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HUMAN HAIR SHAFT VOLUME AND MITOCHONDRIAL DNA RECOVERY

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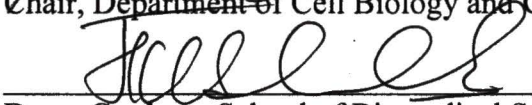

Dean, Graduate School of Biomedical Sciences

HUMAN HAIR SHAFT VOLUME AND MITOCHONDRIAL DNA RECOVERY

INTERNSHIP PRACTICUM REPORT

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By

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CHAPTER I

INTRODUCTION

Hairs are one of the most common types of biological evidence encountered in criminal investigations. Hairs can add valuable probative information to a case by demonstrating physical contact between a suspect and a crime scene or victim. Previously, the only method of forensic hair analysis was the physical comparison of hairs by microscopic examination, which involves looking at distinguishing features of hair, such as color, size, pigment density, and other morphological characteristics. The advances in PCR technology have made DNA analysis the method of choice when it comes to biological evidence. The majority of current DNA profiling methods focus on chromosomal DNA found in the cell nucleus. Short tandem repeat (STR) analysis has been in use since the mid-1990's[1, 2] and focuses on length differences at 13 different locations in the nuclear genome. This method has high discriminatory power and has become indispensable in forensic DNA analysis, but it is not always successful. Samples that are highly degraded, or samples that are known to contain low amounts of nuclear DNA, such bones and teeth, will not always work with autosomal DNA testing. Telogen, or shed, hairs also contain low amounts of nuclear DNA and are suited for a different type of DNA testing.

Mitochondria are energy producing organelles found in the cytoplasm of cells and contain a circular genome that is distinct from nuclear DNA. The number of

mitochondrial DNA molecules within a cell is highly variable and typically ranges from 200-1000 copies.[3] This characteristic gives mitochondrial DNA analysis a higher sensitivity when compared to STRs. The mitochondrial genome is also maternally inherited, with siblings and maternal relatives all sharing the same mtDNA haplotype. Due to the lack of recombination, even maternal relatives several generations apart will most likely share the same mtDNA sequence, greatly expanding the number of family reference samples that can be used in forensic investigations.[4] Higher copy number and maternal inheritance are the primary features of mitochondrial DNA analysis that demonstrate an advantage over nuclear DNA for low copy number samples.

The human mitochondrial genome was first sequenced in 1981 by Anderson et al[5], and has been designated the Cambridge Reference Sequence. The mitochondrial genome consists of approximately 16,569 base pairs coding for 37 genes. Twenty-two of these genes code for transfer RNAs, two encode ribosomal RNAs, and 13 encode gene products involved in oxidative phosphorylation.[5] The control region consists of approximately 1,122 base pairs that do not code for any gene products. Forensic mitochondrial DNA analysis focuses on this non-coding control region, or D loop. This area of the genome contains two hypervariable regions, designated HV1 and HV2. The low fidelity and lack of proofreading ability of the DNA polymerase during mitochondrial DNA replication give these regions a high substitution rate of five to ten times that of single copy genes.[6-8] A high substitution rate leads to polymorphisms among individuals of different maternal lineages.

Due to its unique characteristics, mitochondrial DNA analysis has a wide variety of applications, including several outside the field of forensics. Human evolution and migration have been extensively studied using mitochondrial DNA techniques,[9-18] as well as determining that Neanderthals are not directly related to modern humans.[19] Multiple population studies on different world populations have also been done, producing valuable population data.[20-23]

Mitochondrial DNA analysis has also been used to identify human remains, including those of a 7000-year-old brain,[24] and can be a valuable tool in missing persons investigations and mass disasters. Several well-known historical identifications have also been made. Russian Tsar Nicholas II and several members of his family and household were identified after the discovery of human remains of nine individuals along a road in Ekaterinburg, Russia, in 1991.[25] Autosomal STR analysis established familial relationships among five of the skeletons, but was unable to confirm that the remains were descendants of the Russian royal family. Mitochondrial DNA was used to compare the remains to a known maternal relative of the Romanov family, HRH Prince Philip, Duke of Edinburgh. His blood sample confirmed that the remains were of the Tsarina and her children. The mtDNA from the putative Tsar was compared to his brother, Grand Duke Georgij Romanov, and found to be a match as well.[26]

Mitochondrial DNA analysis is also used to identify casualties of past U.S. military conflicts. One of the goals of the Armed Forces DNA Identification Laboratory (AFDIL) in Rockville, Maryland, is to identify unknown human remains of United States soldiers. One of the first military identifications using mitochondrial DNA analysis was

done in 1993 after nuclear DNA analysis did not yield any results.[27] The Vietnamese government had returned the putative remains of an American Marine Corps aviator to the United States almost 24 years after the Vietnam War. The family requested mtDNA analysis by AFDIL and donated reference samples, which shared an identical haplotype with the aviator. AFDIL has also identified the remains one of the soldiers at the Tomb of the Unknown Soldier in Washington, DC in 1998. Due to other evidence found with the remains, it was believed that the Vietnam Unknown was Michael Blassie, a Lieutenant in the United States Air Force. Samples obtained from family members matched that of the remains.

Mitochondrial DNA analysis has also been compared to microscopic examination of hairs to see if there is a difference in accuracy between the two methods.[28] Out of 80 microscopic examinations, nine were excluded by mtDNA analysis methods, producing an error rate of 11% for the microscopy method. While this number may seem high, forensic hair examination has a high exculpatory rate, which is an important aspect of mtDNA analysis. Samples that are counted as inclusions can be subjected to mtDNA analysis. The authors suggest that while mtDNA analysis is more accurate, a combination of these two methods is an ideal approach for forensic hair analysis.

Hairs are slender, thread-like outgrowths from a follicle embedded in the skin of mammals. Hairs consist of three main morphological regions: the cortex, the cuticle, and the medulla. The dermal papilla, found at the bottom of the hair follicle embedded in the skin, is the site of new cell production for all of these regions. As the newly formed cells are forced upward by mitotic pressure, they begin to differentiate into the cells of the hair

shaft. While still in the root bulb, cortical cells begin the keratinization process by rapidly producing proteins in the cytoplasm. Cortical cells make up the bulk of the hair shaft and contain high numbers of mitochondria. As these cells continue to migrate upwards, keratin fibrils fill the cytoplasm until it is completely full of fibril bundles. When the cell is completely keratinized, cytolysis occurs. The nucleus and organelles completely disintegrate, resulting in the loss of nuclear DNA. Some mitochondria are also lost, but due to their higher numbers and the protective mitochondrial membrane, many are still found in the keratinized root shaft.[29]

Cortical cells also contain the pigment granules that determine the color of the hair shaft. Melanocytes are the pigment producing cells found in the root bulb. Melanocytes produce melanosomes, or pigment granules, that are engulfed by the pre-cortical cells before they are forced up into the root shaft. Melanosomes synthesize two types of melanin: brown/black eumelanin and red/blonde phaeomelanin. These pigments produce the wide variety of hair colors seen on different individuals.

Melanin is also a known inhibitor of the polymerase chain reaction, which is a component of both nuclear and mitochondrial DNA analysis. Melanin reversibly binds to thermostable DNA polymerase and inhibits its activity.[30] There are other known inhibitors of PCR, including heme, plant materials, indigo dyes, and humic acid, but melanin is the only natural PCR inhibitor found in hair.[31] Melanin is derived from amino acids, and is a complex polymer composed of dihydroxyphenylalanine (DOPA) and/or cysteinyl-DOPA.[30] Addition of bovine serum albumin (BSA) to the PCR reaction mix is the current method used to try and reduce the inhibitory effects of melanin.

Cuticle cells, which make up the outer covering of the hair shaft, have a different cellular morphology than cortical cells. Cuticle cells produce copious amounts of keratin in a manner similar to cortical cells. However, cuticle cells become elongated and flat before migrating laterally in the hair shaft, and emerge as overlapping scales on the surface of the hair shaft.[29]

Medullary cells are the third cell type produced by the dermal papilla. These cells are centrally located in the hair shaft. These cells do produce structural proteins, but in much lower amounts when compared to cortical and cuticle cells. Some of the medullary cells completely disintegrate before they even reach the mature hair shaft, leaving a central core of air in the center of the hair. This empty core appears as a discontinuous blackened structure called the medulla upon microscopic examination. Other medullary cells continue to produce keratin fibrils and remain in the center of the hair shaft and no medulla will be present in the hair shaft.

Mitochondrial DNA analysis was first validated for use in forensic casework in the mid-1990's[32-34] establishing guidelines that laboratories using mtDNA analysis could follow. Specific guidelines have also been developed for the mitochondrial DNA analysis of hairs, which will be discussed later.

Mitochondrial DNA analysis is very labor intensive and time consuming when compared to autosomal DNA analysis. Autosomal analysis consists of quantification and amplification of extracts using commercial multiplex kits and subsequent capillary electrophoresis for sizing of the PCR fragments. For mitochondrial DNA analysis, HV1 and HV2 are amplified using the polymerase chain reaction from total genomic DNA

preparations using primers specific for each of these two regions. The amplified product is then purified and quantified. The quantified product is subjected to cycle sequencing using dye terminator chemistry, purified again using gel filtration columns, and the sequence is visualized using a fluorescent sequencing instrument. The forward and reverse sequences are aligned and compared to the revised Cambridge Reference Sequence[35] using a computer software package to determine any sequence differences. Results from evidentiary and reference materials are then compared. The mitochondrial genome is treated as a single locus, or haplotype, due to its uniparental inheritance. Point estimates are calculated using established mitochondrial DNA databases to generate statistical weight.

Special attention is paid to quality control and quality assurance when doing mitochondrial DNA analysis due to the sensitivity of the technique. Contamination must be tightly controlled and monitored. Specific areas of the laboratory should be designated for use only for mitochondrial DNA analysis, and disposable personal protective equipment and laboratory supplies should be used if at all possible. The pre- and post-amplification areas should be physically separated and work surfaces should be cleaned thoroughly before and after use.[34]

Mitochondrial DNA was first isolated from hair in 1988.[36] Since then, special guidelines have been developed for the mtDNA analysis from hair shafts due to its unique composition, which consists mostly of dead, keratinized cells. During the extraction process, extra steps are required to break down the hair shaft and release the DNA due to the high keratin content.[37] Keratin is an extremely strong protein composed

of amino acids that contain high numbers of cysteine side chains. These cysteine residues contain sulphur molecules that are able to form strong disulfide bonds across the keratin molecules, giving hair a very resilient, insoluble structure. Guidelines for mtDNA hair analysis[33] recommend using a DNA extraction method that involves the physical crushing of the hair by homogenization using a 0.5 ml micro-tissue grinder. These grinders require an extensive cleaning and sterilization process, involving overnight soaks of 100% ethanol, washing with hot detergent, a 4N H_2SO_4 soak, and microwaving prior to use. Once homogenized, the hair is then transferred to another tube for organic extraction. Amplification, post-amplification processing, and cycle sequencing then proceed according to the validated methods for mitochondrial DNA analysis employed in the laboratory. The entire process takes approximately 24-26 hours.

A recently described method for hair extraction uses an alkaline digestion to break up the keratinized material in the hair shaft.[38] Incubating the hairs at an alkaline pH has a strong denaturing effect on the keratin fibril bundles and will disrupt the cellular and nuclear membranes and dissolve the DNA.[39] The DNA is then concentrated and washed using Microcon-30 microconcentrators. The entire alkaline extraction process takes approximately six to seven hours from start to finish and has a higher amplification success rate when compared to the glass-grinding method.[38]

Before the hair can even be extracted, it is necessary to determine how much of the sample should be consumed. Autosomal DNA testing requires that the quantity of human DNA must be determined when using PCR based analysis techniques. In mitochondrial DNA analysis, a "less direct method for estimating the amount of

recovered DNA, such as control of sample size is an acceptable approach."[40] A specific amount of a sample must be used for mitochondrial DNA analysis. The previously mentioned guidelines for the analysis of hairs recommend using a two centimeter length of hair for extraction.[33, 34, 37]

Using a two centimeter length of hair has had varying degrees of success. Several studies have found that mitochondrial DNA recovery from hair shafts depends on a variety of factors. Pfeiffer et al[41] explored the correlation of mtDNA amplification success rate to length, volume, diameter, color, sex, and body location (head, pubic, axillary) on a sample of 150 hairs. The authors found that head hairs had the highest average success rate (75%), followed by pubic (66%) and axillary (52%), and suggested this might be due to the higher growth rate of head hairs and the resulting increase in mitochondrial activity in this body area. The authors found no correlation between the amplification success rate and sex, length, diameter, and volume. It should be noted that the focus of this study was on the success rates and sequence comparisons between hairs from different body areas, rather than hair morphology.

Melton et al[42] recently performed a five year retrospective study of mtDNA analysis on 691 casework hairs. Using the accumulated data, the authors evaluated a number of parameters potentially associated with the frequency of obtaining a full, partial, or no profile. These authors note that divisibility of hair evidence can often become a contested issue, and examined how frequently an entire hair is consumed in testing. Out of the 691 hairs, 262 (37.9%) hairs were consumed in analysis. The authors also explored whether the recommended length of two centimeters[37] was arbitrarily

chosen, or whether another size fragment should be used. When comparing hairs 1.0-1.9 cm to 2.0-2.9 cm in length, the authors found no difference in the ability to obtain a profile. They did note that hairs under 1.0 cm were able to obtain full and partial profiles (86.0%) and are often forensically probative, as they could have been broken off under a victim's fingernails or shed from a suspect or victim with extremely short hair.

Roberts and Calloway[43] recently reported on the relationship between hair morphology, age of donor, demographics, cosmetic treatments and amplification success rate. The authors examined 2,554 head hairs, where 95.3% were greater than two centimeters in length and 4.7% were less than two centimeters. The study followed the recommendations for hair length to use for extraction and preferentially chose the hairs that were greater than two centimeters for analysis. A qualitative approach was used to evaluate the amplification success rate of the different parameters. The amplified products were run on an agarose gel stained with ethidium bromide and the presence or absence of mtDNA product was noted. Their results indicated that there was no relationship between diameter, volume, and region of scalp and success rate. They did find that shorter hairs had a significantly lower amplification success rate when compared to hairs greater than two centimeters, as well as between racial groups. Among Caucasians, African-Americans, Hispanics, and Asians, African-Americans had a significantly lower amplification rate. The authors suggested that the higher density of melanin in African-American samples inhibited the PCR process, resulting in the lower success rates. For hair color, hairs containing red pigments had a lower amplification

success rate compared to blonde, brown, and black hairs (40-50% vs 75-80%). No explanation was given for the lower success rate of red hairs.

While the previously mentioned studies give insight into using the recommended length of two centimeters for extraction, as well as other aspects of mtDNA hair analysis, their findings were based on amplification success rate and not the amount of amplified DNA. No attempt was made in any of the studies to correlate the amount of PCR yield to a particular morphological feature, or to determine if a length below two centimeters was sufficient for extraction. Using the recommended length of two centimeters length of hair does not take into account the three-dimensional nature of hair, i.e. the volume of the hair fragment. Not considering volume as a factor when taking samples for extraction may lead to varying results in mitochondrial DNA recovery. There are differences in hair size and volume based on race and body area. Head hair among Mongoloids tends to be more cylindrical, Negroid hair is flat, and Caucasians fall somewhere in the middle (See Figures 1-3).[44]

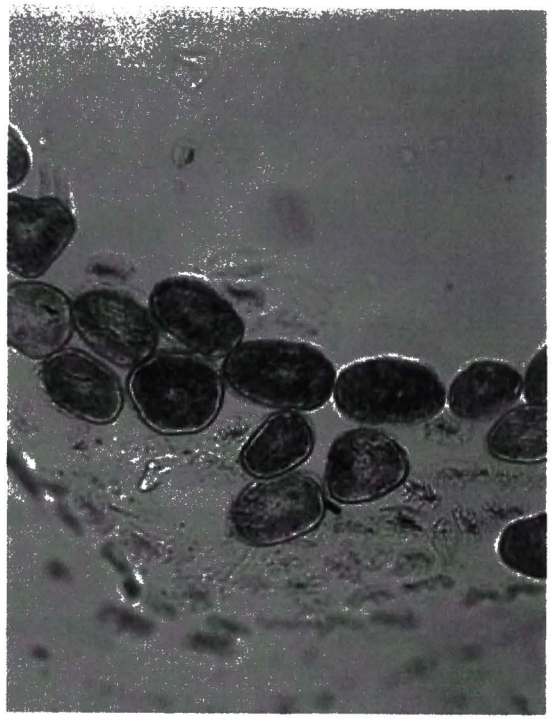

FIG. 1—*Photomicrograph of cross section of Caucasian head hair.*[44] *Caucasian hairs tend to have an oval cross-section.*

FIG. 2—*Photomicrograph of cross section of Negroid head hair.*[44] *Negroid hairs have a very flat cross-section when compared to Caucasoid and Mongoloid hairs.*

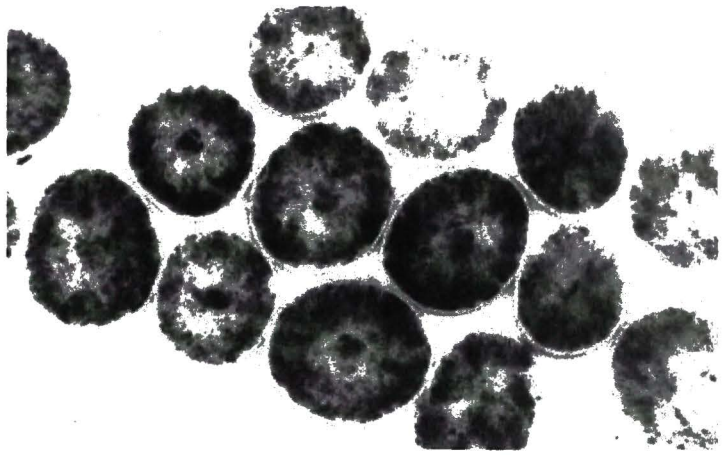

FIG. 3—*Photomicrograph of cross section of Mongoloid head hair.*[44] *Mongoloid hairs tend to be very course with a circular cross-section.*

Given that hairs are common evidentiary items in legal proceedings, the divisibility of hair evidence is an important issue. Federal Standard 7.2 for DNA analysis mandates that a portion of the evidence sample/extract be retained or returned whenever possible.[40] It is therefore ideal to optimize the probability of obtaining a haplotype while preserving as much sample as possible for re-testing.

The goal of this study is to determine if there is a relationship between hair volume and mitochondrial DNA recovery in hair shafts, as well as investigate if there are any differences among DNA recovery within the three major ethnic groups and among different hair colors. These findings may enable forensic scientists to reliably estimate the amount of recovered DNA prior to the extraction process, and therefore prevent unnecessary consumption of valuable forensic evidence.

CHAPTER II

MATERIALS & METHODS

Samples

The study was carried out at the Illinois State Police Research and Development Laboratory in Springfield, Illinois. Head hair samples were obtained from eleven individuals and beard hair samples were obtained from one individual. Pubic hairs were not included in the study due to the difficulty in obtaining an accurate volume for these types of hairs, as they tend to 'buckle' and have a varying diameter along the length of the hair shaft. Donors included employees of the Illinois State Police Forensic Sciences Command and three former Illinois State Police interns. The three major ethnic groups included in this study were Mongoloid, Caucasoid, and Negroid. Hairs were put into one of five color groups based on microscopic analysis. The five main groups were: blonde, brown, black, red, and white. All hairs of a specific color were put in the same group, regardless of the 'lightness' or 'darkness' of the hair, i.e., light, medium, and dark red hairs were all put into the red hair group. Demographic information and hair morphology for each donor can be found in Table 1. The hairs were cut approximately five millimeters from the body surface, so no root material was present. Ten to twenty hairs from each donor were examined microscopically on an Olympus BX50 light microscope. The diameter of each hair shaft was measured using a calibrated micrometer. For head hairs, measurements of the proximal end, mid-shaft, and the distal end were taken to

obtain an average diameter for the entire length of the hair shaft. Proximal and distal measurements were taken for the beard hairs. Hairs from each donor were grouped according to an average diameter that fell between a designated low, middle, and high range. The designated ranges were as follows: 65-79 μm, 80-94 μm, 95-110 μm. Certain exceptions to this procedure are detailed in the following paragraph. A batch consisted of two to four hairs from each donor, with the exact number depending on the starting length of the hairs, i.e. more hairs were needed from male donors, as their hair was shorter in length. An average diameter for the entire batch was calculated and used for the volume calculation. Each batch of hair consisted of lengths of 4, 2, 1, 0.5, and 0.25 centimeters, with the total volumes calculated for each length. Caucasoid and Mongoloid hair was assumed to be cylindrical, and the volume was calculated using $V = \pi r^2 \ell$, where r is half the average diameter and 1 mm is the length (ℓ) of each hair, obtaining an average volume per 1 mm of hair. Due to its specific flat shape, assuming Negroid hair to be a cylinder would be a misrepresentation of the true volume of the hair. A ratio between the average diameter and width of the hair was measured and found to be approximately one-half. The volume for Negroid hair was calculated using the formula for a rectangle, $V = w\ h\ \ell$, where h was the average diameter, w was half the average diameter and 1 mm is the length of (ℓ) each hair, obtaining an average volume per 1mm of hair. Donor 7 had a very high overall average diameter, so her batches did not fit the pre-determined designated ranges. The low, middle, and high ranges for this donor were 100-114 μm, 115-129 μm, 130-145 μm.

The white hairs obtained from Donor 1 were only one centimeter in length and found to have a high amount of intra-individual variability in hair diameter. A batch for this group of hairs consisted of eight hairs spanning a range of diameters. For the beard hair donor, the beard hairs averaged about three centimeters in length. Samples for this donor consisted of five hairs spanning a range of diameters cut into 1 cm and 2 cm lengths. This was done for both the white and the brown hairs.

Table 1—*Demographic information and hair morphology of each donor (Sex: M = Male, F = Female; Ethnicity: C = Caucasoid, M = Mongoloid, N = Negroid)*

Donor	Sex	Ethnicity	Color	Average Diameter Range (μm)
1	M	C	Brown/White	66.7-103.3
2	F	C	Dark Blonde	73.3-109.2
3	F	M	Black	71.7-120.8
4	F	C	Blonde	65.8-105.8
5	F	C	Dark Brown	45.8-118.33
6	M	M	Black	61.25-115
7*	F	N	Black	94.2-138.3
8	F	N	Black	74.2-112.5
9	M	C	Light Red/White	49.2-120.8
10	F	C	Medium Red	64.2-112.5
11	F	C	Dark Red	69.2-118.33
12**	M	C	Brown/White	91.6-163.8

*Donor 7 was later dropped from the volume study. See Results section.
**Donor 12 donated beard hairs only.

Extraction

Hair samples were extracted according to the protocol suggested by Graffy and Foran.[38] Hairs were cut into one centimeter fragments and placed into sterile 1.5 ml microcentrifuge tubes. Hairs were cleaned by successive 1 ml rinses of 5% Terg-A-Zyme (Alconox, Inc., White Plains, NY), 95% ethanol, and sterile tissue culture water (Sigma Aldrich, St. Louis, MO) in a sterile 1.5 microcentrifuge tube, shaking each for five minutes. Hairs were digested by adding 500 µl of freshly prepared 5N NaOH (Fisher Scientific, Pittsburgh, PA) and incubating at room temperature until the hairs are no longer visible. Four hundred microliters of a 1:1 neutralizing solution of concentrated HCl (Fisher Scientific) and 2M Tris base (pH 10; Fisher Scientific) were added to stop digestion. The extracted DNA was recovered and concentrated using a Microcon-30 column, utilizing three 300 µl washes of TE^{-4} buffer, and eluting in a final volume of 25 µl TE^{-4}.[38] A reagent blank was processed with each batch of samples.

Protein filtration

Hair samples that were known to be inhibited or demonstrated low levels of amplification product were used to evaluate the usefulness of protein filtration prior to amplification using Micropure EZ protein filters (Millipore, Billerica, MA). Hair extracts for the selected samples were added to the membrane side of the protein filter and spun in a microcentrifuge until dryness, followed by a 10 µl wash with sterile tissue culture water.

Mitochondrial DNA amplification, Purification, and Electrophoresis

PCR amplification of HV1 was carried out using 10 µl of hair extraction product in a 25 µl reaction containing 0.5 µl of each dNTP (10mM), 0.5 µl of each primer (30mM), 1.0 µl of AmpliTaq Gold © DNA polymerase (5U/µl), 2.5 µl of GeneAmp © 10x PCR buffer (all from Applied Biosystems Foster City, CA), and 2.5 µl of BSA (1.6 µg/µl, Sigma Aldrich). The reaction was brought to volume with sterile tissue culture water purchased from Sigma Aldrich. Positive (9948 control DNA, Promega, Madison, WI) and negative PCR controls, as well as the reagent blank from the extraction process, were amplified with each set of reactions. Thermalcycling conditions were 1 cycle of 95° C for 9 minutes, 36 cycles of 95° C for 10 seconds, 60° C for 30 seconds, 72° C for 30 seconds, 1 cycle of 15° C for 10 minutes. The forward (A1) and reverse primers (B1) for HV1 are:

A1: (L 15997) 5'-CACCATTAGCACCCAAAGCT-3'
B1: (H 16237) 5'-GGCTTTGGAGTTGCAGTTGAT-3'

Both HV1 and HV2 are used in forensic mtDNA analysis, but for the purposes of this study, only HV1 was amplified in order to conserve reagents. The amplified mtDNA product was purified by adding 5 µl of ExoSAP-IT© (USB, Cleveland, OH) to each 25 µl PCR reaction tube at the following reaction conditions: 15 minutes at 37° C, 15 minutes at 80° C. Four microliters of the purified PCR product was added to 1.5 µl of gel loading buffer consisting of glycerol and bromophenol blue dye and analyzed by agarose gel electrophoresis using a 2.5% Nu-Sieve (Cambrex, Rockland, ME) GTG yield gel (2.5 g NuSieve GTG agarose in 100 mL of 1x TBE (prepared in-house) gel running buffer). The gel was electrophoresed at 85 volts for approximately 40 minutes and stained with 1 µl of

5mg/ml of ethidium bromide (Sigma Aldrich) per 10 ml of gel solution. The amount of PCR product per microliter was determined by visually comparing the PCR product to a DNA low mass ladder (Invitrogen, Carlsbad, CA, Product Number: 10068-013) using a transilluminator. The total mass for the sample (in ng) was divided by four microliters, in order to determine the amount of ng/μl for each sample. The mass ladder had lengths of 100, 200, 400, 800, 1200, and 2000 base pairs, and corresponding masses of 10, 20, 40, 80, 120, and 200 ng. The amount of PCR product present was used to determine if there is a relationship between extracted hair volume (rather than length) and the amount of PCR product.

Statistical Analysis

Regression analysis was performed on all 152 head hair samples to determine if there is a relationship between the hair volume and the amount of mtDNA amplification product. Regression analysis was also performed for the three major ethnic groups, as well as for each hair color and donor. Success rates were also calculated for each category and group. Samples were considered successful if they showed detectable mtDNA amplification product on the yield gel (See Figure 4).

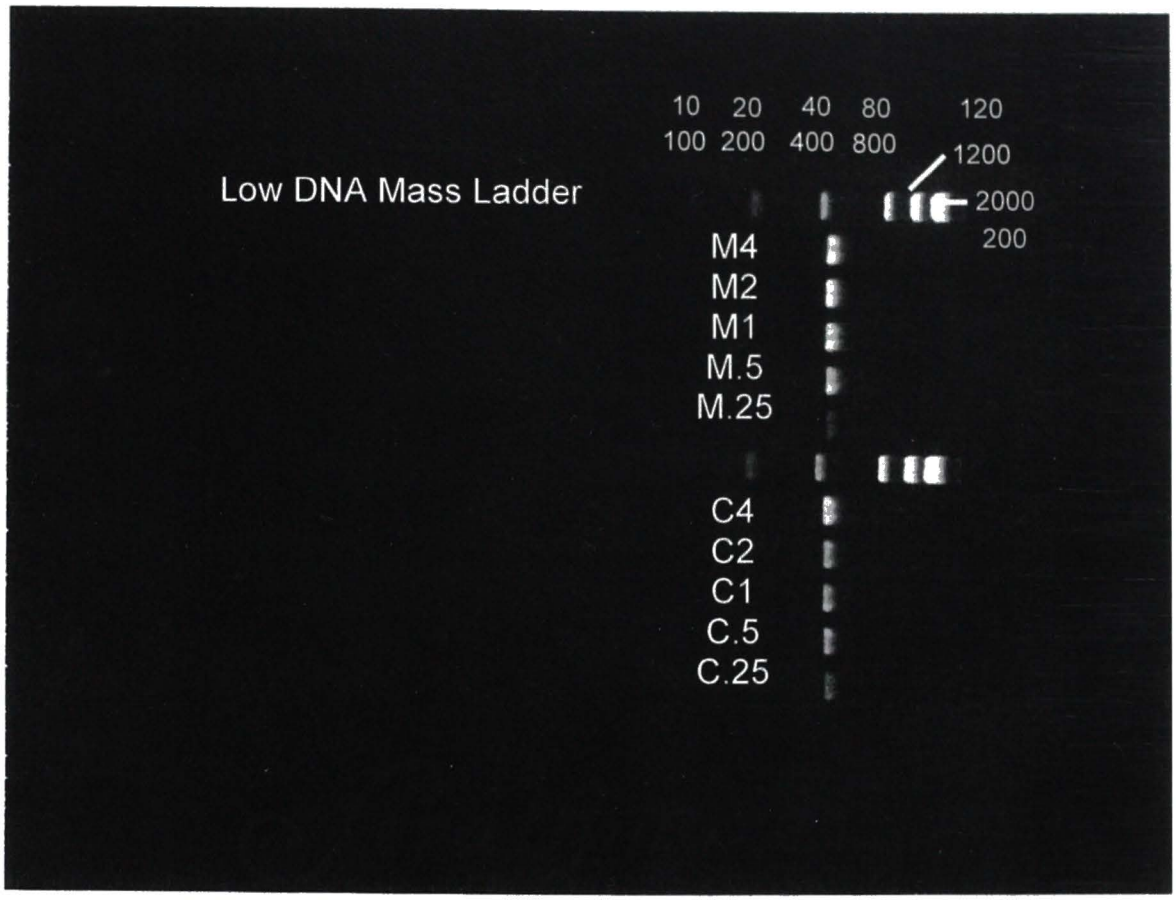

FIG. 4—*Successful samples for two batches of head hair from Donor 2. The 'M' and 'C' designations indicate sample numbers. Numbers above the low DNA Mass Ladder indicate the amount and length of DNA present. The amount of mtDNA amplification product was determined by visually comparing each sample to the ladder and dividing the mass (in ng) of the sample by four μl to get a concentration of ng/ul.*

CHAPTER III

RESULTS

In total, 33 batches of hair were extracted for a total of 172 samples. Hair samples took between three and 10 hours to digest using the alkaline digestion method. Donor 7's ten hour digestion was almost double the five hours reported by Graffy and Foran.[38] As a result, digested samples were neutralized and kept at room temperature for an additional 12 hours in the neutralized solution, at a pH of approximately 9-10. No amplification product was detected, and it is believed to be due to the extended incubation at an alkaline pH. This resulted in the removal of Donor 7 from the volume study. Beard hairs from Donor 12 were also problematic to extract. Upon addition of the neutralization solution, all samples became extremely acidic (pH 1.0-2.0). Addition of 2M Tris solution was added as per instructions, and extraction proceeded according to the recommended protocol. Samples remained at an acidic pH, and gave inconsistent results. Due to these extraction issues, beard hairs were analyzed separately from the head hairs.

Volume Study

For the head hairs, 101 out of 152 samples amplified successfully, for an overall success rate of 66.4%. Success rates were also calculated by ethnic group, hair color, and donor. Among the three major ethnic groups, the Mongoloid samples had the highest success rate of 75.9%, while the Negroid samples had the lowest, at 0%. It should be

noted that the 14 Negroid samples consisted of only one individual. For hair color, brown hairs had the highest success rate, at 100%, followed by white and blonde. Red and black hairs both had the lowest success rates of 51.2%.

Regression analysis was performed on all 152 head hair samples, indicating the lack of relationship between hair volume and amplified mtDNA product ($R^2 = 0.08$; See Figure 5). The low R^2 value is likely due to the low amplification success rate of the Negroid samples, as well as several low volume samples having high amounts of mtDNA product. The relationship between hair volume and amount of DNA was also further explored by performing regression analysis for each ethnic group, hair color, and donor.

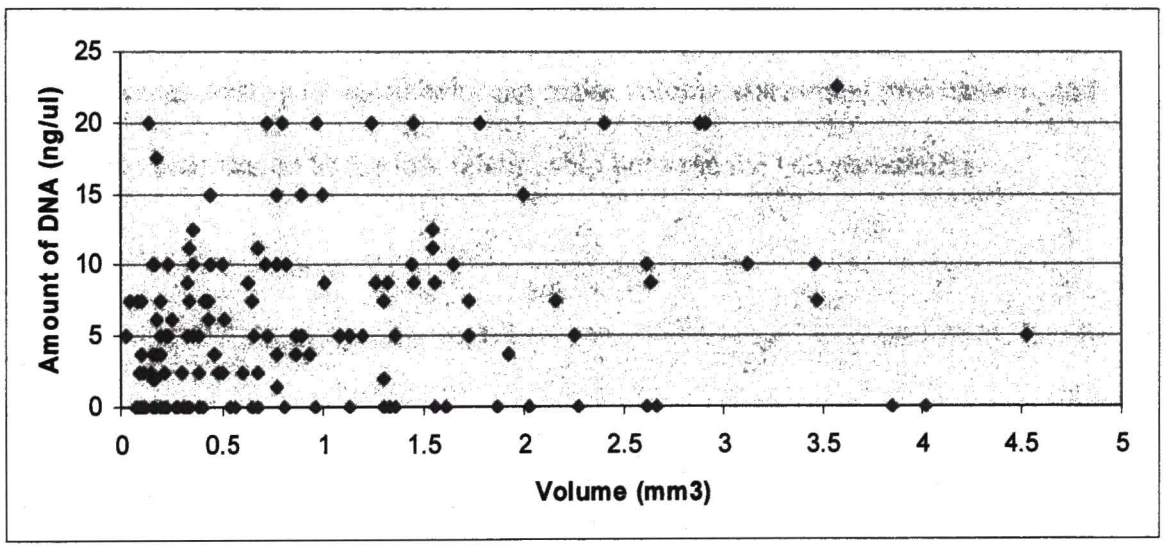

FIG. 5—*All 152 head hair samples ($R^2 = 0.08$). Unsuccessful samples contributed to the low correlation coefficient between the two variables.*

Table 2 summarizes the amplification success rates and R^2 values for each of these categories. The R^2 values for the ethnic groups are also low, indicating the lack of a

relationship between hair volume and amount of DNA in this category. The R^2 values increase for the blonde and white hairs, and remain low for the other three hair colors. (See Figures 6-12). R^2 values were also calculated for each donor, indicating varying amounts of strength in the relationship of hair volume and amount of DNA present. There was a wide range of strengths for the relationship for the hair donors. (See Figures 13-16)

Thirteen of the 20 beard hair samples had amplified product, for a success rate of 65%. All of the brown beard hairs amplified, and only three of the 10 white hairs amplified. Between both the head hairs and the beard hairs, the beard hairs had a higher average amount of mtDNA product. Regression analysis indicated a lack of relationship between hair volume and mtDNA amplification product ($R^2 = 0.07$; see Figure 17). The irregular cross-section of beard hairs can make volume assessment problematic, and could have contributed to the low relationship between the two parameters.

Table 2—*Amplification success rates and R^2 values for each ethnic group, hair color, and donor.*

Category	Group	Successful Samples	Total Samples	% Success	R2
Ethnicity					
	Mongoloid	22	29	75.9	0.04
	Caucasoid	79	108	73.1	0.11
	Negroid	0	14	0	**
Hair Color					
	Blonde	21	30	70	0.57
	Red	21	41	51.2	0.04
	Brown	30	30	100	0.16
	Black	22	43	51.2	0.06
	White	7	8	87.5	0.49
Donor					
	1	22	23	95.6	0.26
	2	14	15	93.3	0.66
	3	14	14	100	0.49
	4	8	15	53.3	0.48
	5	14	15	93.3	0.04
	6	8	15	53.3	0.01
	8	0	14	0	**
	9	5	11	45.5	0.009
	10	5	15	33.3	0.26
	11	12	15	80	0.05
Overall		101	152	66.4	0.08

***Regression analysis not performed.*

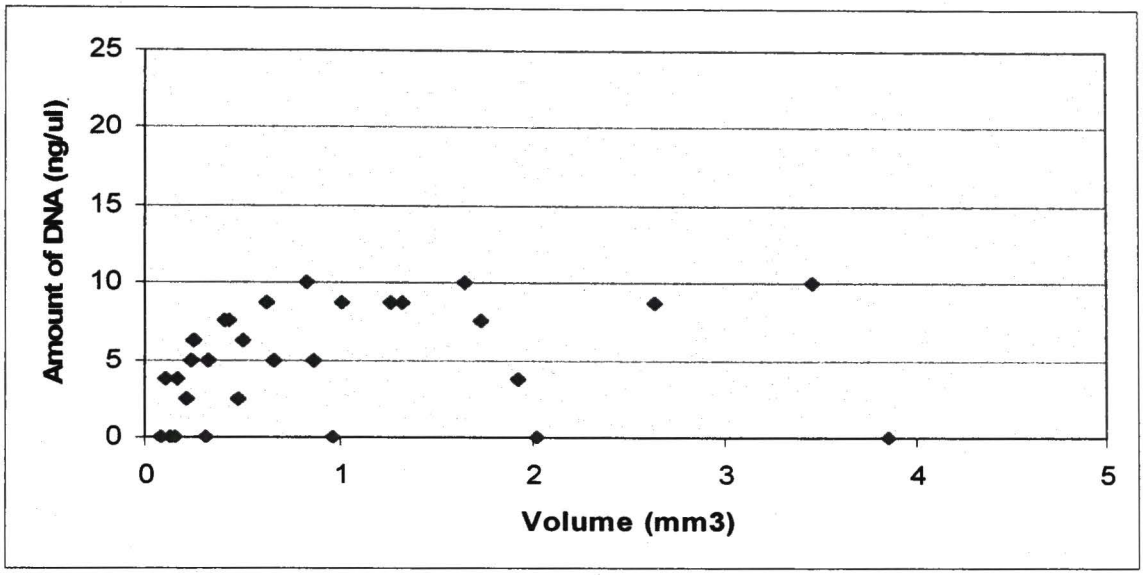

FIG. 6—*Relationship between hair volume and amount of mtDNA product for all 29 Mongoloid head hair samples (R^2 = 0.04). The unsuccessful high volume samples contributed to the low correlation between these two parameters.*

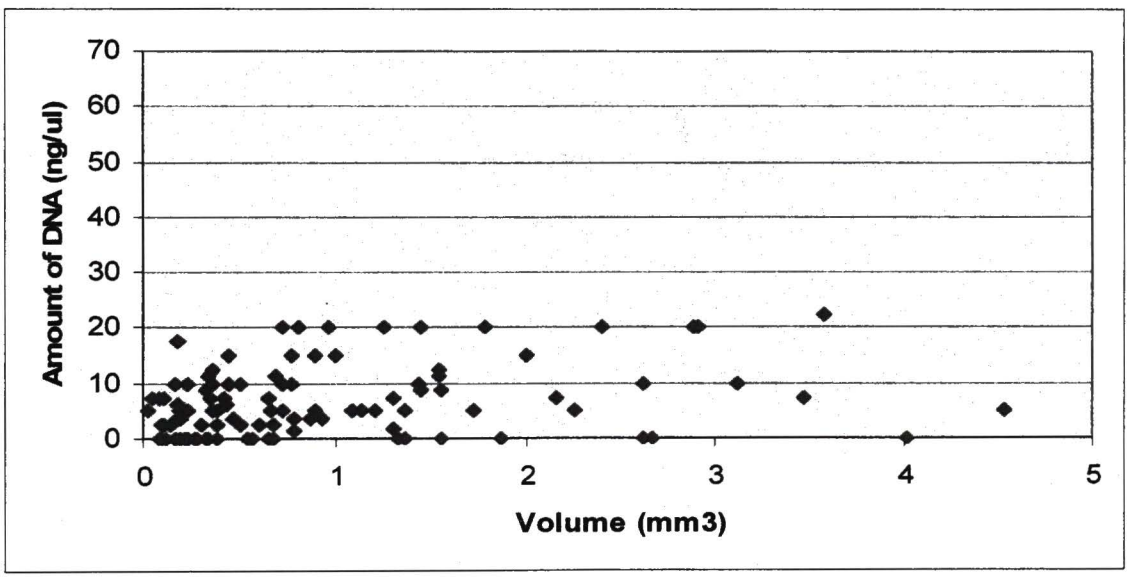

FIG. 7—*Relationship between hair volume and amount of mtDNA product for all 108 Caucasoid head hair sample (R^2 = 0.11). The higher volume samples tended to 'plateau' instead of producing higher amounts of mtDNA product.*

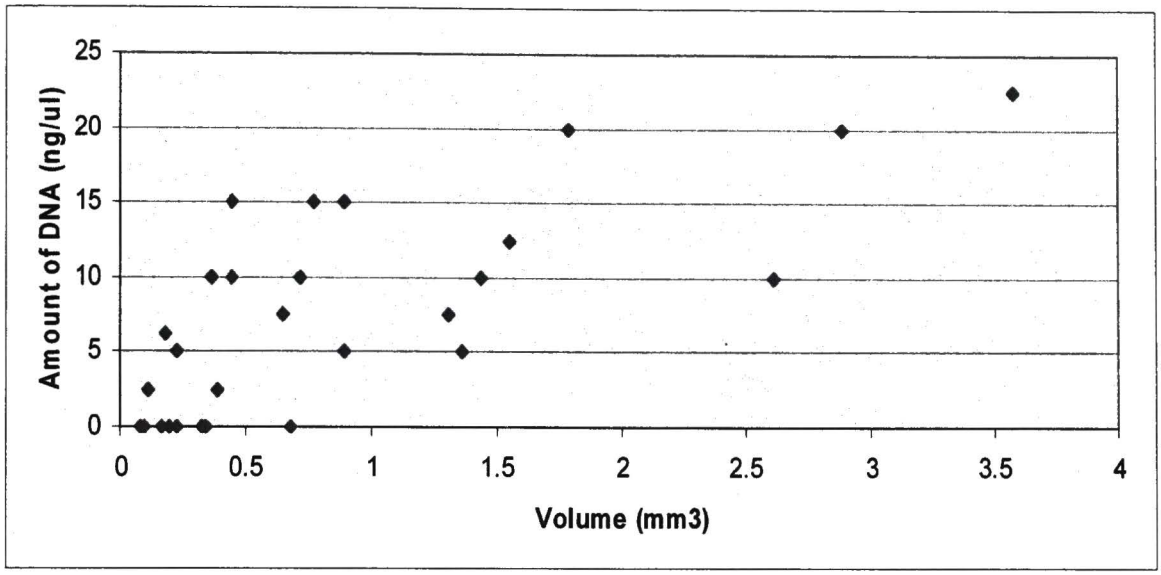

FIG. 8—*Relationship between hair volume and amount of mtDNA product for all blonde head hair samples (R^2 = 0.56). Out of all the hair colors, blonde hairs had the strongest relationship between hair volume and amount of mtDNA product.*

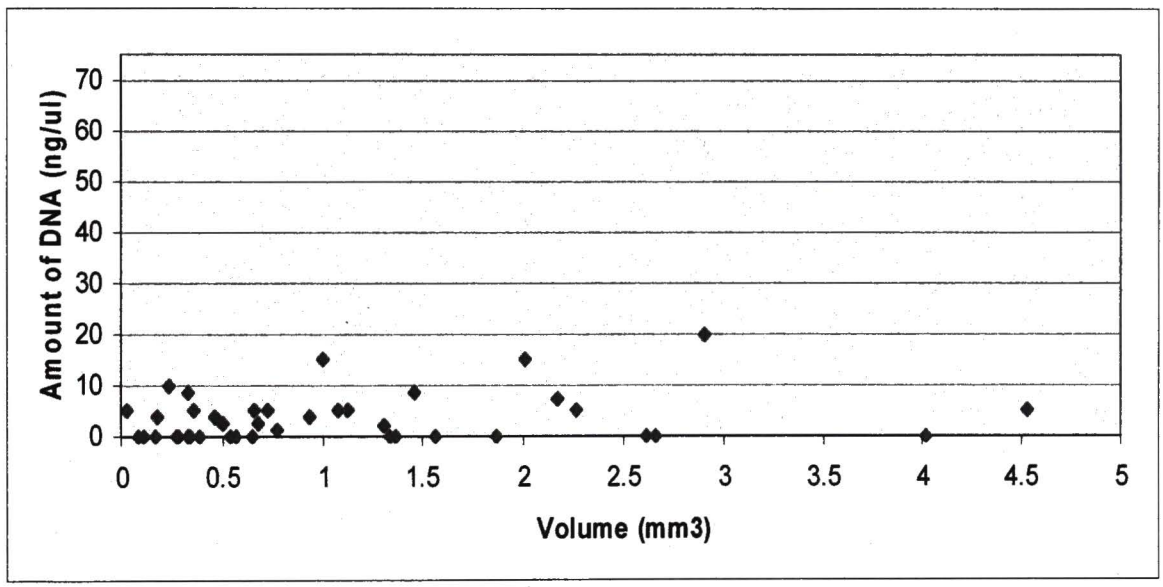

FIG 9—*Relationship between hair volume and amount of DNA for all red head hairs (R^2 = 0. 04) Several of the longer red hair samples were unsuccessful and produced no detectable mtDNA product.*

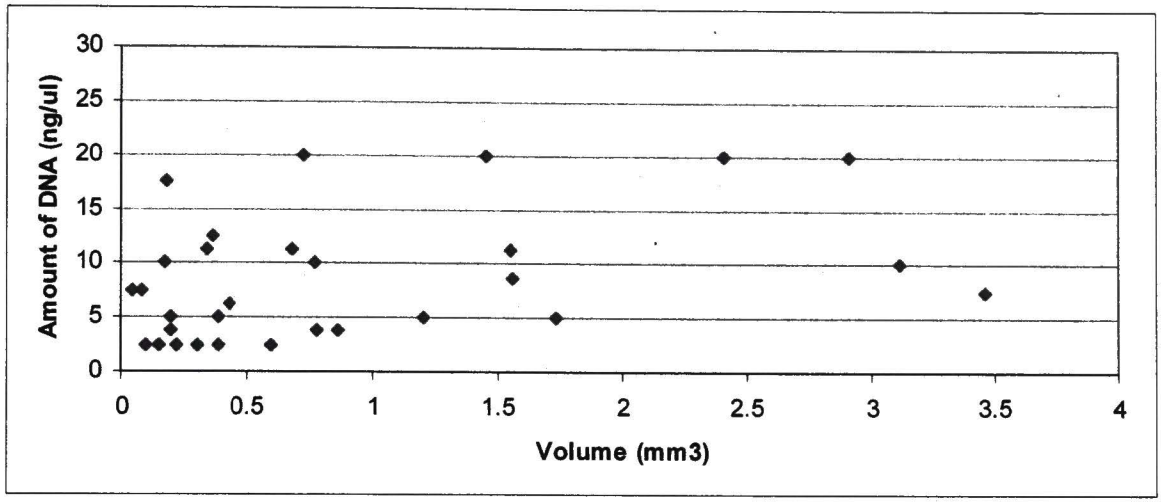

FIG. 10—*Relationship between hair volume and amount of mtDNA product for all brown hairs (R^2= 0.15).*

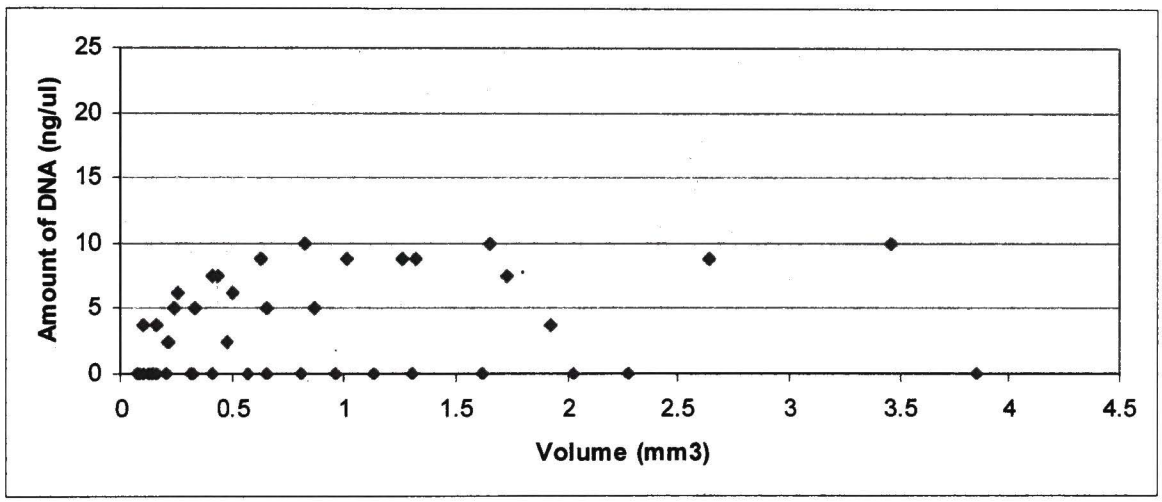

FIG. 11—*Relationship between hair volume and amount of DNA for all black head hairs (R^2 = 0.05). The unsuccessful samples from the Negroid donor lowered the correlation coefficient for this group.*

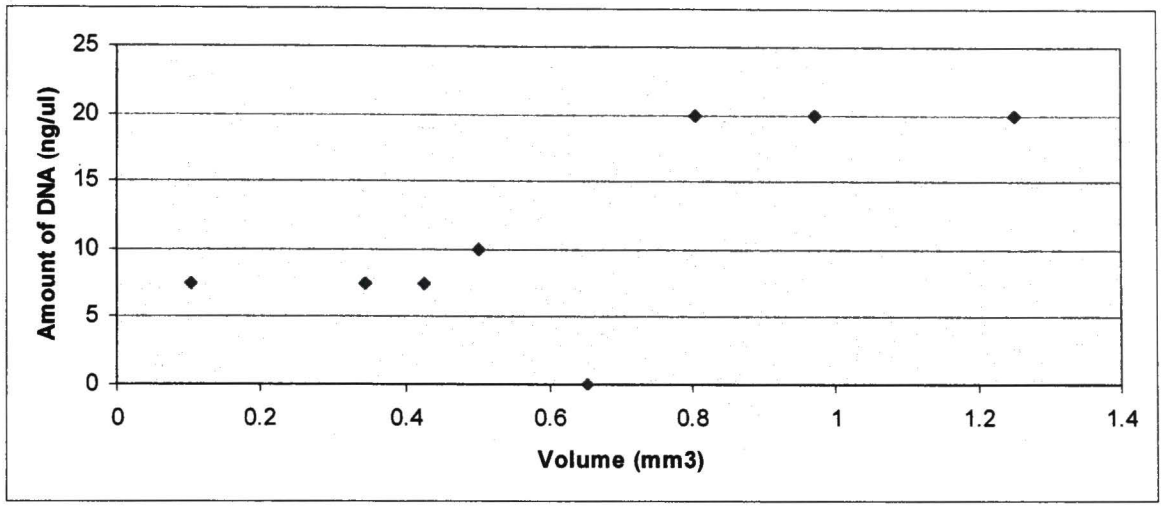

FIG. 12—*Relationship between hair volume and amount of DNA for white head hairs (R^2 = 0.49). It is likely the lone unsuccessful sample contributed to the lower than expected correlation coefficient.*

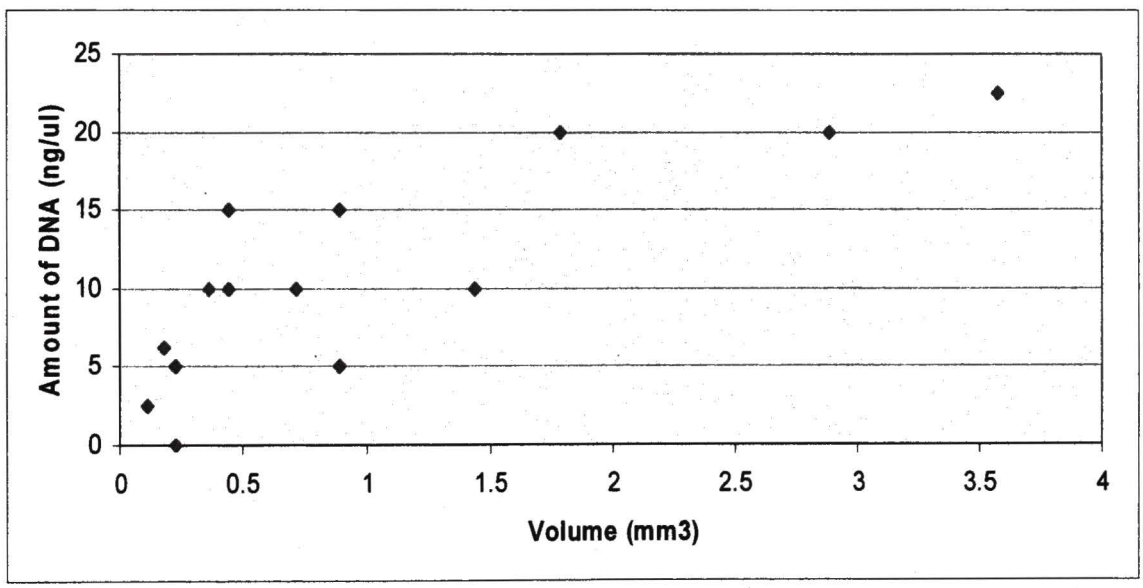

FIG. 13—*Relationship between hair volume and amount of mtDNA for Donor 2 (R^2 value = 0.66.) Donor 2 had the strongest relationship between these two parameters for all donors.*

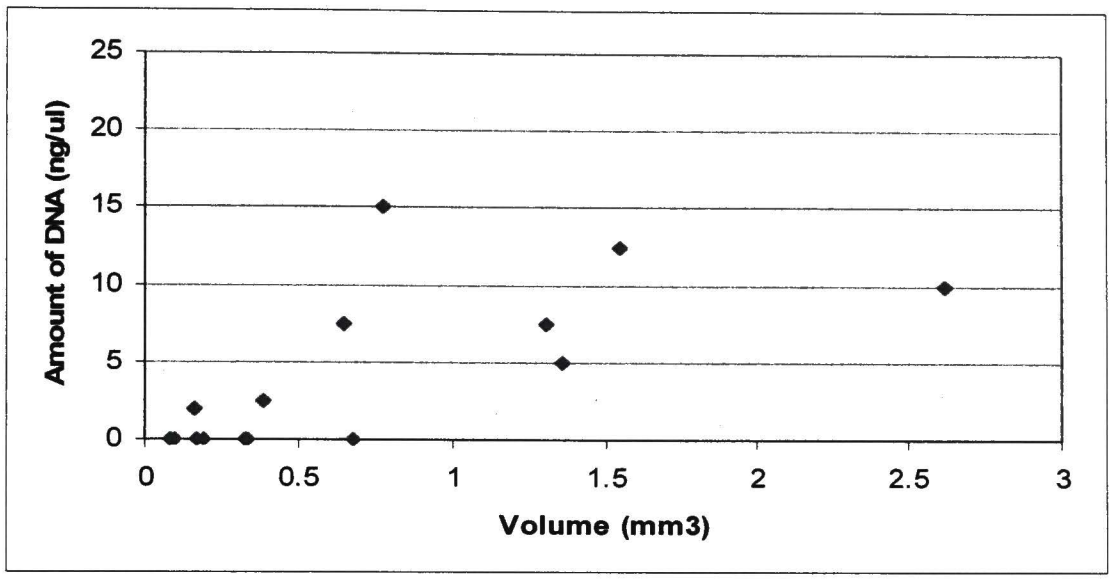

FIG. 14—*Relationship between hair volume and mtDNA amplification product for Donor 4 (R^2 = 0.48).*

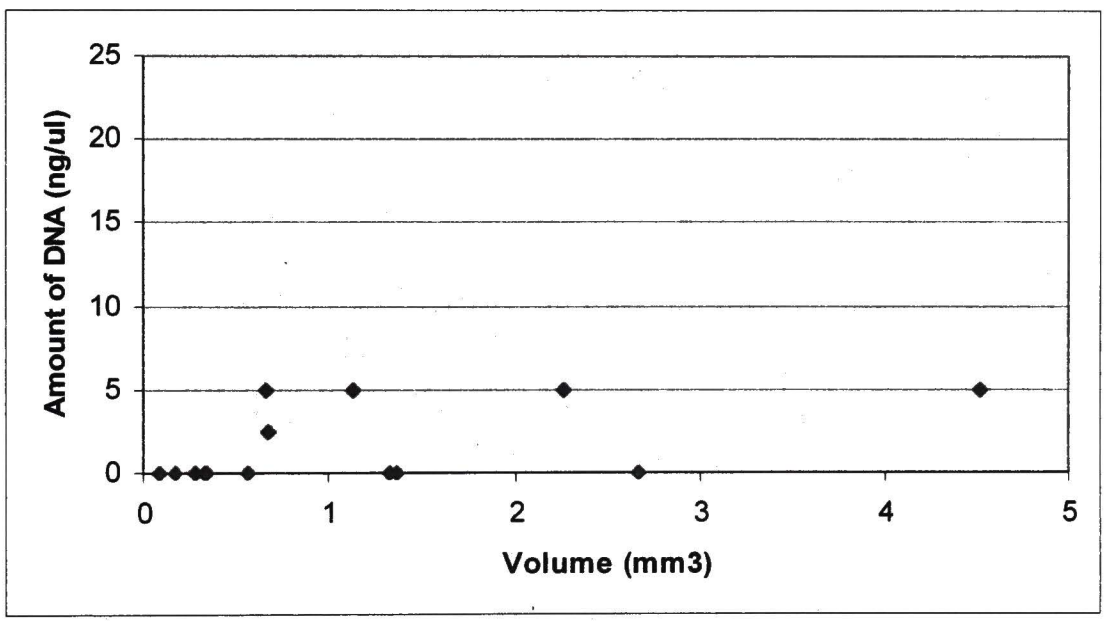

FIG. 15—*Relationship between hair volume and amount of DNA for Donor 10 (R^2 = 0.26).*

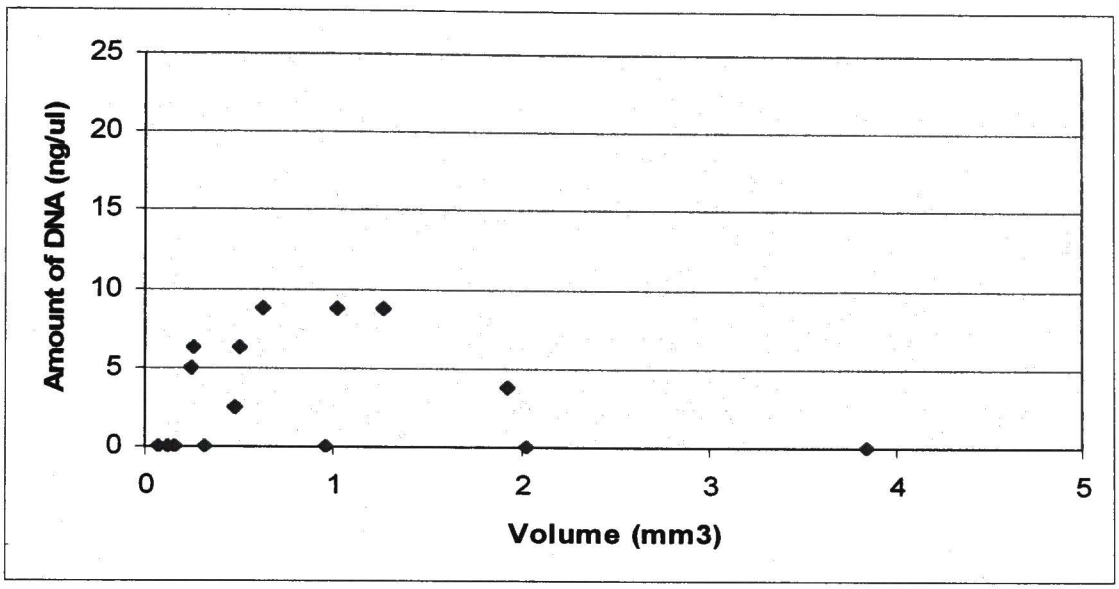

FIG.16—*Relationship between hair volume and amount of DNA for Donor 6 (R^2 = 0.01). Donor 6 had one of the lowest correlation coefficients for all the donors. This result is likely due to the lack of amplification success for the high volume samples.*

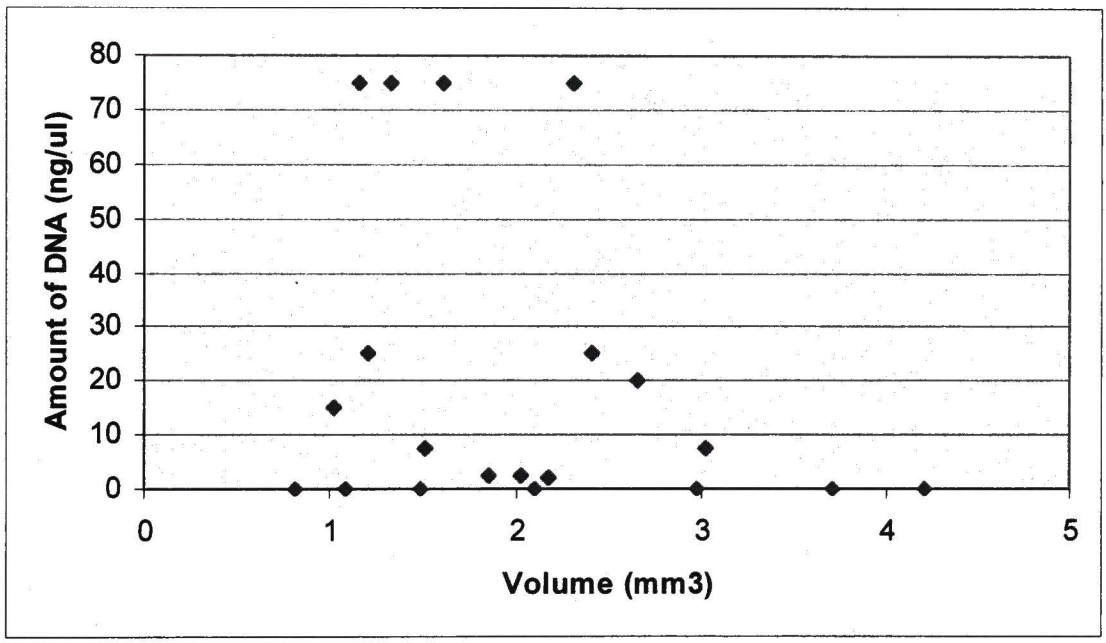

FIG. 17—*Relationship between volume and amount of DNA for all 20 beard hairs (R^2 = 0.07). Only three of the ten white beard hairs amplified.*

Evaluation of protein filtration

After all hair samples had been extracted, amplified, and quantified on agarose gels, inhibition of specific samples became apparent. Several four centimeter samples failed to amplify, while the shorter lengths produced mtDNA product (See Figure 18). Thirty-seven samples that did not amplify due to possible inhibition were subjected to filtration using Micropure EZ protein filters. Micropure EZ filters are typically used to remove restriction enzymes from cell digests, but have been previously used to purify and remove inhibitors for STR analysis.[45] Filtered samples were then re-amplified following the previous protocol and run on 2.5% agarose gels and the amount of mtDNA product was assessed as before. Out of the 37 samples, 25 successfully amplified, for a success rate of 68.4% (See Figure 19). Success rates for each category were also calculated (See Table 3).

Another set of 26 hair samples that had low amounts of DNA were also subjected to the above procedure to see if protein filtration could increase mtDNA product yield. Out of these samples, all but three increased in the amount of mtDNA amplification product, with two samples remaining the same, and one sample decreasing from 2 ng/ul to 0. Pre- and post-filtration values were compared and found to have average percent increase in DNA yield of 234% (See Figure 20 and Table 4).

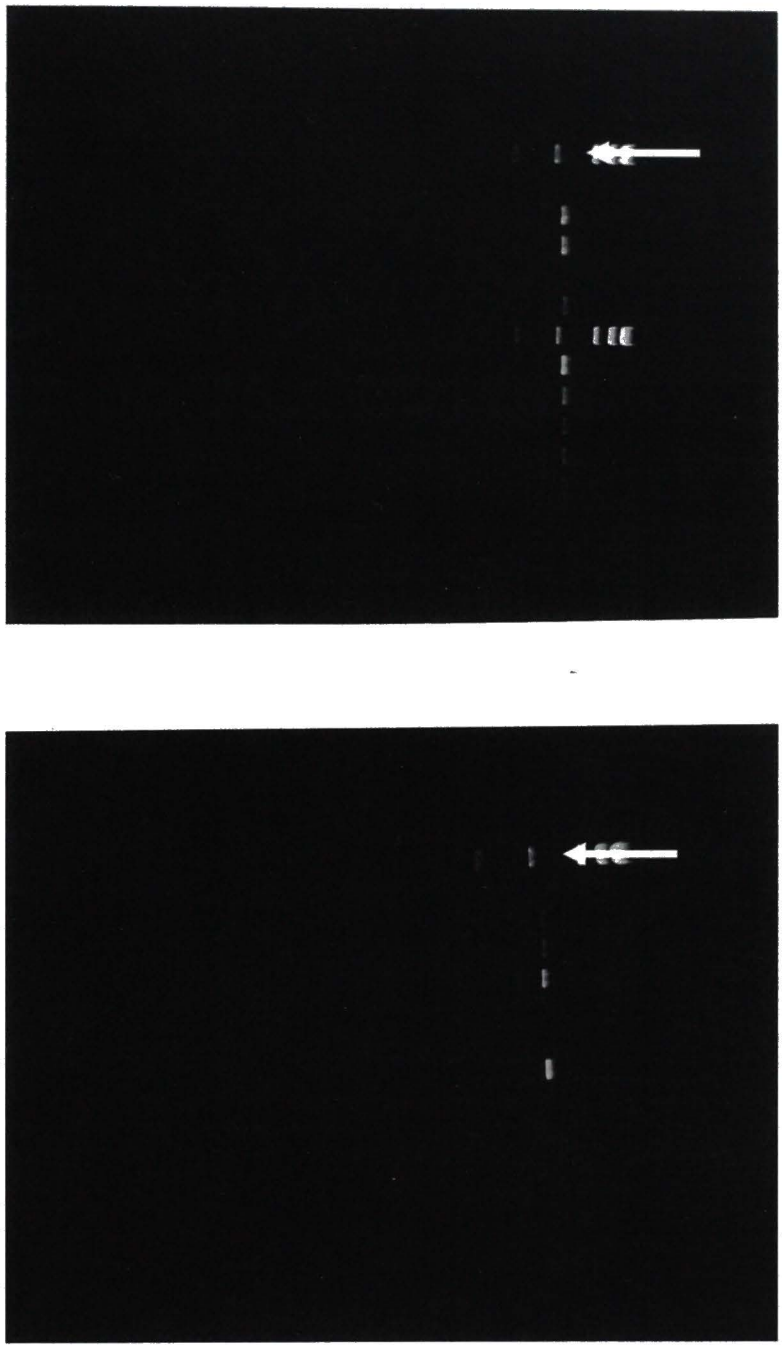

FIG. 18—*Examples of inhibition in two batches of red hair samples as indicated by the white arrows.*

Table 3—*Amplification success rates by category and within each group after protein filtration on samples that had no previous amplification success.*

Category	Group	Samples Filtered 0 ng/µl DNA	% Success
Hair Color			
	Blonde	5	40
	Red	13	68.4
	Brown	1	100
	Black	16	81.3
	White	1	0
Ethnicity			
	Mongoloid	3	100
	Caucasoid	22	40.9
	Negroid	13	100
Overall		37	68.4

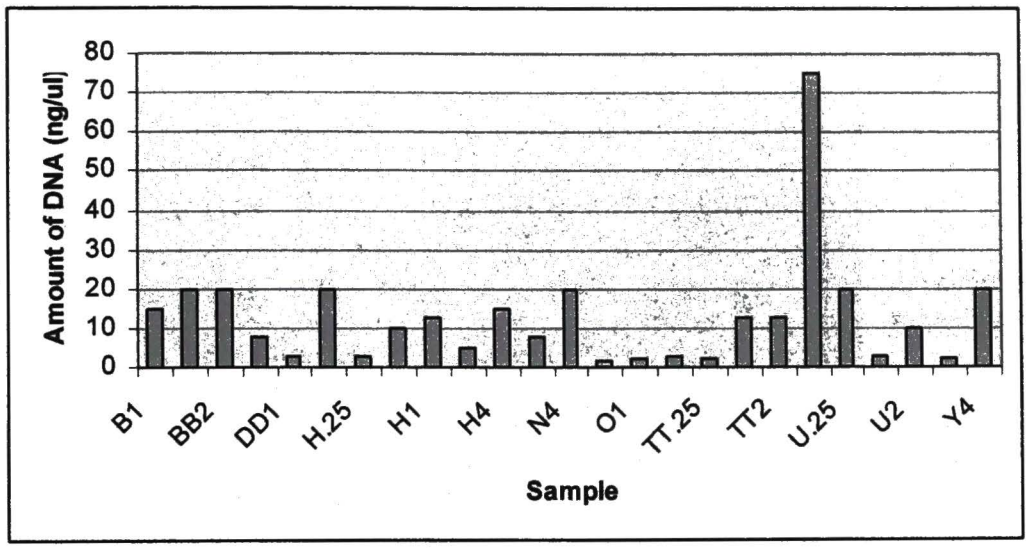

FIG. 19—*Amount of mtDNA product for all 25 successful samples that prior to protein filtration had no detectable mtDNA product.*

Table 4—*Number and average percent increase by group for samples that previously had low amounts of mtDNA amplification product.*

Category	Group	Number Filtered <15 ng/µl DNA	Average % Increase
Hair Color			
	Blonde	7	104.8
	Red	12	202.6
	Brown	7	419
	Black	0	*
	White	0	*
Ethnicity			
	Mongoloid	0	*
	Caucasoid	26	234
	Negroid	0	*
Overall		26	234

**These groups did not have any filtered samples.*

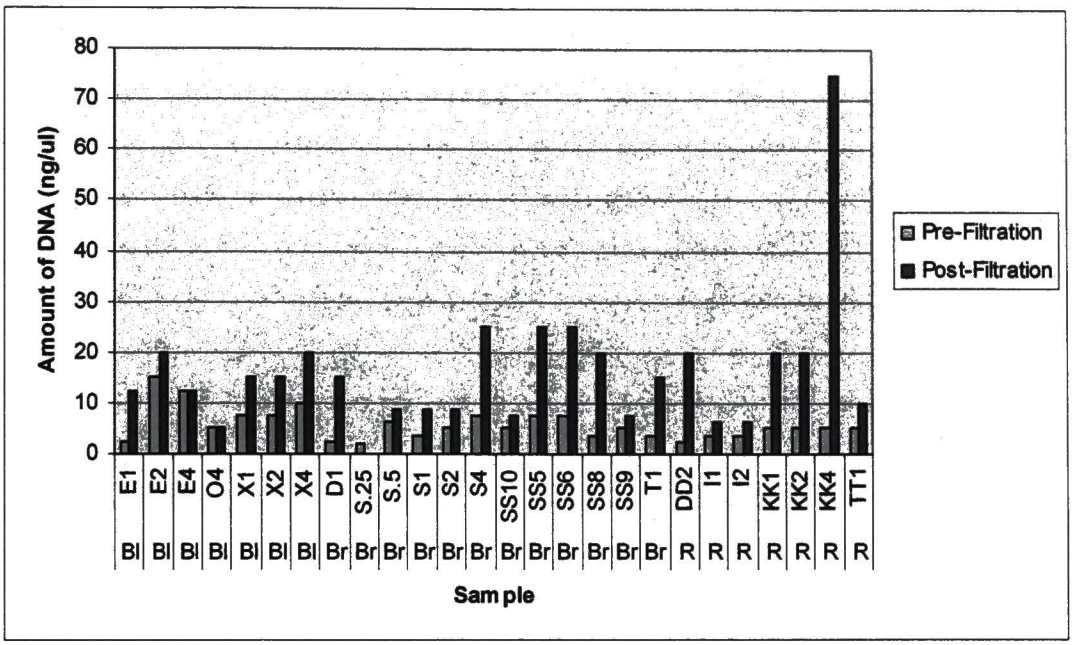

FIG. 20—*Pre- and post-filtration amounts for all 26 samples that had low amounts of mtDNA product prior to protein filtration. All but three samples had an increase in mtDNA product yield.*

In total, 63 samples were subjected to protein filtration. The majority of the hairs were either red Caucasoid hairs or black Negroid hairs. Since just under half of all the head hair samples were subjected to protein filtration (41.4%), new success rates and regression analysis were calculated using the post-filtration data. The relationship between hair volume and amount of mtDNA product strengthened after the re-evaluation of the data (See Figure 21).

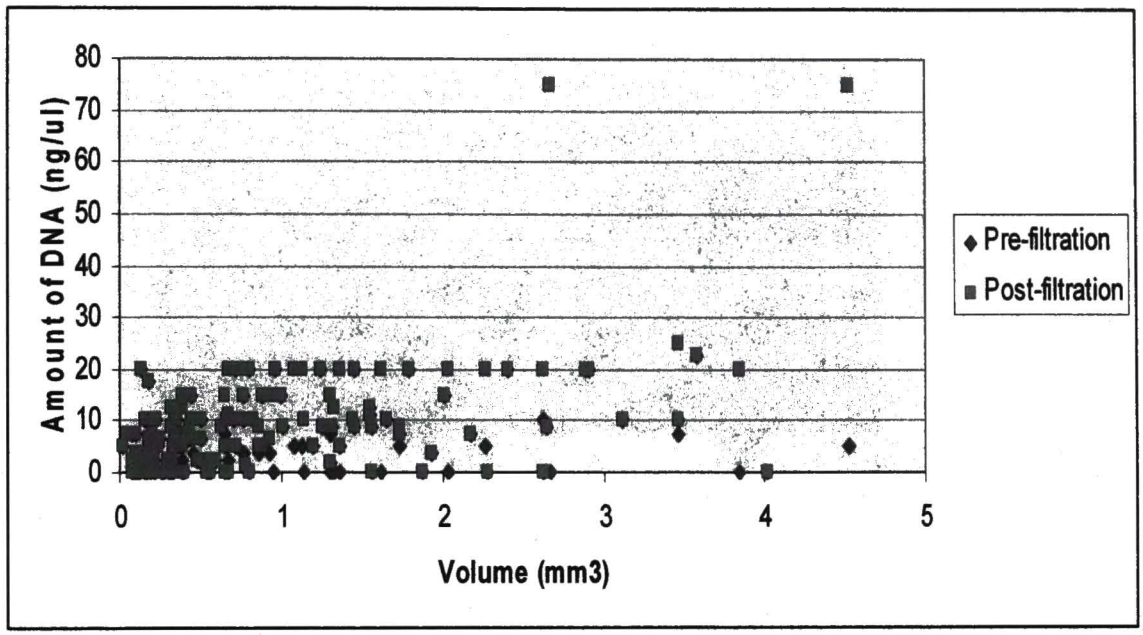

FIG. 21—*Relationship of hair volume versus amount of DNA for both pre-filtered and post-filtered hair samples (Post-filtration R^2 = 0.28)*

The protein filtration increased the overall amplification success rate from 64.4% to 84% and the new R^2 value for all the head hair samples was 0.28. The success rates and R^2 values for all the ethnic groups, hair colors, and donors increased as well (See Table 5, Figures 22-25). A new R^2 value was also calculated for samples that did not amplify after protein filtration and were known to be inhibited.

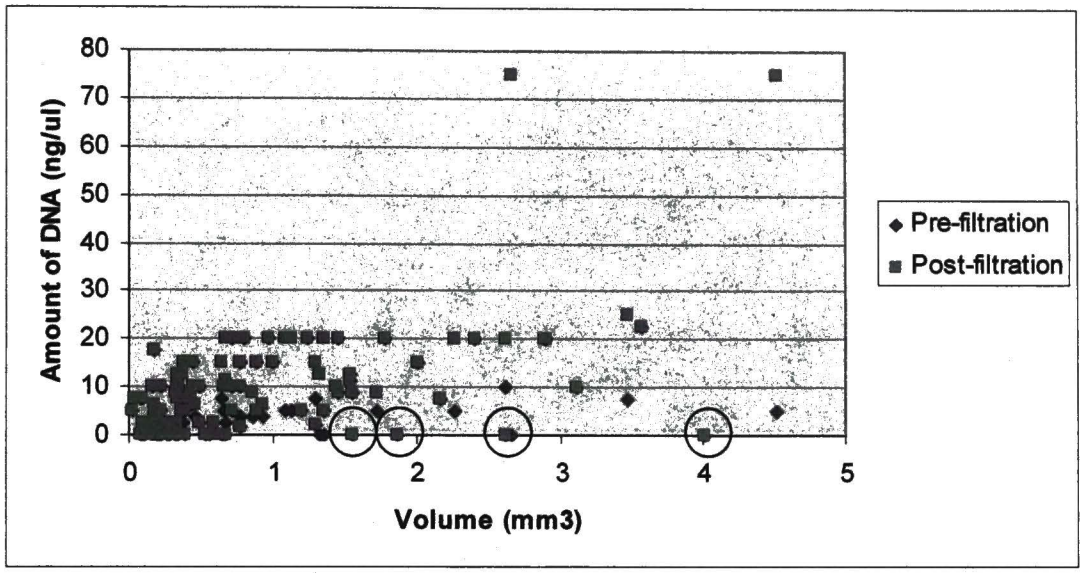

FIG. 22—*Pre-filtration and post-filtration data points for the Caucasoid ethnic group. The R^2 value increased from 0.11 to 0.13 after protein filtration. Circled data points indicated inhibited samples that were removed for the R^2 'no inhibitor' calculation, resulting in a stronger relationship between the two parameters (R^2 = 0.46).*

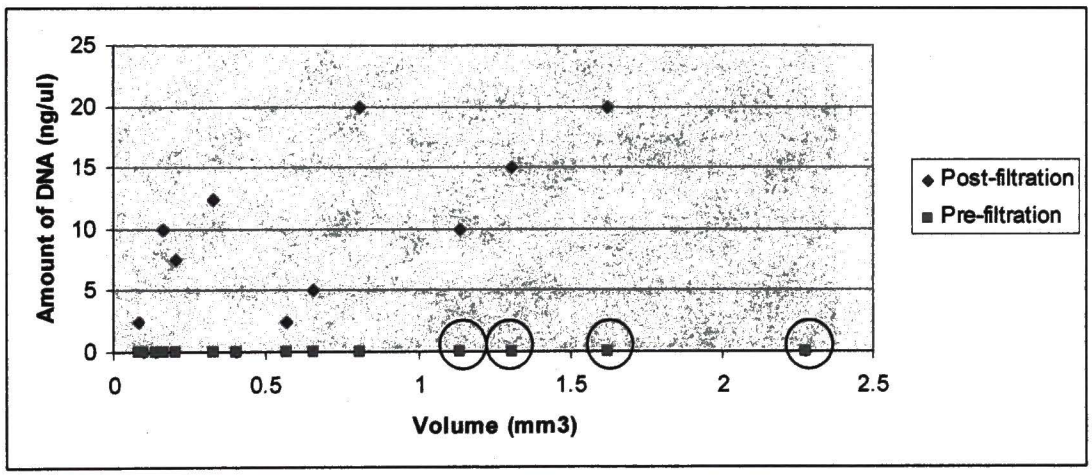

FIG. 23—*Pre-filtration and post-filtration data points for the Negroid ethnic group. Circled data points indicate inhibited samples that were removed for the R^2 'no inhibitors' calculation (R^2 = 0.50). Prior to protein filtration, none of the Negroid samples successfully amplified.*

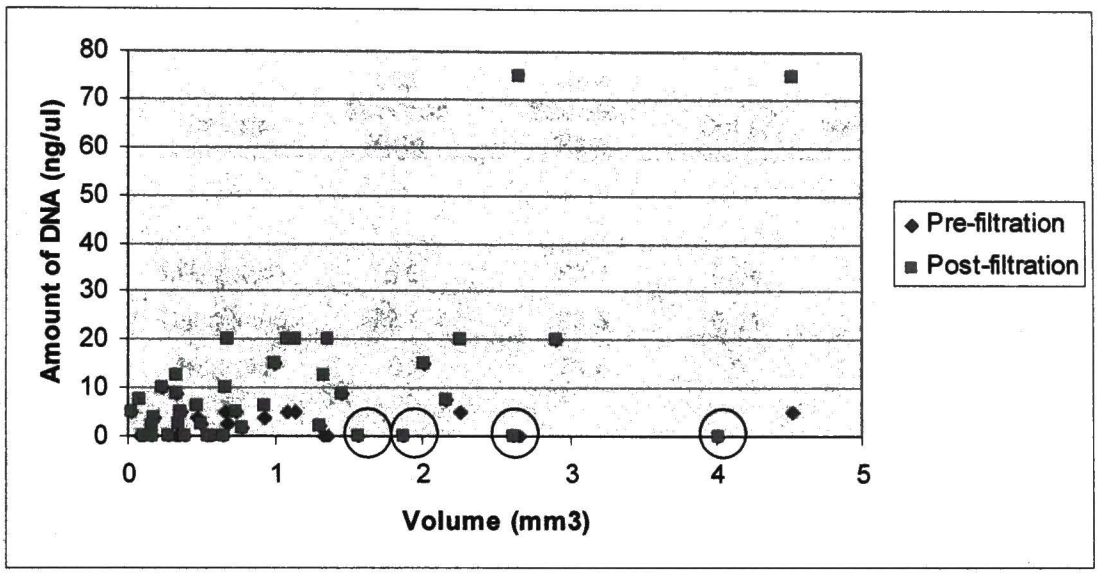

FIG. 24—*Pre-filtration and post-filtration data points for red head hair samples. The R^2 value increased from 0.04 to 0.32 after protein filtration. Circled data points indicate samples that were not included in the R^2 'no inhibitors' calculation (R^2 = 0.60).*

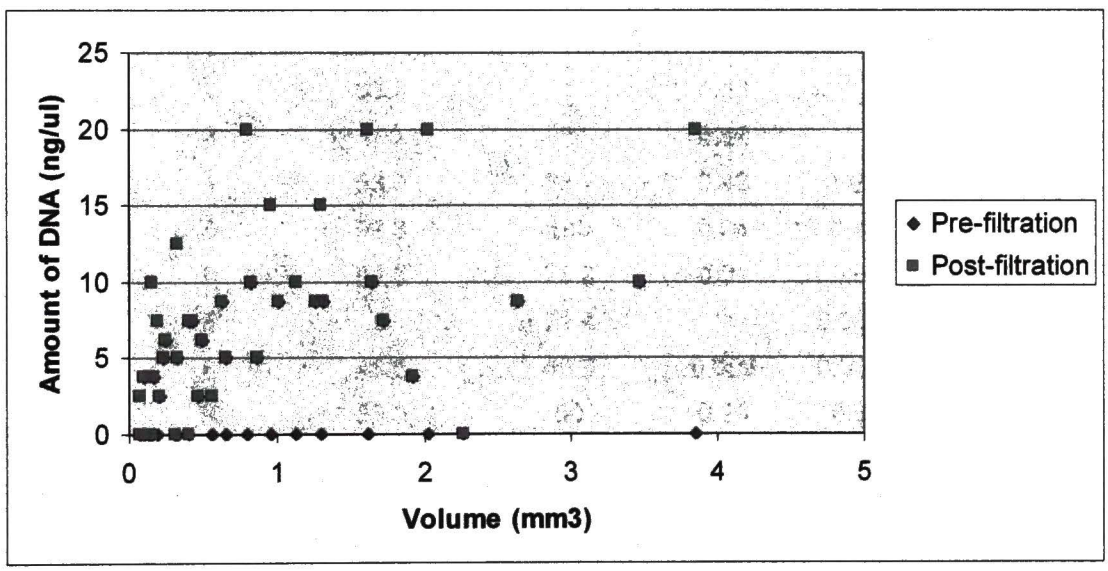

FIG. 25—*Pre-filtration and post-filtration for black head hair samples. The correlation coefficient increased from 0.05 to 0.35 after protein filtration and known inhibited samples were removed.*

Table 5—*New amplification success rates and R^2 values after protein filtration for each category.*

Category	Group	Old Success Rate	New Success Rate	Old R^2	New R^2	New R^2 w/o inhibition
Ethnicity						
	Mongoloid	75.9	86.2	0.04	0.48	*
	Caucasoid	73.1	83.3	0.11	0.13	0.46
	Negroid	0	71.4	**	0.08	0.47
Hair Color						
	Blonde	70	80	0.56	0.57	0.58
	Red	51.2	70.7	0.04	0.32	0.62
	Brown	100	100	0.15	0.38	*
	Black	51.2	83.7	0.05	0.27	0.35
	White	87.5	87.5	0.49	0.49	*
Donor						
	1	95.6	*	0.26	*	*
	2	93.3	*	0.66	*	*
	3	100	*	0.49	*	*
	4	53.3	66.7	0.48	0.48	*
	5	93.3	100	0.04	0.4	*
	6	53.3	73.3	0.01	0.6	*
	8	0	71.4	**	0.006	*
	9	45.5	45.5	0.009	0.002	*
	10	33.3	80	0.26	0.8	*
	11	80	80	0.05	0.39	*
	9	45.5	45.5	0.009	0.002	*
	10	33.3	80	0.26	0.8	*
	11	80	80	0.05	0.39	*

**A new R^2 value without inhibitors was not calculated for these samples because no inhibited samples were present.*
***An R^2 value was not calculated for this sample, as the success rate was 0%.*

Success rates were also calculated for each length of hair (See Table 6). Two centimeters had the highest success rate, at 90%. These results indicate that the guidelines recommending a length of hair were accurate in choosing a two centimeter length. However, the success rates for the two centimeter fragments were followed closely by four and one centimeters, with 86.7% and 84.2%, respectively. One half and one quarter centimeter lengths both had success rates of 74.1%. The high success rate of the one centimeter fragments and the thicker 0.5 centimeter fragments indicates that other factors, such as hair volume, may be influencing the success rates for the shorter hair lengths.

Table 6—*Amplification success rates and volume ranges for all hair lengths.*

Length	Volume Range (mm^3)	Number Amplified	Total	Success Rate
4 cm	0.68-4.62	26	30	86.7
2 cm	0.34-2.26	27	30	90
1 cm	0.10-1.25	32	38	84.2
0.5 cm	0.22-0.44	20	27	74.1
0.25 cm	0.03-0.28	20	27	74.1

CHAPTER IV

DISCUSSION

The goal of this study was to determine if there was a relationship between hair volume and amount of amplified mtDNA for all head hair samples, as well as among the three major ethnic groups, different hair colors, and different donors. This relationship could be used to determine how much of a hair sample is needed for extraction so that enough mtDNA PCR product is obtained, while preserving forensic hair samples and avoiding unnecessary consumption of evidence. Amplification success rates were also calculated for each of these categories to determine if the findings coincided with previously published literature.[41-43]

The pre-filtration R^2 value was 0.13, which indicates almost no correlation between hair volume and mtDNA amplification product recovery. The post-protein filtration value was 0.27, and this improved to 0.40 when correcting for known inhibitors. While this value is still lower than necessary to report a true relationship between the two parameters, it is still feasible that there is a connection when certain considerations are taken into account.

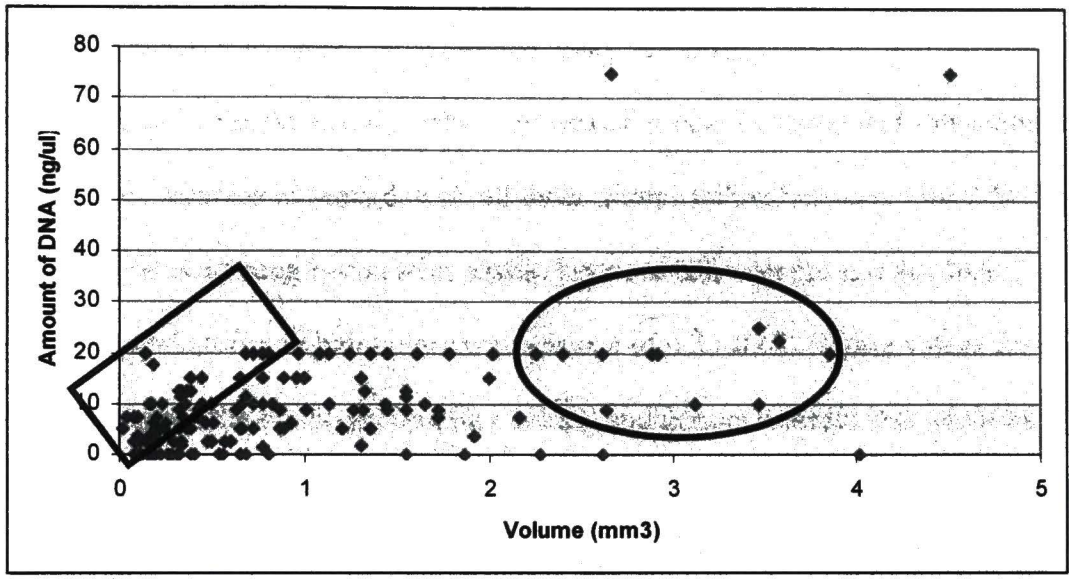

FIG. 26—*Relationship between hair volume and amount of mtDNA product for all head hair samples after protein filtration . The correlation coefficient for these values was 0.40, which indicates a minimal relationship between these two parameters. Data points indicated by the rectangle are low volume samples that had high amounts of DNA, which lowered the R^2 value, but is not an undesirable result. Data points indicated by the oval are higher volume samples that demonstrate the limitations of the yield gel quantitation method, as it is more difficult to estimate mtDNA product yield at the higher end of the DNA mass ladder.*

There are several low volume samples indicated on the graph that had high amounts of DNA, which are indicated on the scatterplot by the rectangle. These samples lower the R^2 value and the overall hair volume/DNA recovery relationship, but obtaining high amounts of DNA from small samples is not an undesirable result. Samples within the oval are high volume samples that are not at the expected DNA recovery amounts if there was a linear relationship between hair volume and mtDNA product recovery. These samples demonstrate the limited nature of determining mtDNA amplification product using a yield gel. Samples that had concentrations of mtDNA product of 40 ng/ul or

higher are difficult to interpret and contain an inherent subjectivity due to the nature of the low DNA mass ladder. Volume estimates also become less accurate for longer lengths of hair, which would also affect the regression analysis values.

R^2 values for the three major ethnic groups after post-filtration and correction for known inhibited samples improved, with all three groups falling between 0.46-0.50. Hair color also indicates a strengthened relationship between hair volume and mtDNA amplification product for all hair colors with a range of 0.35-0.62. Higher values for each of the groups within the ethnic groups, hair colors, and donors indicate that while the overall relationship has an R^2 value worth noting, the relationship between hair volume and mtDNA product recovery might be stronger and more useful when looking at the previously mentioned categories.

While the reported correlation coefficients for the donors are not applicable for predicting DNA recovery amounts, it is both necessary and interesting to note these particular values for each donor. All donors had R^2 values at least 0.39, with the exception of Donor 1, who donated both white and brown hairs and had a value of 0.29, and Donors 8 and 9, both of whom had R^2 values below 0.10. Donor 8 is the only Negroid donor in the study and after protein filtration, had several low volume samples with high amounts of mtDNA, which threw off the R^2 value. Donor 9 is a red-haired individual and had the lowest success rate of all the donors at 45.5%, with only six of his 11 samples successfully amplifying.

In order to determine if the results of this study corroborated with previously published findings, the amplification success rates were calculated for all hair samples

and for each category. The overall post-filtration success rate using the alkaline digestion extraction method was approximately 84%. This success rate is slightly lower than the success rate of 90% as reported Graffy and Foran[38], but higher than previous studies using the glass grinding method.[41, 43] Graffy and Foran did have a lower sample size (N = 30) compared to this study, which has 152. Out of their 30 samples, 27 were six to seven centimeters, and the remaining three were less than two. This study had lengths of four, two, one, one-half, and one-quarter centimeters. Having a larger sample size and more hair lengths that were less than one centimeter contributed to the lower amplification success rates for this study.

For the three major ethnic groups, the Negroid population had the lowest success rate of 71.4%, which is consistent with previously reported findings.[43] Caucasoids tend to have the highest amplification success rate. However, the Mongoloid success rate was 3% higher than the Caucasoid success rate, which contradicts previous studies.

For hair color, all results obtained in this study follow Roberts and Calloway's reported success rate for hair color, with a few exceptions. According to the authors, red-haired individuals have the lowest success rate, at least 20% lower than the other hair colors (58.4%). The current study had the lowest success rate for red hair samples, but with a higher success rate of 70.7%. A contradiction to Roberts and Calloway's study was the high success rate of the brown-haired samples found in this study (100%), compared to 72.3-83.5% for the authors. In both studies, hair with no pigment had the highest success rate.

Different lengths of hair were also examined to determine if the recommended length of two centimeters[37] was a reliable length for extraction. Out of the five lengths chosen for this study, two centimeters did have the highest overall success rate at 90%. This was followed very closely by four and one centimeters, at 86.7% and 84.2%, respectively. These results indicate that the recommended length of two centimeters does have the overall highest success rate of all the lengths studied. However, for the one centimeter hairs, almost 85% were able to successfully amplify using less sample. A success rate of 74.1% was obtained for both 0.5 and 0.25 centimeter hairs in this study and out of these smaller hairs, the higher volume samples are the ones that successfully amplified. These success rates for the shorter length hairs raise the question of whether a specific volume, rather than length, can be used for extraction purposes.

When all hair samples were ordered by volume, a recommended volume range of 0.40-0.50 mm^3 was determined to be a sufficient volume for extraction. Out of the 92 samples that were within and above this range, 11 had no detectable mtDNA product, although of these 11, seven were four centimeter hair samples that were known to be inhibited because lower volume samples in the same batch had detectable mtDNA product. This leaves only four samples out of 81 that failed to amplify. Out of these 81 samples, 18 were one centimeter and six were only 0.5 centimeter, clearly demonstrating that a length of two centimeters is more than needed for extraction for certain hairs. In order to be confident that an analyst would get amplified product, this recommended volume could be adjusted for ethnicity and hair color, as these categories indicated a stronger overall relationship between hair volume and amount of mtDNA. For example,

if an analyst needed to determine how much of a red Caucasian hair to extract, it might be advisable to use more than the recommended range of 0.40-0.50 mm^3, and instead opt for a higher volume to ensure amplification success. White or brown hairs, which demonstrate a higher success rate, might use a volume at the lower end of the recommended range. These results can also be used to determine if two centimeters might not be enough sample. If the hair in question is from an infant, or is a very fine, thin hair (< 50 μm), it might be a good idea to measure the hair and calculate the volume, as well as taking into account ethnicity and color, to ensure amplification success.

The effectiveness of protein filtration using the Micropure EZ© protein filters to remove possible PCR inhibitors prior to amplification was also evaluated. Of the 37 samples without any previous mtDNA product, 25 were successfully amplified. It is interesting to note that of these 37 samples, 14 were from the lone Negroid donor, who, prior to filtration had a success rate of 0%. When the protein filtration was applied to 25 samples that had low levels of mtDNA product, all but three had an increase in yield. These findings, as well as an increase of the overall success rate from 66.4% to almost 84% after filtration, clearly indicate that the use of these filters is an effective means of improving mtDNA product yield and are a beneficial addition to the mitochondrial DNA analysis process.

The question that now must be addressed is the exact nature of the removed PCR inhibitor. Without further experimentation, it is impossible to ascertain a positive identification of the inhibitor at this time. However, after careful observation, one is able to note specific characteristics of this potential inhibitor and suggest possible candidates.

As stated previously, known inhibitors of the polymerase chain reaction are plant material, indigo dyes, melanin[30], hemoglobin, and humic acid, among others. Out of these, only melanin is naturally found in hair. It is also interesting to note that while melanin is not a protein in and of itself, the polymer contains monomeric building blocks of dihydroxyphenylaline (DOPA) and/or cysteinyl-DOPA, which are derivatives of the amino acids cytosine and tyrosine. Out of the 63 samples that were filtered, the majority were red and black hairs, which contain the highest amounts of phaeomelanin and eumelanin, respectively. The color change of the extract from a brownish color to clear between pre- and post-protein filtration was noticeable as well, indicating that some sort of cleansing process had taken place during filtration. Based on the above observations and lack of other potential candidates, it is suggested that the Micropure EZ filters are possibly filtering out phaeomelanin and eumelanin, thus improving the polymerase chain reaction and increasing yield of PCR product during the amplification portion of mitochondrial DNA analysis.

This study evaluated the relationship of hair volume and mitochondrial DNA product recovery to determine if a specific volume of hair could be used in order to preserve valuable forensic evidence. While the overall relationship between these two parameters is minimal, the relationship increases when one looks at the specific categories of ethnicity and hair color. A range of 0.40-0.50 mm^3 of hair is suggested for extraction. This study found that with the exception of samples with known inhibition, 95% (77 out of 81 samples) within and above this range gave detectable mtDNA product. Adjustments can be made to this range based on the previously mentioned morphological

characteristics of hair. Success rates of the different categories of hairs also followed previous literature, with a few minor exceptions. The results of this study also indicate the use of the Micropure EZ filters as beneficial addition to mitochondrial DNA analysis by increasing the yield of PCR product. Based on specific characteristics of the potential inhibitor, melanin is suggested as the substance that the protein filters are removing, but further experimentation is needed to address this issue.

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