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DEVELOPMENT AND VALIDATION OF AN ALKALINE EXTRACTION METHOD FOR ISOLATING MITOCHONDRIAL DNA FROM HUMAN HAIR SHAFTS

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DEVELOPMENT AND VALIDATION OF AN ALKALINE EXTRACTION METHOD FOR ISOLATING MITOCHONDRIAL DNA FROM HUMAN HAIR SHAFTS

By

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ABSTRACT

DEVELOPMENT AND VALIDATION OF AN ALKALINE EXTRACTION METHOD FOR ISOLATING MITOCHONDRIAL DNA FROM HUMAN HAIR SHAFTS

By

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Human hair is one of the most common types of forensic biological evidence; thus, the reliable identification of its source is important to the outcome of criminal investigations and court proceedings. Recent post-conviction exonerations in cases involving hair evidence (Dwyer et al. 2000) have shifted the preferred method of hair analysis from microscopic comparison of morphological features to analysis of nuclear DNA or, more often, mitochondrial DNA (mtDNA). Current methods for isolating mtDNA from hair are tedious and pose substantial risk of sample contamination. The goal of this research was to develop a simplified protocol for mtDNA extraction from hair shafts, using a strong alkaline solution, that performed as well or better than the method currently used by forensic laboratories. Hair and reference DNA samples were collected from thirty volunteers, and mtDNA was extracted from hairs using both techniques. The quantity and quality of DNA was consistent between the two methods, while amplification success rates for the alkaline protocol exceeded those of the standard method. Further, mtDNA sequence results from alkaline-extracted hairs matched the reference samples in all cases, confirming the accuracy of mtDNA testing from hairs using alkaline digestion. The simplicity and efficiency of alkaline extraction, and its comparable, if not improved, results make it worthwhile to implement and may expand the capabilities of forensic laboratories conducting mtDNA analysis from hair.

To my parents, for their unwavering support of all my pursuits

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INTRODUCTION

Human hair has long been regarded as one of the most frequently recovered types of biological evidence (Bisbing 1982), and the accurate identification of its origin can be paramount to the success of a criminal investigation. Traditional forensic analysis of hair involves microscopic comparison between an evidence specimen and a collection of exemplar hairs—measuring structural dimensions and examining morphological features such as color, cross section shape, pigmentation, cosmetic treatment and disease. While these criteria have been used since the early 20th century to include or exclude a person as a potential source of an evidence hair (Bisbing 1982), the method is incapable of supporting its conclusions with statistical data. Even with a large and seemingly representative collection of exemplars from a suspected source, morphological hair comparison remains a highly subjective technique, as "little data exist to aid the examiner in assessing the significance of a hair comparison" (Bisbing 1982).

Recent post-conviction exonerations in cases where morphological comparison of evidence hairs played a key role have also cast doubt on the reliability of this form of forensic analysis. A large proportion of these exonerations have been handled by the Innocence Project, a group of attorneys and law students that provide pro bono legal assistance to convicted persons, often pursuing DNA testing that was not available at the time of trial. The Innocence Project estimates that evidence from morphological hair analysis was involved in 29% of the wrongful convictions taken on by the group through 1999 (Dwyer et al. 2000). Problems with hair evidence in these cases ranged from examiner incompetence, to the invention of spurious statistics, to inflated testimony about

conclusions drawn from microscopic analysis. While some of these transgressions can be assigned to unscrupulous examiners who were aware of their deceit (e.g. Fred Zain, who gave testimony regarding examinations he never conducted, Holliday 1993), others have their origins in the shortcomings of morphological hair comparison. For example, in the case of two men convicted of rape and murder in Oklahoma (*State of Oklahoma v. Ronald Keith Williamson and Dennis Leon Fritz*, CRF87-90), DNA testing of seventeen hairs attributed to the pair by microscopic analysis found that not one of them originated from the convicted men. In fact, the results indicated that DNA from one pubic hair matched the victim's DNA type, and two other hairs could be tied to a prosecution witness, both of whom had supposedly been eliminated as possible sources of the hairs in the original analysis. One of the wrongly convicted men was five days from execution when a stay was issued and the DNA tests ordered on the hair evidence that exonerated him (Dwyer et al. 2000).

Forensic DNA testing of hairs takes one of two forms: analysis of nuclear DNA or mitochondrial DNA (mtDNA). Nuclear DNA typing requires a relatively intact sample, and is usually limited to the root portion of the hair. MtDNA analysis, while less discriminating than nuclear DNA testing, is amenable to trace and/or compromised biological samples, including hair shafts. Both forms of DNA analysis are supported by population data, allowing the probability of a random DNA match to be calculated for each association.

DNA analysis of hairs is independent of morphological characteristics and therefore may be used to evaluate the accuracy of microscopic hair comparison. Houck and Budowle (2002) used mtDNA testing to review the results of 170 hair examinations

performed by the Federal Bureau of Investigation (FBI) Laboratory between 1996 and 2000. Nine of 80 hairs (11%) associated with a known source by FBI hair examiners using morphological comparison could be excluded by mtDNA analysis. Furthermore, while 71 of these 170 hairs (42%) were either insufficient for microscopic examination or gave inconclusive results, 161 of 170 hairs (95%) produced mtDNA results. Even while exercising the caution to draw conclusions from only about 60% of their examinations, 1 of every 9 associations made by the FBI was incorrect. The subjectivity and potential for error in morphological hair comparisons present a special problem to the forensic science and criminal justice communities. Controversy over the technique's reliability has made this type of evidence subject to attack by attorneys, and there are movements among some members of the criminal justice community to keep results of morphological hair comparisons out of court unless they are supported by DNA evidence (Dwyer et al. 2000, Foran and Rowe 2001).

Microscopic examination of hair remains useful as a screening process for gleaning information on species of origin, body area of origin (scalp, pubic, axillary, etc.), the presence of any tissue or foreign substance adhering to the hair, and potentially the ancestry of the donor. However, the amenability of mtDNA testing to trace samples and the availability of population statistics make it the preferred method for source identification of forensic hair samples. The move toward DNA testing of all evidentiary hair samples is not without problems, however. Some state forensic laboratories may lack the funds, personnel, and expertise to carry out their own mtDNA testing. Furthermore, implementation of a new technique is a slow process, and many laboratories may be unwilling to undertake a change when the opportunity exists to pass the analysis

on to the FBI or contract it out to private companies, the latter at a cost of several thousand dollars per comparison. In either case, mtDNA testing of hair shafts is an expensive and lengthy process. Mitotyping Technologies, a private mtDNA testing facility, estimates its production at 1–2 cases per analyst per month (Melton and Nelson 2001).

Concurrent with the growing trend toward mtDNA testing of hairs prior to trial, increasing numbers of past cases involving hair evidence are now coming under review. Laws setting forth specific guidelines for post-conviction DNA testing, including mtDNA testing of hairs, are in effect in over 30 U.S. states; thus, the re-examination of post-conviction evidence has the potential to dramatically expand the mtDNA caseload of forensic laboratories (Melton and Nelson 2001). Given that many forensic laboratories already face large backlogs, an improvement in the ease and efficiency of mtDNA testing is needed to accommodate this potential growth in case submissions.

Hair Anatomy and Physiology

The suitability of nuclear DNA and mtDNA for forensic analysis differs over the course of hair development. A basic knowledge of hair biology is useful to fully appreciate both the capabilities of DNA testing of hair samples and the situations in which a certain extraction or analysis technique is appropriate. While hair is an outgrowth of soft tissue, its complex structure requires special considerations for forensic biological examination. Linch et al. (2001) produced a description of hair histogenesis so as to better inform the mtDNA analyst how these processes might affect forensic

examinations. The description that follows, except where other references are noted, can be attributed to their review of the subject.

Hair consists of three primary structures (Figure 1). The medulla is a central shaft that in humans is usually filled with air. Surrounding the medulla is the cortex, the layer of cells that contains the various pigments (mostly melanin) that confer color to the hair. The primary component of the cortex is keratin—the protein that gives hair its hardened quality—also the major component in fingernails, toenails, and animal hoofs and horns. The outermost layer of the hair shaft is the cuticle, whose function is to protect the inner components and anchor the hair in the skin during growth (Harkey 1993).

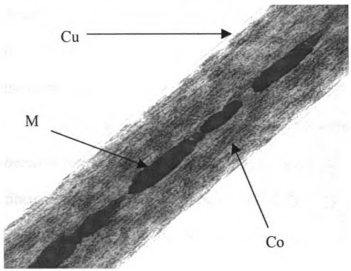


Figure 1. Structure of Human Hair Shaft

The medulla (M) is fragmented and appears dark in the photomicrograph's transmitted light because it is filled with air. Within the cortex (Co), the long, thin longitudinal layers are bundles of keratin, interspersed with melanin (dark areas). The cuticle (Cu) is largely transparent and barely visible along the edges of the hair. (Photo courtesy of Jay Siegel)

Each hair on the human body grows from an epithelial organ lying just below the skin surface, called a follicle. The follicle's structure consists of underlying mesodermal tissue, termed the dermal papilla, and an enlarged bulb of ectodermal cells called the matrix. Within the matrix are two types of cells: actively-multiplying germinal cells, which in their mature form comprise the entirety of the hair shaft, and slowly-dividing cells called melanocytes. Melanocytes remain in the bulb matrix throughout the life

cycle of the hair but contribute cellular products such as pigment grains, and organelles such as mitochondria, to the developing shaft.

Hair development

Stimulated by growth factors secreted by the dermal papilla, germinal cells multiply in the hair bulb at the base of the follicle. This germination feeds the growing hair with new cells, contributing to the lengthening of the hair shaft. As germinal cells are released from the matrix, they begin the process of differentiation into medullary, cortical, and cuticle cells. Pre-cortical cells passing through the bulb matrix engulf dendritic projections of melanocytes by endocytosis and carry the absorbed material into the hair shaft. This process distributes pigments produced by the melanocytes throughout the cortex and may also create a mixture of mitochondria within the cortical cells.

As the hair lengthens and cells move away from the root bulb, synthesis of keratin becomes the major cellular activity. Eventually the cell is filled with bundles of keratin fibers to the point of cytolysis, and many of the cellular components disintegrate. Before a region of hair even emerges from the skin surface, the shaft consists of a mass of keratin fibers, interspersed with the debris of dead cells. Nuclear DNA has been lost, and mitochondria and other membrane-bound organelles, while mostly intact, are subject to extensive damage.

Growth Stages

Hair growth and regeneration is a cyclical process encompassing three distinct stages: anagen, catagen, and telogen. The active growth stage (anagen) is described

above, consisting of the genesis of a new hair in the follicle, followed by lengthening of the hair up to the point where the hair stops growing. This phase lasts several years for human head hairs, 11–18 months for pubic hairs, and only about 6 months for eyebrow, limb and trunk hairs (Harkey 1993, Linch et al. 2001). Melanocytes cease releasing pigment granules prior to the end of growth, so the basal segment of hairs in the subsequent stages are nonpigmented. Forced removal of the hair during the anagen stage often produces a specimen with follicular tissue adhering to the root end that can harbor nuclear DNA ideal for forensic analysis.

The catagen phase is characterized by the termination of cell division in the matrix and the regression of the root bulb toward the skin surface. The root bulb shrinks and rounds off to produce a club-like structure of keratinized cells. The catagen phase is fleeting, and hairs in this stage are not likely to be recovered as forensic specimens.

The telogen phase is considered the resting period before a hair is shed, and lasts several months for head hairs, 12–17 months for pubic hairs, and 2–6 years for other body hairs (Harkey 1993, Linch et al. 2001). Some tissue does serve to anchor the root club in the skin during this stage, but removal of the hair is relatively simple by mechanical means (e.g. brushing) or due to growth of a new anagen hair beneath it. An estimated 100–150 telogen hairs may be shed from a human head each day (Bisbing 1982, Linch et al. 2001). Not surprisingly, it is widely held that these represent the majority of hairs recovered from crime scenes (Higuchi et al. 1988, Wilson et al. 1995a, Allen et al. 1998, Jehaes et al. 1998, Pfeiffer et al. 1999, Savolainen & Lundeberg 1999), although no actual data exist to verify this notion.

Mitochondrial DNA Analysis of Hair

Human mitochondria contain a small, circular DNA of ~16569 base pairs (bp), first sequenced in its entirety by Anderson et al. (1981), that is found in hundreds to thousands of copies per cell (in contrast to the diploid nature of nuclear DNA). Whereas nuclear DNA is inherited from both parents, mtDNA is transmitted to offspring via the egg, and thus all maternal relatives have the same mtDNA type. The genes in mtDNA encode several important mitochondrial proteins, and thus these DNA areas vary little among individuals and are of minimal forensic utility. The segment of mtDNA used in identity testing, including forensic mtDNA analysis (reviewed by Holland and Parsons 1999), is known as the D-loop or control region, because it controls the replication and gene expression of the molecule. The control region does not encode proteins, hence this segment of the mtDNA is generally free to mutate, and thus vary among unrelated individuals. Two sections of the control region show a large amount of variation and are called the "hypervariable regions," coined HVI and HVII. Forensic mtDNA analysis is generally performed by amplifying the control region using the polymerase chain reaction (PCR), in which small amounts of DNA are copied millions of times, and determining the exact DNA sequence of HVI and HVII. The collection of nucleotide differences between a given sample and a human reference sequence comprise the mtDNA profile of the sample.

Another distinction of mtDNA is its base composition. From studies in mouse (Grossman et al. 1973, Brennicke and Clayton 1981) and human tissue culture cells (Grossman et al. 1973), mtDNA is known to contain between 10–30 ribonucleotides per molecule. These RNA building blocks were discovered due to their sensitivity to alkaline

conditions, and are concentrated in two regions around the origins of replication for each DNA strand, one of which is located in the D-loop. As ribonucleotides are normally components of RNA rather than DNA, and in view of their placement on the DNA molecule, they are theorized to be remnants of primers for mtDNA replication (Bogenhagen and Clayton 2003).

Although its power of discrimination is less than state of the art nuclear DNA analyses, mtDNA sequencing is often the best option for DNA typing of trace samples such as finger or toe nails, or compromised biological material, such as putrefied, skeletonized or ancient remains (Holland and Parsons 1999). Why mtDNA analysis remains viable long after nuclear DNA has apparently degraded in such samples is unknown. Although this phenomenon is frequently attributed to the high copy number of mtDNA in the cell, its location within the mitochondrion itself or other factors could just as easily contribute to its hardiness. Whatever the case, mtDNA seems to survive the process of keratinization well enough to adequately allow its amplification from single hair shafts. While nuclear DNA testing is preferred for its greater specificity, this form of analysis is often unsuccessful on telogen hairs; thus, the only option available for genetic testing of these samples is mtDNA analysis.

Extraction of mtDNA from Hair Shafts

Biological samples of evidentiary value are often recovered in less than ideal condition. Forensic hair specimens may have traces of soil, blood, semen, saliva, and any number of other contaminating substances adhering to the shaft, all of which may generate confounding DNA results or inhibit the amplification and sequencing processes.

In many instances these contaminants can be detected through microscopic examination and be isolated from the hair, usually by swabbing. Even with meticulous treatment, traces of the contaminating material are commonly left behind and must be removed prior to DNA extraction from the hair itself. For this reason most extraction methods are preceded by some form of cleaning or rinsing. These protocols vary by laboratory, but generally involve soaking the hair in sterile water, detergents, alcohol, or the like (Higuchi et al. 1988, Wilson et al. 1995a, Jehaes et al. 1998, Pfeiffer et al. 1999). Some protocols call for extended wash periods in a sonic bath or shaker (Wilson et al. 1995b), while others are fulfilled with a brief vortex or vigorous shaking (Morley et al. 1999, Savolainen and Lundeberg 1999). The cleaning method of choice is based on the equipment and supplies available, the degree and type of contamination suspected, and the protocols that have been tested and validated in each laboratory.

DNA extraction from hair roots is relatively straightforward and follows standard forensic methods like those used for blood, buccal swabs, and other typical biological samples. The abundance of keratin makes mtDNA extraction from a hair shaft more problematic, and this and other unique challenges of hair as a substrate for DNA analysis must be considered when developing an extraction protocol. Yoshii et al. (1992, original reference in Japanese, cited in Pfeiffer et al. 1999, Baker et al. 2001) and Uchihi et al. (1992) demonstrated that the abundant melanin in hair shafts acts as a PCR inhibitor. The success of mtDNA typing from heavily pigmented hair often depends on neutralizing these effects, either by removing inhibitors during extraction or modifying PCR additives to counteract their activity (Giambernardi et al. 1998).

Both chemical and mechanical means have been employed to break down the hair shaft and release mtDNA into solution. Allen et al. (1998) digested 1–2cm of hair in PCR buffer with proteinase K (proK) and dithiothreitol (DTT) and used this solution in nested PCR without further purification. Higuchi et al. (1988) utilized a relatively standard organic extraction, incubating the hair in Tris buffer with proK, DTT, and the detergent sodium dodecyl sulfate (SDS), and went on to extract the solution with phenol-chloroform and n-butanol followed by purification in a centrifugal filter device. Savolainen and Lundeberg (1999) adopted the protocol of Higuchi et al. but added carrier tRNA to the extraction mix. A vortex step to break apart the hair was followed by addition of more proK and several extra extraction steps with phenol and back-extractions with buffer. Morley et al. (1999) adapted a Chelex protocol to hair shafts with the addition of proK, DTT, and SDS. Baker et al. (2001) employed a glass tissue homogenizer to mechanically grind the hair, followed by a silica clean-up method adapted from techniques for ancient DNA.

The extraction method most prevalent in the United States, used by both the FBI and the Armed Forces DNA Identification Laboratory (AFDIL), uses mechanical and chemical means to break down the hair shaft. Hairs are physically macerated in extraction buffer using glass grinders, followed by multiple reagent additions (proK and DTT), organic extraction, and filtration and washing with a centrifugal filtration column (Wilson et al.1995a, 1995b, http://www.afip.org/Departments/oafme/dna/afdil/protocols.html). The method employs multiple containers (tubes), with the expectation of some loss of sample with each transfer. Given that sample size can be a limiting factor in forensic analysis, maximum retention is a priority for successful mtDNA typing. While

all other containers are disposable and therefore pose minimal cross-contamination risk, glass grinders are expensive and therefore ideally reused. The FBI has established a policy of using each grinder for three extractions before it is discarded (Rebekah Gundry, personal communication). This practice, along with multiple sample transfers and reagent additions (see Methods & Materials below) results in an obvious potential for sample contamination, "the most critical potential source of error in mtDNA" analysis (Wilson et al. 1993). Although labor-intensive cleaning and sterilizing steps are necessarily performed between extractions in order to guard against carry-over of DNA, the FBI reports low levels of signal in reagent blanks and negative controls after PCR amplification of mtDNA. A sample-to-contaminant ratio of 10:1 is considered by the FBI as sufficient to produce correct mtDNA typing results (Wilson et al. 1995a).

Research Goals

An extraction method that minimizes reagent additions, sample transfers, and sample handling, while still effectively and efficiently degrading the hair shaft and neutralizing inhibitors, would be ideally suited for forensic analysis of mtDNA from hair. The use of a strong alkaline solution to directly break down keratin could provide an alternative approach for mtDNA extraction from hair shafts. High-pH solutions readily hydrolyze proteins, even those as structured as keratin, potentially eliminating the need for reagents such as proK and DTT and the subsequent organic extraction steps required to remove them. The time and cost required for DNA extraction and the potential for sample loss and contamination are reduced, as the entire procedure takes place in one microcentrifuge tube and one spin column. While acidic conditions (low pH) will

damage DNA, extraction under high pH will simply denature it (make it single-stranded, which is the same as the first step in the PCR process), leaving its sequence information intact. Washing DNA solutions with sodium hydroxide (NaOH) has been demonstrated to reduce inhibition of DNA amplification, possibly by reducing DNA's affinity for intercalating inhibitors (Bourke et al. 1999). The known PCR inhibitor melanin, while insoluble in organic solvents, is soluble in strong alkali (Bisbing 1982).

Alkaline lysis has been used to extract DNA from other forensic samples, including whole blood, bloodstains, saliva and semen stains (Klintschar and Neuhuber 2000), and highly-keratinized samples such as fingernails (Cline et al. 2003). The objective of this research was to develop an alkaline extraction protocol for hair shafts that produced results comparable to the methods currently in use, but with fewer steps, low contamination potential, and reduced time and cost requirements. Even with these advantages, alkaline extraction must produce equal or better results in DNA yield, DNA quality, and sequence accuracy in order to be a viable option for forensic mtDNA testing of hair shafts.

METHODS AND MATERIALS

Sample Collection from Volunteers

Several strands of shed head hair and buccal swabs (to serve as reference DNA samples) were collected anonymously from 30 human volunteers. Subjects were asked, in the form of a questionnaire, to provide information on their sex, self-declared ethnic background/population ancestry, and any treatments recently done to their hair (Appendix A). Numbered stickers were used to associate samples and paperwork; a set of samples could not be associated with a volunteer. Samples were stored in manila envelopes at room temperature until DNA was extracted. All sample collection procedures and forms were approved by the University Committee on Research Involving Human Subjects.

DNA Extraction and Sequencing from Buccal Swabs

Buccal swabs were halved lengthwise with a flame-sterilized scalpel. One half of the cotton tip was transferred to a Spin-EASE extraction tube (Gibco BRL), to which was added 500µl digestion buffer (20mM Tris, 100mM EDTA, 0.1% SDS) and 3µl proK (20mg/ml). These were incubated overnight at 56°C. The cotton material was next transferred to a spin basket, and receiver tubes with baskets were centrifuged 2 min at 5000rpm to collect the extraction liquid. Baskets and dry cotton were discarded. The liquid was extracted with 500µl phenol:chloroform:isoamyl alcohol (25:24:1), followed by centrifugation for 5 min at 14000rpm. The aqueous layer was transferred to a clean tube, and DNA was precipitated using 50µl sodium acetate (3M) and 1ml cold 95% ethanol. Tubes were stored at –20°C approximately 1.5 hr, then centrifuged 15 min at

14000rpm. Pellets were washed once with 1ml 70% ethanol, vacuum-dried and resuspended overnight in 25µl 10mM Tris pH 7.6, 1mM EDTA (TE) buffer.

Buccal sample mtDNA control regions were amplified using an Eppendorf Mastercycler in two 20µl reaction volumes using 1µl of DNA template and a 1:20 DNA dilution, with PCR primers F15989 and R484 (see Table 1 for primer sequences) under the following conditions: 2 min at 94°C, 40 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, followed by 5 min at 72°C. The amplified DNA yield was estimated by electrophoresis of 5µl of each reaction on a 0.8% agarose gel. The remaining 15µl of successful reactions were purified using a Microcon-100 column (Millipore) with 3 washes of 300µl TE, and DNA was eluted in 15µl TE. If both PCR reactions for a sample were successful, then the DNAs were pooled for the purification and eluted in 30µl TE.

Table 1. MtDNA Primer Sequences (5'–3')

F15989	CCCAAAGCTAAGATTCTAAT
F16190	CCCCATGCTTACAAGCAAGT
R16410	GAGGATGGTGGTCAAGGGAC
F15	CACCCTATTAACCACTCACG
F82	ATAGCATTGCGAGACGCTGG
R285	GTTATGATGTCTGTGTGGAA
R484	TGAGATTAGTAGTATGGGAG

F=forward; R=reverse; numbers given correspond to the first nucleotide at the 5' ends of each primer

DNA sequences of HVI and HVII were generated for 15 of the reference DNA samples, using primers F15989 and R16410 or primers F15 and R285, respectively. Based on the ability to differentiate samples using HVII, the remaining 15 reference DNA samples were sequenced only for this region. Sequencing was carried out in 10µl

reactions using ~50–100fmol of DNA template, 4μl Quick Start Master Mix (Beckman-Coulter), and 1μl of 20μM primer, with the following thermal conditions: 30 cycles of 96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min. DNA was precipitated in a 1.5ml tube with 2.5μl stop solution (1.2M sodium acetate, 20mM EDTA, 4mg/mL glycogen) and 30μl cold 95% ethanol. Tubes were centrifuged 15 min at 14000rpm. DNA pellets were washed twice with 200μl cold 70% ethanol and centrifuged 4 min at 14000rpm after each wash. Pellets were vacuum-dried and resuspended in 40μl Sample Loading Solution (Beckman-Coulter). Sequencing samples were electrophoresed on a CEQ8000 (Beckman-Coulter) capillary electrophoresis instrument, using program LFR-1 (capillary temperature 50°C, denature 120 sec at 90°C, inject 15 sec at 2.0kV, and separate 85 min at 4.2kV), and sequences aligned using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide differences from the Anderson reference sequence in the interval 82–285 were noted for each sample.

Development of the Alkaline Extraction Protocol

Varying parameters were tested throughout this study in order to develop a working protocol for mtDNA extraction from hair shafts by alkaline treatment, and for its subsequent analysis. Sodium hydroxide concentrations from 0.5N–10N were evaluated for their ability and time required to digest several centimeters (cm) of hair. A variety of spin columns were tested to determine if they could withstand high pH conditions and/or how quickly a sample (either at high pH or neutralized, see below) flowed through their membranes. This was done using a control DNA (lambda DNA digested with HindIII) at a known concentration, examining on an agarose gel how much DNA was recovered, and

later with known human DNA, whose recovery was tested by mtDNA amplification. The columns tested included Whatman Vectaspin Micro columns and Millipore Ultrafree MC columns, both with polysulphone membranes, and Microcon-100 and Microcon YM-30 columns, both with cellulose membranes.

DNA Extraction and Sequencing from Hairs

Hair Sample Preparation:

Hairs were removed from sealed envelopes using UV-irradiated and flame-sterilized forceps. Hairs were visually inspected to identify the root and tip ends; any adhering follicular tissue was removed and discarded. Hairs were cut into 1cm fragments, starting at the root end of the shaft. Fragments were dropped into two UV-irradiated 1.5ml tubes in an alternating fashion, so as to equitably distribute the hair sample between the two extraction methods tested. Twenty-seven samples contained 6–7 cm of hair per tube; the remaining three samples (22, 46, 55) were analyzed in lesser amounts.

Prior to the DNA extraction procedures below, the hairs were cleaned by rinsing them successively with 1mL of 5% Terg-a-zyme (Alconox), ethanol, and water, all previously UV-irradiated to ~6 J/cm^2. For each liquid, tubes were agitated for 5 min on a platform rocker and liquid discarded.

DNA Extraction using Glass Grinders:

Hairs were extracted in batches of ten, following the AFDIL protocol (www.afip.org/Departments/oafme/dna/afdil/protocols.html), with exceptions noted

below. Glass tissue grinders (0.2ml, Kontes Glass) were cleaned successively with 10% bleach, water, and 100% ethanol for 5 min each. Tissue grinders, 1.5ml tubes, Centricon retentate vials, forceps, pipettors, filter pipet tips, digestion buffer and TE were UV-irradiated to ~6 J/cm^2. Eight grinders were rinsed with 200µl TE (grinder blanks, to ensure the grinder had no DNA contamination) and grinding was briefly simulated. These blanks were precipitated in 1.5ml tubes with 20µl 3M sodium acetate and 400µl cold 95% ethanol and centrifuged 15 min at 14000rpm. Samples were vacuum-dried and resuspended in 10µl TE. The remaining two grinders were filled with 187µl digestion buffer each (reagent blanks, as per the AFDIL protocol, to ensure that no reagents used in the extraction process were contaminated), grinding was briefly simulated, and the blank solutions were transferred to 1.5ml tubes. In succession, each of 10 hair samples was then transferred to a glass grinder containing 187µl of digestion buffer. Hair fragments were ground until no longer visible, and the homogenate was transferred to a 1.5ml tube. To each reagent blank and hair sample, 5µl of proteinase K (20 mg/ml) and 8µl dithiothreitol (1M) were added. All extractions and reagent blanks were incubated overnight at 56°C. These were extracted with 200µl phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged 5 min at 14000rpm. Aqueous layers were transferred to 1.5ml tubes, re-extracted with 200µl chloroform (AFDIL: 1-butanol), centrifuged 5 min at 14000rpm, and transferred to Centricon YM-50 columns (Millipore; AFDIL: YM-30 columns) with 1.5ml TE. Centricon columns were centrifuged ~15 min at maximum speed (~3750g, AFDIL: 4000g) in a Beckman GPR swinging bucket centrifuge. Membranes were washed once with 2ml TE, and DNA was eluted in 25µl TE (AFDIL: 50µl).

DNA Extraction using Alkaline Solution:

Hairs were extracted in sets of ten. Microcon vials, 1.5ml tubes, pipettors, filter pipet tips, 5N sodium hydroxide, 2M Tris base buffer (pH 8), and TE were UV-irradiated to ~6 J/cm^2. A 1.5ml tube was rinsed with 500µl of each of the cleaning reagents (see above) and filled with 250µl 5N sodium hydroxide to serve as a reagent blank. Five hundred microliters of 5N sodium hydroxide were added to each tube containing hair fragments. Hair samples and the reagent blank were agitated on a platform rocker and vortexed ~10 sec hourly until hair fragments were no longer visible. Concentrated hydrochloric acid (11.6M) and 2M Tris were mixed in equal volumes, and 400µl of this neutralization solution were added to each extraction (200µl to the reagent blank). The pH of the neutralized solution was measured by dropping 0.5–1µl onto pH paper to ensure a pH between 7 and 8. Neutralized solutions were passed through a Microcon YM-30 column in two centrifugations of 10 min at 14000g, filtering 400–500µl per spin. Membranes were washed three times with 300µl TE, and DNA was eluted in 25µl TE into a Microcon elution vial.

Amplification and Sequencing of Hair Samples:

Hair samples extracted by glass grinding were PCR amplified in 10µl reactions, using mtDNA primers F82 and R285, and 0.5 or 0.05µl of DNA as template. Blanks and positive and negative controls were amplified using 1µl as template. PCR reactions were cycled through the following conditions: 2 min at 94°C, 35 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, followed by 7 min at 72°C. Amplification results were verified by running 5µl of each PCR reaction on a 2% agarose gel. Any reagent

blank that generated PCR product was re-amplified to confirm the result. Blanks that were positive in the second amplification were amplified in 20µl volumes for DNA sequencing.

Alkaline-extracted samples were amplified in 20µl volumes, using 1 or 0.05µl of DNA as template, under the same thermal conditions as glass grinding samples. Any reagent blank that was positive for amplification was re-amplified to confirm the result. PCR additives were assessed for their ability to reverse inhibition and/or improve amplification of samples that were amplifiable but produced no sequence, including bovine serum albumin (BSA, 8µg per 10µl reaction), Eppendorf HotMaster Taq polymerase, and Eppendorf TaqMaster PCR Enhancer. Amplification results were verified by running 5µl of each PCR reaction on a 2% agarose gel, and the remaining 15µl of successful amplifications were purified using a Microcon YM-30 column and eluted in TE, as described for the buccal samples. HVII was sequenced as described for the reference buccal samples, except using primers F82 and R285. Sequencing reactions were precipitated and resuspended as described above, and electrophoresed using CEQ8000 program LFR-1-60 (capillary temperature 50°C, denature 120 sec at 90°C, inject 15 sec at 2.0kV, and separate 60 min at 4.2kV). Sequences were aligned using the BioEdit software, and nucleotide differences from the Anderson sequence were noted. Finally, mtDNA profiles from alkaline-extracted hairs were compared with those obtained from buccal swabs to ensure that a correct DNA sequence had been generated. Differences among test criteria (demographic information, hair features, etc.) within and between the two methods were examined for statistical significance using the two-sided z-test for comparing proportions (http://math.uc.edu/~brycw/classes/149/wang.htm).

DNA Yield Comparison between Glass Grinding and Alkaline Digestion Techniques

Ten samples that had readily amplified at a 1:20 dilution using both extraction methods (8, 9, 24, 27, 28, 29, 32, 35, 36, 56) were chosen for an assessment of comparative DNA yields. One microliter of an extraction was used as template in a 10μl PCR reaction with primers F82 and R285. Each sample was amplified in three serial dilutions, representing 1, 0.1, and 0.01μl of template DNA (see preceding section, Amplification and Sequencing of Hair Samples, for PCR conditions). Results were evaluated by running 5μl of each reaction on a 2% agarose gel. Samples were scored as a band present or absent, or as showing evidence of inhibition (a condition where no DNA amplification is possible because of interference by a contaminating substance).

DNA quality comparison between Glass Grinding and Alkaline Digestion Techniques

Two samples that reliably amplified using both extraction methods (samples 24 and 27) were tested to determine the largest product size (664bp: F16190/R285; 865bp: F15989/R285; 1064bp: F15989/R484) that could be obtained. Based on these results, the same samples used for the yield comparison were amplified in 10μl reactions using three primer combinations that generate amplicons of 469bp (F15/R484), 664bp, and 865bp. The amount of DNA template used was based on dilutions that successfully amplified in previous experiments. MtDNA was amplified using the same conditions as for buccal swabs, and amplification success was evaluated by running 5μl of the reaction on a 2% agarose gel. Samples were scored as a band present or absent.

RESULTS

Sample collection

Thirty complete sample packets were collected from volunteers: 16 Caucasian (6 male, 10 female); 6 African American (1 male, 5 female); 2 Hispanic (both female); and 6 Asian (1 male, 5 female). All participants included enough hair to allow DNA extraction from 6–7cm of shaft, except samples 22, 46, and 55, which were limited and thus were analyzed in lesser amounts (3cm, 4cm, and <1cm, respectively). Table 2 shows the demographic information for each sample, along with the hair color and treatments each subject used within the last year. Half of the respondents indicated blow-drying their hair either daily or often. Seven participants had dyed their hair within the last month; five within the last year. Five subjects had undergone a permanent or relaxer treatment (3 within the previous month, 2 within a year). Other hair treatments described on the questionnaires included gel, hair spray, curling or straightening irons, henna, Malibu 2000 (for removing mineral build-up from hair) and Nizoral shampoo (anti-dandruff).

Reference sample sequencing

The mtDNA region 15989–484 was successfully amplified from all 30 buccal swabs. To determine what sequence information was necessary to ensure that DNA from a hair sample matched the correct donor, sequence data from both HV regions were obtained for 15 buccal samples. All but two pairs of individuals could be differentiated using mtDNA interval 82–285: samples 9 and 36 (sequence common to 38.4% of Caucasians) and samples 23 and 27 (sequence common to 0.35% of African Americans

Table 2: All information presented, except hair color, was provided by participants on the questionnaires included in their sample packets. Hair color data were determined from observations when hair was cut and transferred to tubes in preparation for extraction.

Table 2: Demography and Hair Descriptions of Study Participants

Sample ID	Sex	Ethnic group	Hair Color	Blow dry	Dye	Perm/relaxer	Other treatment
8	F	Af Amer	dk brown/black	rarely	--	within year	
9	F	Cauc	lt brown	rarely	within year	--	
10	F	Cauc	lt brown	often	--	--	
11	M	Cauc	lt brown	rarely	--	--	
12	F	Cauc	lt brown	daily	--	--	
13	F	Hisp	dk brown	daily	within month	--	straightening gel, creme to shine and take out frizz
14	F	Cauc	dk brown	rarely	within month (henna)	--	
16	F	Asian	dk brown/black	often	within year	--	
17	M	Cauc	dk brown/black	rarely	--	--	
19	F	Cauc	dk brown	rarely	within month	--	
20	F	Asian	dk brown/black	daily	--	--	henna
22	M	Cauc	dk brown	rarely	--	--	
23	F	Af Amer	dk brown/black	often	--	within year	curling iron
24	M	Cauc	dk brown	often	--	--	
27	M	Asian	dk brown/black	daily	--	--	
28	F	Cauc	blond	often	within year	--	
29	M	Cauc	dk brown	rarely	--	--	
32	F	Cauc	dk brown	rarely	--	--	
35	F	Cauc	lt brown	daily	within year	within month	
36	F	Cauc	dk brown	daily	within month	--	Malibu 2000 demineralizing treatment
37	F	Cauc	lt brown	daily	within month	--	
38	F	Hisp	lt brown	daily	within year	--	
43	F	Asian	dk brown	often	within month	--	
46	M	Cauc	lt brown	rarely	--	--	gel
54	F	Af Amer	dk brown/black	often	--	--	hot comb for pressing hair
55	M	Af Amer	dk brown	rarely	--	--	Nizoral dandruff shampoo
56	F	Af Amer	dk brown	rarely	--	within month	straightening iron
57	F	Asian	dk brown/black	rarely	--	--	
59	F	Asian	dk brown	rarely	--	--	curling iron, hair spray on occasion
60	F	Af Amer	dk brown/black	rarely	within month	within month	

and 7.85% of persons of Asian descent). Because region 82–285 contained enough inter-individual variation to adequately indicate if a mtDNA sequence obtained from a hair shaft was actually from the donor, the remaining 15 buccal swabs were sequenced only through this region (see Table 3 for HVII mtDNA profiles). Additional samples with identical sequences in the interval 82–285 were observed: samples 38 and 43 (sequence common to 35.7% of Hispanics and 40.9% of persons of Asian descent) and samples 54 and 59 (sequence common to 4.18% of African Americans and 7.71% of persons of Asian descent). Sequence data were obtained for the interval 82–285 in all cases except sample 24, from which sequence was obtained only to nucleotide 239.

Development of the Alkaline Extraction Protocol

The lowest NaOH concentrations tested (0.5N, 1N) were slow to dissolve hair shafts; in fact, no effect was observed after 48 hours of exposure to 0.5N NaOH. Thinning of the hair shaft was observed within 4 hours of exposure to 2N NaOH, but the hair did not fragment and dissolve until the sample was vortexed at 48 hours. Structural changes were most readily observed using 5N and 10N NaOH, which thinned and fragmented the hairs within the first hours of exposure. Neutralization of 10N NaOH solutions using equimolar amounts of concentrated HCl with 1M Tris was often problematic, overshooting neutral pH into the acidic range. When using fresh 5N NaOH (prepared immediately before extraction), neutralization with a slightly less than equimolar amount of HCl with 2M Tris buffer reliably produced pH 7–8.

Table 3: MtDNA Profiles from Buccal Samples and Alkaline-digested Hair

The sequence interval available from hair for comparison to the buccal control is given for each sample, with nucleotide differences from the human mtDNA reference sequence for both sample types; Y=pyrimidine (C or T); R=purine (A or G); Ø = no differences from the human mtDNA reference sequence within the interval listed; N/A= not available for comparison (buccal sequence only available to nucleotide position 239).

Sample ID	Hair sequence interval	Hair sequence polymorphisms	Buccal sequence polymorphisms
8	82-283	120Y	--
		128Y	--
		143A	143A
		146C	146C
		152C	152C
		195C	195C
		263G	263G
9	182-285	263G	263G
10	83-182	150T	150T
		152C	152C
11	82-284	263G	263G
12	82-173,	202N	--
	180-285	224N	--
14	82-174	146C	146C
17	87-183	Ø	Ø
19	85-285	153G	153G
		202N	--
		218N	--
		224N	--
		246N	--
		263G	263G
22	82-284	150T	150T
		263G	263G
24	97-285	185A	185A
		188G	188G
		228A	228A
		263G	N/A
27	85-284	D249	D249
		263G	263G
28	90-274	185A	185A
		188G	188G
		195N	--
		196N	--
		228A	228A
		263G	263G

Sample ID	Hair seqence interval	Hair sequence polymorphisms	Buccal sequence polymorphisms
29	84-279	146C	146C
		152C	152C
		195C	195C
		209N	--
		249G	249G
		263G	263G
32	85-285	Ø	Ø
35	84-284	150T	150T
		152C	152C
		195C	195C
		215G	215G
		263G	263G
36	85-285	263G	263G
37	85-285	224N	--
		235G	235G
		263G	263G
38	83-199	Ø	Ø
43	83-174	Ø	Ø
46	83-284	185A	185A
		228A	228A
		263G	263G
54	83-285	152C	152C
		263G	263G
55	82-184,	152C	152C
	190-285	182T	182T
		195C	195C
		247A	247A
		263G	263G
56	85-182,	200G	200G
	197-285	263G	263G
59	83-285	152C	152C
		263G	263G
60	82-176,	143R	143A
	197-283	146C	146C
		152C	152C
		263G	263G

When treated only with TE buffer, Whatman VectaSpin Micro columns were found to be losing small amounts of lambda DNA into the rinsate, while Millipore Ultrafree MC columns retained all of the control DNA. Both Whatman and Millipore columns required increased centrifugation times as the NaOH solutions were made more concentrated: the former between 5 and 30 minutes for 0.5–10N solutions, the latter requiring 1.5 hours for 2N NaOH and longer for 5N and 10N solutions (these were discarded after this period). Lambda DNA was not visible on an agarose gel after elution from either of the columns (see Discussion). In experiments using human DNA in neutralized solutions, Millipore Ultrafree MC columns and Microcon-100 columns required over an hour to filter the same volumes of liquid (neutralized solution and several TE washes) as could be filtered by Microcon YM-30 columns in under 30 minutes. All three columns produced amplifiable mtDNA from the neutralization experiments.

Using the technique developed through preliminary experiments (see Appendix B for the complete protocol), it was noted that all hair samples were fully dissolved (no longer visible) within 5 hours of exposure to 5N NaOH; some disappeared within 2 hours (9, 32, 36, 37, 43, 57). Eight samples extracted by glass grinding (14, 16, 20, 23, 27, 29, 59, 60) were discolored (tan/brown) during the extraction process and in the final DNA elution. These samples remained discolored, even after several freeze-thaw cycles. Eleven hair samples extracted by alkaline digestion (13, 14, 16, 20, 23, 24, 27, 43, 54, 59, 60) also demonstrated discoloration. Following freezing, much of the brown color in the alkaline-extracted samples could be observed in a clump at the bottom of the tubes,

leaving the solution relatively colorless (see Discussion for relationship between discoloration and amplification success).

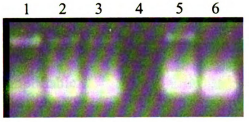
Amplification and Sequencing of the 203bp mtDNA fragment (82–285) from Hair DNA

Twenty-two of thirty (73%) hair samples extracted by glass grinding successfully generated the 203bp PCR product. MtDNA from eight hair samples (8, 17, 19, 24, 27–29, 36) was amplifiable both when 0.5µl of sample was used in a 10µl PCR reaction and at a 1:20 dilution (Table 3). Fourteen samples (9, 11, 22, 23, 32, 35, 37, 43, 46, 54–57, 59) generated the PCR product at only one of two template concentrations. MtDNA from eight hair samples (10, 12–14, 16, 20, 38, 60) extracted by glass grinding could not be amplified at either DNA concentration. All of these samples showed evidence of PCR inhibition, three of them at both template concentrations, as no primer activity could be observed (see example in Figure 2). The reagent blank for sample 8 showed amplification of a 203bp product in two 10µl PCR reactions, but when amplified in 20µl for sequencing, no product was observed. Grinder blanks for samples 36 and 37 showed faint amplified product in an initial amplification, but no PCR product was observed in repeat amplifications. All other reagent blanks and grinder blanks from glass grinding extractions were negative.

Twenty-seven of thirty (90%) hair samples extracted by alkaline digestion produced the 203bp PCR product. Nine mtDNA samples (11, 22, 24, 27–29, 32, 36, 46) generated amplified product when 1µl of DNA was used in a 20µl PCR reaction and at a 1:20 dilution of template DNA (Table 4). Eighteen samples (8–10, 12–14, 17, 19, 23, 35, 37, 38, 43, 54–56, 59, 60) demonstrated amplification in only one PCR reaction. Three

DNA samples (16, 20, 57) could not be amplified at either DNA concentration. One reagent blank showed faint amplified product in a preliminary amplification, but was negative when it was re-amplified for sequencing.

Figure 2: Amplification Results: Positive, Negative, and Inhibited



Example of amplification results observed. Lanes 1–3, 5: positive (PCR product amplified); Lane 4: inhibited (no primer activity observed at low molecular weight toward bottom of gel); Lane 6: negative (primer activity present, but no product band visible). PB= product band; PA= primer activity

Table 4: Amplification of a 203bp Product from Hair mtDNA at Two Concentrations

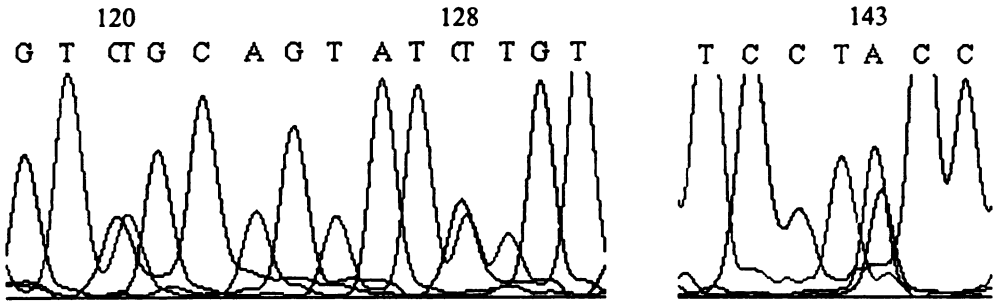
	Glass grinding		Alkaline digestion			Glass grinding		Alkaline digestion	
	1	1:20	1	1:20		1	1:20	1	1:20
8	+	+	[–]	+	28	+	+	+	+
9	[–]	+	[–]	+	29	+	+	+	+
10	[–]	–	[–]	+	32	[–]	+	+	+
11	+	–	+	+	35	[–]	+	[–]	+
12	[–]	–	[–]	+	36	+	+	+	+
13	[–]	–	[–]	+	37	[–]	+	[–]	+
14	[–]	[–]	[–]	+	38	[–]	–	[–]	+
16	[–]	[–]	[–]	[–]	43	[–]	+	[–]	+
17	+	+	+	–	46	+	–	+	+
19	+	+	+	–	54	+	–	+	–
20	[–]	–	[–]	–	55	+	–	+	–
22	+	–	+	+	56	[–]	+	[–]	+
23	[–]	+	[–]	+	57	[–]	+	[–]	–
24	+	+	+	+	59	[–]	+	[–]	+
27	+	+	+	+	60	[–]	[–]	[–]	+

Amplification of glass grinding samples was carried out in 10µl using 0.5 and 0.05µl of DNA template; alkaline digestion samples were amplified in 20µl, using 1 and 0.05µl of template. + = band present; – = band absent; [–] = inhibited

In attempts to improve amplification in inhibited PCR reactions, two samples (23—extracted using both methods) of 13 tested were amplifiable with the addition of BSA. Eleven samples (14, 16, 20, 60—extracted by both methods, and 37, 43, 57—extracted by alkaline digestion) remained inhibited. Two samples tested using HotMaster Taq (23—extracted by both methods) generated PCR product, while four others tested (16, 60—extracted by both methods) remained inhibited. None of the six samples tested with TaqMaster PCR Enhancer (14, 16, 60—extracted by both methods) were amplifiable.

To ensure that results obtained from hairs extracted by alkaline digestion actually originated from the hair and were not the result of contamination, region 82–285 was sequenced for comparison to buccal samples. Sequence data were obtained from 25 of the 30 hair samples extracted by alkaline digestion (Table 3). The short segments amplified often produced incomplete sequence due to poor quality data at the 5' ends of the fragments generated in the forward and reverse reactions. When the two sequencing reactions did not produce sufficient data to allow sequence overlap, a small stretch of nucleotides from the middle of the interval could not be determined (e.g. sample 12). Examination of the chromatographs revealed a total of 11 bases in regions of clean sequence that could not be conclusively called. Three of these were possible cases of heteroplasmy (a condition in which more than one mtDNA sequence exists in an individual), including sample 8 (positions 120 and 128) and sample 60 (position 143). These are shown in Figure 3. Aside from these 11 uncalled nucleotides, all 25 hair sequences were consistent with their corresponding buccal sequence within the interval available for comparison for each sample.

Figure 3: Electropherograms of Possible Heteroplasmic mtDNA Sites

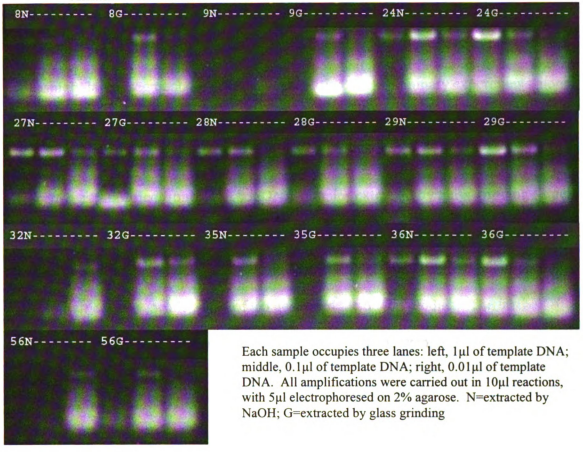


Left: Portion of electropherogram for sample 8, exhibiting possible heteroplasmy at nucleotide positions 120 and 128 (both sites either C or T); Right: Portion of electropherogram for sample 60, with possible heteroplasmy at nucleotide position 143 (either A or G).

DNA Yield Comparison between Glass Grinding and Alkaline Digestion Techniques

In order to compare the amount of mtDNA recovered from hair shafts using each extraction method, a subset of samples was amplified using 1µl of DNA template and DNA dilutions of 1:10 and 1:100. Five of ten glass grinding samples generated amplified product using 1µl of DNA template; the remaining five samples showed PCR inhibition (Figure 4, Table 5). All ten samples generated product using a 1:10 dilution, and five samples amplified at 1:100. Six of ten DNA samples extracted by alkaline digestion amplified using 1µl of DNA template; the remaining 4 samples showed PCR inhibition. Seven samples could be amplified using a 1:10 dilution, while three showed evidence of PCR inhibition. Six of ten samples amplified at a 1:100 dilution, while one sample continued to demonstrate PCR inhibition (sample 9, see Discussion).

Figure 4: Amplification of DNA Dilutions to Compare Yield of Glass Grinding and Alkaline Extraction Methods



Each sample occupies three lanes: left, 1µl of template DNA; middle, 0.1µl of template DNA; right, 0.01µl of template DNA. All amplifications were carried out in 10µl reactions, with 5µl electrophoresed on 2% agarose. N=extracted by NaOH; G=extracted by glass grinding

Table 5: Amplification of DNA Dilutions to Assess Yield

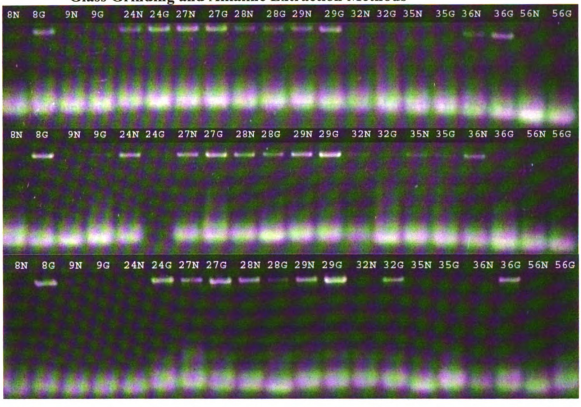
	Glass grinding			Alkaline digestion		
	1	1:10	1:100	1	1:10	1:100
8	[–]	+	+	+	+	–
9	[–]	+	–	[–]	[–]	[–]
24	+	+	+	+	+	+
27	+	+	–	+	+	+
28	+	+	–	+	+	–
29	+	+	+	+	+	+
32	[–]	+	+	[–]	[–]	+
35	[–]	+	+	[–]	+	–
36	+	+	–	+	+	+
56	[–]	+	–	[–]	[–]	+

Tabular representation of data from Figure 4. + = band present; – = band absent; [–] = inhibited

DNA Quality Comparison between Glass Grinding and Alkaline Digestion Techniques

In order to discern if there was a difference in mtDNA quality (the length of mtDNA that could be recovered from a hair) using the two extraction techniques, increasingly larger segments of DNA were amplified. In preliminary experiments, none of the samples tested produced a fragment as long as 1064bp (15989–484); therefore, the 10 samples examined from each extraction method were tested up to 864bp. A 469bp fragment was amplified from six of ten DNA samples extracted by glass grinding (Figure 5, Table 6). For both the 664bp and the 865bp amplifications, seven of ten glass grinding samples gave positive results. A 469bp product was amplified from five of ten alkaline-digested hair samples, while seven of ten generated the 664bp product. The 864bp product was amplified from four of ten alkaline-digested hair samples.

Figure 5: Amplification of Long PCR Products to Compare mtDNA Quality from Glass Grinding and Alkaline Extraction Methods



All amplifications were carried out in 10µl PCR reactions using 1µl of the template DNA dilution that amplified successfully in previous amplifications of 203bp. Top, 469bp (15–484); middle, 664bp (16190–285); bottom, 865bp (15989–285). N=extracted by NaOH; G=extracted by glass grinding

Table 6: Amplification of 469, 664, and 865bp Products to Assess DNA Quality

	Glass grinding			Alkaline digestion		
	469	664	865	469	664	865
8	+	+	+	—	—	—
9	—	+	—	—	—	+
24	+	+ *	+	+	+	—
27	+	+	+	+	+	+
28	+	+	+	+	+	+
29	+	+	+	+	+	+
32	—	—	+	—	+	—
35	—	+	—	—	+	—
36	+	—	+	+	+	—
56	—	—	—	—	—	—

Tabular representation of data from Figure 5. + = band present; — = band absent; * = sample evaporated and not loaded on gel in Figure 5; data given here from preliminary experiment with sample 24.

DISCUSSION

The goal of this study was to develop a simplified protocol for mtDNA extraction from human hair using a strong alkaline solution to efficiently digest the keratin-rich hair shaft. Quick hydrolysis of keratin and other proteins would free the mtDNA within the hair, which could be readily recovered following neutralization by filtering the extraction solution through a spin column. The potential advantages of such a method over extraction techniques presently used are many: reduction in the time and cost required to extract mtDNA from hair shafts; digestion of the hair shaft without addition of multiple reagents (e.g. proK and DTT); no need for toxic organic solvents; minimization of sample transfers and thus sample loss; reduced possibility of contamination. These benefits must be balanced with the need to produce results as good or better than those obtained with the most prevalent extraction method currently used, the glass grinding protocol, namely retaining the quantity and quality of recovered DNA. As important, the accuracy of any data obtained from hair DNA extracted using alkaline digestion must also be assured.

As in many fields, forensic DNA analysts generally have a large backlog of work to be completed. Each sample they receive must be examined in an objective and accurate manner. At the same time, the urgency of prosecutors, defense attorneys, and impending trials places added pressure on analysts to complete work quickly, and any technique that can reduce the time needed to acquire results is welcomed. In this regard, the alkaline digestion protocol for hair shafts designed and tested in this study can be performed in its entirety over the course of an 8-hour workday. From submersion in 5N sodium hydroxide to elution from the MicroconYM-30 column, approximately 6–7 hours

were required to extract DNA from 10 hair samples. Including the overnight incubation, the glass grinding method required approximately 22–24 hours from the preparation of blanks to elution of DNA from the Centricon columns. The period in which the hair is dissolving in NaOH (usually 2–5 hours) is virtually labor-free, requiring only an hourly vortexing. In contrast, the glass grinding protocol requires the awkward transfer of hairs into the grinder, the physical maceration of the hair, addition of multiple reagents, and several organic extraction steps, all of which demand a commitment of a large amount of time and labor from the analyst.

While maximum efficiency was a priority in the development of the protocol, maintaining the integrity of the DNA within the hair was as important. The need to sacrifice some speed for the sake of maintaining DNA quality became apparent during early experiments with NaOH concentrations and neutralization. The original plan for the alkaline protocol was to digest the hair in sodium hydroxide and transfer the solution directly to an alkaline-resistant column for filtration. Spin columns purify and concentrate DNA by passing particles smaller than the membrane's pore size into the rinsate, while larger molecules (like DNA) are retained and are able to be rinsed and eluted from the column. The Whatman VectaSpin Micro and Millipore Ultrafree MC column membranes are made of polysulphone, a material described to be resistant to damage by high pH up to a sodium hydroxide concentration of 6N (Whatman Product Guide, 2002–2003). The Whatman columns were found to be losing DNA into the rinsate, even when exposed only to neutral pH TE buffer. The Millipore columns sufficiently retained DNA but had to be centrifuged for extended periods to filter the

strong NaOH solutions. These results necessitated the neutralization of the extraction solution before filtration.

The neutralization step eliminated the need to use a sodium hydroxide concentration below the threshold of tolerance for the column membranes, allowing the rapid degradation of hair shaft with stronger alkaline solutions (i.e. 5–10N). While 10N NaOH would likely have reduced the time required to dissolve the hair samples, difficulties with neutralization and the heightened possibility of sample loss due to degradation in acidic conditions precluded its use. It is unclear why the 10N solution was so unreliable in neutralization experiments, but it is possible that its concentration was diminished over the course of storage. Highly concentrated solutes can precipitate from solution, as may have occurred for the 10N NaOH solution. Upon neutralization, the decreased concentration of the alkaline solution would be overwhelmed by the concentrated acid, producing the low pH often observed after addition of Tris and HCl in the 10N experiments. Although 5N NaOH did not exhibit these complications, it should be noted that the solution was made fresh prior to each extraction so as to ensure an accurate concentration and minimize the possibility of sample degradation due to overshooting neutral pH.

The use of Microcon YM-30 columns over the other membranes examined was a noteworthy advantage in the efficiency and applicability of the alkaline digestion procedure. These columns can be centrifuged at up to 14000g, while other membranes require slower speeds (Centricon YM-50: 5000g, Millipore Ultrafree MC: 5000g, Microcon-100: 500g) and thus longer time to filter solutions. Centricon columns, though they can accommodate larger volumes than Microcon columns, require centrifuges that

have the capacity for their size, while the latter are amenable to use in any standard microcentrifuge common in forensic laboratories.

The speed of the extraction is only meaningful if sequence results are reliably obtained and if these results are accurate. MtDNA control region sequences were obtained from 25 of 30 samples (83%), exceeding previously reported success rates of 75% (Pfeiffer et al. 1999) and 71% of initial typing attempts (Wilson et al. 1995b) for human hair shafts. In all cases, the sequence obtained from the hair sample was consistent with its buccal reference sample (Table 3). While the sequence intervals obtained in these experiments were limited, the only goal here was to verify that the sequences obtained from the hair were correct. This could be accomplished with the 82–285 interval, and no further effort was made to increase the length of sequence acquired. The method is capable of producing longer amplified fragments (Figure 5, Table 6), and maximization of sequence length (as would be desirable in a forensic situation) is therefore certainly possible.

As noted in Results, 11 bases could not be called from the electropherograms, including 3 possible instances of heteroplasmy. Some have argued that the level of heteroplasmy in hair is inordinately elevated over that in other tissues (Grzybowski 2000), while others maintain that it is comparable throughout the body and that observations of heightened rates of heteroplasmy in hair are due to experimental bias (Budowle et al. 2002). Grzybowski (2000) found evidence of heteroplasmy in 19 of 100 hair roots sequenced for HVI, and reported several instances of multiple heteroplasmic sites in a single hair root. His surprising result of up to six heteroplasmic sites within HVI from a single hair root was the first report of such high mutational activity. In a

critique of the study, Budowle et al. (2002) noted that Grzybowski used approximately three orders of magnitude more DNA in his PCR amplifications than is common in forensic practice, and that his nested PCR technique employs more amplification cycles than standard forensic mtDNA testing. They also point out several inconsistencies among Grzybowski's data and mtDNA sequence information and population statistics, and attribute Grzybowski's unusual results to inadvertent amplification of nuclear DNA or sample contamination.

The three nucleotides in question in the current study (sample 8: positions 120 and 128; sample 60: position 143) are good candidates for heteroplasmy, as each represents a potential transition event, in which a purine (A or G) is replaced by a purine, or a pyrimidine (C or T) is replaced by a pyrimidine. Transitions are the most common mtDNA mutation observed (Brown et al. 1982). While heteroplasmic mtDNA molecules may be present in any ratio, the signal strengths from the two bases at these three candidate positions are relatively balanced, increasing their likelihood of being true heteroplasmy, as opposed to background signal noise. Other than the 11 sites where the base could not be called, all nucleotides matched the reference sample, a confirmation of the accuracy of mtDNA typing from hair shafts using alkaline digestion.

Among the 10 samples chosen for the yield comparison between the two extraction methods (Figure 4), similar results were observed: five samples were amplifiable at a 1:100 dilution using the glass grinding method, and six for the alkaline digestion protocol (Table 5). It should be noted that sample 9 from the NaOH method demonstrated PCR inhibition at all three DNA concentrations, inconsistent with data from initial 203bp amplifications (Table 4). In these data, amplification was observed at

a 1:20 dilution. It is unclear what caused the inhibition in the yield experiment, nor is it possible to predict whether the DNA yield for sample 9 was sufficient for amplification at 1:100 had this inhibition effect been absent. In any case, within the limits of the small sample size in this experiment, the two extraction methods appear to yield similar amounts of DNA.

While all of the samples used for the yield comparison were extracted from 6–7cm of hair shaft, another perspective on DNA yield may be found in the amplification results (Table 4) from the three samples analyzed in smaller amounts (22, 46, 55). Glass grinding samples 22 and 46 (extracted from 3 and 4cm of hair, respectively) were amplifiable using 0.5µl of sample as template, but not at 1:20 dilution. These same samples extracted by alkaline digestion generated amplified product at both template concentrations. These results, though limited in scope, may point to an advantage of the alkaline method, namely its minimal sample transfers and thus reduced opportunity for sample loss. While 6–7cm of shaft appears to have been plentiful enough to mask any disparity in yield between the two procedures, extractions from 3–4cm of hair may have revealed a distinction. However, more experiments with varying lengths of hair shaft would have to be conducted to confirm any differences in sample loss between glass grinding and alkaline digestion. While amplification of DNA from sample 55 (<1cm) was successful using 0.5 or 1µl of sample as template, neither technique could produce enough DNA to amplify at 1:20. Given that the sample was similar to the size of an eyelash, it may be that even maximal DNA recovery was not enough to generate amplified product at a template dilution from such a small hair fragment. In any case, the

results from sample 55 indicate that the NaOH method is applicable to even the smallest of samples.

The two methods also produce similar results in the DNA quality assessment, save for the largest fragment amplified (865bp). Amplification results for the 469bp and 664bp products were consistent between the two extraction methods (Figure 5, Table 6), but a potential difference was noted in the results for the 865bp product: 7 positive amplifications for the glass grinding method, and 4 for alkaline digestion. While these results are accurate, the small sample size means the difference is not statistically significant ($p=0.1775$) and may result from chance. Again, more experiments would have to be conducted to verify this observation.

One plausible explanation for the difference in the 865bp amplification results lies in the potential presence of ribonucleotides (in lieu of the normal deoxyribonucleotides) in the mtDNA molecule. MtDNAs of mammals and amphibians have been reported to be alkali labile, or prone to breakage at certain sites when exposed to an alkaline environment (Grossman et al. 1973). Kinetics experiments assaying the sensitivity of mtDNA to alkaline conditions indicated the presence of 10–30 ribonucleotides and other alkali-sensitive sites (including nucleotides that have lost their nitrogenous base and other areas of damage) within the molecule (Grossman et al. 1973). Noting that the majority of mtDNA strands produced by hydrolysis at alkali-sensitive sites were high molecular weight, Brennicke and Clayton (1981) determined that these sites are clustered at the two DNA strands' origins of replication. Alkali labile sites around the heavy strand origin of replication (O_H) at nucleotide position 191 (Anderson et al. 1981) would be within the intervals amplified for the DNA quality experiment and could have influenced the results

from the alkaline-extracted samples; however, this scenario seems unlikely given that all of the intervals tested in the quality experiment included this region. It is unclear what caused the discrepancy between the data for the 664bp and 865bp amplifications, unless an as-yet-undiscovered cluster of alkali-labile sites exists between mtDNA nucleotide positions 15989 and 16190 (the 201bp difference between the 664bp and 865bp products). Since the smaller products were amplified across the alkali labile region at O_H and the data are not statistically different, the presence of ribonucleotides in mtDNA does not appear to be an obstacle to the use of sodium hydroxide for forensic mtDNA testing of hairs.

Bourke et al. (1999) used other methods of DNA quantity and quality testing in their study of NaOH treatment to neutralize inhibitors of PCR in nuclear DNA, namely gel electrophoresis of DNA samples (without amplification) using ethidium bromide staining. A known quantity of DNA from PCR-inhibited samples was placed in a Microcon-100 column, and several washes of 0.4N NaOH were filtered through the membrane by centrifugation. Following elution from the column, DNA was quantified to determine recovery rates for the method. In contrast to the results discussed above, the authors described problems with sample loss and degradation. They estimated that ~50% of DNA was recovered after denaturing and washing with NaOH. The authors also reported shearing of DNA with repeated washes of 0.4N NaOH, although only with their "field" sample (which they suggested may have been previously damaged) and not their high molecular weight control DNA. According to their data, NaOH treatment was effective in neutralizing PCR inhibitors but may not be advisable for limited samples due to loss of DNA.

Although Bourke et al. (1999) stated that both agarose gel electrophoresis and the Quantiblot DNA quantification method were used to determine the amount of DNA recovered after NaOH treatment, no results were given for the latter technique. The authors acknowledged that "stains such as ethidium bromide intercalate into dsDNA (*double-stranded DNA*) with high affinity, yet [these stains] have considerably lower affinity for ssDNA" (*single-stranded DNA*; italics my addition). Oddly, they based their estimate of a 50% post-treatment DNA recovery rate on agarose gel electrophoresis, which uses an intercalating dye to visualize the DNA. In the study presented here, early experiments attempted to measure DNA recovery by gel electrophoresis and ethidium bromide staining, with similar results as those reported by Bourke et al. (1999) (i.e. a large "loss" of DNA). However, when control DNA was denatured by boiling and electrophoresed in a similar manner, it also appeared to be lost, even though it had undergone no treatment; thus, agarose gel electrophoresis was deemed an inaccurate measure of DNA recovery for denatured samples. Since a Quantiblot-like procedure (which uses a DNA probe for quantitation and is more applicable to denatured samples) is not yet validated for mtDNA, the detection method for this study was changed to serial dilution and PCR analysis of the DNA.

Bourke et al. (1999) purported that some DNA loss could be due to low molecular weight fragments (generated by shearing of damaged DNA) passing through the membrane (Microcon-100) into the rinsate, and proposed using a smaller molecular weight cut-off (MWCO) filtration unit (e.g. 50kD) to minimize this possibility. The protocol developed for the study presented here uses a Microcon YM-30 column (30kD MWCO). It is not known whether more DNA loss would have been observed with a

50kD or 100kD MWCO membrane. Despite the caveat given by Bourke et al. (1999) regarding NaOH treatment of limited DNA samples, results of the experiments conducted in the development and validation of the alkaline extraction protocol described here (especially those on small amounts of hair sample) suggest that sodium hydroxide is safe to use for mtDNA extraction from hair shafts.

A challenge to mtDNA testing from hairs in which the alkaline digestion method appears to have an advantage over glass grinding is amplification success. Eight of 30 DNA samples extracted by glass grinding could not be amplified, in contrast to three for the alkaline digestion method (Table 4). Although equal amounts of hair were used for each extraction procedure and the samples were cut alternately so as not to provide one technique with more "fresh" shaft closer to the root end, the number of samples that were able to be amplified from each method were different ($p=0.0953$), though not at a 95% confidence level.

The eight glass grinding samples that could not be amplified showed no discernible connection to any of the demographic information collected about each sample (Table 2). Among these samples, all four of the ethnic groups represented in this study were present. Likewise, hair color did not seem to have an influence, as three samples were light brown, two were dark brown, and three were dark brown/black.

A potential pattern emerges in the three alkaline-digested samples that could not be amplified, as all were from Asian females with dark brown/black hair. Three other hair samples from Asian volunteers and six characterized as dark brown/black were amplifiable (a 50% success rate for Asian samples, 62.5% for dark brown/black hairs). The success rate of alkaline extraction for Asian samples was statistically different than

that for Caucasians (p=0.0023) and African Americans (p=0.0455). Similarly, the success rate for dark brown/black hair was statistically different than that for less-pigmented hair (p=0.0053). This does not indicate that the alkaline extraction technique works particularly poorly on heavily-pigmented hair; indeed, two of these dark brown/black samples also were not amplifiable using the glass grinding technique.

Of the hair treatments specifically inquired about in this study, two (dye and blow drying) were sufficiently represented in the sample population to assess their effect on amplification success. For the glass grinding technique, five of eight (63%) negative samples were treated with dye, while seven of 22 (32%) amplifiable samples had been dyed. One of three (33%) negative alkaline-extracted samples was dyed (one of the other two participants indicated using henna under "other treatments," not specifically as a dye), while 11 of 27 (41%) of the successful samples were treated with dye. Six of the eight (75%) negative glass grinding samples were treated by blow drying, while nine of 22 (41%) successful samples were treated similarly. Two of three (67%) negative alkaline-extracted samples were treated by blow drying, while 13 of 27 (48%) successful samples were subjected to the same treatment.

Although there is no statistical difference among the success rates for treated versus untreated hairs given above, these data do indicate that hair treatments such as dyeing and blow drying may have a negative influence on mtDNA isolation from hair shafts, regardless of the extraction technique used. The success rate for treated hairs extracted by glass grinding in this study (62%) was similar to the success rate reported by the FBI (71%) in their validation studies for mtDNA analysis (Wilson et al. 1995b). The success rate for dyed hairs extracted by glass grinding was 58% compared to that

reported by the FBI (75%), although the FBI figure arises from a small sample size (four amplifications). The alkaline extraction method's success rate for treated hairs (90%) and dyed hairs (92%) was a marked improvement in both cases. In the experiments conducted as part of this study, the NaOH method's amplification success rates were equal to or better than those for the glass grinding method for all demographic (sex, ethnic group/population ancestry, hair color) and cosmetic treatment (blow drying, dye, permanent/relaxer) criteria examined, save one: Asian ancestry (50% success for alkaline digestion, 66.7% for glass grinding). However, this is a difference of a single sample.

The alkaline digestion method was also more successful than glass grinding in producing amplifiable DNA from discolored extraction samples. While four of the eight samples (50%) observed to be discolored during extraction by glass grinding generated a 203bp PCR product, nine of the 11 samples (81.8%) demonstrating discoloration during alkaline digestion were amplifiable. All of the hair samples producing discolored extraction solutions were dark brown or dark brown/black, corroboration that the discoloration was due to a large amount of melanin pigment in the extraction. Fourteen of the 19 discolored samples showed evidence of PCR inhibition; all of the samples that did not amplify were among them. The observation that the dark-colored contaminant precipitated out of solution in the alkaline-digested samples, and the fact that a larger proportion of these samples were amplifiable than for those extracted by glass grinding may provide support for the hypothesis of Bourke et al. (1999) concerning intercalating PCR inhibitors. If, in fact, the substance imparting the brown color to these samples was an inhibitor (such as melanin), then it is possible that denaturing the DNA by treatment with NaOH allowed it to be released from the DNA molecule. Freezing temperatures

might have contributed to the precipitation of this substance from the solution, resulting in improved amplification success over double-stranded DNA samples still bound to the inhibitor. It is also possible that the contaminant was hydrolyzed by the strong alkaline solution, diminishing its ability to hinder PCR amplification.

Other benefits of the alkaline digestion method arise from the reduction in sample transfers, reagent additions, and supplies required. When aqueous solution containing DNA is transferred away from undesired material (e.g. organic solvents), some aqueous liquid (and thus DNA) is necessarily left behind so as not to contaminate the next container with the waste material. It follows that for every transfer step in a protocol, more DNA is lost; therefore, fewer sample transfers can result in greater sample retention. Further, every tube, pipet tip, and reagent that comes in contact with the sample has the prospect of carrying exogenous DNA that could confound sequence results. Fewer transfers and reagent additions can reduce the potential for contamination of the sample, always a concern with mtDNA analysis. The simplicity of the alkaline digestion method results in fewer supplies and reagents being consumed, and will reduce the cost of mtDNA extraction from hair shafts. With the success of this study, both public and private forensic laboratories would benefit from reductions in the time and labor required of analysts. Lower costs and fewer man-hours have the potential to increase the sample capacity of publicly-funded facilities and to make mtDNA testing available to a wider array of interested parties.

In order for the alkaline digestion method of mtDNA extraction from hair shafts to be implemented in forensic laboratories presently using the glass grinding method, or those that have not previously undertaken mtDNA testing, a set of validation experiments

similar to those conducted here must be done. Evaluation of the method on the various types of equipment used in forensic laboratories will ensure that the protocol is adaptable to the types and conditions of hair samples regularly encountered. While implementing a new protocol can be an undertaking, the ease of the technique developed in this study and the comparable, if not improved, results should be incentive to pursue its validation in forensic mtDNA testing facilities.

APPENDICES

APPENDIX A

Forms Included in the Sample Packet for Volunteers:

Consent, Instructions, Questionnaire

Consent Form for participation in the study entitled:
"A simplified extraction method for isolating mitochondrial DNA from hair shafts"

The study in which you are being asked to participate is a thesis project being undertaken by a student and her advisor in the Forensic Science program at Michigan State University. The aim of the project is to develop a quicker and simpler method of getting DNA out of human hair shafts.

You will be asked to donate samples of shed head hairs, collected from a comb or brush, or by running your fingers through your hair. You will also be asked to rub the inside of your cheek with a Q-tip-like swab. Finally, you will be asked to complete a short questionnaire asking your gender, ethnic background, and any treatments you apply to your hair. We estimate that this process will consume no more than 10–20 minutes of your time.

Your participation in this study is completely voluntary, and you may choose to refuse participation altogether or participation in any part of the process (i.e. donation of either sample or answering of any question) without penalty. The investigator(s) will not be present when you are reading this form or contributing your samples. You will label your own samples and questionnaire with a random number that will not be linked to you; it will only be used to match a hair, swab and questionnaire to each other. The investigators will not know which number corresponds to any study participant. Your privacy will be protected to the maximum extent allowable by law.

If you have any questions about this study, please contact the Responsible Project Investigator David Foran, Ph.D., by phone: (517) 432-5439, email: foran@msu.edu, or regular mail: 560 Baker Hall, East Lansing, MI 48824. If you have questions or concerns regarding your rights as a study participant, or are dissatisfied at any time with any aspect of this study, you may contact— anonymously, if you wish—Ashir Kumar, M.D., Chair of the University Committee on Research Involving Human Subjects (UCRIHS) by phone: (517) 355-2180, fax: (517) 432-4503, email: ucrihs@msu.edu, or regular mail: 202 Olds Hall, East Lansing, MI 48824.

Your signature below indicates your voluntary agreement to participate in this study.

_____ _____

Signature Date

Instructions for Sample Donation

1. Shed head hairs:

   Collect 6–10 head hairs, either from a comb or brush or by running your fingers through your hair to gather any loose strands. Place the hairs carefully into the small white envelope and seal the envelope.

2. Buccal (cheek) swab:

   Open the package and carefully remove the swab, taking care not to brush the cotton tip against anything. Holding the wooden end in your hand, place the cotton tip against the inside of your cheek. Rub the swab against your inner cheek in a circular motion for approximately 30 seconds. Place the cotton tip into the bottom of the blue-capped tube and break off the wooden stick so that the entire swab fits inside the closed tube. Cap the tube and snap it closed. The small holes in the tube are there so that the swab can air-dry.

3. Questionnaire:

   Answer the questions asked to the best of your ability. DO NOT write your name on the questionnaire.

4. Labeling samples:

   Inside your packet is a set of small orange stickers marked with identification numbers. Place one sticker on the sealed envelope containing your hair, one sticker on the tube containing your cheek swab, and finally one sticker on your questionnaire. DO NOT place a sticker on your signed consent form or on the outside of the large envelope.

Place your labeled small envelope, tube, and questionnaire inside the large envelope. Keep your consent form separate. Seal the envelope and return both the packet and the signed consent form to the investigator(s) or their laboratory at 426 Giltner Hall.

Thank you for your participation!

Questionnaire for the study entitled:
"A simplified extraction method for isolating mitochondrial DNA from hair shafts"

The following questions are designed to account for differences in the ability to isolate DNA from hair. Please circle the most appropriate answer.

1. What is your gender? Male Female

2. What is your ethnic/racial group?

White/Caucasian Non-Hispanic Black/African American Non-Hispanic

Chicano/Mexican American Hispanic

American Indian/Alaskan Native Asian/Pacific Islander/Asian American

3. Please circle any treatments that have been applied to the hair that you are donating (keep in mind the time that has elapsed since the treatment, and that hair grows approximately 6 inches per year). For blow drying, indicate how often your hair is blown dry. For the other treatments, please indicate how long ago (to your best estimation) the treatment was performed.

Blow drying: daily often rarely

Dye/highlights/lowlights:

within the last month within the last year beyond 1 year

Permanent/relaxer:

within the last month within the last year beyond 1 year

Other (please describe): _____

within the last month within the last year beyond 1 year

APPENDIX B

Protocol for Alkaline Digestion of Hair Shafts

Alkaline Extraction Protocol

1. UV-irradiate to ~6 J/cm^2:

   5% Terg-a-zyme, 100% EtOH, H_2O, 5N NaOH, 2M Tris (pH 8.0), TE
   Filter tips: P10, P20, P200, P1000
   Pipets: P2, P20, P200, P1000
   Microcon tubes: 2n + 2 n= # hair samples
   1.5mL microcentrifuge tube (for reagent blank—RB)
   Culture tube (for Tris/HCl)

2. Add 1ml 5% Terg-a-zyme to each tube containing hair (500µl to RB). Place on shaker 5 min. Draw off Terg-a-zyme.

3. Add 1ml 100% EtOH to each tube containing hair (500µl to RB). Place on shaker 5 min. Draw off EtOH.

4. Add 1ml H_2O to each tube containing hair (500µl to RB). Place on shaker 5 min. Draw off water.

5. Add 500µl of 5N NaOH to each tube containing hair (250µl to RB). Place on shaker. Vortex for ~10 sec each hour. Maintain shaking/vortexing until hairs are completely digested. Note time and any tint or color of the samples.

6. Mix 2M Tris and conc. HCl in culture tube: 200µl of each per sample, plus extra.

7. Neutralize all samples with 400µl Tris/HCl per sample (200µl for RB). Vortex to mix. Test for neutral pH by spotting 0.5–1µl of solution onto Hydrion pH paper. If necessary, add 100µl Tris to solutions with unsatisfactory pH (outside 7–8).

8. Transfer 500µl of each solution (except RB) to corresponding Microcon YM-30 column, in Microcon tube. Spin ~10 min at 14000g.

9. Empty rinsate from Microcon tubes. Transfer remaining volume of neutralized solution to Microcon columns (including 450µl of RB). Spin ~10 min at 14000g.

10. Empty rinsate from Microcon tubes. Wash columns 3x, each with 300µl TE, emptying rinsate between each wash. Spin 6–8 min, making sure final wash spins through all liquid.

11. Add 15–20µl TE to each column. Allow columns to sit undisturbed for ~2–3 minutes. Invert columns over UV-treated Microcon tubes. Spin 3 min at 1000g to elute DNA.

12. Measure volumes of all samples and add necessary amounts of TE to bring all samples to desired final volume.

REFERENCES

REFERENCES

Allen M, Engstrom A-S, Meyers S, Handt O, Saldeen T, von Haesler A, Paabo S, Gyllensten U. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. J Forensic Sci 1998;43(3):453-464.

Anderson S, Bankier AT, Barrell BG, deBruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Rose BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457–465.

Baker LE, McCormick WF, Matteson KJ. A silica-based mitochondrial DNA extraction method applied to forensic hair shafts and teeth. J Forensic Sci 2001;46(1):126–130.

Bisbing, RE. The forensic identification and association of human hair. In: Saferstein R, editor. Forensic Science Handbook, Vol. I. Englewood Cliffs, New Jersey: Prentice Hall Regents, 1982; 184–221.

Bogenhagen DF, Clayton DA. The mitochondrial DNA replication bubble has not burst. Trends Biochem Sci 2003;28(7):357–360.

Bourke MT, Scherczinger CA, Ladd C, Lee HC. NaOH treatment to neutralize inhibitors of Taq polymerase. J Forensic Sci 1999;44(5):1046–1050.

Brown WM, Prager EM, Wang A, Wilson AC. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 1982;18:225–239.

Budowle B, Allard MW, Wilson MR. Critique of interpretation of high levels of heteroplasmy in the human mitochondrial DNA hypervariable region I from hair. Forensic Sci Int 2002;126(1): 30–33.

Brennicke A, Clayton DA. Nucelotide assignment of alkali-senstitive sites in mouse mitochondrial DNA. J Biol Chem 1981;256(20):10613–10617.

Cline RE, Laurent NM, Foran DR. The fingernails of Mary Sullivan: developing reliable methods for selectively isolating endogenous and exogenous DNA from evidence. J Forensic Sci 2003;48(2):328–333.

Dwyer J, Neufeld P, Scheck B. Actual innocence: five days to execution and other dispatches from the wrongly convicted. New York: Doubleday, 2000.

Foran DR, Rowe WF. Is it time to stop microscopic hair comparisons? 53rd Annual Meeting of the American Academy of Forensic Sciences, Seattle, WA. 2001.

Giambernardi TA, Rodeck U, Klebe RJ. Bovine serum albumin reverses inhibition of RT-PCR by melanin. BioTechniques 1998;25(4):564–566.

Grossman LI, Watson R, Vinograd J. The presence of ribonucleotides in mature closed-circular mitochondrial DNA. P Natl Acad Sci USA 1973;70(12):3339–3343.

Grzybowski T. Extremely high levels of human mitochondrial DNA heteroplasmy in single hair roots. Electrophoresis 2000;21(3):548–553.

Harkey MR. Anatomy and physiology of hair. Forensic Sci Int 1993;63:9–18.

Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA. DNA typing from single hairs. Nature 1988;332(7):543–546.

Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis—validation and use for forensic casework. Forensic Sci Rev 1999;11(1):22–50.

Holliday JO. In the matter of an investigation of the West Virginia state police crime laboratory, serology division. Kanawha County (WV): Circuit Court of Kanawha County; 1993 Nov. Civil Action No. 93-MISC-402.

Houck MM, Budowle B. Correlation of microscopic and mitochondrial DNA hair comparisons. J Forensic Sci 2002;47(5):964–967.

Jehaes E, Gilissen A, Cassiman J-J, Decorte R. Evaluation of a decontamination protocol for hair shafts before mtDNA sequencing. Forensic Sci Int 1998;94:65–71.

Klintschar M, Neuhuber F. Evaluation of an alkaline lysis method for the extraction of DNA from whole blood and forensic stains for STR analysis. J Forensic Sci 2000;45(3):669–673.

Linch CA, Whiting DA, Holland MM. Human hair histogenesis for the mitochondrial DNA forensic scientist. J Forensic Sci 2001;46(4):844–853.

Melton T, Nelson K. Forensic mitochondrial DNA analysis: two years of commercial casework experience in the United States. Croat Med J 2001;42(3):298–303.

Morley JM, Bark JE, Evans CE, Perry JG, Hewitt CA, Tully G. Validation of mitochondrial DNA minisequencing for forensic casework. Int J Legal Med 1999;112:241–248.

Pfeiffer H, Huhne J, Ortmann C, Waterkamp K, Brinkmann B. Mitochondrial DNA typing from human axillary, pubic and head hair shafts—success rates and sequence comparison. Int J Legal Med 1999;112:287–290.

Savolainen P, Lundeberg J. Forensic evidence based on mtDNA from dog and wolf hairs. J Forensic Sci 1999;44(1):77–81.

Uchihi R, Tamaki K, Kojima T, Yamamoto T, Katsumata Y. Deoxyribonucleic acid (DNA) typing of human leukocyte antigen (HLA)-DQA1 from single hairs in Japanese. J Forensic Sci 1992;37(3):853–859.

Wilson MR, Holland MM, Stoneking M, DiZinno JA, Budowle B. Guidelines for the use of mitochondrial DNA sequencing in forensic science. Crime Lab Digest 1993;20(4):68–77.

Wilson MR, Polanskey D, Butler J, DiZinno JA, Repogle J, Budowle B. Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. BioTechniques 1995a;108:68–74.

Wilson MR, DiZinno JA, Polanskey D, Repogle J, Budowle B. Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J Legal Med 1995b;108:68–74.

Yoshii T, Tamura K, Ishiyama I. Presence of a PCR-inhibitor in hairs. Nippon Hoigaku Zasshi 1992;46:313–316.