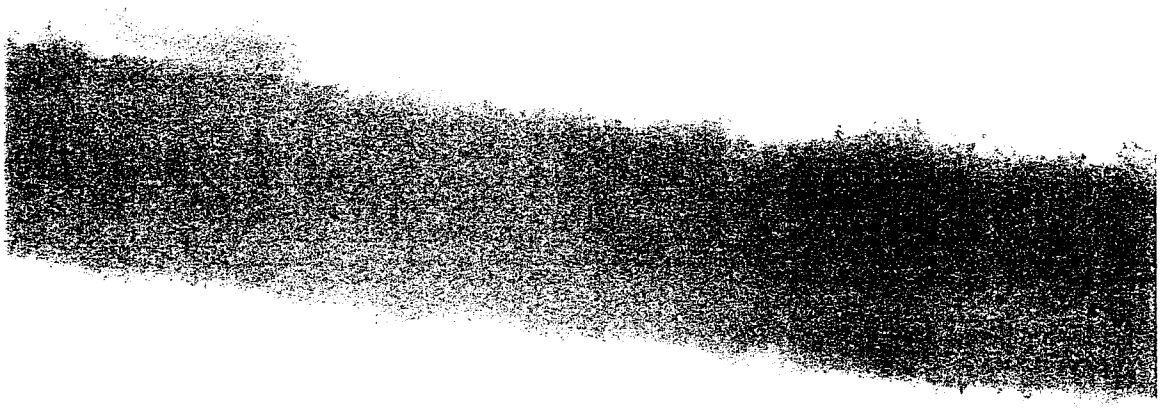

Figure 15. Micrograph of hair shaft from burial G10-3. Fungal contamination on hair shaft exterior (?). 400X magnification

Evaluation of Three Purification Methods for the Retrieval of DNA from Modern Human Hair Shafts

The highest DNA yields (mean=66.98ng/mL, standard deviation=33.78) obtained from modern hair shafts were retrieved using phenyl chloroform isoamyl alcohol (PCIA) followed by microfiltration. Silica column purification (mean=16.69ng/mL, standard deviation=14.75) and magnetic bead purification (mean=12.63ng/mL, standard deviation 14.23) had significantly lower DNA yields (Table 5). The FBI standard DNA extraction procedure (physical grinding of hair followed by proteinase K/TE buffer incubation) was used in conjunction with each of the three purification methods. Negative controls run in parallel to the extraction and purification process were used as an indicator of experimental contamination. Using the aforementioned DNA extraction and purification protocols, the average amount of DNA contaminating the negative control was 15.53ng/mL. The amount of DNA contamination obtained did not differ significantly between the three purification protocols. The average amount of DNA retrieved from the modern hair shafts using the aforementioned extraction and purification protocols was 32.1ng/mL.

Table 5. DNA Yields from Modern Hair Shafts using Three Different Purification Methods

Sample	Extraction/Purification Method**	DNA Yield (ng/mL)*
C.Murray	PCIA and Microfiltration (FBI protocol)	33.89
C.Murray	PCIA and Microfiltration (FBI protocol)	98.78
C.Murray	PCIA and Microfiltration (FBI protocol)	95.6
C.Murray	PCIA and Microfiltration (FBI protocol)	28.15
C.Murray	PCIA and Microfiltration (FBI protocol)	78.47
C.Murray	Silica Column	0.62
C.Murray	Silica Column	7.05
C.Murray	Silica Column	20.51
C.Murray	Silica Column	16.12
C.Murray	Silica Column	39.14
C.Murray	Magnetic Beads	4.63
C.Murray	Magnetic Beads	8.58
C.Murray	Magnetic Beads	0.64
C.Murray	Magnetic Beads	12.46
C.Murray	Magnetic Beads	36.86

**Sample quantity= 2cm hair shaft*

***All hair shafts were ground using glass microtissue grinders prior to the extraction process*

**HVI was successfully amplified and sequenced from DNA purified by each method.*

Evaluation of the Gilbert et al (2004) Protocol for the Retrieval of DNA from Modern Human Hair Shafts

The Gilbert *et al* (2004) protocol on average retrieved 173.17ng/mL (standard deviation=108) of DNA from 2cm of modern human hair shaft (Table 6). The average amount of DNA contamination present in the extraction and purification negative controls was 1.1ng/mL. On average, the FBI standard protocol (66.98ng/mL) yielded a significantly lower amount of DNA than the Gilbert *et al* (2004) protocol (173.17ng/mL). The protocols that involved physical grinding of the hair shaft prior to the DNA extraction and purification process had higher amounts of contamination (mean=15.53ng/mL) than the Gilbert *et al* protocol (2004) (mean=1.1ng/mL) which uses a chemical method instead of a physical method to break down the hair shaft.

Table 6. DNA Yields from Modern Hair Shafts using the Gilbert *et al* (2004) Protocol

Sample	DNA Yield (ng/mL)*
C.Murray	360
C.Murray	140.8
C.Murray	116.1
C.Murray	87.65
C.Murray	161.3

**Sample quantity= 2cm hair shaft*

**HVI was successfully amplified and sequenced from DNA extract*

Assessment of the Efficacy of Keratinase for the Retrieval of DNA from Modern Hair Shafts

In this preliminary assessment, DNA yields using keratinase did not prove to significantly differ from DNA yields using the control enzyme, proteinase K (Table 7). The average DNA yield using keratinase was 48.1ng/mL. The average DNA yield using proteinase K was 54.3ng/mL.

Table 7. DNA Yields from Modern Hair Shafts using Proteinase K and Keratinase

Sample	*DNA Yield (ng/mL)**	*Enzyme*	*Protocol*
C. Hildedrandt	53.1	Proteinase K	FBI
C.Hildebrandt	43.15	Proteinase K	FBI
L.Murray	66.6	Proteinase K	FBI
L.Murray	83.4	Keratinase	FBI
C. Hildedrandt	21.2	Keratinase	FBI
C. Hildebrandt	39.68	Keratinase	FBI

**Sample quantity=2cm*

Assessment of DNA Fragmentation in Modern Hair Shafts

DNA fragments from the mtDNA control region ranging from 86bp to 807bp in size were amplified from DNA extracted from modern hair shafts (Figure 16). There is an inverse correlation between the amplicon size and the gel band intensity. The band intensities of

the control DNA extract (DNA from buccal cells) are much stronger than the band intensities of DNA amplified from the hair shaft extract.

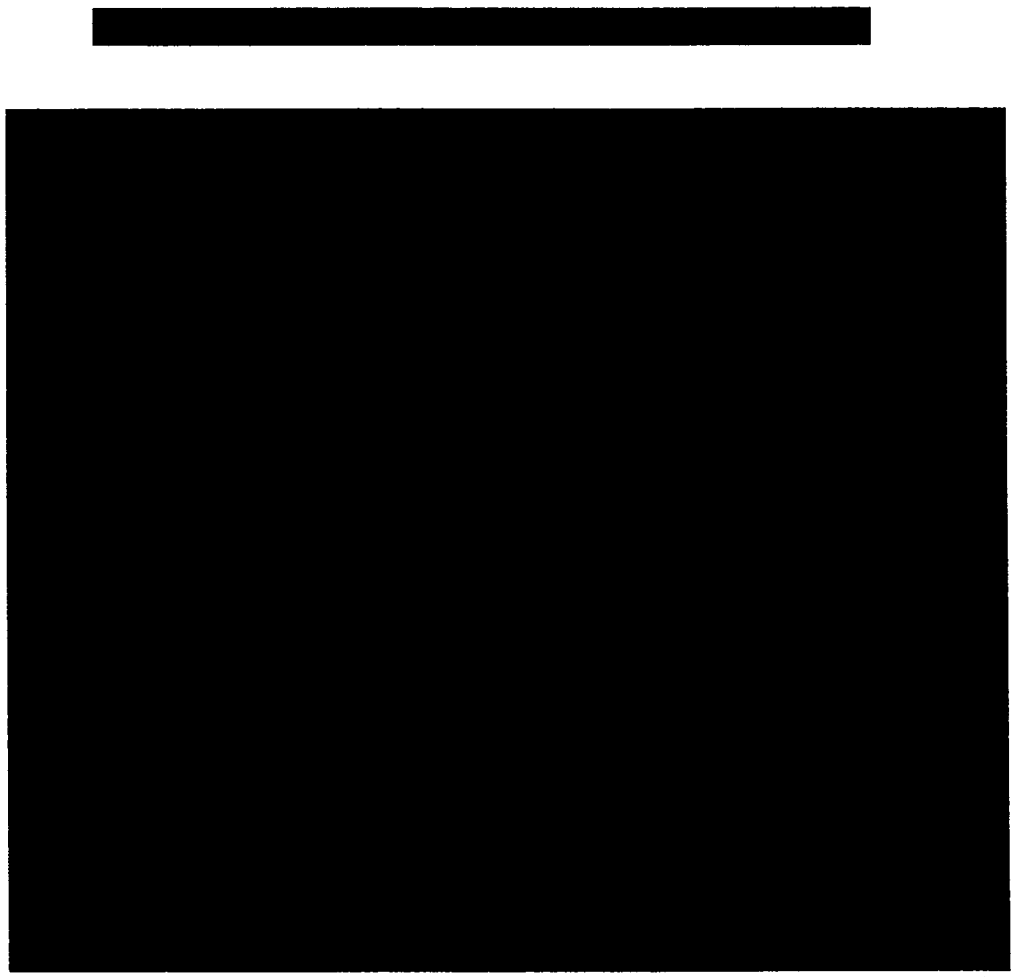

Figure 16. DNA fragmentation in a modern human hair shaft. Lane 1- 100bp DNA ladder, lanes 2 to 6-PCR and extract negative controls, lane 7- hair DNA extract HV2 (162-248), lane 8- hair DNA extract HV1 (16193-16425), lane-9 hair DNA extract HV2 (15f-431), lane 10-hair DNA extract HVI/HV2 (16193-431). Lane 11 to14-buccal DNA extract/positive controls.

DNA ANALYSIS OF ANCIENT EGYPTIAN HAIR SHAFTS

Evaluation of Three Purification Methods for the Retrieval of DNA from Ancient Egyptian Hair Shafts

The highest DNA yields (mean=28.8ng/mL, standard deviation=12.71) obtained from the ancient Egyptian hair shafts were retrieved using phenyl chloroform isoamyl alcohol (PCIA) followed by microfiltration. Silica column purification (mean=4.69ng/mL, standard deviation=4.15) and magnetic bead purification (mean=2.21ng/mL, standard deviation=1.04) had significantly lower DNA yields (Table 8). The FBI standard DNA extraction procedure (physical grinding of hair followed by proteinase K/TE buffer incubation) was used in conjunction with each of the three purification methods. Negative controls run in parallel to the extraction and purification process were used as an indicator of experimental contamination. Using the aforementioned DNA extraction and purification protocols, the average amount of DNA contaminating the negative control was 13.45ng/mL. The amount of DNA contamination obtained did not differ significantly between the three purification protocols. The average amount of DNA retrieved from the ancient Egyptian hair shafts using the aforementioned extraction and purification protocols was 11.19ng/mL.

Table 8. DNA Yields from Ancient Egyptian Hair Shafts using Three Different Purification Methods

Sample	Extraction/Purification Method*	DNA Yield (ng/mL)**
K2 B271	PCIA and Microfiltration (FBI protocol)	26.2
K2 B271	PCIA and Microfiltration (FBI protocol)	17.62
K2 B458	PCIA and Microfiltration (FBI protocol)	42.63
K2 B259	Silica Column	2.2
K2 B259	Silica Column	0.87
K2 B269	Silica Column	5.52
K2 B271	Silica Column	10.18
K2 B458	Magnetic Beads	2.1
K2 B458	Magnetic Beads	1.23
K2 B458	Magnetic Beads	3.3

**All hair shafts were ground using glass microtissue grinders prior to the extraction process*

***Sample quantity=2cm*

Evaluation of the Gilbert et al (2004) Protocol for the Retrieval of DNA from Ancient Egyptian Hair Shafts

The average DNA yield obtained from the ancient Egyptian hair shafts using the Gilbert *et al* (2004) protocol was 116.42ng/mL (standard deviation=85.53)(Table 9). The average amount of DNA contamination present in the extraction and purification negative controls was 6.2ng/mL. On average, the FBI standard protocol yielded a significantly lower amount of DNA (28.ng/mL) than the Gilbert *et al* (2004) protocol (116.42ng/mL). The protocols that involved physical grinding of the hair prior to the extraction and purification process (FBI Protocol, Silica Column Protocol, and Magnetic Bead Protocol) had a higher amount of contamination (mean=13.45ng/mL) than the Gilbert *et al* (2004) protocol (mean=6.2ng/mL) which uses a chemical means to break down the hair shaft.

Table 9. DNA Yields from Ancient Egyptian Hair Shafts using the Gilbert *et al* (2004) Protocol

Sample	DNA Yield (ng/mL)*
K2 B76	112.3
K2 B76	124.9
K2 B76	116
K2 B101	55.42
K2 B101	30.3
K2 B101	40.8
K2 B101	67.4
K2 B124	176.7
K2 B124	166.8
K2 B124	53.62
K2 B259	93.4
K2 B269	72.53
K2 B269	99.1
K2 B458	18.79
K2 B458	76.17
K2 B491	107.4
G10-6	222.5
G10-6	125.7
G10-6	416.5
G10-6	124.3
NT6-2-28	144.2

**Sample quantity=2cm*

PCR Amplification of DNA Retrieved from Ancient Egyptian Hair Shafts

A 246bp fragment of HVI (15997-16223) was successfully amplified using 1:20 dilution of DNA extract from K2 B458. However, amplification as indicated by gel band intensity was weak. 178bp of the HVI fragment was sequenced and a polymorphism was observed at position 16103 (C to T) (Figure 17). However, it was not possible to successfully replicate this data. The polymorphism at 16103 is not shared by myself, laboratory personnel nor the individuals who collected the ancient hair samples. The DNA extract was obtained using the FBI standard protocol (Wilson *et al*, 1995)

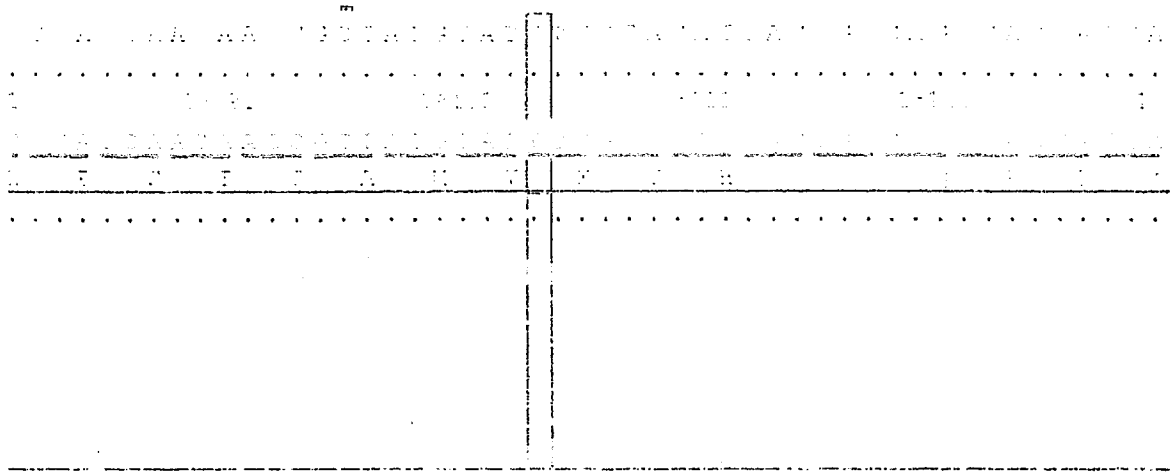

Figure 17. Burial K2 458 electropherogram. A 178bp region of HVI was amplified and sequenced. A Polymorphism is located at nucleotide position 16103 (C to T).

A 96bp fragment of HV2 (162-258) was successfully amplified using a 1:10 dilution of DNA extracts from K2 B491 and K2 B76 (Figure 18). However, amplification was minimal and unamenable to the sequencing process. The DNA extract was obtained from both samples using the Gilbert *et al* (2004) protocol. An attempt was made to re-PCR the amplified 96bp fragment, but was unsuccessful.

A 100bp fragment of the 28S rRNA gene was successfully amplified using diluted DNA extracts from several ancient Egyptian hair samples (K2 B269, K2 B101, K2 B458, G10-3, NT-2-28). These fragments were unable to be successfully sequenced (mixed sequences, poor quality template). The DNA extracts were obtained using the Gilbert *et al* (2004) protocol.

No human DNA was amplified from K2 B271, K2 B259, K2 B269, K2 B101, K2 B491, K2 B124, G10-3 or NT-2-28 (Table 10). Primer dimer was not apparent on the detection gel for each PCR involving the aforementioned DNA extracts. Positive controls and positive controls spiked with ancient DNA extract were run in parallel with each PCR. PCR amplification of the spiked positive control was invariably compromised (Figure

19). The spiked control either did not amplify at all, or only amplified minimally (i.e. did not amplify to the extent of the non-spiked positive control).

Table 10. PCR amplification of MtDNA from Ancient Egyptian hair shafts

Sample	HVI/HV2	28S rRNA	Sequence
K2 B76	Yes*	no	n/a
K2 B101	No	yes*	n/a
K2 B124	No	no	n/a
K2 B259	No	no	n/a
K2 B269	No	yes*	n/a
K2 B271	No	no	n/a
K2 B458	Yes*	yes*	yes**
K2 B491	Yes*	no	n/a
G10-3	No	yes*	n/a
NT6-2-28	No	yes*	n/a

**Presence of amplifiable mtDNA in extract, either HVI, HV2 or 28S*

***unable to replicate*

[redacted]

Figure 18. Minimal PCR amplification (HV2: 162-258) of DNA extracts from K2 B491 and K2 B76. *Lane 11-* K2 B76, *lane 12*-K2 B491, *lane 13*- positive control (modern human)

[redacted]

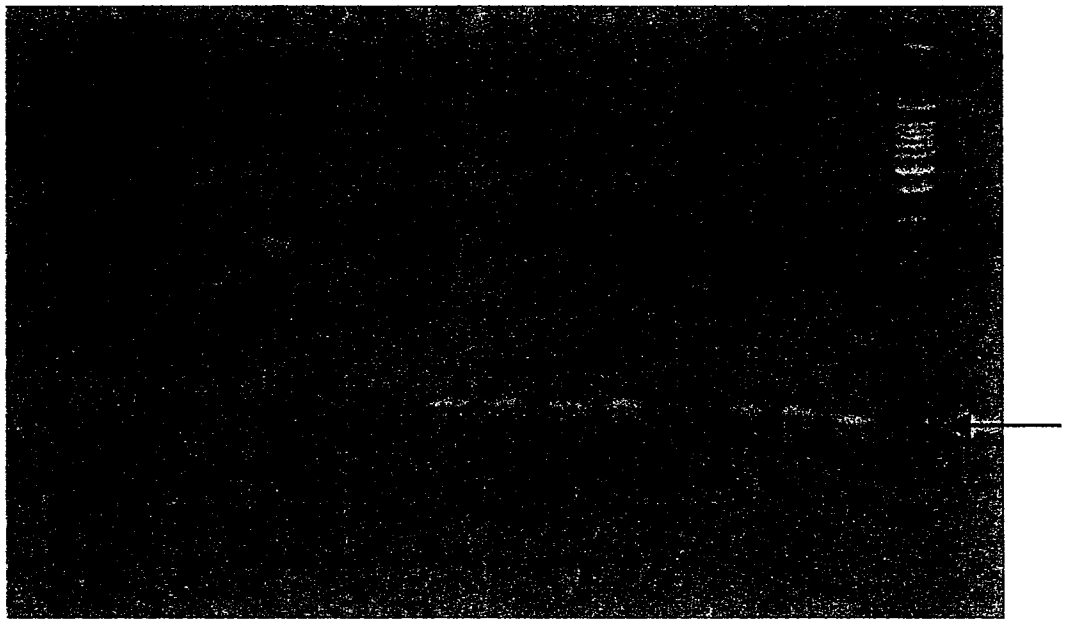

Figure 19. PCR amplification (HV1: 16193-16425) of spiked positive controls is compromised. Arrow indicates presence of primer dimer. *Lane-10* Spiked (K2 B101) positive, *lane –11* Spiked (K2 B101) positive, *lane 12*- positive control (modern human).

Quantification of DNA

The fluorometer registered total DNA yields of 124.3ng/mL for G10-3 and 53.62ng/mL for K2 B124. The negative extract control registered as –0.008ng/mL. The initial quantity of target template was calculated by interpolating from the standard curve (Table 11).

Melting Curve Analysis

A 238bp DNA fragment was successfully amplified from both ancient DNA extracts (G10-3 and K2 B124) using human specific *12S rRNA* primers.

Melting curves were acquired for both ancient and modern comparative PCR products (*12S rRNA*: 708-946) (Table 12 and Figure 20, 21, 22). The melting profiles of the ancient extracts (G10-3 and K2 B124) were compared with those of the known controls and it was determined that the ancient extracts were of human origin. The melting curve profile of K2 B124 matched the melting curve of the modern Asian control. The Asian control template differs from the Caucasian template in that it contains a polymorphism in the primer-binding region at nucleotide position 709 (Figure 23).

Table 11. Real-time PCR DNA Quantification of Ancient Egyptian and Modern Human Control Extracts

Sample	Initial mtDNA Quantity (ng/μL)
G10-3*	1.291×10^{-12}
K2 B124 *	3.764×10^{-12}
Modern Asian	7.216×10^{-6}
Modern Caucasian	6.494×10^{-6}
Extract negative	N/A
PCR negative	N/A

* G10-3 1:10 dilution; K2 B124 1:5 dilution

Table 12. Melting Temperatures of Ancient Egyptian and Modern Human Control PCR Amplicons (12S rRNA gene: 708-946)

Sample	Melting Temperature (°C)
G10-3	82
K2 B124	83
Modern Asian	83
Modern Caucasian	84

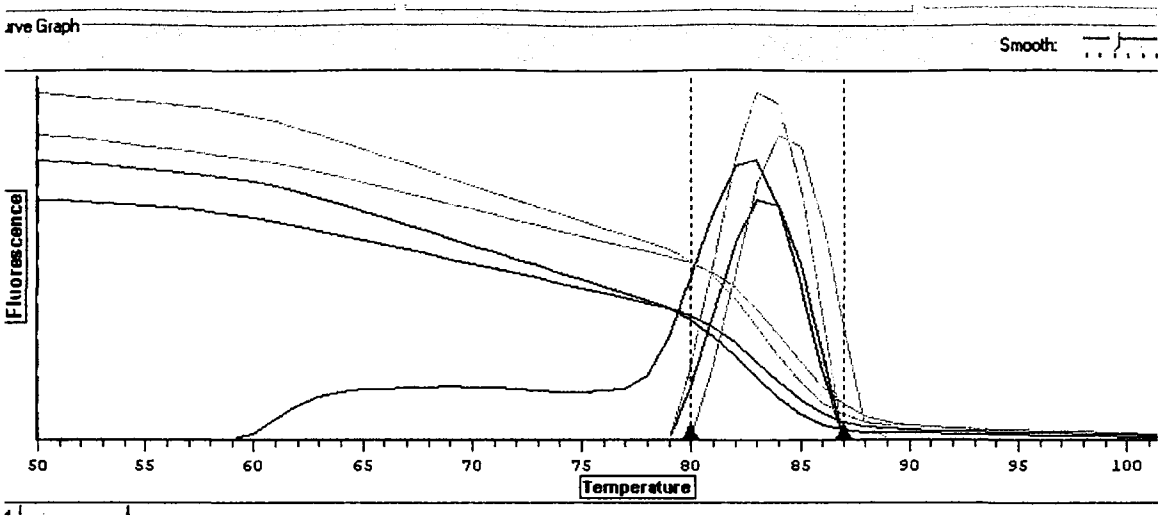

Figure 20. Melting curve profiles of ancient Egyptian amplicons and modern controls. K2 B124-orange, G10-3- blue, Asian modern control-red, Caucasian modern control-green.

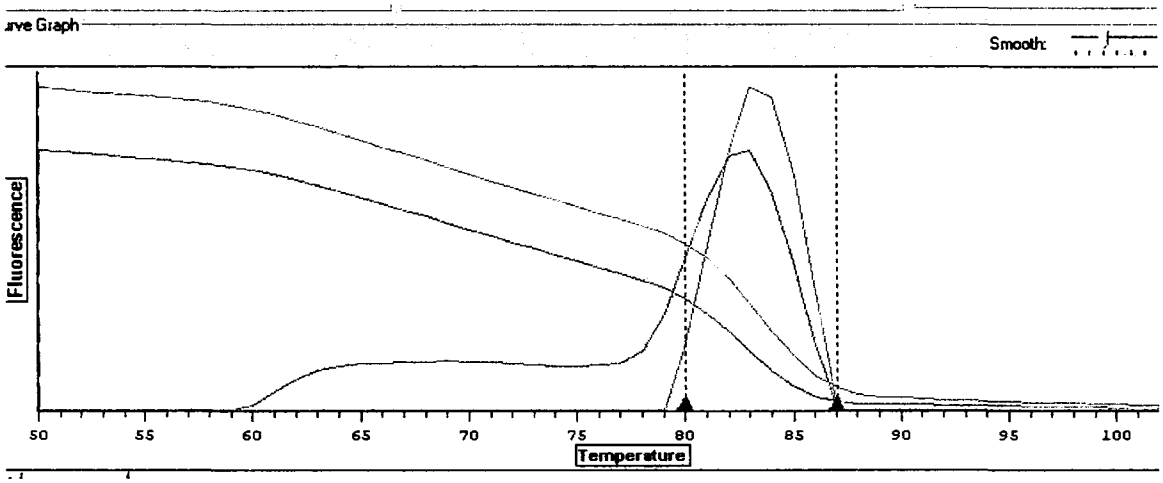

Figure 21. Melting curve profiles of G10-3 (Nubian) and Caucasian modern control. G10-3-blue, Caucasian modern control-green.

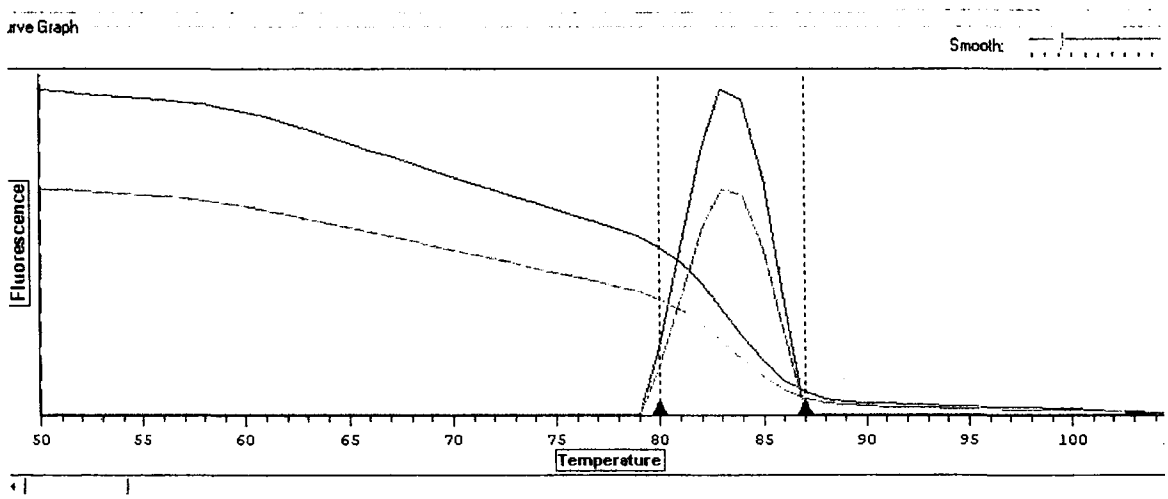

Figure 22. Melting curve profiles of K2 B124 and Asian modern control. K2 B124-green, Asian modern control-red.

Asian *12S rRNA* Sequence (708-946)

CATTCCAGTGAGTTCACCCTCTAAATCACCACGATCAAAAGGGACAAGCA
TCAAGCACGCAGCAATGCAGCTCAAAACGCTTAGCCTAGCCACACCCCCA

CGGGAAACAGCAGTGATTAACCTTTAGCAATAAACGAAAGTTTAACTAAG
CTATACTAACCCCAGGGTTGGTCAATTTCGTGCCAGCCACCGCGGTCACA
CGATTAACCCAAGTCAATAGAAGCCGGCGTAAAGAGTGT

Caucasian *12S rRNA* Sequence (708-946)

CGTTCCAGTGAGTTCACCCTCTAAATCACCACGATCAAAAGGGACAAGCA
TCAAGCACGCAGCAATGCAGCTCAAAACGCTTAGCCTAGCCACACCCCCA
CGGGAAACAGCAGTGATTAACCTTTAGCAATAAACGAAAGTTTAACTAAG
CTATACTAACCCCAGGGTTGGTCAATTTCGTGCCAGCCACCGCGGTCACA
CGATTAACCCAAGTCAATAGAAGCCGGCGTAAAGAGTGT

Figure 23. 12S rRNA (708-946) sequence data for modern Asian and Caucasian controls. A polymorphism is located at nucleotide position 908 (G to A). It is likely that K2 B124 also possesses a polymorphism at this nucleotide position.

An Investigation of Potential PCR Inhibitors Present in Ancient Egyptian Hair

Detection of Organic Contaminants (e.g. henna dye molecule)

Gas Chromatography-Mass Spectrometry (GC-MS)

Benzenesulfonic acid was the only organic molecule detected by GC-MS that is not biologically inherent in hair (Figure 23). Benzenesulfonic acid is commonly present in cosmetics and industrial solvents.

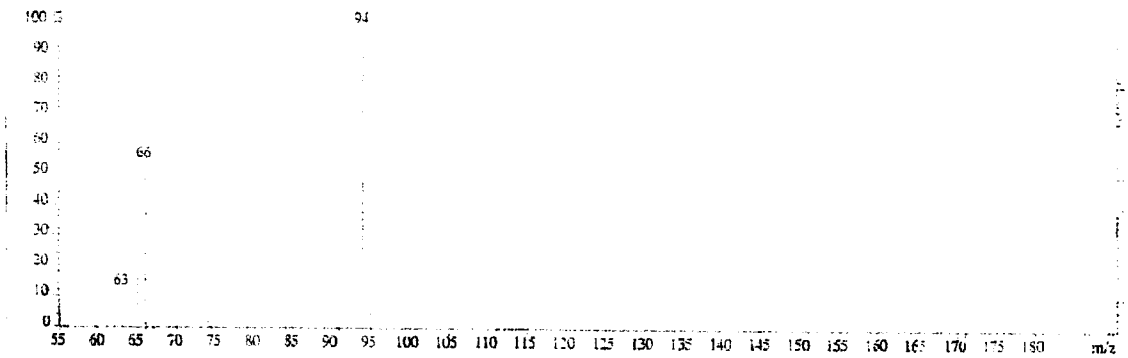

Figure 24. GC-MS revealed the presence of benzenesulfonic acid in the hair of Egyptian burial K2 268.

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS did not identify the presence of any specific organic compound (e.g. henna dye molecule) in hair samples from burials K2 76 or K2 124. Fragments detected had very low mass weights and thus had very low powers of discrimination. The low mass fragments were not unusual to those that are typical of hair proteins.

Raman Spectroscopy

Raman spectroscopy failed to identify the presence of henna (2-hydroxy-1,4 napthoquinone) in hair samples from burials K2 76 and K2 124. The pigment naturally

found within hair (melanin) saturated the spectrum hiding smaller spectral features that may have provided evidence of the henna dye.

Detection of Inorganic Contaminants

Inductively Coupled Argon Plasma Spectrometry (ICAP/ICP)

i) Hair Samples

ICP analysis of purified DNA extract from hair samples NT6-2-28 and G10-3 revealed an abundance of Na and S. Ca, K, Li, Mg and W were also present in slight amounts (Table 13). The negative control of the purified extract did not contain the aforementioned elements.

ii) *Soil Samples*

ICP analysis of soil associated with burial NT6-2-28 revealed the presence of large amounts of Na, Ca, Al, Fe, S, Mg and K. Soil associated with burial K2 269 contained large amounts of Ca, Al, Fe, S, P, Na, Mg and K. K2 B269 soil contained a significantly higher amount of Ca and S and a significantly lower amount of Na than NT6-2-28 soil (Table 14).

Hair and soil associated with burial NT6-2-28 both contained large amounts of Na. Both sample types also possessed the following common elements at detectable levels: S, Ca, Mg and K.

Table 13. Elemental Analysis of Ancient Egyptian Hair Sampies (NT6-2-28 and G10-3) using ICP

Element	Hair Samples(ug/mL)	
	NT6-2-28	*G10-3*
Al	<DL	<DL
Ca	0.1	0.31
Cu	<DL	<DL
Fe	<DL	<DL
K	1.15	0.37
Li	0.05	0.05
Na	32.9	10
P	<DL	<DL
S	85	32.5
Sr	<DL	<DL
Ti	<DL	<DL
Tl	<DL	<DL
V	<DL	<DL
Mo	<DL	<DL
Mg	0.05	0.02
Mn	<DL	<DL
W	<DL	<DL
Zn	<DL	<DL

DL=below detection limit

TABLE 14. Elemental Analysis of Soil from Kellis 2, Dakhleh Oasis, Egypt using ICP

Element	Soil Samples (ug/g)	
	NT6-2-28	*K2 B269*
Al	14154.1	15471.9
B	20.9	29.7
Ba	49	47.6
Be	<DL	<DL
Ca	27253.5	47372.3
Cd	0.7	2
Co	1.9	3.5
Cr	19.8	23.1
Cu	3.6	22
Fe	8872.6	13393.9
K	3565	3087.1
Mg	5066.5	4775.6
Mn	149	146
Mo	<DL	<DL
Na	176648	9046.5
Ni	6.2	7.6
P	825.4	9306
Pb	0.3	6.3
S	5068.6	10881.3
Si	574.5	591.4
Sr	132.8	208.6
Ti	148.5	116.9
Tl	<DL	<DL
V	<DL	<DL
Zn	26.3	62.68

DL= below detection limit

DISCUSSION

This thesis tested the efficacy of hair as a potential and better source of ancient DNA than other tissues, especially bone and dentin. The hypothesis was posited on advantages of hair in terms of its structure, namely being avascular, having a protective outer cuticle (anhydrodic) layer and an inner cortex made up of keratin which binds and protects the mtDNA. Also, mtDNA yields should be higher because the hair shaft is outside the body and free from the apoptotic autolytic enzymes that destroy the DNA in living tissues. The research design initially included the use of keratinase as a substitute for proteinase K in the extraction protocol, since its specificity for keratin was hypothesized to be more effective in liberating the mtDNA. This hypothesis was not supported as the mtDNA yields were similar in the three modern control samples. This protocol was abandoned in the experiments on the ancient hair samples from the Kellis 2 population sample from the Roman Period Dakhleh Oasis, Egypt. However, keratinase may possibly prove to be effective for the extraction of mtDNA from hair shafts when used with alternative buffers and/or in conjunction with proteinase K.

Four protocols were tested on the ancient Dakhleh hair samples. Three protocols involve a mechanical preparation (mortar and pestle) phase (the FBI protocol, proteinase K-silica column, proteinase K-magnetic beads) while the Gilbert *et al* (2004) protocol utilizes chemical digestion. The latter protocol performed the best in terms of mtDNA yields measured by the fluorometer. The FBI method (Wilson *et al*, 1995) ranked second, while the silica and magnetic bead methods performed poorly. The lower DNA yields using silica column and magnetic beads could be attributed to the high DNA binding

affinity of both matrices. Once DNA is bound to the silica column and/or magnetic beads, it is difficult to recover in totality. Heat can be used as a catalyst to release the bound DNA and may improve recovery but this is not entirely effective. PCIA-micro-filtration recovered significantly more mtDNA than the previous two methods. Unfortunately, microfiltration is not selective for DNA it filters molecules on the basis of size and thus the probability of inhibitors being included in the extract is increased. If a particular PCR inhibitor is below the filter cut-off size, it will move through the membrane along with the DNA. Conversely, although both magnetic bead and silica column purification yielded significantly lower amounts of DNA, these purified extracts may contain significantly lower levels of PCR inhibitors. I recommend using PCIA in conjunction with microfiltration to purify DNA extracts from ancient hair shafts for maximal DNA recovery.

As noted, the Gilbert *et al* (2004) extraction protocol had the highest DNA yields likely because of the chemical digestion step. The mechanical method probably compromised DNA yields for several reasons. If the hair shaft is not sufficiently ground prior to the incubation step, yields will be limited as a result of a reduced surface area exposure to the DNA extraction reagents. Also, DNA yields may be sub-maximal because the total ground sample will likely not be fully recoverable from the mortar and pestle (i.e. adherence of sample to glass surfaces). The significantly higher level of DNA contamination with the FBI protocol also is attributable to the mechanical extraction process because it is difficult to avoid generating aerosols while grinding the hair shafts with microtissue grinders. This can be especially problematic while processing two or more samples in parallel and may result in cross-contamination. An additional problem

occurs when reusing the microtissue grinders, since even with stringent cleansing and sterilization contaminating DNA is not always effectively removed. Although costly (each microtissue grinder costs approximately $65.00), it would be recommended to discard microtissue grinders after each use, since contaminated samples ultimately are more costly in terms of both money and time.

PCR amplification is typically unsuccessful with ancient samples and this was the case with the ancient hair. Two main reasons account for this: (1) PCR inhibition and (2) poor template quantity and quality. PCR inhibitors that co-purified with mtDNA extracted from the ancient hair shafts contributed to PCR failure herein. The presence of a PCR inhibitor in the ancient hair DNA extracts was confirmed by "spiking" a modern positive control with the ancient DNA extracts. The "spiked" PCR can be used to distinguish between amplification failure due to the degradation of DNA or from failure due to the presence of an inhibitor (Reiss *et al*, 1999). If a PCR inhibitor is present in the extract, the "spiked" control band will be reduced or eliminated. For comparative purposes, a control lane containing pure modern template without ancient extract is run parallel to the "spiked" control.

An inverse correlation was observed between sample quantity and PCR amplification. When used as template for PCR, DNA extracts from hair shaft quantities >10cm did not even weakly amplify. Although, PCR inhibition to varying degrees was universal with all ancient DNA extracts, all DNA extracts retrieved from hair shafts in excess of 2cm typically resulted in complete PCR inhibition.

Inhibitor molecules may have been introduced to the hair shaft by diffusion of organic and/or inorganic molecules from the burial microenvironment, or perhaps by

ancient cultural or cosmetic practices (Zink *et al*, 2005). There are molecules biologically inherent to the hair shaft, such as melanin, that also act as PCR inhibitors. PCR inhibitors are ubiquitous and can be found in soil, wood, textiles, leather, dyes and blood (Bourke *et al*, 1998). Maillard products, glucose derived protein cross-links, are another type of PCR inhibitor commonly found within ancient samples (Poinar *et al*, 1998).

In most cases, the identity and the mechanism of the inhibitor are unknown. Inhibitors can act through several mechanisms, but primarily are known to target Taq polymerase (Eckhart *et al*, 2000). They can also impede the PCR process by degrading or capturing the DNA or by interfering with other components of the PCR reaction such as $MgCl_2$ (Weissensteiner *et al*, 2004). PCR inhibitors may be diffusible in the PCR reaction or may be bound to the DNA template. If the inhibitor is bound to the template (e.g. intercalated) it can interfere with the denaturation and/or annealing step of PCR (Reiss *et al*, 1999).

Most methods commonly used to remove inhibitors from DNA extracts are not suitable for highly degraded or low yield DNA samples such as ancient hair shafts (Bourke *et al*, 1999). However, it may prove effective to include PCR additives such as bovine serum albumin (BSA), T4 gene 32 protein (gp32), single strand DNA binding protein or dimethyl sulfoxide (DMSO) in PCR reactions with ancient hair DNA extracts. The mode of action of both BSA and gp32 is to bind phenolic compounds (Kreader *et al*, 1996). Phenolic compounds include humic and tannic substances which are produced by the decomposition of organic matter in soil. However, it is possible that the inhibitor present in the ancient hair DNA extract will not respond to the addition of PCR

adjuvants. Another means in which PCR inhibition could potentially be eliminated is by purification of the DNA extract using agarose gel electrophoresis followed by band excision and passage through a microconcentrator (Zhou, 1996). The appropriate equipment and reagents to pursue these experiments were unavailable for this study. Ultimately, in order to be efficiently and effectively removed the PCR inhibitor (s) must be identified.

There are a variety of techniques that can be used to eliminate PCR inhibition. This problem was addressed herein by reducing sample quantity to a minimum, using PTB, and a variety of purification methods, diluting the DNA extract, and by increasing Taq polymerase. To combat the potential presence of maillard products, PTB was included in the extraction buffer. PTB is capable of breaking down maillard products by cleaving glucose-derived protein cross-links (Poinar *et al*, 1998). Progressive dilutions of the extract were prepared with the hope of being able to dilute away the inhibitor(s). However, there is a fine balance that must be maintained when diluting ancient DNA extracts in order to retain sufficient amount of template for analysis.

Numerous analytical techniques were used to test for the presence of PCR inhibitors. Gas-chromatography-Mass spectrometry (GC-MS), Secondary Ion Mass Spectrometry (SIMS) and Raman Spectrometry were used to detect the presence of organic contaminants. It was hypothesized that henna may be a potential inhibiter because it is grown in Egypt (Egyptian privet) and many of the Dakhleh hairs are red, the colour in which the natural dye produces. I tested for the presence of '2-hydroxy-1, 4 napthoquinone' the active dye molecule in henna. Initially, GC-MS was used but was unsuccessful possibly because hydroxy and oxygen groups on the napthoquinone are

active sites from a chromatographic perspective (Mr. Ain Raitsakas, Personal Communication). Furthermore, GC-MS may have likely been unsuccessful because it required a larger quantity of hair sample to meet its detection threshold. SIMS and Raman spectroscopy were subsequently used because these techniques are extremely sensitive and only require a minimal amount of sample for analysis. None of these techniques however, proved successful in identifying henna or any other organic inhibitor. The reddish colouration in the Dakhleh hair samples may be a response to hyperthermia in the tombs, not cosmetic treatment of the hair (Dr. Dave Chapman, Personal Communication).

Inductively Coupled Plasma (ICP) analysis was used to identify potential inorganic contaminants. High levels of Na and S were found in hair shafts from burials NT6-2-28 and G10-3, which was not unexpected. Na is a common biological and environmental constituent and likely diffused into the hair shaft from the burial microenvironment. Perhaps the high level of Na found within the hair shaft acted as preservative and/or anti-microbial agent maintaining the structural integrity of the hair shaft. Sulphur is abundant within these samples because the main constituent of hair, keratin, is composed of the sulphur-based amino acid cysteine. Ca, K, Li, Mg and W were also present within the hair extracts.

Soil from Kellis 2 was also analyzed using ICP. ICP analysis of soil associated with burial NT6-2-28 and G10-3 revealed an abundance of Na, Ca, Al, Fe, S, P, Mg and K. Some of these elements found within the soil may have contaminated the hair shafts and contributed to PCR inhibition. Hair seems to be highly absorbent rather than protected against environmental contaminants as was hypothesized.

One question that deserves special consideration is the DNA content measured by the fluorometer. Historically in ancient DNA research quantification, of the DNA was not attempted because of the highly degraded nature of the product. The high yields of DNA found in the ancient DNA product from the hair samples, especially the yields from the Gilbert *et al* (2004) protocol, suggest contamination from microbial DNA and/or modern human DNA. This was revealed by the use of real time PCR. Real-time quantity PCR (qPCR) indicated that human mtDNA is present within the DNA extracts from burials G10-3 and K2-124. However, the concentration of amplifiable human mtDNA as expected, is low. Compared to the DNA quantification using a fluorometer, qPCR quantifies the total amount of target template (e.g. *12S rRNA* 708-946bp) not the total amount of cellular DNA. Consider the following: The amplicon of interest, *12S rRNA* 708-946 represents about 1.4% of the total mtDNA genome (238/16,569). Although the fluorometer readings are reflective of total cellular DNA, due to the nature of the sample type (i.e. hair shaft) the contribution of nuclear DNA to the readings is likely insignificant. Thus in theory, the qPCR readings should be equivalent to 1.4% of each fluorometer readings. The fluorometer reading for the DNA extracts from burials G10-3 and K2 124 were 0.123ng/μL and 0.0536ng/μL, respectively. 1.4% of the fluorometer readings are as follows, burial G10-3, 1.851×10^{-3} ng/μL and burial K2 124, 8.043×10^{-4} ng/μL. The qPCR readings for the DNA extracts from burials G10-3 and K2 124 were 1.291×10^{-12} ng/μL and 3.764×10^{-12} ng/μL, respectively. Thus, hypothetically, the fluorometer readings of the ancient DNA extracts are significantly higher ($>10^{8}$) than the qPCR readings. The fluorometer readings are likely higher than the qPCR readings due to the contribution of non-human (e.g. microbial) DNA present in the ancient extracts.

Also possible is that the ancient hair shafts may suffer from fungal contamination as a result of antemortem infection. In ancient times, due to lack of inadequate hygiene, it is likely that fungal infections of the hair shaft were common. Fungi can invade the hair shaft and leave contaminating spores in the cortex. More likely however, is non-human DNA present in the ancient extracts from soil fungi. To investigate this, the ancient hair extracts could be examined for the presence of DNA from soil fungi indigenous to the Dakhleh Oasis using Real-time PCR. The presence of fungi could also be determined by using a longitudinal sectioning method (Kempson *et al*, 2001) and subsequently viewing the internal and external structure of the hair shaft with SEM. This technique would reveal the presence of contaminants as well as the state of histological preservation.

The fluorometer readings may also be reflective of highly fragmented and/or damaged mitochondrial and nuclear DNA that is unamenable to the PCR process. Poor template quality is a common challenge when analyzing DNA extracts from ancient remains. The DNA quality may be intrinsically compromised during keratinization of the hair shaft and extrinsically by the extraction and PCR processes. Common DNA lesions include DNA strand breaks and apurinic/apyrimidinic sites (Weissensteiner *et al*, 2004).

The melting curve profiles of the ancient DNA (G10-3 and K2 B124) amplicons were compared with the melting curve profiles of known modern human controls to test if the ancient amplicons were of human origin. Melting curve profiles differentiate amplicons on the basis of GC/AT ratio, length and nucleotide sequence (Ririe *et al*, 1996). Amplicons that have the same length and GC/AT ratio, but differ only in their GC distribution will have different melting curve profiles. Melting curves can even be affected by a single base mutation (Marziliano *et al*, 2000). The melting curve profile of

K2 B124 is most closely associated with the melting curve profile of the modern Asian control. The difference between the modern Asian and modern Caucasian melting curves is the result of a polymorphism at nucleotide position 709 (G to A). This polymorphism is indicative of Asian haplotype M (Kong *et al*, 2003). Due to the similarity of the melting profiles of the modern Asian and K2 B124 templates, it is likely that K2 B124 also possesses a polymorphism at nucleotide position 709. However, this cannot be confirmed without sequencing or probing the ancient amplicon. It should be noted that in a study by Graver *et al* (2001), it was determined that on the basis of the restriction markers *AluI* and *DdeI*, that none of the tested (N= 18) K2 burials possessed the Asian mtDNA haplotype M. However, 44% (8/18) of the K2 sample possessed a C to T transition at position 16223, a polymorphism that is common in Asian populations (Graver, 2000).

The melting curve profile of burial G10-3 did not match the profile for Asian or Caucasian affinity. This result, which was done in blind, is noteworthy since the osteometric and qualitative morphological traits, in conjunction with the hair structure (kinky black Negroid hair), suggest that this young adult female in her early 20s was a foreigner probably brought into the Dakhleh Oasis from Nubia or some other sub-Saharan region as part of a marital arrangement. The possible area of origin for this individual is currently being addressed using stable oxygen isotopes (Dr. Tosha Dupras, Personal Communication). This data provides further support of the hypothesis that the DNA quantified using Real-time PCR is authentic ancient DNA.

CONCLUSION

This thesis has demonstrated that authentic ancient human DNA can be recovered from hair. Since the Dakhleh samples are almost 1800 years old, this challenges the interpretation of Marota *et al,* (2002) that states DNA can not survive longer than 800 years in Egypt because of the xerophilic environment. The hypothesis that hair is a better archive of mtDNA than bone or dentin is however, is rejected at this time. Hair seems just as likely to be degraded and contaminated as other tissues. The preliminary results, particularly that showing the Negroid ancestry of burial G10-3 using a novel approach (melting curve) in conjunction with Real-time PCR, are encouraging and indicate mtDNA is present in low yet analyzable levels in ancient human hair shafts. Future research should utilize the Real-time methodology. Perhaps sequencing results could be obtained from the melting curve data by removing the SYBR green dye which, because it bonds to the minor groove of the DNA helix, acts as an inhibitor. From this research I recommend the use of the Gilbert *et al* (2004) extraction protocol in conjunction with PCIA-microfiltration for optimal DNA recovery in human hair shafts. In particular, this protocol would be recommended when using ancient or degraded specimens where sample contamination poses a significant risk. The role of proteolytic enzymes (e.g. proteinase K, keratinase) in the DNA extraction process also needs to be addressed. These enzymes digest proteinaceous material (e.g. cell membrane proteins, histones) within the tissue of interest, however, they may not be of great importance in the extraction of mtDNA from hair shafts, bone and dentin. MtDNA does not have histones and therefore the proteolytic enzymes may in fact damage ancient DNA. Sample preparation, non-enzymatic extraction buffer components (e.g. DTT, SDS), and

purification methods may play a more significant role in the recovery of optimal DNA yields than proteolytic enzymes. An experimental approach to validate this hypothesis would be to assess DNA yields from hair shafts using an extraction protocol without proteolytic enzymes. Finally, this thesis has shown that ancient hair samples are highly susceptible to products that can cause PCR inhibition. Future research should be designed to identify specific inhibitors in order to maximize the recovery of targeted ancient DNA from hair.

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