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ALKALINE DIGESTION OF HEAD AND PUBIC HAIRS FOR NUCLEAR AND MITOCHONDRIAL DNA ANALYSIS

By

Shannon A Soltysiak

A THESIS

Submitted to
Michigan State University
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for the degree of

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ABSTRACT

ALKALINE DIGESTION OF HEAD AND PUBIC HAIRS FOR NUCLEAR AND MITOCHONDRIAL DNA ANALYSIS

By

Shannon A Soltysiak

Hair is a common form of evidence found at crime scenes, and may be the sole trace evidence to tie a suspect or victim to a location or crime. Isolating DNA from head or pubic hairs is an attractive means of placing a suspect and/or victim at a crime scene. Nuclear DNA is thought to be degraded or not present in shed (telogen) hairs, making STR analysis using commercially available kits difficult or impossible. MtDNA analysis of hair shafts is often successful, but many labs have not validated the method, and in the end, it is not an absolute identifier. Likewise, DNA isolation from hair shafts involves laborious extraction techniques, which can increase the likelihood of contamination. An alternative to standard DNA isolation from hair shafts is alkaline extraction, in which keratin from hair is hydrolyzed but DNA is kept intact. This method was used to extract DNA from head and pubic hair shafts. Hairs were washed in an enzymatic detergent, and then rinsed with ethanol and water. The hairs were then incubated in concentrated sodium hydroxide until completely dissolved, neutralized, and DNA eluted in TE on a spin column. A 220bp product of mtDNA was obtainable from 68% of alkaline digested head hairs and from 98% of alkaline digested pubic hairs. Hair DNA that successfully generated mtDNA product was tested on single and multi-copy nuclear markers. Multi-copy markers show promise as possible sex determinates, while small single copy loci have real-time PCR applications.

To my parents,

who never doubted my ability to achieve anything I set out to accomplish

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INTRODUCTION

Trace evidence such as fibers, hair, glass, inks, toners, and paint is commonly collected as class evidence in criminal investigations. Such evidence connects items to a group, lot, or type of a larger subset, and, as such, is not individualizing. However, class evidence can be helpful in criminal cases to narrow the search field to a particular group or geographic location. Hairs examined as trace evidence are commonly restricted to class evidence as well—such as from which mammal the hair originated, or ethnicity if the suspected donor is human. DNA short tandem repeat (STR) profiling and advancing knowledge of mitochondrial DNA (mtDNA) and its distribution in the population have allowed hair, which is commonly collected at crime scene locations and found on victims and suspects of violent crimes, to be individualizing evidence.

Hair evidence collected in relationship to a crime is microscopically examined and compared to reference hairs from the victim or suspect. The first documented instance of hair being used in a forensic analysis was in 1861 by Rudolf Virchow, a professor at the Dead House of Berlin Charite Hospital (Bisbing 1982). He noted that "...the hairs of the victim represent a so thorough and complete accord with the hairs found on the defendant...however, the hairs found on the defendant do not possess any so pronounced peculiarities or individualities, that no one with certainty has the right to assert that they must have originated from the head of the victim". Virchow's interpretation of the hair evidence nearly 150 years ago is eerily similar to hair analysis capabilities today. While this lengthy and labor-intensive process can, in instances, lead to powerful class evidence, all too often there are not enough distinguishing characteristics among hairs to definitively associate them with an individual.

Furthermore, putative homologies lack substantial support due to limited (if any) population data on hair associations.

Genetic Analysis of Hair

Genetic analysis of hair can take two forms—nuclear DNA analysis in the form of STR typing, or mtDNA sequence analysis. Nuclear DNA analysis of hair evidence is usually preferred, as STR profiles are highly individualizing, but normally require an anagen hair that has been forcefully removed and thus has an intact root (Linch et al. 2000). However, because it is telogen (shed) hair that is most commonly found at crime scenes, nuclear DNA analysis is usually not an option for the forensic analyst. MtDNA analysis, however, has a much higher success rate because of the hundreds of mitochondria and thousands of copies of mtDNA present in each cell (Linch et al. 2000). In addition, the protective environment within the mitochondrion helps maintain mtDNA (Foran, 2006). The discriminatory power of a mitochondrial profile is not nearly that of an STR profile because of the limited genetic variability of the mtDNA genome among individuals. The mitochondrial genome is passed from mother to offspring, so all maternal relatives share common mtDNA profiles (Hutchinson et al. 1974, Stoneking et al. 1991). Unrelated individuals can also share mtDNA profiles due to the slow mutation rate of the genome. Despite the limited differentiation ability, there are a number of attainable haplotypes that can provide useful inclusionary or exclusionary evidence.

Microscopic Analysis of Hair

Forensic microscopic hair examination begins with species identification, ethnic origin (Caucasoid, Mongoloid, Negroid) and somatic origin of the hair (i.e. scalp, facial, pubic, etc.). The hair structure and color are examined, both of which can indicate racial background via hue, cross-sectional shape, hair diameter, pigment density and pigment aggregation. Hair length and features of the outer sheath, or cuticle, are noted, along with the condition of the root (if present) and tip. Lastly, any treatments to the hair or possible disease are assessed. The exemplar hairs are compared to the hair(s) in question using a variety of microscopic techniques such as stereoscopic microscopy, comparison microscopy, and polarized light microscopy (Bisbing 1982). Despite this lengthy and labor-intensive process, absolute matches to an individual are impossible.

The analysis of hair DNA in conjunction with microscopic examination can be more objective and provides additional information when routine examinations are inconclusive or when the hair itself is too damaged to be useful. A study by Houck and Budowle (2002) reviewed 170 microscopic hair examinations at the FBI laboratory between 1996 and 2000 and compared them with data obtained from mtDNA sequencing. They found that neither analysis was a unique identifier (nor are they one together), but that the two complement one another. Hair that is too damaged to be microscopically compared to a reference hair—one that is too small (<1 cm), one lacking in any distinguishable characteristics, or one that is burned, charred, or otherwise deteriorated—may yet yield critical information when the mitochondrial genome is sequenced. Conversely, when the donors of the hairs are maternally related, mtDNA will be identical (Stoneking et al. 1991) and thus microscopic examination may be the only possible

differentiator. Microscopic comparisons of hairs in the Houck and Budowle (2002) study fell into three categories: positive association (hair cannot be excluded as originating from the reference), negative association (hair is excluded as originating from the reference), or inconclusive (insufficient information). Results of the study showed that with microscopic comparisons, 58.2% of the analyses gave conclusive results, whereas with mtDNA analyses, the percentage of conclusive results rose to 94.7%. Most significantly, 9 of 80 (~11%) hairs deemed to be associated by microscopic examination were found to be dissimilar (having conflicting mtDNA profiles) once DNA analysis was performed.

DNA Sequence Analysis

The mitochondria are the energy providers for the cell, the "powerhouses", providing about 90% of the body's energy (Bisbing 1982). The mitochondrial genome is circular, replicates independently, and has a sequence length of about 16,596 base pairs (Anderson et al. 1981). Many of the proteins that comprise the mitochondrion are encoded by the nucleus, but the mitochondrion does code for a few of its own proteins along with various tRNAs and rRNAs (Anderson et al. 1981). Mutations in mtDNA over time (deletions, insertions, and transitions/transversions) result in the polymorphisms that make up an individual's mitochondrial haplotype. The power in mtDNA analysis lies in the control region, a 1.1 kb segment housing the two hypervariable regions, termed HVI and HVII, that have a higher mutation rate than the rest of the genome and can therefore be used to potentially discriminate among individuals (Morley et al. 1999).

MtDNA typing of hair evidence has been very successful when other biological material is not available. In a comprehensive analysis of casework hairs over five years, Melton et al. (2005) found a full or partial mtDNA profile was obtainable from 92.8% of hairs cases. The average amount of hair taken for testing was approximately 2 cm, with more hair being used if difficulty arose when obtaining a profile. A small percentage (8.7%) of hair DNA sequenced exhibited a "mixed" profile—the presence of two or more nucleotide positions that display different nucleotides within a profile. This is opposed to sequence heteroplasmy where a single nucleotide position differs in an mtDNA profile. While the two appear to be similar, it has been shown that mtDNA sequence heteroplasmy is relatively uncommon and is unlikely to occur at multiple locations in an mtDNA profile (Bendall et al. 1996, Morley et al. 1999, D'Eustachio 2002 in response to Budowle et al. 2002). A mixed profile suggests that exogenous DNA, such as that attained by contact with another material (blood, saliva, etc.), is not being removed by the washing process. Wilson et al. (1995b) found hair shafts contaminated with body fluids to type correctly at a rate of 60%, but that subsequent typing attempts could increase that success to 88%. Melton et al. (2005) observed the incidence of mixed profiles to be increasingly common with aged hairs, presumably owing to the decline in total mtDNA due to sample degradation. This underscores the necessity of maintaining strict washing protocols for all hairs subject to DNA analysis. An antimicrobial detergent, Terg-A-Zyme (Alconox), has been utilized in past research to cleanse hairs (Wilson et al. 1995a, 1995b; Melton et al. 2005), but other methods have also proven effective—such as that evaluated by Jehaes et al. (1998) in which a differential lysis buffer was successful in removing saliva and blood from hairs before mtDNA sequencing.

Nuclear DNA testing of biological specimens in forensic cases (such as blood, saliva, urine, semen, etc.) is commonly referred to as "DNA profiling". This "profile" is a genetic fingerprint of the donor of the biological material. The profile is generated by analyzing fragments of highly variable repeated regions, or STRs, of the human genome, which vary in the number of times they are repeated from person to person. Thirteen separate DNA locations (loci) are analyzed in the generation of the profile—each locus having several combinations of repeats possible (one set of repeats inherited maternally, another paternally). Each pair of STRs has a frequency of occurrence (based on population genetics) associated with that combination. The multiplicative law of probabilities allows the frequency of having a specific combination of repeats at a single locus to be multiplied across all loci, resulting in an extremely individualizing profile, and thus a highly significant low probability that the DNA profile could belong to another individual (other than an identical twin), often on the order of one in a quadrillion.

Nuclear DNA analysis from shed hairs has been a topic of debate for several years. Common belief is that telogen hairs contain little if any nuclear DNA (Allen et al. 1998, Higuchi et al. 1998, Pfeiffer et al. 1999). Considering that DNA analysis of hair is destructive, if microscopic examination is desired, it must be performed prior to STR analysis. In addition, hair without root material is often not processed for STR analysis (personal correspondence, Julie Howenstine, Michigan State Police), leaving microscopic examination or mitochondrial sequencing as the only alternatives.

One method of DNA analysis that may be successful in amplifying highly degraded (subjected to fire, oxidation, bacteria, or biochemical agents), aged, or otherwise inhibited DNA is the use of miniSTRs (Butler et al. 2003). MiniSTR markers

are an attractive means of overcoming the boundaries of nuclear DNA in poor condition, as they are based on the 13 STR loci currently in use for DNA profiling. The variable loci used in the analysis are the same as the standard STR loci but are reduced in size, moving the primer annealing sites as close as possible to the repeated region. The human genome is not limited to the currently used 13 highly variable regions—thousands of repeated areas of non-coding DNA have been identified as potential high-variability candidates (Coble and Butler 2005). These too can be useful in the amplification of small segments of nuclear DNA, especially when STR typing and miniSTR typing fail or only some of the loci amplify, resulting in a partial profile (Coble and Butler 2005). Statistics can be applied to newly developed miniSTRs by determining observed heterozygosity from a random sampling of the population. In the study by Coble and Butler (2005), new miniSTRs were developed (each under 125bp) in an attempt to discriminate among degraded DNA samples. A set of 474 individuals (170 Caucasian, 164 African American, and 140 Hispanic) was used to test the six loci—all were found to be in Hardy-Weinberg equilibrium (save one in the African American group) and observed heterozygosities were between 0.5 and 0.8. These promising data were further expounded upon by the authors' identification of 41 additional suitable miniSTRs for the analysis of highly degraded DNA that have yet to be tested on forensic specimens.

Hair Morphology and Biology

Hair is a complex structure that varies across the human body as much as it does among individuals. Mammalian hair has been described as a "thread of protein" which grows from follicles in the skin (Linch et al. 2000). Human hair is comprised of three

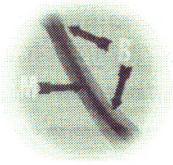
primary components—an outer sheath (cuticle), a densely packed core containing the pigment melanin and the protein keratin (cortex), and a central canal filled with air (medulla). Two reviews of hair anatomy, physiology, and histology (Harkey 1993; Linch et al. 2000) serve to educate the forensic hair examiner on the growth and formation of human hair for use in trace evidence evaluation or DNA analysis.

The entire human body is covered with hair except for the palms of the hands and the soles and heels of the feet (Harkey 1993). The three types of hair that coat the body are different in their texture, length, color, and shape (Figure 1). Terminal hair refers to the areas most commonly thought of as being "hairy"—the scalp, pubic area, armpit (axillary hair), and facial hair (beard and eyebrow)—all are long, coarse, and pigmented (Harkey 1993). Vellus hair is the opposite; it corresponds to parts of the body that one would consider hairless—the eyelids, forehead, and bald scalp. Between those hair types are intermediate hairs, those found on the arms and legs.

Figure 1 – Pubic hair structure.

Human pubic hair is often characterized as such by microscopic observation of increased thickness, larger medulla (M), and buckling (B) of the hair along the shaft.

Photo taken from the Forensic Science Handbook (Petraco and DeForest 1993).



Melanin and keratin affect the appearance and texture of human hair, detailed in the article by Linch et al. (2000). Melanin, produced from melanocytes in the root bulb, varies in concentration and distribution based on the ethnicity of the individual. Its presence (or absence) and the manner in which it is positioned in the shaft gives hair its

color. Keratin, a durable protein produced from keratinocytes, gives hair its rigid shape and robust structure during the growth process known as keratinization. It is also the major component of fingernails and toenails, as well as animal horns and hoofs. The amino acid cysteine in keratin contains sulfur molecules that form covalent bonds (disulfide bridges) and accounts for the overall structure of hair. It is the disulfide bonds that are either created or destroyed when a permanent treatment is applied. The cortical cells elongate as keratin fibril production increases until the cytoplasm is overtaken by bundles of the thick material. The cytoplasm is consumed by keratin and the hair shaft dehydrates and shrinks slightly. At this point, complete keratinization has occurred and nuclear DNA is lost when the cell ruptures (cytolysis). The mitochondria also begin to disintegrate, but can still be seen among the dead cells and filaments of keratinized cells. While the nucleus is destroyed and nuclear DNA thought to be lost, mitochondria, which far outnumber nuclei, remain—albeit often in poor condition.

Hair growth in humans begins in the womb approximately three months into the gestational period (Olsen 1994). Hair emerges in a cylindrical form from the follicle, a small organ located approximately 3 – 4 mm below the surface of the skin (Harkey 1993). The bottom of the follicle, referred to as the bulb, is the site where hair cells are synthesized. A germinal layer of cells, or the matrix, is responsible for initiating the growth of new cells destined to become the components of hair. Above the bulb is the keratogenous zone where hair cells are keratinized and hardened (Harkey 1993). The synthesis of the molecule responsible for hair pigment color, melanin, also occurs at this step in the growth process. As cells grow and divide, they stack on top of one another until emerging from the skin in the form of a chemically robust and stable cylindrical

structure. This final segment of the follicle is the permanent hair region where the fused, fibrous hair cells forming the shaft emerge from the skin.

The three components of hair can be easily described as resembling a pencil—the yellow paint representing the cuticle, wood core as the cortex, and graphite center as the medulla (Figure 2). The cuticle functions to protect the shaft and to anchor the hair into the follicle, but can be easily damaged or even destroyed if subjected to harsh treatments such as heat and chemicals, or those used to color, perm, or relax the hair (Harkey 1993). The cortex (which makes up the bulk of the hair shaft) contains fiber-like keratinized clusters that adhere tightly to one another, resulting in hairs' durable nature (Harkey 1993). Melanin pigment granules are also located in the cortex. Melanin pigment distribution and quantity can later impart inhibitory properties during the amplification of extracted hair DNA, which can be troubling for the DNA analyst (Giambernardi et al. 1998). The final component of the hair shaft is the medulla, which in some cases may be fragmented (discontinuous) or missing altogether (Harkey 1993). In human hair, the medulla is usually the least abundant of the three components. In other species, the medulla can comprise a large portion of the hair shaft, aiding in the distinction between human and animal hair (Harkey 1993).

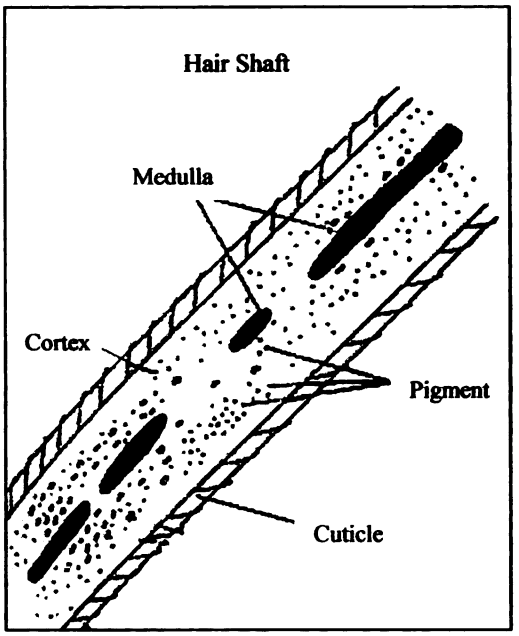


Figure 2 – Human hair shaft components.

The hair shaft is composed of an outer sheath, or cuticle; the body of the hair, or cortex; and a central canal, the medulla, which may be continuous or fragmented (as shown). Pigment granules are dispersed throughout the cortex in varying densities depending on the ethnic origin of the hair and its location on the body.

Figure taken from the Forensic Science Handbook (Petraco and DeForest 1993).

Hair is not in a constant state of growth. At any given time period, various hairs are actively growing, some are in a dormant state, while others are being shed to make way for new growth—therefore hair growth follows a mosaic-like organization. The three phases, common to all mammals, are termed anagen, catagen, and telogen (Figure 3) and are well described in the paper by Harkey (1993) on the anatomy and physiology of hair.

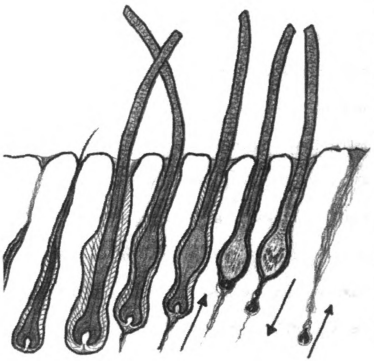


Figure 3 – Hair growth stages.

The life of a hair consists of three stages—growth, quiescence, and exodus. Anagen hairs are in the active growth phase of the cycle. Growth ceases and hairs enter a resting state in the catagen phase. Telogen hairs have stopped growing, and are easily shed or removed by gentle pulling, and are thus most often recovered as trace evidence from a crime scene. The process takes 2 – 7 years for head hairs, but only 1 year for pubic hairs.

Figure taken from Linch et al. 2000.

The anagen phase is the active growth period. A hair enters this phase after the matrix layer at the base of the follicle is stimulated by growth factors. Once the production of hair begins, the follicle is driven deeper into the dermis to accommodate the dividing cells. A thin chain-like series of cells, or filament, is formed as the cells elongate, stack upon one another, and force their way toward the epidermis. Cell division in the root bulb is estimated to occur once every 23 – 72 hrs (Olsen 1994). Differentiation into the three cellular components (cuticle, cortex, and medulla) and keratinization begin before the hair emerges from the follicular canal.

Harkey (1993) detailed the catagen and telogen phases, which hair enters after a period of active growth. Depending on the type of hair (head, pubic, axillary, etc.), the phase might not be reached for weeks, months, or years after cell growth initiates. This

transitory period is marked by a halt in cell division and complete keratinization of the base of the shaft. The bulb of the follicle begins to break down and shrinks in size. The hair then enters a resting period termed the telogen phase—a quiescent period where growth is no longer occurring. The majority of hairs found at crime scenes are telogen hairs, as they are easily shed, or removed by gentle pulling (forcibly removed hairs still containing an intact root are anagen hairs) (Deedrick 2000). The amount of time a hair spends in the resting period depends on the origin of the hair, but varies from about 10 weeks (head hair) to as long as 6 years (body hair). An average adult has about 15% telogen head hairs and 85% in the growth stages (Harkey 1993).

Estimating hair growth rate can be difficult given that not all hairs on the body are growing at the same time. Different locations of hair grow at different rates—scalp hair grows more quickly than pubic or axillary hair. Ethnicity, sex, and age also affect the average growth rate for hairs covering the human body (Harkey 1993). Head hair growth averages a rate of just less than 2 centimeters each month while pubic hairs average only 9 millimeters per month (Harkey 1993).

Wrongful Convictions

Various differences in hair morphology (i.e. shape, length, hue) can exclude one from being the donor of a questioned hair. However, an undiscriminating "cannot exclude" determination has led to wrongful convictions in several instances. Some of the most infamous of all erroneous convictions based on faulty hair comparisons are the scandals surrounding the testimonies of Arnold Melnikoff. Melnikoff opened Montana's first forensic crime laboratory in 1970. Over the years, he claimed that he had "analyzed

hair in 500 to 700 cases and found matches between unrelated people only a few times" (Possley et al. 2004). Based on these examinations, Melnikoff came up with statistics on hair comparisons, which he routinely presented in court testimonies. Such statistics specified that the odds of a person other than the defendant donating the hair in question was "1 in 100" (and in the child rape case of Jimmy Ray Bromgard, 1 in 10,000) (Possley et al. 2004). He also applied the multiplicative law of probabilities when more than one hair was in question (as in the child rape case)—treating head and pubic hair as independent events—resulting in enormous probabilities that were misleading and scientifically unsound. Melnikoff's testimonies lead to the convictions of Chester Bauer in 1983, Jimmy Ray Bromgard in 1987, and Paul Kordonowy in 1989 (Possley et al. 2004). Each of these men has since been exonerated following the exposure of Melnikoff's faulty analyses.

There has been an increasing need for an organization able to investigate past cases where DNA evidence could have resulted in more sound rulings for those on trial. The Innocence Project is a non-profit organization that aims to exonerate those wrongly convicted before the availability of highly discriminatory DNA evidence or in cases where DNA evidence was not used. The project "only handles cases where post conviction DNA testing of evidence can yield conclusive proof of innocence" (http://www.innocenceproject.org). Incorrect identifications based on faulty eyewitness testimony, official misconduct, unsound scientific processes, uninformed or unethical legal counsel, and false testimonies are the leading causes of wrongful imprisonment (http://www.innocenceproject.org). Twenty-one of the Innocence Project's first 130

exonerations (currently over 180) were cases where an individual was convicted based on a faulty microscopic hair comparison match.

Similar organizations exist outside the United States. The work of The Association in Defense of the Wrongly Convicted (AIDWYC), a Canadian group, investigated the case of James Driskell, a man imprisoned more than a decade for the murder of his friend Perry Harder (http://www.aidwyc.org). Although Driskell denied any involvement in the crime, three hairs collected at the scene were used to pronounce a sentence of life in prison. Expert witnesses from the Winnipeg Royal Canadian Mounted Police (RCMP) crime lab claimed that the hairs found in Driskell's vehicle belonged to the victim, but subsequent testing by Forensic Science Services in England found none of the hairs belonged to Harder. The AIDWYC made it possible for Driskell's case to be reviewed—he has recently been granted a stay (as of March 2005) and is seeking reparations for the more than 12 years he spent in prison (http://www.aidwyc.org).

Extraction of DNA from Hair Shafts

The technique used to liberate DNA from shed hairs in the present study was an alkaline solution incubation designed by Graffy and Foran (2005). An alkaline solution (5N NaOH) serves to degrade the makeup of the hair shaft by hydrolyzing the keratin. Alkaline hydrolysis has been used to obtain DNA from forensic samples in addition to hair, such as whole blood, semen, and saliva (Klintschar and Neuhuber 1999). The alkaline digestion procedure has fewer steps than traditional extractions, and thus fewer opportunities for analyst error and/or sample contamination. In addition, treatment with sodium hydroxide has been shown to neutralize inhibitors of Taq DNA polymerase, the

enzyme responsible for assembling strands of DNA from primers and template DNA in a process termed the "polymerase chain reaction (PCR)" (Bourke et al. 1998). The addition of bovine serum albumin (BSA) has also been shown to help overcome PCR inhibition (Giambernardi et al. 1998) and was employed in this project.

Research Goals

The current study expanded upon the research published by Graffy and Foran (2005) on alkaline extraction of DNA from shed hairs by increasing the type of hairs tested to include pubic hairs, and augmented sample numbers. A rapid DNA extraction procedure for pubic hairs collected from sexual assault cases, or those found at crime scenes, would be helpful to the forensic DNA analyst by reducing preparation time and decreasing the number of steps involved.

A secondary goal of the study was to follow successful mtDNA analysis with the analysis of nuclear DNA from shed hairs. To date, this has proved exceedingly difficult, but some success has been claimed (Estacio et al. 2005) using smaller amplicons such as miniSTRs. Extracts of shed pubic and head hairs, if shown to be successfully amplifiable using mtDNA primers, were to be further tested with nuclear DNA primers 100 – 200bp in size. If amplification of single copy nuclear loci proved unsuccessful, the use of multi-copy nuclear genes was to be examined to assess the presence of nuclear DNA in telogen hairs.

MATERIALS AND METHODS

Sample Collection

Head hairs, pubic hairs, and buccal swabs (to serve as reference DNA samples) were anonymously collected from 56 human volunteers. Sample kits contained envelopes for head and pubic hair donations, and a plastic culture tube with sterile swab for buccal sample collection and storage. Along with the biological samples, the kits also contained a questionnaire (Appendix A) asking donors to detail their sex, ethnicity, blow drying frequency, treatments to hair (such as application of color, highlights, permanents or relaxers), and the use of styling product. Donors applied numbered stickers (provided in the donation kit) to each biological specimen and the questionnaire. No sample specimen could be traced back to any individual. Consent forms approved by the Institutional Review Board (IRB) of the University Committee on Research Involving Human Subjects (UCRIHS) were submitted separately from the donations so as to ensure donor anonymity (IRB approval # 05-282).

Hair Preparation and Cleansing

Reagents for hair cleansing included sterile water, 95% ethanol, and an antimicrobial solution (5% Terg-A-Zyme)—each was UV treated before use, as were various mechanical pipettors. Disposable 1.5mL microcentrifuge tubes and filtered pipette tips were autoclaved and UV treated before use. Scissors and forceps were UV treated, then sterilized with 70% ethanol. Ethanol-flamed forceps were used to remove hairs from sample envelopes and hairs were examined for the presence of root material. Forceps and scissors were soaked in ethanol between sampling envelopes. If a root was

observed, it was removed by trimming—however, to ensure no root material was transferred to sample tubes, hairs were trimmed at each end before measurements were taken. Two-centimeter clippings of pubic hair were used for alkaline digestion and manual grinding comparison experiments. Two 1 cm trimmings from a single hair were split between the 1.5mL microcentrifuge tube designated for each method, if possible, so as to equally distribute a single hair between each extraction method. In seven instances, hair was donated in fragments less than 1 cm in length, making equal distribution impossible. Alkaline digestion of head hair (used in nuclear DNA analysis) utilized slightly larger segments (2 – 4 cm), adding 1 cm increments into 1.5mL microcentrifuge tubes. Hairs were cleansed with successive washes for 5 min each of: 1mL 5% Terg-A-Zyme, 1mL 95% ethanol, and 1mL sterile water. Hairs proceeded to an alkaline digestion or a manual grinding digestion following the wash step. In total, 49 pubic hair comparison digestions were performed, while 25 head hairs underwent alkaline digestion.

DNA Extraction from Pubic Hairs by Alkaline Digestion

Pubic hairs were digested in groups of ten. TE buffer (10mM Tris at pH 7.5, 1mM EDTA), 2M Tris base buffer (pH 8), and freshly prepared 5N sodium hydroxide were UV treated before use as were YM-30 Microcon spin columns. An empty 1.5mL tube was subjected to a wash with 1mL 5% Terg-A-Zyme, 1mL 95% ethanol, and 1mL sterile water for 5 min each to simulate the cleansing process undergone by the pubic hairs. Following the wash, 500µL of 5N sodium hydroxide were added to serve as a reagent blank. Each sample tube of cleansed pubic hairs received 500µL of 5N sodium hydroxide solution. Hairs and reagent blank were incubated at room temperature on a

rocking platform and were occasionally vortexed (to aid in hydrolysis) until hairs were no longer visible. The incubation step was often carried out overnight as previous testing showed approximately 5 hrs of incubation were required to dissolve hairs (Graffy and Foran 2005).

Once the hair was fully dissolved, the sodium hydroxide solution was neutralized with 2M Tris base (pH 8) and concentrated HCl (11.6M). Two hundred microliters of 2M Tris base were added to each sample and all tubes were transferred to a fume hood. One hundred eighty microliters of concentrated HCl were slowly added to each sample and verification of neutral pH (6 – 8) was accomplished by spotting 1– 2μL on pH paper. If the solution was found to be basic, 10μL increments of HCl were added until neutrality was reached. Samples were concentrated and filtered via centrifugation using a Microcon YM-30 spin column. Neutralized hydrolyzed hair samples were loaded into a Microcon filter vial in 400 – 500μL increments and centrifuged for 10 min at 14000g. Filter membranes were subsequently washed three times with 300μL TE buffer. Each was eluted in 20μL TE, and transferred to a clean 1.5mL microcentrifuge tube, and stored at –20°C until amplification.

DNA Extraction from Pubic Hairs by Manual Grinding

Hairs were digested in batches of five or ten, using the AFDIL protocol (www.afip.org/Departments/oafme/dna/afdil/protocols.html) as a basic guide. Micro-tissue grinders (0.2μL, Kontes Glass) were sanitized with 10% bleach, water, and ethanol before use and allowed to dry. Prior to the addition of pubic hair, micro-tissue grinders were irradiated in an ultraviolet crosslinker for 10 – 20 min.

A reagent/grinder blank was collected from each micro-tissue grinder before hair was added. Twenty microliters of TE buffer were added and grinding was simulated. The solution was transferred to a labeled 0.5mL microcentrifuge tube and stored at –20°C until amplification experiments were performed. One hundred eighty-seven microliters of digestion buffer (20mM Tris pH 8, 100mM EDTA, 0.1% SDS) were added to each grinder. An additional grinder containing an equal amount of digestion buffer was prepared as a reagent blank to be processed alongside the sample group, and undergoing the same treatments as the pubic hair.

Hairs were transferred to micro-tissue grinders using ethanol-flamed forceps. Grinding was carried out until fragments of hair were no longer visible. The solution in each grinder (including the reagent blank) was transferred to a labeled 1.5mL microcentrifuge tube. Five microliters of proteinase K (ProK; 20mg/mL) and 8μL of 1M dithiothreitol (DTT) were added to each sample tube and the single reagent blank. Tubes were incubated at 55°C for 18 – 24 hours. Following incubation, samples were purified via standard organic extraction. Two hundred microliters phenol were added to each digestion and sample tubes were vortexed vigorously. Samples were centrifuged for 5 min at 13000g and the aqueous layer was transferred to a clean 1.5mL microcentrifuge tube. Two hundred microliters of chloroform were added to each sample and tubes were vortexed vigorously. Samples were centrifuged for 5 min at 13000g and the aqueous layer was transferred to a YM-30 Microcon spin column for concentration and filtration. Extracts were concentrated by centrifuging for 10 min at 14000g and washed with 300mL TE buffer in triplicate under the same centrifugation settings. Samples were eluted in 20μL TE and stored at –20°C until further use.

DNA Extraction from Head Hairs by Alkaline Digestion

Head hairs were digested in groups of five. All materials and solutions were UV sterilized before use. One sample tube was washed with equal amounts of solutions used in the cleansing process and filled with 500µL 5N sodium hydroxide to serve as a reagent blank. Five hundred microliters 5N sodium hydroxide were added to each sample tube of cleansed head hairs. Hairs and reagent blank were incubated at room temperature on a rocking platform until hairs were no longer visible. Occasional vortexing aided in the hydrolysis of the hairs. The incubation step was carried out overnight.

Once the hair was fully dissolved, the sodium hydroxide solution was neutralized with 2M Tris base (pH 8) and concentrated HCl (11.6M) in the same manner as for the pubic hair digests. Samples were eluted in 20µL TE and transferred to a clean 1.5mL microcentrifuge tube and stored at –20°C until use in amplification analyses.

DNA Extraction from Buccal Swabs

Buccal swabs were halved lengthwise using an ethanol flame-sterilized disposable scalpel and transferred to a clean 1.5mL microcentrifuge tube with ethanol-flamed forceps. Two hundred microliters of digestion buffer and 2µL ProK were added to the swabs and tubes were incubated overnight at 55°C. Swabs were moved to a spin basket and placed in 2mL tubes, which were centrifuged for 5 min at 13000g to collect liquid. Baskets and dry swabs were discarded and remaining liquid was pooled with liquid from the overnight incubation. All samples proceeded to DNA purification via phenol/chloroform extraction identical to the method used to isolate DNA from manually

digested pubic hairs. Following the chloroform extraction, the aqueous layer was transferred to a 1.5mL microcentrifuge tube and DNA was precipitated using 20μL sodium acetate (3M) and 400μL cold 95% ethanol. Tubes were stored at –20°C for 2 – 24 hrs, then centrifuged for 15 min at 14000g. Pellets were washed with 1mL 70% ethanol, vacuum-dried for 20 min, and dissolved in 20μL TE. Samples were stored at –20°C until amplification.

PCR Amplification of MtDNA from Head and Pubic Hairs

Preliminary confirmation of mtDNA acquisition was performed using a PCR mix containing HotMaster Buffer (1X; Eppendorf), dNTPs (0.2μM), sterile water, and mitochondrial primer pairs F16190 (2μM) and R16410 (2μM) (Table 1), amplifying a 220bp segment. If samples showed possible PCR inhibition, 1μL of extracted pubic hair DNA was transferred to a clean tube and 9μL TE buffer was added to create a 1:10 dilution of the initial concentration. Ten μg/μL BSA was added to the PCR reaction in an attempt to overcome inhibition. Verification of target product amplification was performed via gel electrophoresis using a 1.5 – 2.0% agarose gel and ethidium bromide staining.

DNA quality differences between the two digestion methods applied to pubic hairs was assayed using increasingly larger segments of mtDNA. One microliter each of 2μM primer pairs F15989/R16410 (~421bp), F16190/R285 (~664bp), and F15989/R285 (~865bp) (Table 1) were used in PCR reactions, along with 1μL of template DNA and PCR reagents identical to those used in the 220bp mtDNA amplification. Verification of

target product amplification was performed via gel electrophoresis using a 0.8 – 1.0% agarose gel and ethidium bromide staining.

Head hair DNA extracts were amplified to determine the presence of a 220bp mtDNA amplicon. Samples were stored at –20°C for 10 months, and were retested for the same target product to assess stability.

Table 1 – MtDNA primer sequences (5'→ 3').

F15989	CCCAAAGCTAAGATTCTAAT
F16190	CCCCATGCTTACAAGCAAGT
R16410	GAGGATGGTGGTCAAGGGAC
R285	GTTATGATGTCTGTGTGGAA
R484	TGAGATTAGTAGTATGGGAG

KEY: F=Forward primer, R=Reverse primer
(Edson et al. 2004).

Table 2 – Cycling parameters for 220bp and 421bp mtDNA amplicon.

Temperature (°C)	Time	
94	2m	
94	30s	⎫
55	1m	⎬ 38 Cycles
72	1m	⎭
72	5m	
4	∞	

Table 3 – Cycling parameters for 664bp and 865bp mtDNA amplicon.

Temperature (°C)	Time	
94	2m	
94	30s	38 Cycles
55	1m	
72	1.5m	
72	5m	
4	∞	

PCR Amplification of Nuclear DNA

PCR amplification of nuclear DNA was performed on alkaline digested head hairs using a reduced-size amelogenin primer set (designed by L. Ramos; amplifying ~65bp) and two high copy number alpha satellite primer pairs. Two loci were chosen—one (designed by C. Jackson, personal correspondence) on chromosome 17 (D17Z1) present in 500 to 1000 copies (147bp in length) per chromosome, and one (designed by Koganm et al. 1987) on the Y chromosome (DYZ1) present in 2000 – 4000 copies (154bp in length).

Table 4 – Nuclear DNA primer sequences (5'→ 3').

FD17Z1[1]	GATCATTGCACTCTTTGAGGAG
RD17Z1[1]	GTGTTTCTAAACTGCTACATCGC
FDYZ1[2]	TCCACTTTATTCCAGGCCTGTCC
RDYZ1[2]	TTGAATGGAATGGGAACGAATGG
FAmel Int[3]	AAGAATAGTGTGTGGATTCTTTATCCCA
RAmel Int[3]	GGAACTGTAAAATCGGGACCACTTGAG

KEY: F=Forward primer, R=Reverse primer (C. Jackson[1], Koganm et al. 2000[2], L. Ramos 2006[3]).

Table 5 – Cycling parameters for D17Z1 amplicon.

Temperature (°C)	Time	
94	4m	
94	30s	] 35 Cycles
60	30s	
72	45s	
72	5m	
4	∞	

Table 6 – Cycling parameters for DYZ1 amplicon.

Temperature (°C)	Time	
94	4m	
94	30s	] 38 Cycles
60	30s	
72	45s	
72	5m	
4	∞	

Real-Time Amplification Using SYBR-Green

Real-time PCR analysis of alkaline extracted head hairs (samples A102 and A124), alkaline digested pubic hair (B122), and manually digested pubic hair (B103) were performed using an iQ5 (Bio-Rad) real-time PCR detection system. Samples were tested in triplicate using mtDNA primers F16190/R16410, reduced-size amelogenin primers, and D17Z1 primers (only samples A102 and B122 were analyzed in duplicate with D17Z1 primers due to lack of primer stock) (Table 7, PCR Program 1). Each sample well included 2.5μL template DNA, 12.5μL of IQ SYBR-Green Supermix, 2.5μL of 2μM primer, 2.5μL 10 μg/L BSA, and 2.5μL of sterile water.

DNA quantity analysis of five alkaline digested pubic hairs and five manually digested pubic hairs (103, 117, 122, 123, 127) was performed using the same mtDNA primer pairs and the amelogenin primers using a higher annealing temperature during the reaction cycle (Table 7, PCR program 2). Reaction contents were identical to those listed above.

Table 7 – Cycling parameters for real-time PCR.

PCR Program 1

Temperature (°C)	Time	
95	3m	
95	10s	40 Cycles
55	30s	

PCR Program 2

Temperature (°C)	Time	
95	3m	
95	10s	40 Cycles
56.5	30s	
95	1m	
55	1m	

DNA Sequencing Analysis of Buccal Swab Extracts and Pubic Hair Extracts

A 421bp segment of the hypervariable region of the mitochondrial genome (HVI) was sequenced to verify that pubic hair alkaline extracts were consistent with the accompanying buccal swab extracts. PCR product was purified via centrifugation for 15 min at 1000g using a Montage (Millipore) spin column and 100µL water rinse. Fifty to

100 fmol of template DNA was added to the sequencing reaction otherwise consisting of 4μL Quick Start Master Mix (Beckman-Coulter) and 1μL of 2μM primer (F15989 or R16410). Sequencing reaction thermocycler parameters are detailed in Table 8. Upon completion, 2.5μL stop solution (1.2M sodium acetate, 20mM EDTA, 4mg/mL glycogen, sterile water) and 30μL cold 95% ethanol were added to each reaction to precipitate DNA. Sample tubes were centrifuged for 15 min at 14000g and supernatant was removed from the DNA pellet. Two washes with 100μL of cold 70% ethanol were performed, centrifuging each for 3 min at 14000g. Supernatant was removed from the DNA pellet and samples were vacuum dried for 15 – 20 min. Samples were loaded onto a Beckman-Coulter CEQ 8000 after dissolving the DNA pellets in 40μL Sample Loading Solution (SLS; Beckman-Coulter). Sequence data were obtained using the LFR-7 program (85 min, capillary temperature 50°C, denature 120 sec, inject 4 sec at 2.0kV, and separate at 4.2kV), and the BioEdit Biological Sequence Alignment Editor (Hall 1999).

Table 8 – Cycling parameters for mtDNA sequencing reaction.

PCR Program		
Temperature (°C)	Time	
90	2m	30 Cycles
60	4m	
55	1m	

Comparison Analysis

To gain perspective on the effectiveness of the two digestion methods when run nearly simultaneously, five pubic hair samples were again digested using both

techniques, extracted according to the methods previously specified for each method, and tested for DNA integrity using 220bp, 421bp, 664bp, and 865bp target amplicons. The presence of nuclear DNA was tested using multi-copy markers D17Z1 and DYZ1.

RESULTS

Sample Collection

Sample packets were collected from 56 volunteers: 41 Caucasian head hairs (15 male, 26 female); 38 Caucasian pubic hairs (15 male, 23 female); 10 Asian head hairs (7 male, 3 female), 8 Asian pubic hairs (7 male, 1 female); 5 African American head hairs (2 male, 3 female); 2 African American pubic hairs (1 male, 1 female); 1 Hispanic head hair (male); and 1 Hispanic pubic hair (male). All participants included sufficient hair to allow for collection of 4 cm head hair and 2 cm pubic hair, and all donors returned buccal swabs and enclosed questionnaire, although several were not fully completed. Table 9 shows demographic information for each sample, along with any treatments subjected to the hair such as blow-drying, coloring, highlighting, relaxing, permanents, and styling products. Approximately one-third of donors indicated blow-drying their hair daily or often. Sixteen of the 56 participants had colored or highlighted their hair within the last year, 2 of those being within two months prior to donation. Three individuals had relaxed or received a permanent treatment to their hair within the last month, and an additional participant had done so within the year prior to donation. Other products applied to hair included gel, wax, shine serum, oils, moisturizes, mousse, and curling or flattening iron use.

Sample Preparation

Thirty-nine of the 49 pubic hair samples prepared were divided into two tubes, each corresponding to a different digestion technique. One-centimeter segments were cut from single hairs and added to the two tubes in an alternating manner. If a 2 cm or

greater clipping of pubic hair was not available, as occurred in seven instances, hairs were split between tubes in fragment sizes less than 1 cm. One sample (B177) was unable to be split, as all fragments were <0.5 cm in length.

The 25 head hairs digested via alkaline digestion utilized 4 cm hair pieces and were incubated overnight for 22 to 28 hours. One-centimeter segments were placed in each sample tube, except for one sample (A132), which contained only short hair fragments (<0.5 cm). Sample A124 appeared to be body hair, but could not be verified as such and was processed as head hair.

In the side-by-side comparison study of five alkaline digested and manually ground pubic hairs, 4 cm of hair from each donor was split between two sample tubes, resulting in 2 cm of pubic hair processed per method. The two digestion methods were carried out within 24 hrs of each other so as to remove any time variable that may have biased the results of the study.

The following pages (32-34) contain:

Table 9 – Demographics of hair sample donors.

Sample #	Sex	Ethnic/Racial Group	Pubic Hair color	Blow Drying	Dye/ highlights/lowlights	Permanent/ relaxer	Other
101	F	Caucasian	Lt. Brown	Daily	8 months ago		
102	F	Caucasian	Dk. Brown	Rarely	3 years ago	Never	
103	F	Caucasian	Lt. Brown	Rarely	Beyond 1 year	Beyond 1 year	
105	F	Caucasian	Dk. Brown	Rarely	Within the year		
106	F	Caucasian	Lt. Brown	Rarely	Within the year	Beyond 1 year	
107	M	Hispanic	Dk. Brown	Rarely	Within the year		
108	M	Caucasian	Brown	Never	Beyond 1 year	Never	Gel/wax daily
111	M	Caucasian	Dk. Brown/Black	Rarely	Within the year	Beyond 1 year	
114	F	Caucasian	Lt. Brown	Often			
116	F	Caucasian	Brown	Rarely	Beyond 1 year	Beyond 1 year	Gels/shine serum occasionally
117	M	Caucasian	Brown	Rarely	Beyond 1 year	Beyond 1 year	
118	F	Caucasian	Lt. Brown		6 weeks ago		
122	F	African-American	Reddish Brown	Rarely	Beyond 1 year	Within the month	
123	F	Caucasian	Lt. Brown	Rarely	Beyond 1 year	Beyond 1 year	
124	F	Caucasian	Brown				
126	F	Caucasian	Brown	Daily			
127	F	Caucasian	Brown	Rarely	Beyond 1 year	Beyond 1 year	
128	M	Asian/Pacific Islander	Black				
130	M	Caucasian	Lt. Brown	Rarely	Beyond 1 year	Beyond 1 year	

Sample #	Sex	Ethnic/Racial Group	Pubic Hair color	Blow Drying	Dye/ highlights/lowlights	Permanent/ relaxer	Other
132	M	Asian/Pacific Islander	Dk. Brown	Often	Beyond 1 year	Beyond 1 year	
133	M	Caucasian	Brown	Rarely	Never	Never	
134	F	African-American	NA	Often	Within the year	Within the month	Oil/moisturizer, gel/mousse
135	M	Asian/Pacific Islander	Dk. Brown/Black	Often	Beyond 1 year	Beyond 1 year	
136	F	Caucasian	Dk. Brown/Black	Daily	within the year	Beyond 1 year	
138	F	Caucasian	Lt. Brown	Daily	within the month		
139	M	Caucasian	Dk. Brown	Rarely	Beyond 1 year	Beyond 1 year	
140	F	Caucasian	Brown	Rarely	Never	Never	
143	M	Caucasian	Brown	Rarely	Beyond 1 year	Beyond 1 year	
144	F	Caucasian	NA	Never	within the year	Never	
146	M	Asian/Pacific Islander	Black	Rarely	Beyond 1 year	Beyond 1 year	
148	M	Caucasian	Brown	Rarely	Beyond 1 year	Beyond 1 year	
149	F	Caucasian	Brown	Often	Within the year	Beyond 1 year	
150	F	African-American	NA	Often	Within the year	Within the month	
151	M	Asian/Pacific Islander	Black	Rarely	Beyond 1 year	Beyond 1 year	
152	M	Caucasian	Dk. Brown	Rarely	Beyond 1 year	Beyond 1 year	Hair gel
155	F	Caucasian	Brown	Often	Within the year	Beyond 1 year	
157	M	Caucasian	Dk. Blonde	Rarely	Beyond 1 year	Beyond 1 year	

Sample #	Sex	Ethnic/Racial Group	Pubic Hair color	Blow Drying	Dye/ highlights/lowlights	Permanent/ relaxer	Other
161	F	Asian/Pacific Islander	NA	Rarely			
163	F	Caucasian	Dk. Brown	Rarely	Never	Never	
165	M	Caucasian	Lt. Brown	Rarely	Beyond 1 year	Beyond 1 year	
167	M	Caucasian	Lt. Brown	Rarely	Beyond 1 year	Beyond 1 year	Wash/condition daily
170	F	Caucasian	Brown	Rarely	Never	Never	
171	M	Asian/Pacific Islander	Black	Never	5 Years Ago	Never	
173	F	Caucasian	Blonde	Often	Beyond 1 year	Within the year	
175	M	Asian/Pacific Islander	Black	Rarely	Beyond 1 year	Beyond 1 year	
177	F	Asian/Pacific Islander	Black	Rarely	Never	Never	
178	M	Caucasian	Dk. Brown	Never	Beyond 1 year	Never	Styling product
181	M	Caucasian	Brown	Rarely	Within the year	Beyond 1 year	
187	F	Caucasian	Brown	Often	Beyond 1 year	Beyond 1 year	
188	M	Caucasian	Dk. Brown				
189	F	Caucasian	Dk. Brown	Daily	Within the year	Beyond 1 year	
191	M	African-American	Black	Rarely	Beyond 1 year	Beyond 1 year	
193	F	African-American, Asian/Pacific Islander	NA	Often	Beyond 1 year		
196	F	Caucasian	Brown	Often	Within the year		Curling iron, straightner often
198	F	Caucasian	NA	Rarely	Never	Never	
199	F	Caucasian	NA	Daily	Beyond 1 year		

KEY: M=male, F=female, Lt. Brown= light brown, Dk. Brown=dark brown, Dk. Blonde=dark blonde, NA=not applicable.

Digestion of Hairs and Extraction of DNA

Alkaline digested pubic hairs were fully hydrolyzed within 24 hrs, save one sample (B126) that was not dissolved and was digested for an additional 15 hrs. Several alkaline processed pubic hair DNA extracts exhibited pigment carryover that became visible on the spin column membrane during the TE buffer washes. This phenomenon was never seen in manually digested hair samples, and was assumed to be the result of decreasing salt concentration of the solution affecting pigment solubility. Although no pigment sediment was observed on the spin column membrane of manually ground hairs, discolored final extracts were occasionally observed in both alkaline digested and manually ground hair DNA. In alkaline digested hairs, a precipitate sometimes accompanied discolored samples; those samples were centrifuged prior to amplification to pellet any material. Thirty-two pubic hairs digested by manual grinding had to be reprocessed due to contaminated grinders. A modification to the sterilization method (subsequent water/bleach/ethanol rinses opposed to bleach/water/ethanol rinse) and upright positioning of grinders in the UV crosslinker eliminated contamination.

PCR Amplification of MtDNA

DNA Isolation of MtDNA from Hairs

Nine of the initial ten head hairs processed via alkaline digestion and examined for the presence of mtDNA showed PCR inhibition, failing to produce the 220bp amplicon. Inhibition can be recognized by the lack of primer-dimer activity (Figure 4). Samples were diluted prior to any re-amplification attempts—primarily at 1:10 and then at 1:100 if inhibition was seen after the initial dilution. One-tenth initial concentration

plus BSA addition to the PCR reaction produced the highest number of samples overcoming inhibition (8 of 9), as only one sample amplified at 1:100 (1 of 9). Subsequent batches of alkaline digested head and pubic hair samples were diluted to one-tenth initial concentration immediately after elution in TE, and BSA was used in combination with sample dilutions within the PCR mix to overcome the anticipated inhibitory effects. One sample (B107) failed to amplify at any of the attempted concentrations. The ethnicity of the donor of sample B107 was listed as being of Hispanic decent, and was the only such sample in the set. One group of ten pubic hairs was reprocessed due to a contaminated reagent blank (samples B126, B130, B133, B135, B136, B138, B139, B140, B143, and B146) and rechecked for the presence of the 220bp mtDNA amplicon. The success rate for amplification of mtDNA (220bp amplicon) extracted from pubic hairs via alkaline digestion was 98% (48 of 49 samples) (Figure 5, Table 10).

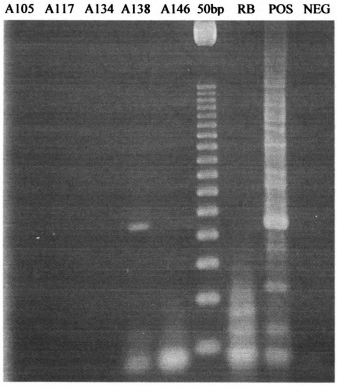


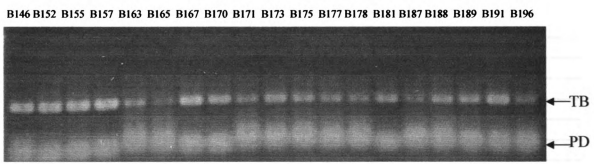
Figure 4 – PCR inhibition.

Sample inhibition is characterized by the absence of the target band and no evidence of primer-dimer activity. Samples A105, A117, and A134 are inhibited. Sample A138 is not inhibited and contains the target band. Sample A146 is not inhibited, and does not contain the target band.

Pubic Hair MtDNA Amplification

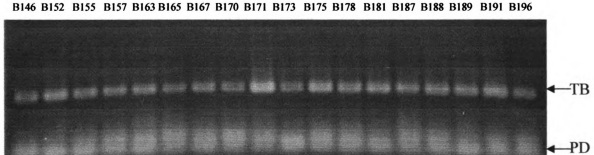
Pubic hair DNA extracted via manual grinding experienced the same inhibition difficulties as those seen with the alkaline digestion technique. Samples were diluted to one-tenth initial concentration and BSA was added to the PCR mix to overcome inhibition. One sample (B108) failed to amplify and one sample (B128) was not counted in the final analysis due to repeated failures. Success rate for mtDNA extracted from pubic hairs via alkaline digestion was 97.9% (47 of 48 samples) (Figure 6, Table 10).

Figure 5 – Alkaline digested pubic hair amplification of 220bp mtDNA amplicon.



KEY: B=Pubic hair, 1XX=sample number, TB=target band, PD=primer-dimer activity.

Figure 6 – Manually digested pubic hair amplification of 220bp mtDNA amplicon.



KEY: B=Pubic hair, 1XX=sample number, TB=target band, PD=primer-dimer activity.

Table 10 – MtDNA amplification results: Alkaline digestion versus manual grinding.

Pubic Hair Sample	Alkaline Digestion	Manual Grinding	Pubic Hair Sample	Alkaline Digestion	Manual Grinding
B101	+	+	B139	+	+
B102	+	+	B140	+	+
B103	+	F	B143	+	+
B105	+	+	B146	+	+
B106	+	+	B148	+*	+
B107	—	+	B149	+*	+
B108	+	S	B151	+	+
B111	+	+	B152	+	+
B114	+	+	B155	+	+
B116	+	+	B157	+	+
B117	F	—	B163	+	+
B118	+	+	B165	+	+
B122	+*	+	B167	+	+
B123	+	+	B170	+	+
B124	+	+	B171	+	+
B126	+	+	B173	+	+
B127	+	F	B175	+	+
B128	+	NA	B177	+	+
B130	+	+	B178	+	+
B132	F	+	B181	+	+
B133	+	+	B187	+	+
B135	+	+	B188	+	+
B136	+	+	B189	+	+
B138	+	+	B191	+	+

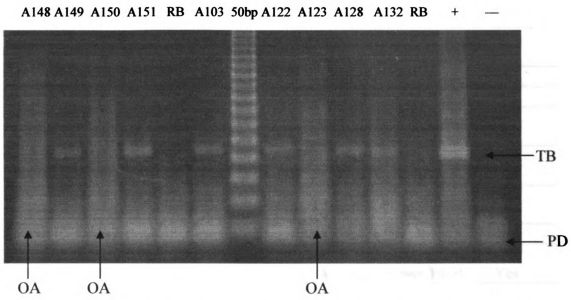
KEY: B=pubic hair, 1XX=sample number, + = target band present, — = target band not present, S=smeary product, F=faint product band seen, * = 2.5 – 3 cm used in digestion, NA=amplification not performed.

Head Hair MtDNA Amplification

Twenty-five head hairs were digested with 5N sodium hydroxide, concentrated on spin columns, and tested for the presence of a 220bp target product of mtDNA. Results visualized on a 2% gel showed heavily over-amplified product (Figure 7) and 16 of 25 samples (64%) generating the target band. When samples were retested approximately

ten months later, 17 of 25 (68%) amplified, with an additional 4 samples producing faint bands (Table 11).

Figure 7 – Head hair 220bp mtDNA amplification.



KEY: A=head hair, 1XX=sample number, OA=over amplification, TB=target band, PD=primer-dimer activity.

Table 11 – Head hair color and mtDNA amplification success of 220bp target product.

Hair Sample	Hair Color	Treated	220bp	Hair Sample	Hair Color	Treated	220bp
A101	Lt. Brown	Yes	F	A127	Reddish Brown	No	+
A102	Reddish Brown	No	+	A128	Reddish Brown	N/A	+
A103	Lt. Brown	No	+	A132	Dk. Brown	No	+
A105	Dk. Blonde/Brown	Yes	F	A133	Dk. Brown/Black	No	—
A107	Brown	Yes	+	A134	Lt. Brown	Yes	—
A111	Brown	Yes	—	A135	Black	No	+
A117	Dk. Brown	No	+	A138	Dk. Brown/Black	Yes	+
A118	Brown	Yes	+	A144	Reddish Brown	Yes	+
A122	Reddish Brown	No	+	A146	Brown	No	F
A123	Dk. Blonde	No	+	A148	Dk. Brown	No	+
A124	Brown	N/A	+	A149	Dk. Brown/Black	Yes	—
A126	Lt. Brown	N/A	+	A150	Brown	Yes	—
				A151	Dk. Brown	No	+

KEY: A=head hair, 1XX=sample number, Treated=any color, highlights, lowlights, permanents or relaxers applied to hair within the last year, + = target band present, — = target band not present, F=faint product band seen, N/A = not answered by donor.

Assessment of Pubic Hair DNA Quality

DNA extracted from pubic hair was tested for quality by amplifying larger segments of the mitochondrial genome. Twenty pubic hair samples, extracted after both alkaline and manual digestion, were compared for amplification of a 421bp and 664bp mtDNA amplicon (Table 12). Because all alkaline digested pubic hair DNA failed to generate the 664bp product, analysis of the large 865bp amplicon was not performed. Twelve of the 20 (60%) alkaline digested pubic hair DNA samples produced the 421bp

target product. Of those 12, all failed to amplify the 664bp segment. Sixteen of the 20 (80%) manually digested pubic hair DNA samples successfully generated the 421bp product. Ten of those 16 samples (62.5%) that went on to 664bp analysis showed amplification of the target band, and an additional two showed faint bands.

Comparison Analysis

In this study, alkaline and manually digested pubic hairs were processed for DNA extraction at different time periods—alkaline digestions were completed before manual grinding of pubic hairs was performed. In addition, the contaminated reagent blank and contaminated grinders that necessitated the re-extraction of several samples further confounded this non-concordance. Thus, a side-by-side comparison study was performed to eliminate time and contamination variables. Five pubic hair DNA samples processed simultaneously via each method were amplified and checked for the presence of four target amplicons of increasing size (Table 13). The five sets were also tested using nuclear markers D17Z1 and DYZ1 for the presence of nuclear DNA (Table 14).

Table 12 – Quality assessment of alkaline and manually digested pubic hairs.

	Alkaline Digestion		Manual Grinding	
Sample	421bp	664bp	421bp	664bp
B126	+	—	+	—
B130	+	—	+	+
B133	+	—	+	F
B135	—	NA	—	NA
B136	+	—	+	F
B138	NA	—	+	+
B139	—	NA	+	F
B140	+	—	+	+
B143	+	—	+	+
B146	+	—	—	—
B152	+	—	+	+
B155	+	—	—	NA
B157	—	NA	—	NA
B163	+	—	+	+
B165	+	—	+	+
B167	—	NA	+	—
B170	—	NA	+	—
B171	—	NA	+	+
B173	—	NA	+	+
B175	—	NA	+	+

KEY: B=pubic hair, 1XX=sample number, + = target band present, — = target band not present, S=smeary product, F=faint product band seen, NA=amplification not performed.

Table 13 – Amplification of increasing size mtDNA amplicons from concurrently extracted alkaline and manually digested pubic hairs.

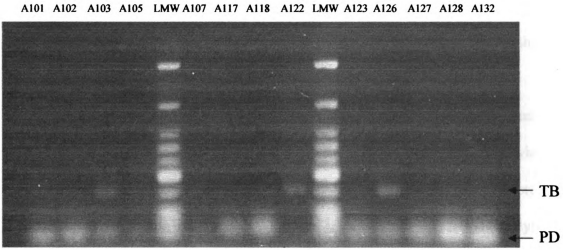
	Alkaline Digestion				Manual Grinding			
Sample	220bp	421bp	664bp	865bp	220bp	421bp	664bp	865bp
B118	+	+	—	—	+	—	—	—
B126	+	+	—	—	+	F	—	—
B143	+	—	—	—	+	+	—	—
B152	+	+	+	+	+	+	—	—
B173	+	+	—	—	+	+	—	—

KEY: B=pubic hair, 1XX=sample number, + = target band present, — = target band not present, F=faint product band seen.

PCR Amplification of Nuclear DNA

Preliminary nuclear DNA results using alkaline digested head hair successfully generated product with both the autosomal D17Z1 and the Y-chromosome DYZ1 multi-copy primer pairs, but demonstrated negative results with the single-copy mini-amelogenin primers. Male head hair DNA produced the multi-copy Y locus target band when the alkaline digestion method was used, but not when manual grinding had been employed. Male and female head hair DNA amplified the D17Z1 target band after alkaline digestion, but not until dilution to one-tenth or one-hundredth initial concentration, and BSA was often needed to overcome inhibition. Four of the 7 donated head hair sample DNAs that were tested for the presence of nuclear DNA (using the D17Z1 primers) successfully amplified the target band. When tested on all 22 alkaline digested head hair DNAs that had successfully generated the 220bp mtDNA amplicon, 32.8% (7 of 22) amplified the target band (Figure 8). Both multi-copy nuclear loci were tested on pubic hair samples that were concurrently digested by alkaline and manual digestion (Table 14).

Figure 8 – Head hair DNA amplification of D17Z1.



KEY: A=head hair, 1XX=sample number, TB=target band, PD=primer-dimer activity.

Table 14 – Amplification of multi-copy nuclear DNA from concurrently extracted alkaline and manually digested pubic hairs.

		Alkaline Digestion		Manual Grinding	
Sample	Sex	D17Z1	DYZ1	D17Z1	DYZ1
B118	♀	+	—	—	+
B126	♀	+	—	—	—
B143	♂	+	+	+	+
B152	♂	F	+	—	—
B173	♀	—	+	F	+

KEY: B=pubic hair, 1XX=sample number, + = target band present, — = target band not present, F=faint product band seen.

Real-Time Amplification Using SYBR-Green

Pubic and head hair DNA extracts were diluted to one-tenth initial concentration for use in real-time PCR analysis. In the first of two real-time analyses, two head hair samples extracted via alkaline digestion were compared to two pubic hair extracts—one extracted by alkaline digestion and the other by manual grinding (Table 15). MtDNA analysis of the four samples showed an average cycle threshold (C_t) within five cycles of

one another. Similar results were seen with the amelogenin target product—all amplifying within 4 cycles of one another. The chromosome 17 target amplicon was tested in duplicate and only on a single head hair and pubic hair extract, but threshold values were similar for the two samples analyzed.

In the second real-time analysis, mtDNA quantity was compared to the quantity of the nuclear amelogenin gene using 5 pubic hair samples, each extracted following alkaline digestion and manual grinding (Table 16). Threshold cycles were consistent between the two digestion methods (with the exception of the B103 mtDNA analysis whose average C_t values between the methods differed by approximately 7 cycles). However, no demonstrable trend could be determined for which hair digestion method resulted in more total mtDNA and nucDNA, as lowest C_t values toggled between the two methods.

Table 15 – Results for samples amplified using real-time PCR cycle parameter 1.

Primer Pair Utilized	Sample	Digestion Method	Average C_t	Melt Temp (°C)
F16190/R16410	A102	Alkaline	27.27	82.67
	A124	Alkaline	29.24	82.83
	B122	Alkaline	23.58	83.17
	B103	Manual	25.60	83.00
	Negative	NA	36.18~	72.67
F/R Amelogenin (int)	A102	Alkaline	36.71*	75.75
	A124	Alkaline	34.53	76.70
	B122	Alkaline	37.18*	74.00
	B103	Manual	37.93	75.36
	Negative	NA	36.96~	80.00~
FD17Z1/RD17Z1	A102	Alkaline	32.62^	79.25^
	B122	Alkaline	32.17^	79.50^
	Negative	NA	32.94^°	72.00^

KEY: F=forward primer, R=reverse primer, A=head hair sample, B=pubic hair sample, 1XX=sample number, *=2 of 3 triplicates were used to obtain an average C_t due to failure of one duplicate to amplify, ~=only 1 of 3 triplicates was used to obtain an average value due to failure of two triplicates to amplify, ^=samples run in duplicate instead of triplicate, °=only 1 of 2 duplicates was used to obtain an average C_t due to failure of one duplicate to amplify.

Table 16 – Results for samples amplified using real-time PCR cycle parameter 2.

Primer Pair Utilized	Sample	Digestion Method	Average C_t	Melt Temp (°C)
F16190/R16410	B103	Alkaline	32.08	82.50
	B103	Manual	25.06	83.17
	B117	Alkaline	27.59	82.50
	B117	Manual	26.40	83.00
	B122	Alkaline	23.62	83.00
	B122	Manual	21.85	83.50
	B123	Alkaline	23.09	83.00
	B123	Manual	24.17	83.50
	B127	Alkaline	24.04	83.00
	B127	Manual	25.31	83.00
	Negative	N/A	35.74^°	81.00^
F/R Amelogenin (int)	B103	Alkaline	37.12*	70.81
	B103	Manual	38.34~	69.08
	B117	Alkaline	37.43*	70.64
	B117	Manual	36.58	74.86
	B122	Alkaline	36.96	74.50
	B122	Manual	36.33*	72.25
	B123	Alkaline	37.70*	70.71
	B123	Manual	36.25	78.00
	B127	Alkaline	36.39	73.38
	B127	Manual	37.59	72.13
	Negative	N/A	37.89^°	72.60^

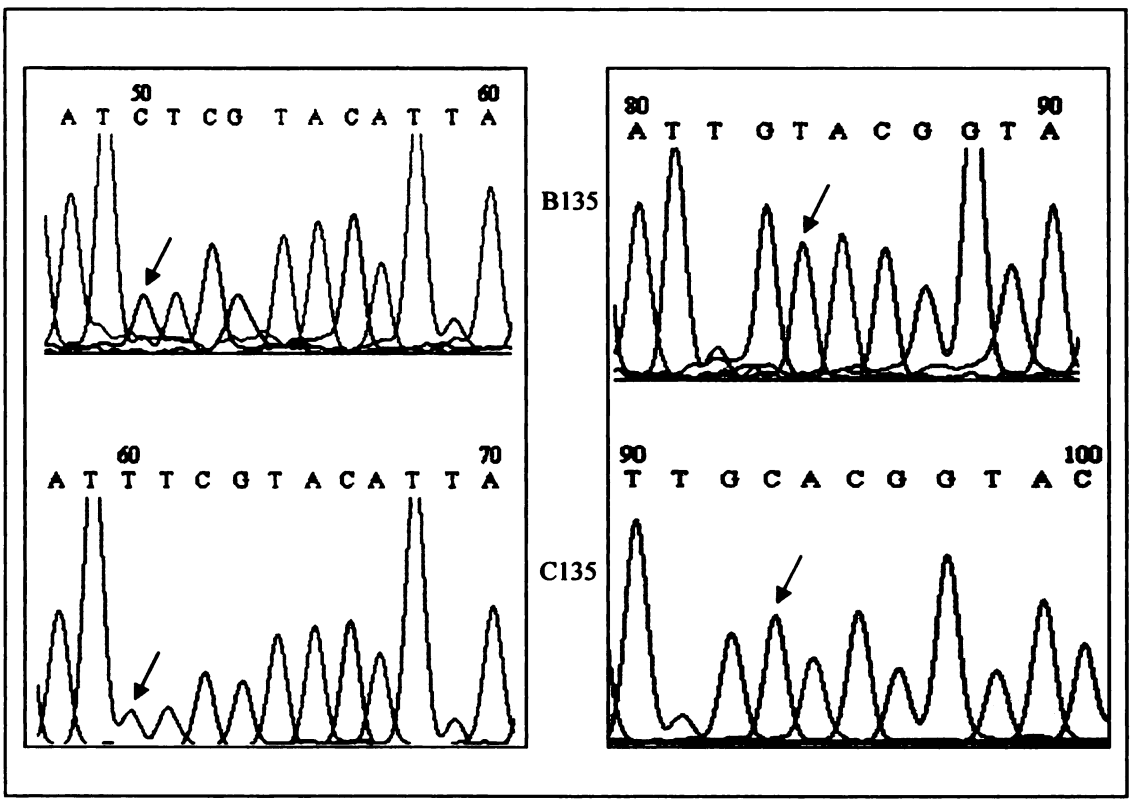
KEY: F=forward primer, R=reverse primer, A=head hair, B=pubic hair, 1XX=sample number, *=only 2 of 3 triplicates were used to obtain an average C_t due to failure of one triplicate to amplify, ~=only 1 of 3 triplicates was used to obtain an average C_t due to failure of two triplicates to amplify, ^=samples run in duplicate instead of triplicate, °=only 1 of 2 duplicates was used to obtain an average C_t due to failure of one duplicate to amplify.

DNA Sequencing Analysis of Buccal Swab and Pubic Hair Extracts

Fifty-two of the 56 donated buccal swab samples were extracted via organic extraction and 33 were sequenced, analyzing a 421bp region of HVI (F15989/R16410). Those that produced viable sequence data were used to select alkaline digested pubic hair DNA extracts to be sequenced over the same mtDNA region. Several alkaline digested pubic hair mtDNA sequences failed to generate the 421bp product, and were re-

sequenced using a 220bp segment of the mtDNA genome (F16190/R16410). Sequence comparisons were made for ten buccal swab and pubic hair DNA extracts (Table 17). One buccal swab and pubic hair extract did not match in their mtDNA sequence—in seven instances, C/T differences were seen between the two extracts (Figure 9).

Figure 9 – MtDNA sequence inconsistency seen between sample 135 pubic hair DNA extract and buccal swab extract.



KEY: Top electropherograms are extracted pubic hair DNA sample B135. Bottom electropherograms are of extracted buccal swab DNA C135. Sequence inconsistencies in two different locations are emphasized by the arrow.

Table 17 – MtDNA profiles from buccal samples and alkaline digested pubic hairs.

Sample Number	Interval Sequenced	Pubic Hair Sequence Polymorphisms	Buccal Sequence Polymorphisms
102	228-304	—	—
108	235-327	**270T**	**270T**
		290C	**290C**
		—	321W
111	79-374	**273T**	**273T**
		275T	**275T**
117	233-358	—	—
118	231-354	**235T**	**235T**
		—	240M
		—	260W
		—	289S
		—	298K
		—	304Y
		—	316M
		—	322M
		—	324W
		—	337M
		—	341Y
130*	235-370	—	—
133	43-357	—	87S
		—	95R
		103C	**103C**
		—	140W
		—	178Y
		270T	**270T**
		273T	**273T**
		275T	**275T**
		—	279W
		—	316M
		—	322M
		343M	—
135	24-305	42W	—
		—	46T
		70C	—
		83R	—
		—	100C
		—	170T
		201C	—
		268T	—
		288T	—

Table 17 (cont'd).

136	226-379	—	248K
		—	294R
		—	320R
		330T	**330T**
		—	359S
152	32-369	—	41K
		—	70K
167	228-285	**270G**	**270G**
		279 R	—
		283M	—

KEY: A=adenine, T=thymine, C=cytosine, G=guanine, M=A or C, R=A or G, W=A or T, S=C or G, Y=C or T, K=G or T; bolded entries indicate sequence similarity.

DISCUSSION

The study presented here expanded upon the applicability of an alkaline digestion technique to liberate DNA from shed head hairs in lieu of the traditional manual glass grinding technique. Graffy and Foran (2005) originally tested the applicability of the alkaline digestion technique on shed head hairs. In the present research project, the sample set was increased in size and focus was shifted to shed pubic hairs. Pubic hairs are among the critical pieces of evidence collected in sexual assault cases—combings are gathered from the victim (in a search for foreign hairs), and plucked hairs are also gathered (if tolerated) to use as references. If a suspect is in custody, pubic hair combings and pluckings may be collected as well. Pubic hairs can also be collected in association with a crime at the location where the sexual assault occurred. In some instances only a single foreign hair may be recovered from a combing or a crime scene—making an effective, reliable, and repeatable genetic analysis technique imperative, especially when microscopic comparison is impossible or inconclusive. The present and past research demonstrated that alkaline digestion of shed hairs to hydrolyze protein and release mitochondrial (and possible trace amounts of nuclear) DNA is as effective as manual grinding, takes less time to complete, and provides less opportunity for contamination.

Research Focus

The focus of the present research was to document the efficiency of an alkaline DNA extraction technique as compared to the manual grinding extraction currently used in crime laboratories to obtain DNA from human hairs. The different methods were

compared on the following criteria: sample integrity, potential for contamination, amount and cost of reagents and supplies needed, and analyst skill/technique required. The duration of the hair DNA isolation procedure was previously established to be significantly shortened when a 5N sodium hydroxide solution was employed instead of a manual maceration by glass grinding (Graffy and Foran 2005). Consequently, the effect of digestion time was not examined in the present study, and all samples were allowed to incubate overnight.

Criteria for Implementation

Forensic crime laboratories are concerned with each of the aforementioned criteria when implementing/performing an extraction technique to obtain DNA from a forensic biological specimen. The transfer of hair or solutions to multiple containers increases the likelihood of sample contamination and is, ideally, avoided if possible. Manual grinding followed by organic extraction of DNA involves several tube transfers, whereas hairs processed via alkaline digestion remain in the same tube until DNA concentration, making it a more desirable method. Several reagents (digestion buffer, ProK, DTT) and hazardous chemicals (phenol, chloroform) are necessary to perform a manual grinding and organic extraction to liberate DNA from hair, adding to laboratory chemical cost. Sodium hydroxide pellets, however, are an inexpensive and commonly stocked laboratory chemical. They can be used to make a 5N solution in minutes and, until neutralization, are the only reagent needed for alkaline DNA extraction from hair. The complexity of the manual digestion along with the increased cost of reagents makes it an undesirable protocol in comparison to the alkaline digestion. The manual digestion

hair protocol also demands a high level of analyst skill and thus requires lengthy personal training.

Despite the positive evaluation of the alkaline digestion technique on several criteria, several concerns must be discussed before an alkaline digestion technique can be employed in a working crime lab. Unexpected results that occurred in each experiment presented here need be addressed, along with possible causes, remedies and/or suggestions for additional experimentation. These concerns are explained below by detailing the unexpected findings in each experiment, and subdivided by specific anomalies. Finally, implementation of the technique in the forensic community is addressed, followed by future research suggestions and conclusions.

DNA Extraction Experiments

Alkaline Extraction Complications

Extraction of head and pubic hair DNA utilizing the alkaline digestion method was non-problematic until DNA elution. Hair DNA was concentrated on a spin column and subsequently washed with TE buffer. After the washes, several samples revealed an artifact that was isolated to the alkaline digestion method—dark residue appearing on the spin column membrane. The residue, believed to be due to melanin carryover, was seen in hair samples that were brown or black in color. This unexpected result could have arisen from the high ion concentration present. After neutralization, the digested hair samples contain sodium ions (Na+) from the 5N sodium hydroxide and chloride ions (Cl–) from the 11.6M hydrochloric acid, resulting in high levels of salt. As the samples were washed during DNA concentration, the salt concentration was reduced and pigment

appeared to precipitate, becoming visible as dark areas on the membrane. Melanin is a documented inhibitor of PCR (Yoshii et al. 1992, 1993) thus the residue was avoided when drawing DNA off the column membrane. Extracted head and pubic hair DNA from alkaline digestions that appeared colored after elution from the spin column were centrifuged to pellet the pigment before 1:10 dilutions were made for use in PCR experiments. Even when these precautionary steps were taken, PCR inhibition was seen, and 1X BSA was used to overcome the effect in later PCR reactions. Experimentation with alternative spin columns may reveal a superior method of removing excess pigment, thus eliminating the need to combat inhibition by diluting samples and adding BSA to PCR reactions. Pigment carryover was not seen on the spin columns of hairs digested with glass grinders, presumably because it is soluble in the organic phases.

Manual Grinding Complications

Grinder contamination was one problem that hindered DNA extraction from pubic hairs and research progress. In grinder controls, only a negative PCR result was acceptable, but initial reactions indicated that there was DNA contamination. To remedy this, a modified sterilization method was employed. Initially, a bleach rinse preceded a deionized water rinse of the grinders; this order was later reversed to prevent the deposit of contaminates present in the deionized (non-sterile) water. The deionized water and bleach rinses were followed by an ethanol rinse for additional decontamination. UV treatment of the grinders was the final step in the sterilization, and grinders with corresponding pestles were originally laid flat in the UV box. UV rays cannot penetrate glass, so while pestles were likely being properly treated, the inside of the grinders was

not. To ensure that the interior was being sterilized, grinders were situated upright in a plastic test tube rack and pestles were placed on end with the grinding surface exposed to UV light. After the sterilization method was improved, grinder contamination ceased. The efficient and clean extraction of DNA was proclaimed to be the most important step in mtDNA sequencing by Wilson et al. (1995b), who also employed a grinding method in their analysis of DNA from hair shafts. They, too, listed their greatest challenge as contamination and stressed the need for stringent hair washing protocols.

Head Hair DNA Extraction Success

In this study, shed head hair DNA was extracted via alkaline digestion to compare amplification success to that for extracted pubic hair DNA. In the research published by Graffy and Foran (2005), head hair digestion success was 90%. The three hair samples that failed to amplify in that research were all Mongoloid (listed as being of Asian decent), and two of the three had been subjected to hair treatments and/or were regularly blown-dry. Graffy and Foran (2005) also listed inhibitory effects and negative results for PCR amplifications with those hairs that had been dyed, chemically treated, or regularly blown-dry, however, because these were of Asian origin, it is not possible to tell the exact cause of the negative result. The same effects/trends were observed in the present research, as all but one of the head hairs that failed to amplify had been treated with dye or other chemicals within the previous year. The one exception, hair A133, belonged to a Caucasoid donor claiming the hair had never been dyed or otherwise treated. The hair was, however, dark brown to black in color, and excess melanin possibly contributed to its failure to amplify. Conversely, the sequencing research by Wilson et al. (1995b)

found dyed hairs to be have an equal or superior typing success rate than other treatments such as the use of shampoo, conditioner, and permanent applications. BSA was included in the Wilson et al. (1995b) study to overcome PCR inhibition, as it was in the present project. The result was a reduced incidence of sample inhibition; this was only attempted on the three Mongoloid hairs that failed to amplify in the Graffy and Foran (2005) study. With all samples in the present research, dilution of the DNA was an additional method used to successfully decrease the concentrations of inhibitors.

It is unclear why a lower percentage of head hair samples successfully amplified in the present research than in the 2005 study. Less head hair was used for DNA extraction (4 cm versus 6 – 7 cm), so it is possible that there was less DNA available/released from the samples. The target amplicon was slightly larger in the present study (220bp versus 203bp), which also may have contributed to this difference. Fourteen of 25 (56%) head hair samples in this study had been chemically treated or blown-dry on a regular basis. This was similar to the hairs subjected to the same conditions in 2005 (63%). Amplification of 1:10 TE diluted head hair DNA in the present study failed to generate the target band in nearly half of the samples, but none were inhibited—several sample lanes had bright "smears" of DNA, indicating over-amplification. These samples were not tested again for 10 months, and at that time no over-amplification was seen and several more produced the target product. This may point to sample degradation over time, but also that less extracted head hair DNA in a PCR reaction produces clearer results. Improved results could also arise from the absence of excess pigment due to precipitation and settling during the storage period.

Head Versus Pubic Hair Extraction Success

Pubic hair DNA extractions from alkaline and manual digestions had a higher rate of amplification success than head hair DNA. For alkaline DNA digestion, the success rate was 68% for head hair versus 98% for pubic hair. Manual digestion of head hairs was not performed, and pubic hairs digested manually had equal amplification success as those alkaline digested. The increased PCR success from pubic hairs could have resulted from various factors. Pubic hair DNA extractions were performed after the head hair extractions, and more familiarity was gained with the alkaline digestion technique, potentially leading to higher yields. Pubic hair DNA extracts were only analyzed under PCR conditions containing BSA, and inhibition was not encountered following alkaline digestion or manual grinding. Less hair was used in pubic hair DNA analyses (2 cm), but the hair itself is more protected from harsh environmental conditions than is head hair, and is most likely not subjected to sunlight, heat-based styling products, and chemical treatments commonly encountered with head hair. The success of pubic hair DNA extraction could also be attributed to its increased robustness. Melton et al. (2005) found that DNA from hairs with increasing hue and diameter is more likely to generate a full mtDNA profile. In their study of 691 casework hairs, all were placed in five categories of robustness—the lowest being thin, light, and/or brittle hairs and the highest being dark, wiry, thick hairs. MtDNA sequencing success increased through each stage of robustness, with the highest scoring hairs producing the most dependable results. This may seem counterintuitive, as increased hair diameter increases extraction difficulty (all hairs in the Melton et al. (2005) study were manually ground). However, while manual digestion of thick or wiry hairs is difficult, it is not impossible, and several were

processed in the present research. Thirty-eight of 49 (77.6%) pubic hair samples in this study were described as being brown, reddish-brown, dark brown, or black; in the Melton et al. (2005) study these hair colors were associated with increased robustness and a sequencing success rate of greater than 80%. Considering the high success in obtaining a full mtDNA profile from extracted hair DNA in the Melton et al. (2005) study, it is not surprising that 97.9% of manually digested pubic hair DNAs and 98% of alkaline digested pubic hair DNAs produced the 220bp mtDNA amplicon in the present research. While excess pigment may make amplification difficult without a component to overcome that inhibition, thicker, coarser hairs may contain more mtDNA, leading to more successful amplifications and sequencing results.

Effect of Incubation Time

The duration of time that hair samples were allowed to digest may have influenced hair DNA extraction success. Graffy and Foran (2005) developed the alkaline extraction technique as a rapid and cost effective alternative to manual grinding in hair DNA extraction. Thus, hairs were only immersed in sodium hydroxide until dissolved, and the average incubation time was approximately 5 hrs (Graffy and Foran 2005). Hairs were allowed to incubate overnight in the present study, and incubation times commonly reached 15 – 24 hrs. While exposure to alkaline conditions is not thought to damage DNA in general, prolonged exposure may have contributed to the lower success rate in head hair amplification in this study. DNA is unwound and denatured into single strands when subjected to an alkaline solution, even one of low molarity (Storer and Conolly 1984). Additionally, mitochondrial DNA is known to have ribonucleotides incorporated

around the origin of replication as well as various other locations throughout the mtDNA genome (Brennicke and Clayton 1981). Storer and Conolly (1984) located sites in mouse mitochondrial DNA exterior to the origin of replication that contained segments of RNA that were alkali-labile and prone to breakage. RNA in the mitochondrial genome is thought to be the remnants of priming activity that is subsequently not completely removed following replication (Brennicke and Clayton 1981). Given that the mitochondrial genome is highly conserved, it is probable that human mtDNA also contains ribonucleotides and, as such, is subject to strand breakage under alkaline conditions. The site damage might increase with prolonged exposure to a highly basic environment, such as the overnight incubation used with all samples in this study. This type of damage could diminish the success in amplifying DNA extracted from hair, and account for the failure of larger amplicons to amplify.

DNA Quality Experiments

Quality assessment of extracted pubic hair DNA was performed by amplification of increasingly larger mtDNA amplicons. As tabulated in the results section, none of the alkaline digested pubic hair DNAs amplified beyond 421bp. Although this trend was not seen in the Graffy and Foran (2005) research, there are several potential reasons for the variation in DNA quality between the two methods in the present study. The prolonged digestion time was a possible factor, as previously mentioned. Additionally, the alkaline digested pubic hair DNA was processed approximately two months prior to the manually digested pubic hairs. Thus, it is feasible that DNA instability during freezer storage was a hindrance to PCR success.

To further examine this, five randomly selected pubic hair samples were re-processed concurrently using both techniques to assess whether storage and stability were, in fact, leading to increased DNA degradation. Results were similar to those of the initial quality assessment when amplifying 220bp and 421bp segments of mtDNA—all of the alkaline digested pubic hair DNA produced a 220bp amplicon, and all but one generated a 421bp product. One sample also generated both the 664bp and 865bp target amplicon, although it had not in the previous experiment. DNA amplification from manually ground pubic hairs had similar success with small amplicons (all produced a 220bp product and 80% produced a 421bp product). However, none of the manually digested pubic hair DNAs generated a product of 664bp or 865bp.

One would anticipate 3 of the 5 manually digested hair samples amplifying beyond 421bp (B118, B152, and B173), as they had amplified in the previous quality experiment (Table 12). Comparison experiments showed that the second round of extractions was poorer for manually ground pubic hairs, and only slightly more successful for those alkaline digested. The failure of the majority of samples prepared by both methods to amplify beyond 421bp possibly resulted from the shortened time period between extraction and amplification. Observations of prior DNA extractions indicated that pigment precipitated out of solution during storage for hairs that were manually ground. This may have aided the amplification of these samples, since melanin is a noted inhibitor of PCR (Giambernardi et al. 1998). Hair samples prepared immediately after DNA elution for the stability experiment may have still contained high concentrations of melanin, which in turn could complicate amplification. This further emphasizes the need for an alternative filtration device to remove excess pigment. If melanin removal proves

difficult or impossible, samples could be tested at defined time intervals post extraction to assess inhibitory effects.

Impact of Storage Conditions

Duration of storage and storage temperature (–20°C) appeared to impact the stability of extracted hair DNA as well as its amplification ability in this study. Freezing is a common method of DNA storage in the forensic community, but improved storage methods for various specimens have been a topic of interest in recent years. A trial conducted by Smith and Morin (2005) compared optimal storage conditions for dilute DNA samples in an attempt to discern how low copy or highly degraded forensic samples should best be preserved. Human and gorilla DNAs were stored over a period of 12 months at temperatures ranging from room temperature to –80°C. DNA quality was assessed using real-time PCR and large fragment (757bp) PCR analysis. Samples were stored in both TE and trehelose; those at –80°C retained the greatest amount of initial DNA (even more so when stored in trehelose), while those stored at refrigerator and freezer temperatures (4°C and –20°C, respectively) in either solution experienced significant DNA degradation (P=0.0001).

In the present study, some alkaline digested head hair DNA samples initially generated mitochondrial and/or nuclear DNA product, but then failed to do so upon subsequent testing. The DNA may have experienced degradation over time at the –20°C storage temperature, as well as additional decay in the form of DNA nicks from repeated freeze/thaw cycles. DNA samples were often used in multiple experiments, and as such were taken in and out of the freezer several times. Experiments involving hair DNA and

deliberate freeze/thaw cycles could measure a decrease (if any) in DNA quality. Testing of potential DNA breakdown as hair samples warm to room temperature may allow for quantifying DNA quality loss each thaw cycle.

Nuclear DNA Experiments

Nuclear DNA analysis of extracted head and pubic hair DNA consisted of PCR experiments designed to amplify short products ranging from 147 – 154bp. Two multi-copy genes (D17Z1 and DYZ1) were tested to determine if nuclear DNA might be present in hair shafts, albeit most likely in a highly degraded state and at undetectable levels using standard PCR methods. Marginal success was seen when locus D17Z1 was tested on alkaline digested head hairs, but success decreased after the DNA samples were subjected to 10 months of freezer storage.

To examine whether head and pubic hair DNA samples had similar nuclear DNA amplification success, PCR of D17Z1 was repeated using the concurrently extracted pubic hair DNA samples. Four of the 5 alkaline digested pubic hair samples successfully generated the target, again indicating pubic hair DNA may contain greater quantities of DNA than head hair. Only 2 of 5 manually ground pubic hair DNAs produced the target, suggesting a potential advantage for alkaline digestion when examining nuclear DNA. The existence of this benefit could be explored with further experimentation on a large group of alkaline and manually digested head and pubic hairs extracted using both methods. An alkaline digestion method may be preferred for multi-copy nuclear DNA testing as the reaction remains in a single tube, and thus any DNA is reserved. A

prolonged storage period, however, may hamper amplification regardless of the extraction method used.

Experiments with the other (male sex-specific) multi-copy locus, DYZ1, were only successful with a single male head hair DNA and were not tested further until experimentation with the concurrently processed pubic hair DNA. Results were rather surprising—two of three female DNA samples amplified, and only one of two male DNA samples amplified. De la Torre et al. (2000) reported that the DYZ1 Y-chromosome sequence has partial homology to other portions of the genome that is present in both sexes. They sequenced a female PCR product 154bp in size found to have 89% identity with the DYZ1 locus. The band appearing in female samples is likely specific to an autosomal region of the genome, and could possibly be remedied by optimizing the primer pair, redesigning the primer pair, or using a TaqMan® assay for greater specificity. Sizing the male and female DNA products with the use of a genetic analyzer may be helpful in determining the exact sizes of the DNA present. While this primer pair requires modification to be a reliable sex determiner, it is nonetheless useful for detecting the presence of nuclear DNA, and could have applications in highly fragmented samples.

Real-Time PCR Experiments

Real-time PCR analysis, though limited in this study, provided some interesting information on the variety of DNA products obtainable from digested head and pubic hairs. MtDNA was found to be abundant in both hair types. C_t values, however, varied among the types of hair and the digestion method employed. In one real-time PCR experiment, an alkaline digested pubic hair (B122) had lower C_t values than the manually

digested pubic hair sample, however it contained an additional 0.5 cm of hair compared to the manually digested sample (B103), thus the exact reason for increased DNA is unknown. Also, the hairs were from different individuals and therefore not directly comparable. Both alkaline digested head hair samples (A102 and A124) had larger C_t values than either pubic hair sample, even though two times the length of hair was used in the alkaline head hair digestion (4 cm versus 2 cm), contradicting the idea that more hair would yield more DNA. Again, this suggests that pubic hairs contain more DNA than head hairs, possibly resulting from the increased thickness and hue of the pubic hairs relative to head hairs. Additional real-time experimentation with a larger group of head and pubic hair samples would better investigate this claim.

A ~65bp version of the single copy nuclear gene amelogenin was tested in real-time PCR experiments with alkaline and manually digested head and pubic hair DNA. C_t values were similar across all samples regardless of the extraction method utilized or target DNA. Amelogenin C_t values were also similar to that of the single amelogenin negative control that generated a threshold value (the other two amelogenin controls did not amplify), indicating possible contamination of a single control triplicate. This finding suggests a small amount of nuclear DNA existed in the hair samples, potentially indistinguishable from trace contamination of a negative control. Background contamination from a tainted master mix was assessed, but results from other negative controls that did not amplify suggests against it.

Real-time PCR experimentation using multi-copy locus D17Z1 was only performed on two hair samples. C_t values were again similar to that of a single negative control that amplified. While contamination was initially suspected, inspection of the

melt curve showed a peak at a lower temperature in the negative control than the hair DNA samples. A melt temperature below that of the hair samples, as seen in the amelogenin analysis, suggests that the C_t was potentially reached from primer-dimer activities than contaminant DNA. Again, repeat testing with positive controls would better elaborate on the findings of this experiment.

The second real-time PCR experiment compared hairs from the same individual. However, single hairs were not split between the two methods (as described in Materials and Methods), as they were prepared before the design of the pubic hair extraction method comparison study. Equal amounts of hair were analyzed (2 cm segments, except for alkaline digested B122, which utilized 2.5 cm of hair) to provide more accurate information as to which extraction method yielded more DNA. Lowest C_t values (indicating greater starting DNA concentration) toggled between the two methods (Tables 15, 16) for both mitochondrial and nuclear DNA. Thus, real-time PCR analysis of alkaline and manually digested pubic hairs could not establish if one method led to more total DNA. However, the real time machine was available for only a limited time, thus a restricted numbers of runs were undertaken. Real-time analysis in the present study was preliminary and requires an increased number of samples for comparison, as well as defined positive and negative controls, before definitive conclusions regarding a superior method of obtaining DNA from hair can be made.

Sequencing Experiments

Sequencing of extracted buccal swab and pubic hair DNA was largely non-problematic. Samples that failed to generate the sequence of the 421bp targeted region

were re-amplified to generate a 220bp product. Sequence comparisons between buccal and pubic hair DNA, 11 in total, all displayed deviation from the researcher's mtDNA sequence. In one instance (sample 135), differences were not only seen from the reference, researcher's, and other donated hair mtDNA sequences, but also between the buccal and pubic hair DNA extracts, characterized by six distinct polymorphisms (Table 17). In each discrepancy, a C/T transition was observed. Explanations for the mismatch in mtDNA sequence are operator error (sample mix-up on the part of the researcher), multiple donors in a single sample kit, or exogenous DNA not belonging to the researcher contaminating one of the samples. Unfortunately, unless the researcher is present at sample collection, hair donations from more than one individual cannot be prevented. Given the clean results obtained from both the buccal swabs and hair, it is suspected that multiple donors were represented in a single sample kit.

Implications for Practice

For a new extraction technique to be implemented in a forensic crime laboratory, not only must the method be proven superior in terms of sample integrity, cost, and ease of execution, but also in repeatability and scientific soundness. The results of this study taken together with those of Graffy and Foran (2005) have established that an alkaline digestion technique is at least as efficient at extracting mtDNA or nuclear DNA from shed hairs as a manual grinding extraction. The technique is also less costly, is time-effective, and involves fewer steps to execute.

Forensic crime laboratories next need to be informed that such a technique is available. This could be accomplished by attending scientific conferences and educating

its attendees on the benefits of the procedure. Publishing research findings in the literature would spread awareness in the forensic community and potentially spur additional large-scale experimentation to support the findings of the present research. Publication of a large-scale validation study performed in a research laboratory may encourage others to use the technique in casework.

The final step in implementing a process such as the alkaline digestion method in a forensic crime lab would be a validation study. Each laboratory is held to strict repeatability standards and must have standard operating procedures for any and all techniques used in the lab. Validation of a new technique is a requirement for criminal investigation DNA facilities, and often consists of the method being performed on numerous occasions by multiple analysts over an extended period of time. Given the investment required to approve the use of a new technique, it is expected that many crime laboratories will be hesitant to change their current practice, if one exists. Numerous laboratories do not currently perform mtDNA testing, therefore notice of a simplified extraction technique may be an incentive to implement this type of analysis. Once the technique has been validated, it would be admissible in a courtroom setting. Subsequently, other labs will have an easier time bringing the procedure in-house and implementing the practice.

Future Research

Further characterization of the genetic content of human hair would be beneficial to the forensic community and to those studying the applicability of mitochondrial DNA analysis in human identification. Increased sample size, greater representation of ethnic

groups, more detailed information about the donated hair, and additional real-time PCR analysis could expand upon the data generated in this study. Sample collection could be increased by soliciting hair from a salon, spa, or barbershop. Much could be learned if the scalp and root end of the hair were noted, as it would be useful for determining DNA concentration as the hair grows away from the scalp. More detailed questionnaires would allow for more concrete determinations to be made regarding amplification success/failure. Information regarding specific hair dye brands and their chemical makeup, for example, may reveal correlations between chemical treatments and DNA damage or other reasons that some dyed hair DNAs were amplifiable while others were not. Illegal drugs and other chemicals ingested into the system are deposited into hair on the body, so assessing personal drug use, vitamin intake, and/or dietary habits may provide some insight into the variable obtainability of DNA from hair.

Conclusions

To conclude, the findings in this research will be weighed in relation to the need for an alternate DNA extraction technique for shed hairs in the forensic community, the ease with which such a technique could be implemented, and the cost and analyst skill required versus the traditional extraction method. Hair is, and will continue to be, a common form of trace evidence recovered from crime scenes. Head and pubic hairs have the potential to reveal a wealth of genetic information, ranging from full STR profiles for those with attached roots to mtDNA sequences for shed hairs. The availability of a simple, effective, reliable DNA extraction technique for shed hairs is critical in the success of these genetic endeavors. An alkaline digestion method allows the extraction to

be performed in the lab in a cost-effective manner, as opposed to contracting services with an independent agency, which are widely used now. Long processing hours are not necessary, nor are expensive chemicals. If an extraction technique is exceedingly difficult to execute without contamination, cost prohibitive, and/or requires considerable man-hours and processing time, crime laboratories will simply look to other evidence or be forced to send the hairs to more well-equipped laboratories with personnel trained solely in hair extraction (e.g., FBI's Mitochondrial DNA Laboratory Unit, Mitotyping Technologies®).

The ability of forensic crime laboratories to implement such a technique is the most limiting variable of those detailed above. The time and commitment required for a new process to be validated can seem unattractive to a facility desiring a hair extraction method. This is unfortunate, as it prevents crime laboratories from using the most current and efficient scientific methods available when processing biological specimens. However, most are not currently invested in a hair DNA extraction protocol, so initiation of one might seem less overwhelming if another organization had done a validation study for their personal use. This would not omit a laboratory from performing a validation of their own, but would serve rather to show that the technique was successfully implemented.

Finally, the results from the present research as well as from Graffy and Foran (2005) suggest that alkaline digestion is as effective, if not more so, as digestion of shed hairs by manual grinding. While further testing using an increased sample size would be beneficial, the procedure should be recommended to the forensic community as a superior method for obtaining DNA from shed hairs, conditional perhaps, that samples

will not be stored for extended periods of time. This work also opens the door for further study on the utility of multi-copy DNA loci in analysis of highly degraded DNA samples. As technology continues to improve and increasingly smaller amplicons can be analyzed by new methods, the likelihood of gaining individualizing genetic information from shed hairs and similar forensic samples only grows more promising.

APPENDICIES

APPENDIX A

Forms Included in Sample Packet for Donors:

Consent Form, Donation Instructions, Questionnaire

Consent Form for participation in the study entitled:

"DNA isolation from hair"

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 method of getting nuclear DNA out of human hair shafts.

You will be asked to donate samples of shed or trimmed head hairs and shed or trimmed pubic hairs. The head hairs can be collected from a comb or brush, by trimming, or by running your fingers through your hair. The pubic hairs can be trimmed from as close to the skin as is comfortable for you. 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 in any part of the process (i.e. donation of any 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 the hairs, 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 any 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—Peter Vasilenko, Ph.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: 204B 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. Pubic hairs:
   Collect 4-6 pubic hairs that are at least one centimeter in length. This can be done by cutting the hairs close to the skin. It is not necessary to pull any hairs from the root. Place the hairs carefully into the small manila envelope and seal the envelope.

3. 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.

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

5. Labeling samples:
   Inside your packet is a set of small orange stickers marked with identification numbers. Place one sticker on the sealed white envelope containing your head hair, one on the sealed manila envelope containing your pubic 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 white envelope, small manila 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:

"DNA isolation from hair"

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 sex? 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

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