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Hair is among the frequently encountered evidence found in crime scenes. The average person loses approximately 100 hairs a day. Because these hairs are telogen strands, or at the end of their life-phase, there is very little tissue present to obtain nuclear DNA. Hair shafts, however, contain mitochondrial DNA that can be used for identification purposes. There are two areas of concern involving mtDNA analysis of hair shafts: 1) will there be enough mtDNA present to obtain a full profile, and 2) and has the integrity of mtDNA been compromised due to oxidative properties, and/or the keratinization of the hair. The purpose of this project is to elucidate whether the amount of mitochondrial DNA changes from the proximal to the distal end of the hair shaft. Five hair samples were obtained from five subjects and the hairs were dissected at every fourth centimeter. DNA was extracted from each hair section, and subjected to mitochondrial DNA quantification (via the control region of the genome), as well as assessed for any deletions seen within the coding region as a sign of damage that may have occurred, using an assay validated by the University of North Texas- Health Science Center (UNTHSC, Fort Worth, Texas). It was found that there was generally a gradual decrease in mitochondria copy number throughout the hair strands from the proximal to the distal end. Also, it was found that mitochondrial DNA is more susceptible to damage towards the distal end. Mitochondrial DNA sequencing was performed on specific samples to observe any relationship between the concentration of mitochondria and the stability of the sequence.

DISCOVERING THE OPTIMAL HAIR SECTIONS FOR MITOCHONDRIAL DNA QUANTIFICATION VIA A MULTIPLEX REAL-TIME PCR ASSAY

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THESIS

Presented to the Graduate Council of the

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CHAPTER I

INTRODUCTION

In forensic casework, hair evidence is regularly analyzed, due to the fact that hairs are easily lost from suspects and victims alike. When an analyst encounters hairs within the submitted evidence, the initial examination is microscopic; this is crucial for separating the hairs into probative and non-probative material. Sometimes microscopic analysis is enough to provide investigative information; however, DNA analysis is necessary objective information as to the actual source of the hair (1).

Hair follicles typically grow in three phases: anagen, catagen, and telogen, as seen in **Figure 1**. The mass majority of human hairs will be found in the anagen phase, which can last anywhere from 48 to 72 months. In the anagen phase, the hair is actively growing because the cells within the dermal papilla are self-proliferating, which requires a large amount of energy (2). The hair follicle then stops growing in the transitory catagen phase, followed by the dormant telogen phase. The hair follicle rests for approximately 2 to 6 months, when the hair strand finally sheds from the scalp (3). There are three major fractions to a hair fiber: the medulla, the cortex, and the cuticle. The cortical cells are within the fiber, ultimately dying through the process of keratinizing and hardening as the hair grows. The cortex contains melanin, the molecule that gives hair its color; a combination of two biochemical pigments, eumelanins (which are associated with brown and black color) and pheomelanins (which are associated with

yellow and red color), are what make up the melanin (2).There may be a strand of cells that are loosely packed in the middle of the cortex known as the medulla. The outermost section of a hair fiber is the cuticle, which is keratinized cells that are arranged in a shingled fashion over the cortex (4).

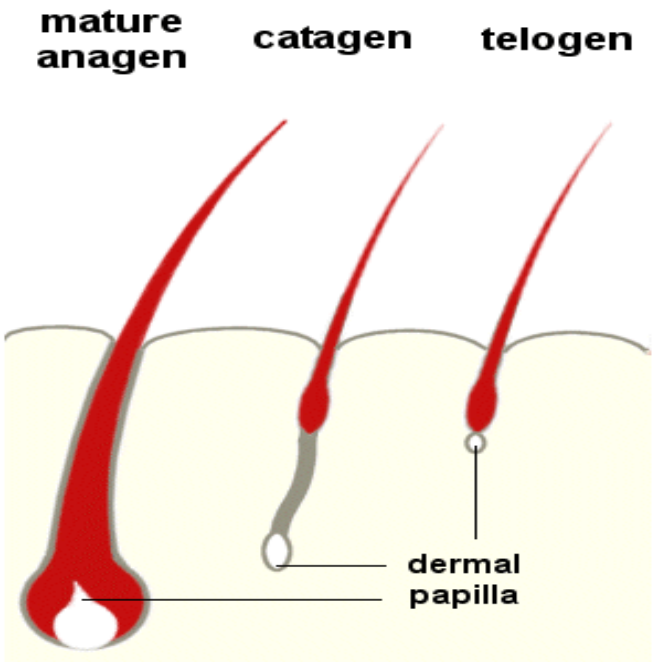


Figure 1: Phases of Hair Growth *(5). The three phases of hair growth are represented. The dermal papilla, or root bulb, is represented by the white circle.*

It is largely acknowledged that nuclei degrade within developing hairs due to the keratinization of hair shafts, but it is unknown as to when. It was found via transmission electron microscopy that nuclei and mitochondria fail to be seen past the dermal papilla, or root bulb, where the cuticle was fully formed (6). Therefore, unless an anagen hair is recovered from a crime scene with the root bulb attached, recovering nuclear DNA (nuDNA) from the evidence is highly difficult and quite unlikely. Consequently, most analysts are constrained to mitochondrial DNA (mtDNA). Mitochondria are organelles found in cells, and account for the majority of

their ATP production by means of cellular respiration and oxidative phosphorylation. Some cells contain multitudes of these double-membraned organelles, and they contain their own genome. It is thought that the capability of extracting and retrieving full mtDNA profiles from hairs, even with a lack of nuDNA, is due to the high copy number found within cells (6). There are approximately 16,569 bases within the circular mtGenome, which were previously sequenced at the Cambridge Research Institute, published by Anderson et al. in 1981 (7). This genome consists of a coding region, which is the majority of the DNA, and non-coding control region, which is highly variable. Due to the hypervariability of the non-coding region, forensic analysis is usually focused on cycle sequencing of the hypervariable region I (HVI) and hypervariable region II (HVII) amplicons in this stretch (8).

It is known that mtDNA is less susceptible to degradation than nuDNA, due to its circular nature disallowing exonucleases to harm it (9). However, because of its role in oxidative phosphorylation, mtDNA is highly vulnerable to damage due to the concentration of reactive oxygen species. The DNA repair mechanisms in mitochondria are insubstantial compared to their nuclear counterpart, resulting in mutations and deletions in the coding region of the genome; this condition is known as heteroplasmy (10). The amount of mutation that the mitochondrial genome experiences is a degree greater than the nuclear genome; however, these mutations occur in some mitochondria and not others. When cells undergo meiosis, a mixture of wild-type and mutant mitochondria will be passed on to the daughter cells, and this is when heteroplasmy is seen (11).

The two classes of heteroplasmy that are typically seen are length and sequence. Length heteroplasmy is attributed to insertions or deletions of nucleotides into the mtDNA, frequently

seen in the control region HVI and HVII amplicons as an expanse of cytosine nucleotides known as "C-stretch," (12) while sequence heteroplasmy is recognized as point mutations. Studies have shown that heteroplasmy can be seen throughout different hairs throughout the scalp, suggesting that mosaicism is erratically distributed. It was also found that Caucasians and Asians display more mosaicism than African-Americans and Hispanics within the US population (11).

It is an ongoing science to optimize quantification of mtDNA. Due to the large variation in copy number within different tissues, quantification has demonstrated to be quite challenging. One of the major problems with earlier quantification assays is the focus on double-stranded DNA (dsDNA), which encompasses the nuclear genome. Accurate quantification is necessary for effective downstream processing, such as amplification and sequencing, especially for forensic purposes (13). Therefore, ensuring this initial process is competent is crucial.

Sequencing mtDNA is a difficult process, because the amplification product needs to be purified before moving onto actual analysis. The samples must first be enzymatically cleaned usually with ExoSAP-IT® (USB Corp., Cleveland, OH) and/or BigDye XTerminator™ (Applied Biosystems) (8) before cycle sequencing can occur. For forensic purposes, sequencing only occurs within the HVI/ HVII amplicons, since they comprise of the control region, and any changes within this sequence would be blatant to the analyst. Once the base calls of the sample mtDNA are assigned, the sequence is then compared to the Cambridge Reference sequence, to discover any disparities between the two, which leads to the sample's profile (12).

The first case that used mitochondrial DNA from hair, *State of Tennessee v. Paul Ware*, was in 1996. The twenty-seven-year-old defendant was accused of sexually assaulting and murdering a four-year-old girl. The defense argued that the other male babysitter, who was

found drunk and sleeping by the child, was guilty, pointing out the fact that there was no evidence of the defendant's seminal fluid on the victim, nor the victim's blood on the defendant. However, there were a few red hairs found at the crime scene, and mitochondrial DNA was extracted from them and then sequenced. The mtDNA sequence of the hairs matched each other, and matched the defendant, thus leading to a guilty verdict (14).

At UNTHSC, a unique protocol is followed for quantification and cycle sequencing of mtDNA. A multiplex real-time PCR assay is used for quantification, highlighting the ratio between mtDNA and nuDNA seen, known as the mtDNA copy number ($mtDNA_{CN}$), and the possibility of deletions seen within the mtDNA genome, known as the deletion ratio ($mtDNA_{DR}$). The number of mitochondria present is an excellent gauge for mitochondrial biogenesis, as well as indicative of the presence of certain deletions, approximately 84% of reported occurring deletions, which are associated with the aging process and certain disorders, such as Kearns-Sayre Syndrome and Alzheimer's disease. These deletions can occur in the minor (mtMinArc) and major arc (mtMajArc), but deletions seen in the mtMinArc are rather rare (15).

This assay measures the $mtDNA_{CN}$ and $mtDNA_{DR}$ by focusing on three areas: a single-copy nuclear (β2M) locus, the mtMinArc, and the mtMajArc, as seen in **Figure 2**. The $mtDNA_{CN}$ is represented as the ratio between the nuclear locus and the mtMinArc, while the $mtDNA_{DR}$ is represented as (mtMinArc-mtMajArc)/mtMinArc (15).

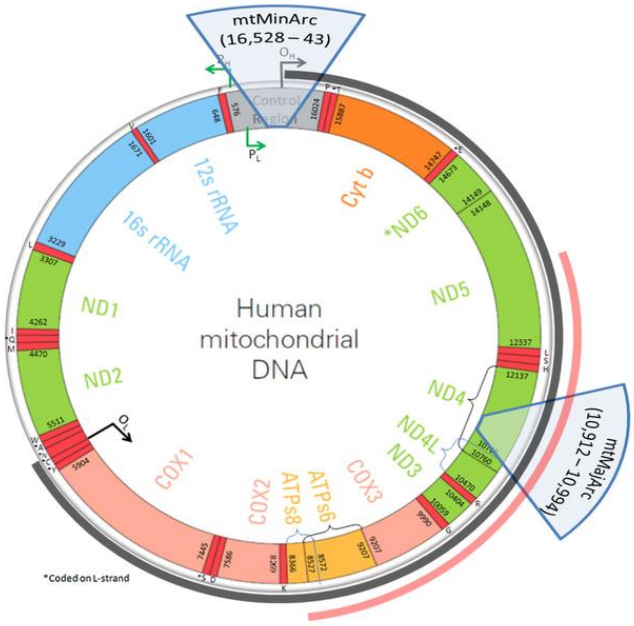


Figure 2: Human mitochondrial DNA *(15). The mtMinArc, which is used to measure the both* $mtDNA_{CN}$ *and* $mtDNA_{DR}$*, contains the HVI and HVII amplicons; it is located within 16,528-43 basepairs. The mtMajArc, which is used in determining* $mtDNA_{DR}$*, is located within 10,912-10,994 basepairs.*

The hindrance with DNA analysis for hair is acquiring an ideal amount for amplification to attain a full mitochondrial DNA profile, as mitochondrial genomes are vulnerable to damage due to its role in oxidative phosphorylation, as well as the keratinization of the hair as it grows. Both of these concerns were resolved by studying various sections along the length of hairs received from different subjects. These hairs were subjected to mitochondrial DNA quantification (via the control region of the genome), as well as assessed for any deletions seen within the coding region as a sign of damage that may have ensued, using an assay validated by UNTHSC.

It was hypothesized that the most quantifiable mitochondrial DNA will be found towards the proximal end of the hair and that these mitochondrial genomes would contain the least amount of damage, as opposed to the mitochondria further down the length of the hair. It was also

hypothesized that there will be a gradual decrease in mitochondria copy number from the proximal to the distal end.

CHAPTER II

MATERIALS AND METHODS

Three hairs were pulled, with the root bulb attached, from five subjects for a total of 15 hairs. The subjects included two blonde females, two brunette females, and one male. The hairs were collected from the top of the head, the left side and the right side. The hair shafts were then sliced into 1 cm-long segments, starting from the proximal end to the distal end, and every fourth segment was collected, i.e. the first centimeter, the fifth centimeter, the ninth centimeter, etc. for analysis, as seen in **Figure 3.** These segments were grinded with a mortar and pestle, and underwent organic extraction, prior to quantification with the $mtDNA_{CN}$/ $mtDNA_{DR}$ assay. A small number of samples were also chosen for sequencing to compare proximal and distal sections to ensure full mitochondrial profiles were obtained, as well as to observe any heteroplasmy that may be present.

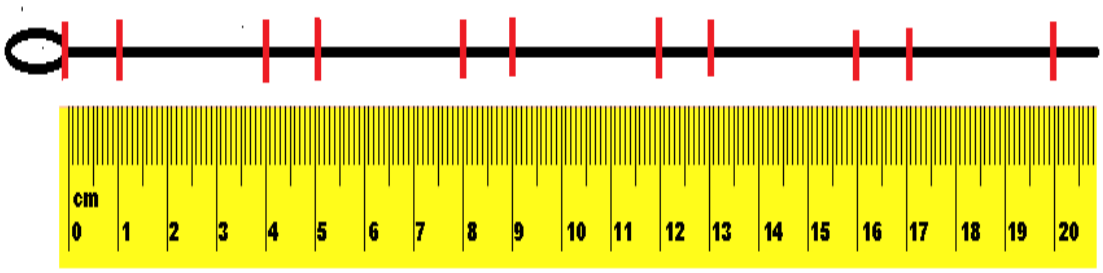


Figure 3: Dissection of Hair Strands. *The hair shafts were sliced into 1 cm-long segments, starting from the proximal end to the distal end, and every fourth segment was collected. The root bulb was taped down, and the first centimeter after the bulb was the first section collected. The sections in red represent the sections that would be collected.*

Organic Extraction via Phenol-Chloroform-Isoamyl Alcohol (PCIA)

To prepare the stain extraction buffer working solution, 300 mg of dithiothreitol (Sigma-Aldrich, St. Louis, MO) was added to 50 ml of stain extraction buffer (SEB) stock solution.

The SEB stock solution consists of:

- 1 ml of TRIS buff (pH 8.0) (Life Technologies, Carlsbad, CA)
- 2 ml of 5M NaCl (Life Technologies, Carlsbad, CA)
- 2 ml of 0.5M EDTA (Life Technologies, Carlsbad, CA)
- 20 ml of 10% SDS (Life Technologies, Carlsbad, CA)
- 60 ml of UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies, Carlsbad, CA)

Each hair strand was then cut into 1 cm sections, starting with the first centimeter right after the root bulb. Every fourth section was then added to a mixture consisting of 200 ul of SEB working solution, and grinded with separate mortars and pestles until the sections dissolved completely. The samples were then vortexed briefly with the addition of 5 ul of Proteinase K, pulse spun, and then incubated at 56°C overnight. When the incubation was complete, 200 ul of buffered PCIA will be added, and then the samples were vortexed and centrifuged for 3 minutes at ~13,300 rpm. The aqueous layer was then removed from each sample and transferred to separate UV irradiated tubes. The DNA obtained from each sample was purified via ethanol precipitation, and resolubilized in 100 ul UltraPure™ DNase/RNase-Free Distilled Water.

A reagent blank for each hair was prepared the same way.

Quantification using a Multiplex Real-Time qPCR Assay

A total of 8 standards were prepared in a dilution series, and were run in duplicate. Each sample well contained the following components for a final reaction volume of 25 ul:

- 20.25 ul TaqMan® Universal Master Mix (Life Technologies, Carlsbad, CA)
- 0.25 ul Forward and Reverse Primers on mtMinArc (Life Technologies, Carlsbad, CA)
- 0.25 ul Forward and Reverse Primers on mtMajArc (Life Technologies, Carlsbad, CA)
- 0.5 ul Forward and Reverse β2M Primers (Life Technologies, Carlsbad, CA)
- 0.25 ul mtMinArc Probe (Life Technologies, Carlsbad, CA)
- 0.25 ul mtMajArc Probe (Life Technologies, Carlsbad, CA)
- 0.25 ul β2M Probe (Life Technologies, Carlsbad, CA)
- 2 ul DNA template

Table 1: Primers and Probes Used in Multiplex Real-Time qPCR Assay. *The sequences of the primers and probes that were used in the $mtDNA_{CN}$/ $mtDNA_{DR}$ assay are represented. The "F" signifies Forward, "R" signifies Reverse, and "P" signifies Probe.*

Primer Name	Binding Site Positions	Sequence (5'-3')
FmtMinArc	mt16,528-16,548	CTAAATAGCCCACACGTTCCC
RmtMinArc	mt23-42	AGAGCTCCCGTGAGTGGTTA
FmtMajArc	mt10,912-10,931	CTGTTCCCCAACCTTTTCCT
RmtMinArc	mt10,975-10,994	CCATGATTGTGAGGGGTAGG
Fβ2M	Chr15 15,798,932-15,798,958	GCTGGGTAGCTCTAAACAAATGTATTCA
Rβ2M	Chr15 15,798,999-15,799,026	CCATGTACTAACAAATGTCTAAAATGGT
Probe Name	**Binding Site Positions**	**Sequence (5'-3')**
PmtMinArc	mt16,560-10	6FAM-CATCACGATGGATCACACAGGT(NFQ)
PmtMajArc	mt10,934-10,951	NED-GACCCCCTAACAACCCCC(NFQ)
Pβ2M	Chr15 15,798,969-15,798,984	VIC-CAGCAGCCTATTCTGC(NFQ)

The master mix was prepared in a UV irradiated 1.5 mL tube. The positive control to be used in this assay will be HL60, and the negative control will be UltraPure™ DNase/RNase-Free Distilled Water. The following conditions will be set on the Applied Biosystems® 7500 Real Time PCR System (Life Technologies, Carlsbad, CA):

- Stage 1: 95°C 10 min, 1 cycle
- Stage 2: 95°C 15 sec, 55°C 15s, 60°C 1 min, 40 cycles
- 9600 Emulation

- Data collection: Stage 2, step 3

mtDNA Amplification

The samples chosen for amplification were then concentrated via a Microcon® DNA Fast Flow Centrifugal Filter (Merck KGaA, Darmstadt, Germany). The samples were then subjected to mtDNA amplification. Each sample well contained the following components for a final reaction volume of 25 ul:

- UltraPure™ DNase/RNase-Free Distilled Water
- 10X PCR Buffer I (Life Technologies, Carlsbad, CA)
- BSA (1.6 g/L) (Life Technologies, Carlsbad, CA)
- 25mM $MgCl_2$ (Life Technologies, Carlsbad, CA)
- 10mM dNTPs (Life Technologies, Carlsbad, CA)
- 10uM Primer A1- forward primer for HVI amplicon (Life Technologies, Carlsbad, CA)
- 10uM Primer B1- reverse primer for HVI amplicon (Life Technologies, Carlsbad, CA)
- 10uM Primer C1- forward primer for HVII amplicon (Life Technologies, Carlsbad, CA)
- 10uM Primer D1- reverse primer for HVII amplicon (Life Technologies, Carlsbad, CA)
- AmpliTaq® Gold Polymerase (Life Technologies, Carlsbad, CA)
- DNA template

Table 2: Amplification and Sequencing Primers. *The sequences of the primers that were used for both amplification and cycle sequencing are represented. The primers were at 10uM concentration for amplification and 3.3uM concentration for cycle sequencing.*

Primer	Direction	Binding Site	Sequence (5'-3')
A1	Forward	mt15,978-15,997	CACCATTAGCACCCAAAGCT
B1	Reverse	mt16,391-16,410	GAGGATGGTGGTCAAGGGAC
C1	Forward	mt29-48	CTCACGGGAGCTCTCCATGC
D1	Reverse	mt408-429	CTGTTAAAAGTGCATACCGCCA

The master mix was prepared in a UV irradiated 1.5 mL tube. The positive control used in this assay was HL60, and the negative control was UltraPure™ DNase/RNase-Free Distilled Water. The following conditions were set on the Applied Biosystems® GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA):

- HOLD 95°C for 11 minutes
- 36 CYCLES
  - 95°C for 10 seconds
  - 61°C for 30 seconds
  - 72°C for 30 seconds
- HOLD 70°C for 10 minutes
- HOLD 4°C indefinitely

Yield Gel

To confirm amplification product was present, a post-PCR yield gel was prepared. The gel was created with 2% DNA Typing Grade® Agarose (Life Technologies, Carlsbad, CA), and bromophenol blue dye was the loading dye to visualize the DNA product. The gel ran for approximately 20 minutes at 100V in 1x TAE buffer on the RunOne™ Electrophoresis Cell System (Embi Tec, San Diego, CA).

Cycle Sequencing

When amplification was complete, 5ul of ExoSAP-IT® (Life Technologies, Carlsbad, CA) was added to the product to be sequenced, and incubated on the Applied Biosystems® GeneAmp® PCR System 9700 at the following settings:

- 37°C for 15 minutes
- 80°C for 15 minutes
- HOLD 4°C indefinitely

Once clean-up of the amplified product was completed, cycle sequencing was performed using the Applied Biosystems BigDye® Terminator™ v1.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA). The optimal mtDNA template concentration for sequencing was between 0.5µL - 7.5µL, and the primers used in this assay are identical in sequence that were used for amplification, as seen in **Table 2**. Each sample well contained the following components for a final reaction volume of 15 ul, including the appropriate DNA template amount:

- BigDye Terminator v1.1
- BetterBuffer
- 3.3uM Primer A1- forward primer for HVI amplicon OR
- 3.3uM Primer B1- reverse primer for HVI amplicon OR
- 3.3uM Primer C1- forward primer for HVII amplicon OR
- 3.3uM Primer D1- reverse primer for HVII amplicon
- UltraPure™ DNase/RNase-Free Distilled Water

The following conditions were set on the GeneAmp® PCR System 9700:

- HOLD 96°C for 1 Minute
- 25 CYCLES
  - 96°C for 15 seconds
  - 50°C for 1 second
  - 60°C for 1 minute
- HOLD 15°C for 10 minutes
- HOLD 4°C indefinitely

Once this was completed, 55ul of XTerminator™ Master Mix was added to each sample well, which contained:

- UltraPure™ DNase/RNase-Free Distilled Water
- SAM™ Solution (Life Technologies, Carlsbad, CA)

- BigDye XTerminator™ (Life Technologies, Carlsbad, CA)

The plate was vortexed for 30 minutes at 2000rpm, and then centrifuged for 2 minutes at 1000 x g. It will then be loaded onto the Applied Biosystems® 3130xL Genetic Analyzer (Life Technologies, Carlsbad, CA) for analysis. The data will be reviewed via the software program MTExpert.

CHAPTER III

RESULTS

Three hairs (labeled 1, 2, and 3) were collected from five different subjects (labeled A-E). Hair 1 was always collected from the left side of the head, Hair 2 from the top, and Hair 3 from the right.

Table 3: Specifications of the Subjects Used. *Subjects are labeled A, B, C, D, and E. Four females and one male were involved in this study, all between the ages of 24-30.*

Subject	Ethnicity	Sex	Age	Hair Color	Texture	Length of Hair 1 (cm)	Length of Hair 2 (cm)	Length of Hair 3 (cm)
A	Caucasian	Female	24	Blonde	Straight	16.0	17.5	17.5
B	Caucasian	Female	24	Blonde	Straight	29.5	13.5	22.5
C	Hispanic	Female	24	Black	Curly	47.0	38.6	54.5
D	Hispanic	Female	28	Dark Brown	Curly	30.4	30.8	29.5
E	Caucasian	Male	30	Light Brown	Straight	26.2	39.0	22.2

Once the hairs were collected and measured, they were cut into sections and every fourth centimeter was collected. These sections underwent individual DNA extractions and then were

subjected to quantification via the multiplex assay. Each sample was quantified once. Subject *D* was concentrated via Microcon® DNA Fast Flow centrifugal filters prior to quantification, while all other subjects were quantified from the original extract.

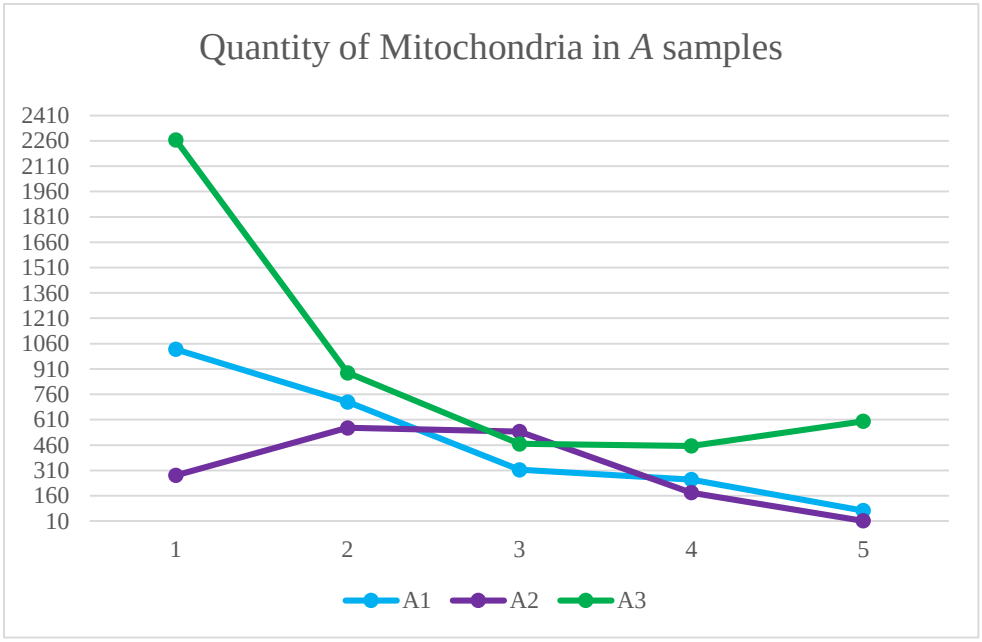
Quantification

SUBJECT A- FEMALE, BLONDE, AGE 24

Table 4: Subject A Quantification Results. *The quantity and deletion ratio of mitochondria found within the hairs collected from of subject A. The deletion ratio is represented as the ratio of mitochondria that have not been compromised. Graphical representation seen of table in* ***Figure 4.***

Quantity of Mitochondria in Samples from Subject *A*				**Deletion Ratio of Samples from Subject *A***			
Section	*A1*	*A2*	*A3*	*Section*	*A1*	*A2*	*A3*
1	1026.76	281.09	2265.65	*1*	0.95	0.68	0.96
2	715.76	562.31	887.23	*2*	0.90	0.93	0.97
3	313.90	540.57	467.19	*3*	0.92	0.93	0.52
4	255.90	178.49	455.40	*4*	0.90	0.91	0.93
5	72.15	11.73	601.01	*5*	0.81	0.80	0.76

a)



b)

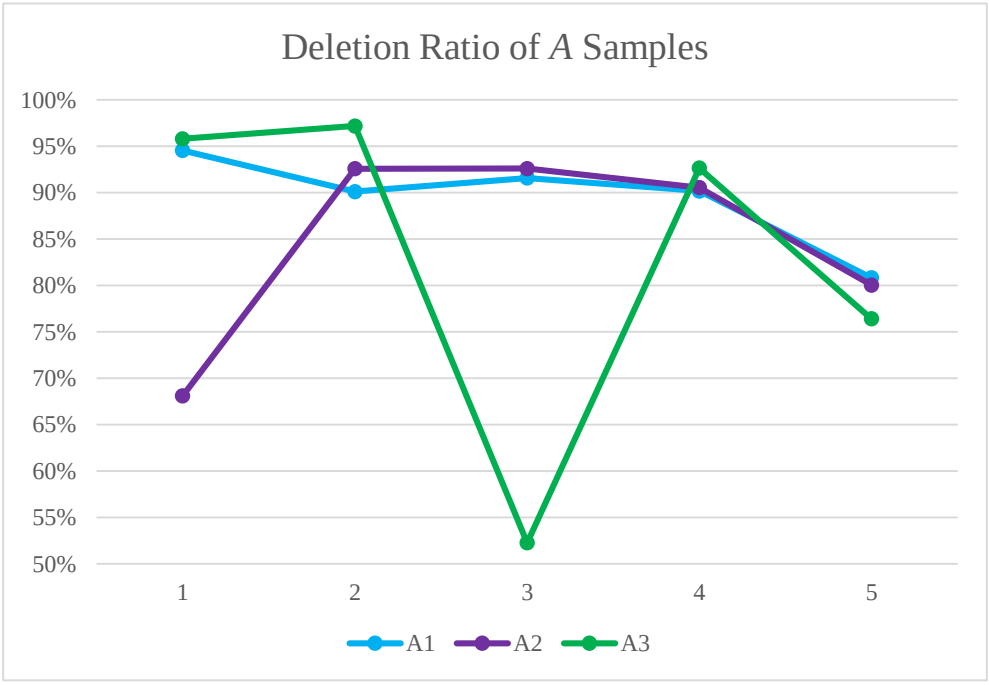


Figure 4: Subject A Quantification Results. *Graphical representation of* ***Table 4****. The X-axis of both graphs represents the sections of the hair. a) Quantity of mitochondria found within the hairs collected from of Subject A. b) Deletion ratio of mitochondria found within the hairs*

collected from Subject A. The deletion ratio is represented as the ratio of mitochondria that have not been compromised.

All hairs collected from Subject A were dissected into five sections, as these hairs ranged in length from approximately 16.0 cm to 17.5 cm, as seen in ***Table 3****. The nuclear DNA within these samples were too negligible to be quantified.*

There seems to be a general decrease in mitochondrial copy number throughout Subject *A*'s hairs. In Hair A1, the quantity of mitochondria in the distal end (section A1.5) was approximately 7% of that found in the proximal end (section A1.1), while in Hair A3, the quantity of mitochondria in the distal end (section A3.5) was approximately 25% of the quantity found in A3.1. In Hair A2, however, the quantity of mitochondria in the proximal end, section A2.1, was about half of the amount found in the following section, A2.2. The subsequent sections decreased in copy number. The distal end in this hair also had much less quantifiable mitochondria than distal ends in the other two hairs.

There was also a general decrease throughout the hair strands in the integrity and stability of the mitochondria. In the proximal section from Hair A1, only 5% of the mitochondria quantified had deletions in the major arc, contrary to the distal end, which had approximately 19% of the mitochondria with deletions present. Comparably, Hair A3 ranged from 4% containing deletions in the first section, to 24% in the last section. In section A3.3, a large drop in the integrity of the mtDNA was seen, though this may be due to human error, because section A3.4 had 41% more mitochondrial stability. In A2.1, the integrity of the mitochondria found

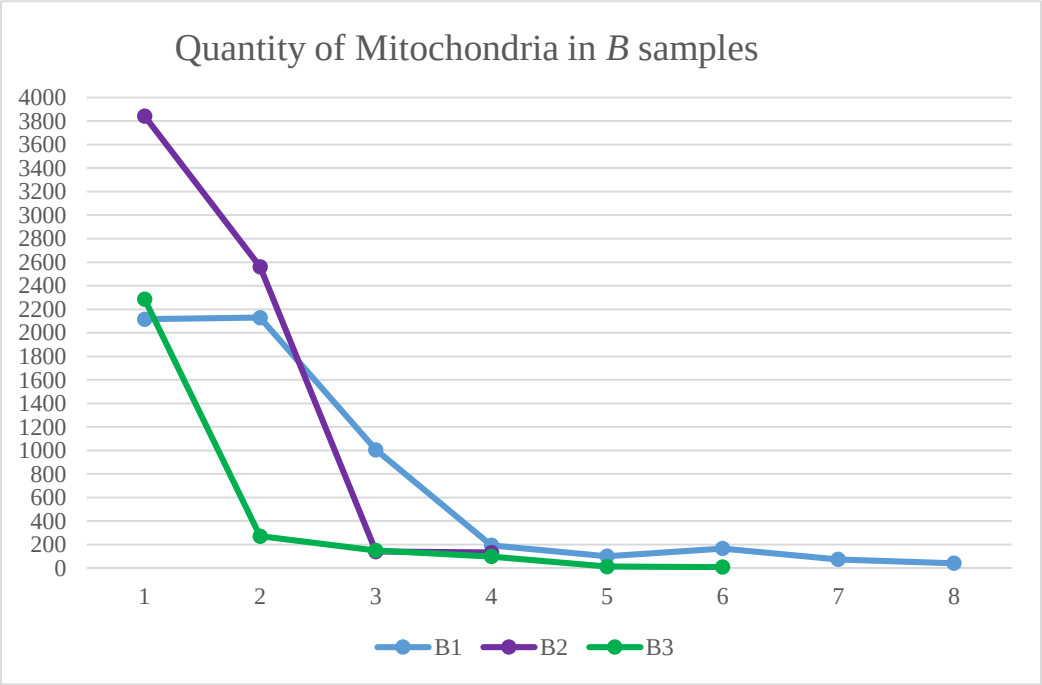
within this sample was much lower than the subsequent sections in Hair A2, which were similar to the analogous sections in the other hairs.

SUBJECT B- FEMALE, BLONDE, AGE 24

Table 5: Subject B Quantification Results. *The quantity and deletion ratio of mitochondria found within the hairs collected from of Subject B. The deletion ratio is represented as the ratio of mitochondria that have not been compromised. Graphical representation seen of table in* ***Figure 5.***

.Quantity of Mitochondria in Samples from Subject *B*				Deletion Ratio of Samples from Subject *B*			
Section	*B1*	*B2*	*B3*	*Section*	*B1*	*B2*	*B3*
1	2115.55	3842.38	2286.25	*1*	0.95	0.95	0.93
2	2128.50	2561.25	270.16	*2*	0.96	0.97	0.90
3	1004.72	139.48	149.14	*3*	0.90	0.96	0.83
4	192.79	131.59	97.84	*4*	0.93	0.84	0.81
5	100.27		11.93	*5*	0.89		0.61
6	165.85		8.11	*6*	0.90		0.61
7	72.83			*7*	0.85		
8	40.75			*8*	0.64		

a)



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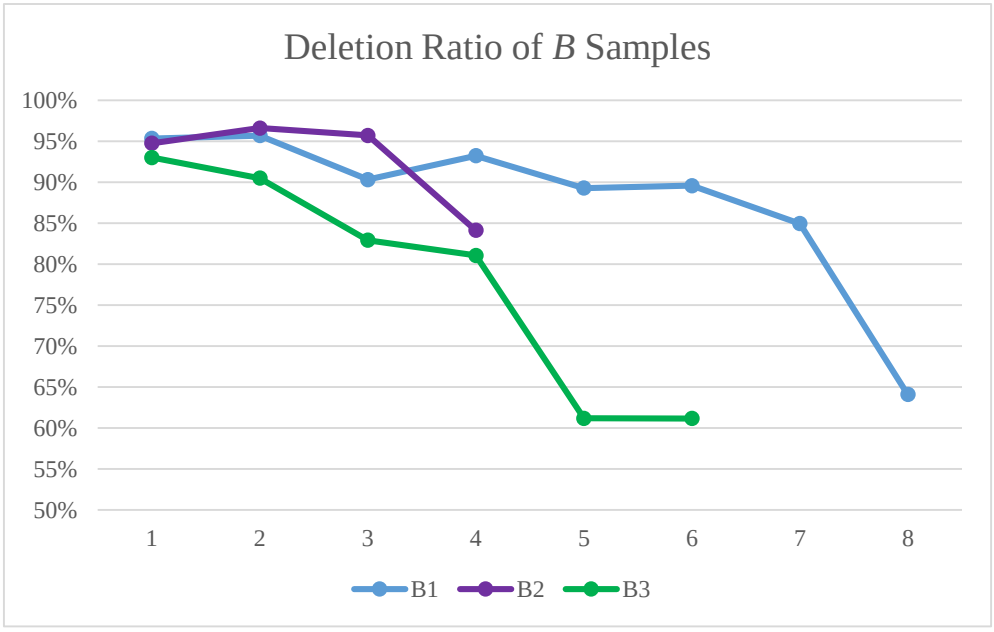


Figure 5: Subject B Quantification Results. *Graphical representation of* ***Table 5****. The X-axis of both graphs represents the sections of the hair. a) Quantity of mitochondria found within the hairs collected from of Subject B. b) Deletion ratio of mitochondria found within the hairs collected from Subject B. The deletion ratio is represented as the ratio of mitochondria that have not been compromised.*

The hair strand collected from the left side of the head from Subject *B* was 29.5 cm, the hair strand collected from the top of the head was 13.5cm, and the hair strand collected from the right side of the head was 22.5 cm, as seen in **Table 3**. The nuclear DNA within these samples were too negligible to be quantified.

There seems to be a general decrease in mitochondrial copy number throughout Subject *B*'s hairs. In Hair B1, the quantity of mitochondria in the distal end, section B1.8, was approximately 2% of that found in the proximal end, section B1.1. Also, the copy number

dropped by half between the second and third sections, and about 80% between the third and fourth sections. Hair B2 had similar results, as the quantity of mitochondria found in the distal end was about 3% of that found in the proximal end, and a significant loss in mitochondria was seen after the second section. However, in Hair B3, the quantity of mitochondria in the distal end was less than 1% of the quantity found in section B3.1, and a substantial loss in copy number was seen in the second section from the proximal end.

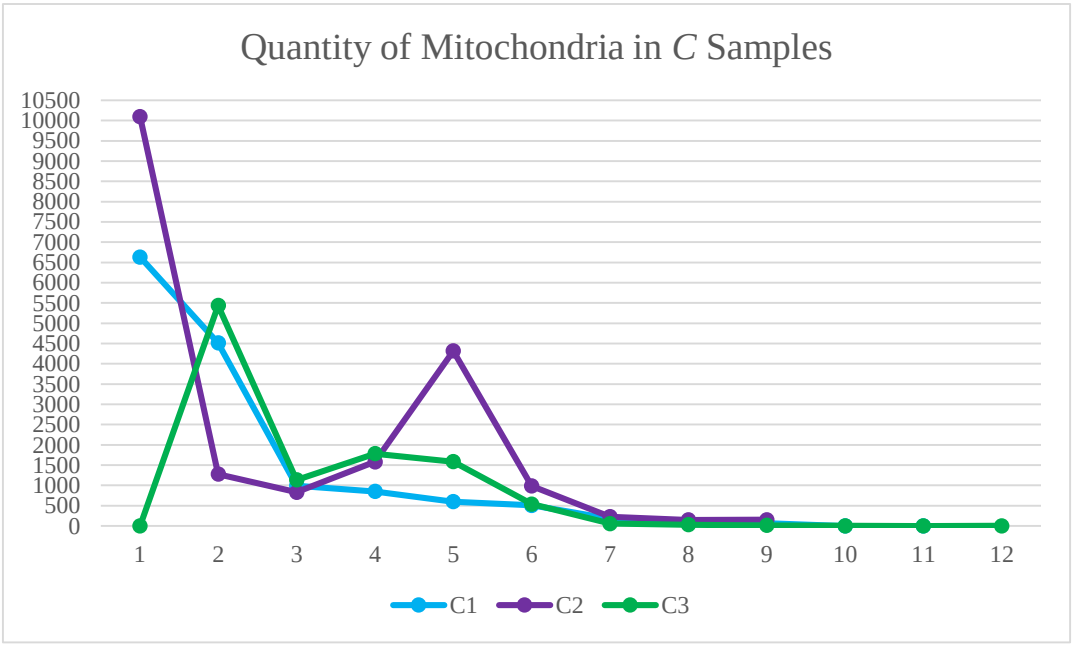
There was also a general decline throughout the hair strands in the integrity and stability of the mitochondria. In the proximal section from Hair B1, only 5% of the mitochondria quantified had deletions in the major arc, contrary to the distal end, which had approximately 37% of the mitochondria with deletions present. Comparably, Hair B3 ranged from 7% containing deletions in the first section, to 39% in the last section. Due to the length of B2, there was only an 11% difference in integrity between the first and last sections, but the drop in mtDNA integrity seen within B2 seems parallel with the other two hairs.

SUBJECT C- FEMALE, BLACK HAIR, AGE 24

Table 6: Subject C Quantification Results. *The quantity and deletion ratio of mitochondria found within the hairs collected from of Subject C. The deletion ratio is represented as the ratio of mitochondria that have not been compromised. Graphical representation seen of table in* ***Figure 6.***

Quantity of Mitochondria in Samples from Subject *C*				**Deletion Ratio of Samples from Subject *C***			
Section	*C1*	*C2*	*C3*	*Section*	*C1*	*C2*	*C3*
1	6631.21	10100.00	0.00	*1*	0.92	0.87	0.00
2	4512.33	1276.45	5438.11	*2*	0.90	0.71	0.90
3	993.10	830.32	1136.68	*3*	0.72	0.68	0.92
4	850.86	1579.29	1783.29	*4*	0.83	0.69	0.93
5	597.56	4314.50	1584.82	*5*	0.83	0.74	0.89
6	507.64	985.54	537.93	*6*	0.83	0.62	0.90
7	174.95	228.62	56.92	*7*	0.83	0.74	0.78
8	98.71	148.64	31.33	*8*	0.62	0.01	0.72
9	69.68	154.04	15.91	*9*	0.51	0.42	0.70
10	0.03		0.43	*10*	0.00	-0.25	0.00
11	0.00		0.03	*11*	0.00		0.00
12			2.89	*12*			0.58

a)



b)

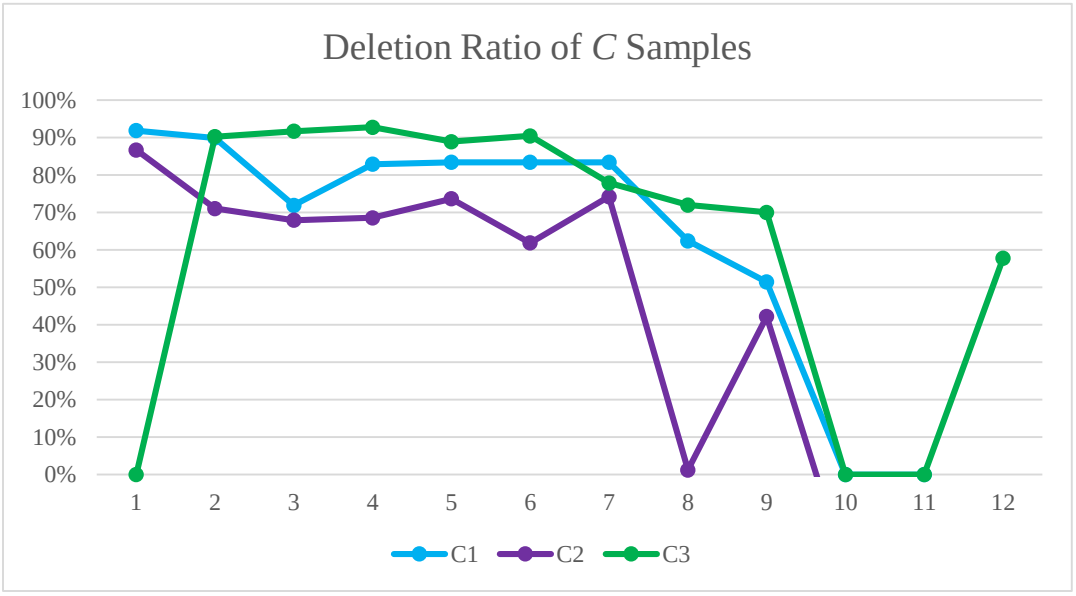


Figure 6: Subject C Quantification Results. *Graphical representation of* ***Table 6****. The X-axis of both graphs represents the sections of the hair. a) Quantity of mitochondria found within the hairs collected from of Subject C. b) Deletion ratio of mitochondria found within the hairs collected from Subject C. The deletion ratio is represented as the ratio of mitochondria that have not been compromised.*

The hair strand collected from the left side of the head from Subject *C* was 49.0 cm, the hair strand collected from the top of the head was 38.6 cm, and the hair strand collected from the right side of the head was 54.5 cm, as seen in **Table 3**. The nuclear DNA within these samples were too negligible to be quantified. The quantity of mitochondria seen within all three hair strands ranged quite widely from the proximal to the distal ends, and more mitochondria overall was found in Subject *C*'s hairs than Subjects *A* or B.

There seems to be a general decrease in mitochondrial copy number throughout Subject *C*'s hairs. In Hair C1, the quantity of mitochondria in the distal end, section C1.11, had negligible amount of quantifiable mtDNA. Also, the copy number dropped by about 78% between the second and third sections, and about 66% between the fifth and sixth sections. Hair C3 had similar results, but there was no quantifiable mitochondria found in the proximal end; the three subsequent sections were found to contain more mitochondria copy number than their counterparts in both C1 and C2. The subsequent section, C3.2, had a large amount of mitochondria, and the following section dropped by 80%. However, in Hair C2, the quantity of mitochondria throughout the hair strand had more erratic results. The proximal section C2.1 had over 10,000 mitochondrial copies, but the next section had about 88% less mitochondria. However, there was an upsurge in mitochondria in the fifth section, then the quantity fell again in the sixth section.

There was also a general decline throughout the hair strands in the integrity and stability of the mitochondria. In the proximal section from Hair C1, only 8% of the mitochondria

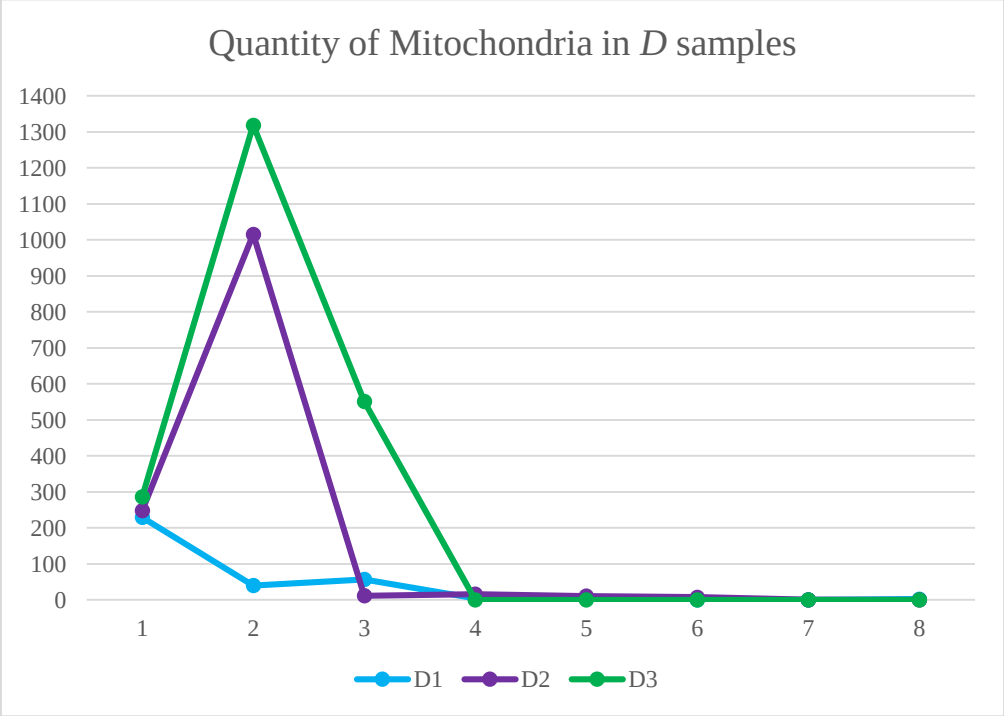
quantified had deletions in the major arc, contrary to the last two sections in the distal end, which showed that all the mitochondria present had compromised integrity. Because no mitochondria was quantified in section C3.1, no deletion ratio was calculated for that section, but starting with C3.2, the stability of the mitochondria slowly dwindled. In Hair C2, the first section had about 13% of the mitochondria with deletions, leading to a slow decline throughout the hair strand. The deletion ratio seen in section C2.10 was negative due to the discrepancies between the standard curves between the major and minor arc probes.

SUBJECT D- FEMALE, DARK BROWN HAIR, AGE 28

Table 7: Subject D Quantification Results. *The quantity and deletion ratio of mitochondria found within the hairs collected from of Subject D. The deletion ratio is represented as the ratio of mitochondria that have not been compromised. Graphical representation seen of table in* ***Figure 7.***

Quantity of Mitochondria in Samples from Subject *D*				**Deletion Ratio of Samples from Subject *D***			
Section	*D1*	*D2*	*D3*	*Section*	*D1*	*D2*	*D3*
1	229.45	248.27	286.56	*1*	0.91	0.90	0.80
2	40.07	1015.76	1318.82	*2*	0.87	0.91	0.86
3	56.96	11.81	551.26	*3*	0.87	0.90	0.85
4	4.86	16.28	0.00	*4*	0.71	0.91	0.00
5	4.86	10.96	0.00	*5*	0.75	0.92	0.00
6	0.00	7.88	0.00	*6*	0.00	0.81	0.00
7	0.00	0.60	0.00	*7*	0.00	0.00	0.00
8	2.32	0.00	0.00	*8*	0.66	0.00	0.00

a)



b)

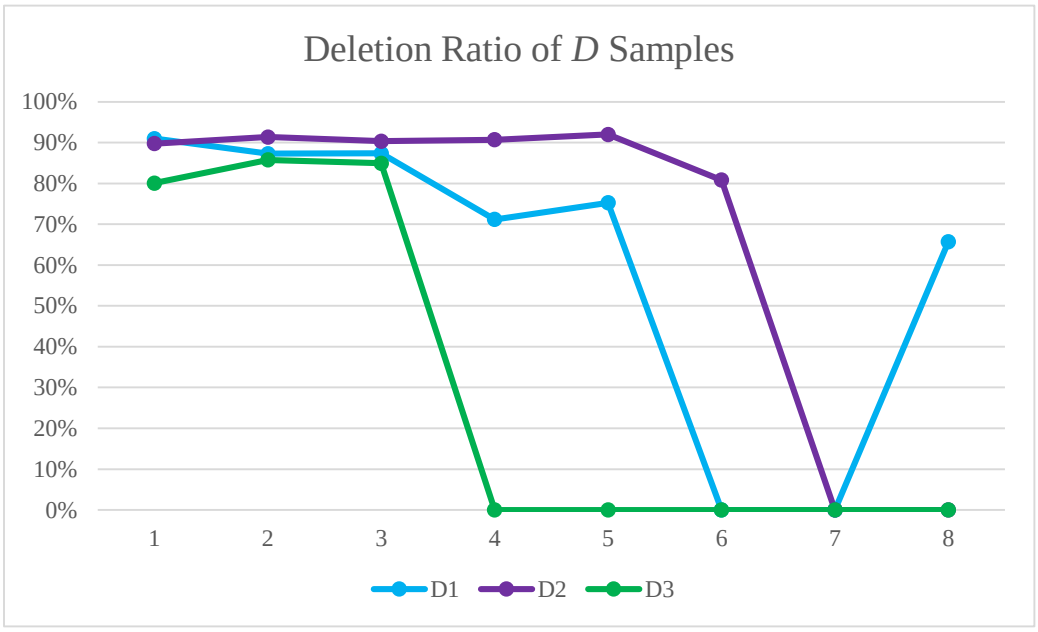


Figure 7: Subject D Quantification Results. *Graphical representation of* ***Table 7****. The X-axis of both graphs represents the sections of the hair. a) Quantity of mitochondria found within the hairs collected from of Subject D. b) Deletion ratio of mitochondria found within the hairs collected from Subject D. The deletion ratio is represented as the ratio of mitochondria that have not been compromised.*

The hair strand collected from the left side of the head from Subject *D* was 30.4 cm, the hair strand collected from the top of the head was 30.8 cm, and the hair strand collected from the right side of the head was 29.5 cm, as seen in **Table 3**. The nuclear DNA within these samples were too negligible to be quantified. The DNA extracts from Subject *D* were concentrated via Microcon® DNA Fast Flow centrifugal filters prior to quantification; however, multiple samples had undeterminable results, thus listed as zero. It was found that the integrity of the mitochondrial that was quantified was mostly intact, as no more than 34% of the quantified results had a deletion in the major arc.

There seems to be a general decrease in mitochondrial copy number throughout Subject *D*'s hairs. In Hair D1, the quantity of mitochondria in the distal end, section D1.8, had approximately 1% of the amount of mitochondria that was found in the proximal section D1.1. Also, the copy number dropped by about 83% between the first and second sections, and 91% between the third and fourth sections. Two of the sections of this hair were unable to be quantified. Hair D2 had similar results, but only the distal end section was unable to be quantified. Section D2.2 had the mitochondria quantified, as here was roughly 24% less quantifiable mitochondria in section D2.1 than D2.2. D2.3 also saw a significant plunge in copy number compared to the preceding section. The rest of the sections in this hair were found to be in the same copy number range as D2.3. In Hair D3, only the first three sections had results. This hair followed the pattern that D2 did, as the second section had 79% more mitochondria than the first section. However, section D3.3 only had about 58% less mitochondria than D3.2, rather than the 99% drop seen in D2.

There was also a general decline throughout the hair strands in the integrity and stability of the mitochondria. In the proximal section from Hair D1, only 9% of the mitochondria quantified had deletions in the major arc, contrary to the last sections in the distal end, which showed that about 34% the mitochondria present had compromised integrity. Because no mitochondria was quantified in sections D1.6 or D1.7, no deletion ratio was calculated for those sections. In Hair D2, the first section had about 10% of the mitochondria with deletions, but the rest of the hair strand did not seem to waiver much until the sixth section that showed about 19%

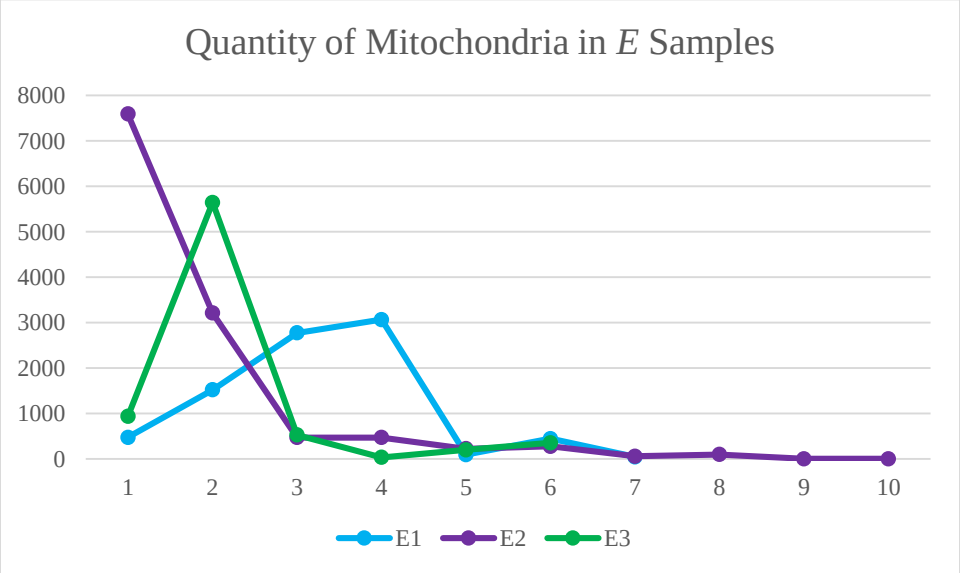
with deletions. Because no mitochondria was quantified in sections D2.7 or D2.8, no deletion ratio was calculated for those sections. The deletion ratio seen in the proximal section of Hair D3 was about 10% lower than its counterparts seen in D1 and D2, but the next two sections seen in this hair, D3.2 and D3.3, the integrity was slightly better. Because no mitochondria was quantified in sections D3.4 through D3.8, no deletion ratio was calculated for those sections.

SUBJECT E- MALE, LIGHT BROWN HAIR, AGE 30

Table 8: Subject E Quantification Results. *The quantity and deletion ratio of mitochondria found within the hairs collected from of Subject E. The deletion ratio is represented as the ratio of mitochondria that have not been compromised. Graphical representation seen of table in* ***Figure 8.***

Quantity of Mitochondria in Samples from Subject *E*				**Deletion Ratio of Samples from Subject *E***			
Section	*E1*	*E2*	*E3*	*Section*	*E1*	*E2*	*E3*
1	472.40	7591.7	932.87	*1*	0.81	0.88	0.93
2	1516.51	3209.27	5644.14	*2*	0.84	0.86	0.83
3	2773.41	468.51	524.46	*3*	0.89	0.86	0.77
4	3064.85	469.42	31.66	*4*	0.90	0.84	-0.11
5	89.83	221.99	196.62	*5*	0.93	0.84	0.96
6	443.16	274.12	350.33	*6*	0.78	0.80	0.82
7	42.57	54.62		*7*	0.46	0.82	
8		95.05		*8*		0.77	
9		0.00		*9*		0.00	
10		0.00		*10*		0.00	

a)



b)

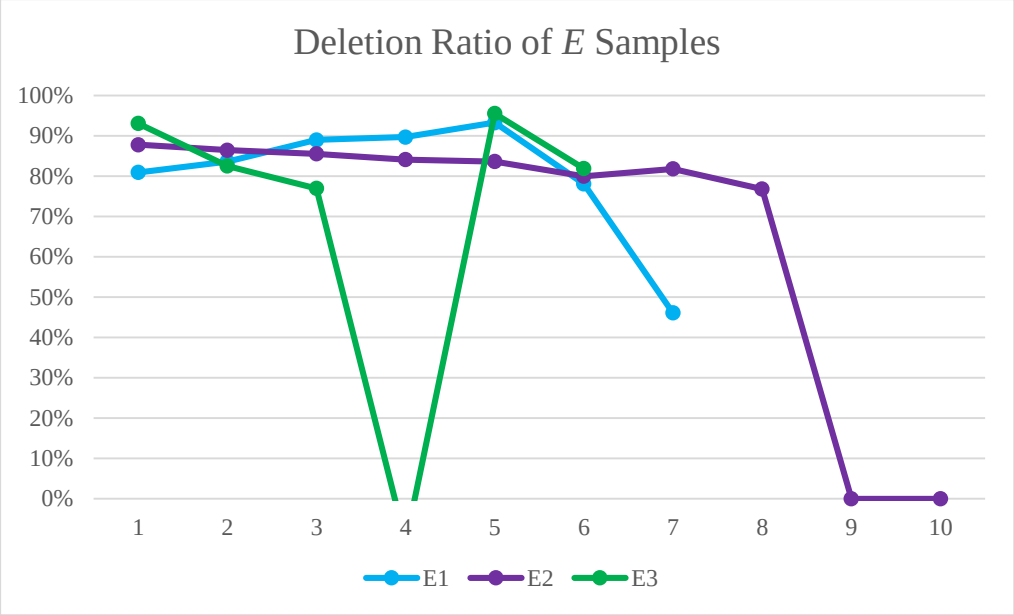


Figure 8: Subject E Quantification Results. *Graphical representation of* ***Table 8****. The X-axis of both graphs represents the sections of the hair. a) Quantity of mitochondria found within the hairs collected from of subject E. b) Deletion ratio of mitochondria found within the hairs collected from subject E. The deletion ratio is represented as the ratio of mitochondria that have not been compromised.*

The hair strand collected from the left side of the head from Subject *E* was 26.2 cm, the hair strand collected from the top of the head was 39.0 cm, and the hair strand collected from the right side of the head was 22.2 cm, as seen in **Table 3**. The nuclear DNA within these samples were too negligible to be quantified. The last two sections from E2 had undeterminable results. The deletion ratio seen in section E3.4 was negative due to the discrepancies between the standard curves between the major and minor arc probes.

In Hair E1, the results were irregular. The section with the most mitochondrial copy number is E1.4, rather than the first or second sections like the hairs from the other four subjects. Though there was a 97% drop in copy number in E1.5, the quantity of mitochondria increased again in the sixth section. In Hair E2, there was a trend of decrease throughout the hair. There was a 58% dip in mitochondrial copy number from section E2.1 to E2.2, and E2.3 had 85% less mitochondrial copy number than E2.2. Hair E3 had the most mitochondria in section E3.2; the results did not seem to follow the trend seen in the other subjects though.

There was also a general decline throughout the hair strands in the integrity and stability of the mitochondria. In the proximal section from Hair E1, about 19% of the mitochondria quantified had deletions in the major arc, contrary to the last sections in the distal end, which showed that about 54% the mitochondria present had compromised integrity. However, there was a slight increase in integrity between sections E1.2 through E1.5, but there was a dive between the fifth and sixth sections. In Hair E2, the first section had about 12% of the mitochondria with deletions, but the rest of the hair strand did not seem to waiver much until the eighth section that showed about 23% with deletions. Because no mitochondria was quantified in sections E2.9 or E2.10, no deletion ratio was calculated for those sections. The deletion ratio seen in the proximal section of Hair E3 showed about 7% of the mitochondria quantified had deletions in the major arc, but the next two sections seen in this hair, E3.2 and E3.3, the stability of the mitochondria dropped 10% and 16% respectively. The fifth section had the most stable mitochondria.

Cycle Sequencing

Another part of this study was to see how mitochondrial DNA behaves throughout the hair strand. Many studies have been performed on multiple hairs strands to test heteroplasmy on various sections of the head, but it was unknown as to whether mitochondrial DNA changes in a single hair strand. One hair from Subjects *A*, *B*, *C*, and *E* was chosen for amplification and ultimately cycle sequencing. Subject *D*'s samples were not involved in this study due to variance in protocol that was followed by the other four subjects. The first, middle, and last section from each hair were amplified and sequenced to note any differences between the proximal and distal ends of these hairs.

Hair A3 was chosen from Subject *A* for this process, as seen in **Table 9**. The signatures seen from the first and middle section were identical; however, section A3.5 had significant differences from the preceding ones. A change in HVI at position 16239 was seen, and the C-Stretch seen in this section had one less cytosine insertion. No heteroplasmy was seen.

For Subject *B*, Hair B1 was chosen for sequencing, as seen in **Table 10**. It was apparent that the signature from the distal end of the hair was quite distinct from the proximal end. Some heteroplasmy was seen in HVI in section B1.5, which increased dramatically towards the distal end. Also, throughout the hair strand, position 150 went from a full substitution in the proximal section, to heteroplasmy in the middle section, back to the full substitution in the distal section.

Hair C3 from Subject *C* was chosen next for sequencing, as seen in **Table 11**. Interestingly, there were very few instances of heteroplasmy seen throughout this hair strand. Furthermore, studying this hair from the proximal to the distal end, it seemed to progressively

become more comparable to the Cambridge Reference Sequence, unlike Hair A3 and Hair B1 which diverged more throughout the hair strand.

When sequencing Hair E3 (**Table 12**) it was originally thought these samples would be the most analogous to the Cambridge Reference Sequence, as the subject was a Caucasian male and the Reference Sequence was also taken from a Caucasian male, but this was hardly the case. In actuality, these samples had the most disparities of all the samples that were studied. Unlike the previous subjects, Subject *E* had many modifications within the HVI region, which lead to a C-Stretch, rather than HVII; this hair was also the only one that had heteroplasmy seen within the proximal section.

Every sample had a cytosine inserted at position 315.1, but MTExpert flagged this as a unique insertion amongst the signatures stored within its database, as seen in **Appendix Figure 1**. This may suggest a polymerase error during amplification.

Table 9: A3 Signatures. *Hair Strand A3 was chosen for sequencing from Subject A. Sections A3.1, A3.3, and A3.5 were chosen for amplification and sequencing. The positions are color-coded for each section. A C-Stretch was seen in HVII in all sections, but in Section A3.5 did not have a C insertion at position 309.1. The percentages calculated were out of 11,768 signatures within the database.*

A3 SIGNATURES				
Section	***Position***	***Base Change***	***% Population Seen***	***Comments***
A3.1	93	G → A	3.3%	
	152	C → T	27.4%	
	263	G → A	99%	
	309.1	C inserted	Unique	C-Stretch
	315.1	C inserted	Unique	C-Stretch
A3.3	93	G → A	3.3%	
	152	C → T	27.4%	
	263	G → A	99%	
	309.1	C inserted	Unique	C-Stretch
	315.1	C inserted	Unique	C-Stretch
A3.5	16239	T → C	1.1%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch

Table 10: B1 Signatures. *Hair Strand B1 was chosen for sequencing from Subject B. Sections B1.1, B1.5, and B1.8 were chosen for amplification and sequencing. The positions are color-coded for each section. At position 150, the Cambridge Reference Sequence contains a T, while in section B1.5, a C was substituted for the T, and heteroplasmy was seen with C as the major base, and T as the minor base. The percentages calculated were out of 11,768 signatures within the database.*

B1 SIGNATURES				
Section	***Position***	***Base Change***	***% Population Seen***	***Comments***
B1.1	150	T → C	13.9%	
	239	C → T	13.0%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch
B1.5	16224	Heteroplasmy	Unique	Major Base-T; Minor Base- C
	16270	Heteroplasmy	Unique	Major Base-C; Minor Base- T
	150	Heteroplasmy	Unique	T → C
	239	C → T	13.0%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch
B1.8	16193	Heteroplasmy	0.02%	Major Base-C; Minor Base- T
	16223	Heteroplasmy	Unique	Major Base-C; Minor Base- T
	16224	Heteroplasmy	Unique	Major Base-T; Minor Base- C
	16270	Heteroplasmy	Unique	Major Base-C; Minor Base- T
	16362	Heteroplasmy	Unique	Major Base-T; Minor Base- C
	150	T → C	13.9%	
	239	C → T	13.0%	
	263	G → A	99%	
	279	Heteroplasmy	0.01%	Major Base T; Minor Base- C
	315.1	C inserted	Unique	C-Stretch

Table 11: C3 Signatures. *Hair Strand C3 was chosen for sequencing from Subject C. Sections C3.1, C3.6, and C3.10 were chosen for amplification and sequencing. The positions are color-coded for each section. At position 16224, the Cambridge Reference Sequence contains a T, while in section C3.6, a C was substituted for the T, and heteroplasmy was seen with C as the major base, and T as the minor base. At position 16239, the Cambridge Reference Sequence contains a C, while in section C3.12, a T was substituted for the C, and heteroplasmy was seen with T as the major base, and C as the minor base. The percentages calculated were out of 11,768 signatures within the database.*

C3 SIGNATURES				
Section	***Position***	***Base Change***	***% Population Seen***	***Comments***
C3.1	16224	C → T	5.1%	
	16270	T → C	6.7%	
	73	G → A	72.3%	
	150	T → C	13.9%	
	263	G → A	99%	
	279	C → T	0.3%	
	315.1	C inserted	Unique	C-Stretch
C3.6	16061	Heteroplasmy	Unique	Major Base- T; Minor Base- C
	16224	Heteroplasmy	Unique	T → C
	16270	T → C	6.70%	
	73	G → A	0.723	
	150	T → C	13.90%	
	263	G → A	0.99	
	279	C → T	0%	
	315.1	C inserted	Unique	C-Stretch
C3.12	16239	Heteroplasmy	Unique	C → T
	263	G → A	0.99	
	315.1	C inserted	Unique	C-Stretch

Table 12: E3 Signatures. *Hair Strand E3 was chosen for sequencing from Subject E. Sections E3.1, E3.4, and E3.6 were chosen for amplification and sequencing. The positions are color-coded for each section. At position 16189, the Cambridge Reference Sequence contains a C, while in section E3.1, a T was substituted for the C, and heteroplasmy was seen with T as the major base, and C as the minor base. At position 16296, the Cambridge Reference Sequence contains a T, while in section E3.1, a C was substituted for the T, and heteroplasmy was seen with C as the major base, and T as the minor base. The percentages calculated were out of 11,768 signatures within the database.*

E3 SIGNATURES				
Section	***Position***	***Base Change***	***% Population Seen***	***Comments***
E3.1	16004	T → C	Unique	
	16126	C → T	15.30%	
	16182	C → A	3.4%	C-Stretch
	16183	C → A	9.6%	C-Stretch
	16189	Heteroplasmy	0.1%	C → T
	16294	T → C	11.9%	
	16296	Heteroplasmy	0.02%	T → C
	73	G → A	72.3%	
	193	C → T	23.2%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch
E3.4	16004	T → C	Unique	
	16182	C → A	3.4%	C-Stretch
	16183	C → A	9.6%	C-Stretch
	16189	C → T	23.5%	
	16223	Heteroplasmy	Unique	Major Base-C; Minor Base- T
	16294	T → C	11.9%	
	16296	T → C	3.4%	
	73	G → A	72.3%	
	193	C → T	23.2%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch

E3.6	16004	T → C	Unique	
	16126	C → T	15.3%	
	16182	C → A	3.4%	C-Stretch
	16183	C → A	9.6%	C-Stretch
	16189	C → T	23.5%	
	16294	T → C	11.9%	
	16296	T → C	3.4%	
	16298	C → T	6.7%	
	73	G → A	72.3%	
	193	C → T	23.2%	
	263	G → A	99%	
	315.1	C inserted	Unique	C-Stretch

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Quantification

When quantifying the mtDNA from all five subjects, Subjects *A* and *B* were the only two that yielded results for every sample. On the other hand, Subjects *C*, *D*, and *E*, had at least one sample yield undeterminable results, meaning the assay was unable to quantify the DNA present. This could be due to many things. One explanation could be that these samples contained negligible amount of DNA, disallowing the probes and primers to anneal properly; another explanation could be PCR inhibition.

The subjects with blonde hair seemed to show a general decrease of mitochondrial copy number, as well as the deletion ratio. Subject *A* showed to have a sudden drop in integrity in section A3.3, but this may be due to human error, as the following sections had much higher ratios. Subject *B* had a large decrease in the quantity of mitochondria throughout the hair, but the integrity of this DNA seemed to be intact, leading to the conclusion that perhaps inhibition was present, rather than the lack of actual DNA.

On the contrary, there was no specific trend followed by the quantity of mitochondria within samples tested from Subjects *C*, *D*, or *E*. In many cases, the proximal section quantified less mitochondrial DNA than in the following section, leading to the belief that inhibition had occurred. There seems to be somewhat of a general decrease of copy number, and deletion ratio

throughout these samples, but there is more uncertainity with analyzing them. It has also been made known that the deletion ratio may be misleading. Since the deletion ratio is dependent on the copy number, it may provide an inflated percentage as to the amount of compromised mitochondria, especially within the samples collected towards the distal end of the hairs.

The $mtDNA_{CN}$/ $mtDNA_{DR}$ multiplex qPCR assay was originally developed for biomedical research on mitochondrial effects on the aging process, and the samples used to develop this assay were blood, which generally have a great amount of DNA. Because the initial intention of this assay was for high-yield samples that are commonly seen within research purposes, the general validation standards for forensic analysis were not considered. Therefore, it is unknown, past this research, how this assay reacts to the type of low-template DNA usually encountered in a forensic setting.

It is well-known that melanin is major inhibitor of PCR, and many validation studies include this in their study when testing for sensitivity to inhibition. There is no previous study to test this assay for inhibition sensitivity, thus it is unspecified how it performs under different amounts of melanin. Both Subjects *A* and *B* have blonde hair, which means their hair contains very little eumelanin, while Subjects *C, D,* and *E* have darker hair, suggesting more eumelanin. It has been suggested that eumelanins can cause DNA strand breaks when not in the presence of other molecules within the cell, (16) which may explain the erratic data seen within the subjects with darker hair.

It should be noted also that melanin has shown to bind to DNA polymerase to construct a complex that slows the overall movement of the polymerase (17). This may explain why

quantifying the darker-haired subjects was challenging. Subjects *A*, *B*, *C*, and *E* were only concentrated with Microcon® DNA Fast Flow centrifugal filters prior to amplification and cycle sequencing to ensure optimal amplification. However, Subject *D*'s samples were concentrated down via the Microcon® filters to further purify them prior to quantification. This was done to evaluate whether further purification will affect the quantification positively. However, the results gathered from Subject *D*'s samples were consistent with those seen with Subject *C*'s and Subject *E*'s, suggesting that either the amount of melanin was too high to be fully removed.

Cycle Sequencing

In order to see how amplification and sequencing are affected by mitochondrial copy number, hairs A3, B1, C3, and E3 were selected, as seen in **Table 13**. Due to the variance in protocol that Subject *D*'s samples underwent, no hairs were chosen for sequencing.

Table 13: Hairs Chosen for Sequencing. *Hairs A3, B1, C3, and E3 were chosen to be sequenced, specifically the first, middle, and last sections of each. The quantity of mitochondria in each section that the multiplex mtDNA$_{CN}$/ mtDNA$_{DR}$ qPCR assay quantified are represented.*

A3		***B1***		***C3***		***E3***	
Section	**Quantity**	**Section**	**Quantity**	**Section**	**Quantity**	**Section**	**Quantity**
1	2265.65	1	2115.55	1	0.00	1	932.87
3	467.19	5	100.27	6	537.93	4	31.66
5	601.01	8	40.75	12	2.89	6	350.33

These hairs were chosen specifically to see a broad range in mitochondrial copy number, and to see if copy number affected the amplification and sequencing process. Hair A3 overall had the most mitochondria compared to the rest of the samples, while hair C3 had the least. The control

region, also known as the HVI and HVII amplicons, were amplified separately, and both forward and reverse primers for each amplicon were amplified separately as well.

Interestingly, all sections were amplified and sequenced successfully, even in section C3.1 which had undeterminable quantification results. This could suggest that when this section, as well as the others with low copy numbers, was concentrated via the Microcon® DNA Fast Flow centrifugal filters, some inhibition was removed, thus allowing for effective amplification. Thus, using some sort of filtration device is recommended for amplification and sequencing of hair samples.

It was also noteworthy to mention that in all samples, the signatures towards the distal end did not match the signatures seen in the proximal end, suggesting that as the hair grows, the mtDNA experiences fluctuations. This could be due to sheer exposure to the environment, that new hair growth has yet to encounter.

Conclusions

It has been recognized that this study was insufficient to truly test the potential of the $mtDNA_{CN}$/ $mtDNA_{DR}$ multiplex qPCR assay. Before it can be used for forensic purposes, it must undergo a full validation study, to test its strengths and weaknesses when utilized for samples commonly seen in the field, such as hair. Also, only five subjects were chosen for this study, and there was very little diversity between them. Both Subjects *A* and *B* were Caucasian, with straight blonde hair; both Subjects *C* and *D* were Hispanic, with curly dark hair, and Subject *E* was the only male involved. It would be in the best interest of understanding this assay if more

subjects with various hair types were involved, as well as test different protocols when inhibition is suspected to be present. It does seem that this assay is sensitive enough to measure minimal amounts of mitochondrial DNA, which can be useful for low-template DNA samples. After gathering much more information regarding this assay, it may be found to be quite helpful in the forensic field.

However, for sequencing purposes, it is recommended to choose a section as close to the proximal end as possible, as these signatures are probably the most accurate; these sections usually were seen to have the highest quantity of mitochondria, as well as the most stable mitochondria of the hair strand. As the hair grows, more heteroplasmy and substitutions were seen, and if comparing an evidence sample to a reference sample, these sections may not provide any conclusive results.

APPENDIX

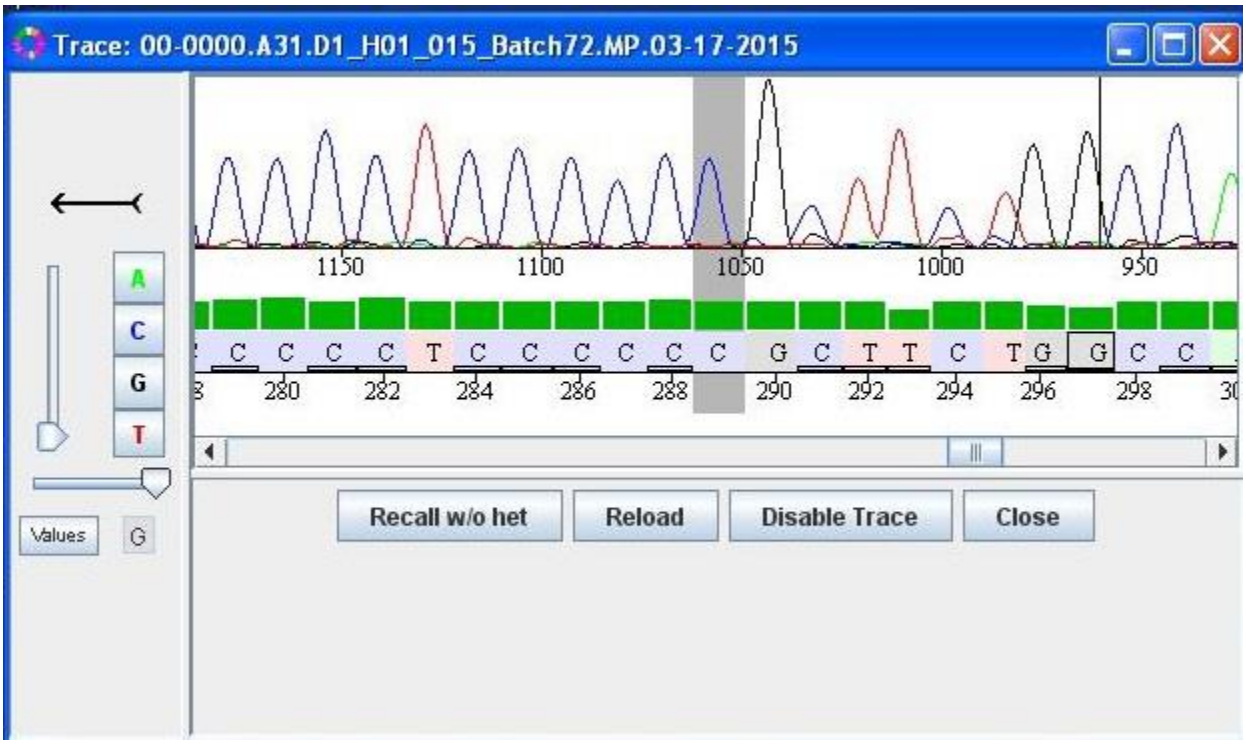


Figure 1: C-Insertion at position 315.1 in Section A3.1. *Every sample that was cycle sequenced had a cytosine inserted at position 315.1, but MTExpert flagged this as a unique insertion amongst the signatures stored within its database. This may suggest a polymerase error during amplification. This example is from section A3.1, from the transcript produced by the reverse primer for the HVII region, D1.*

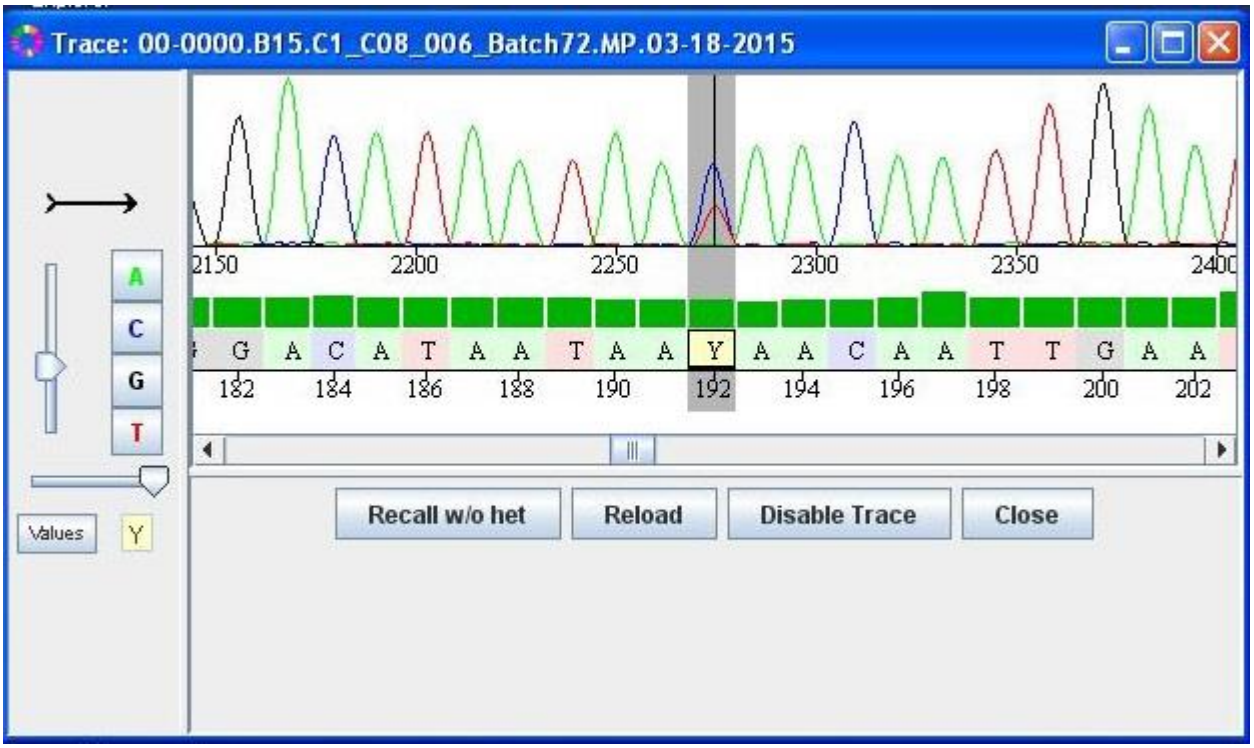


Figure 2: Heteroplasmy seen within Section B1.5. *At position 150, a C was substituted for the T (as seen in the Cambridge Reference Sequence), and heteroplasmy was seen with C as the major base, and T as the minor base. This example is from the transcript produced by the forward primer for the HVII region, C1.*

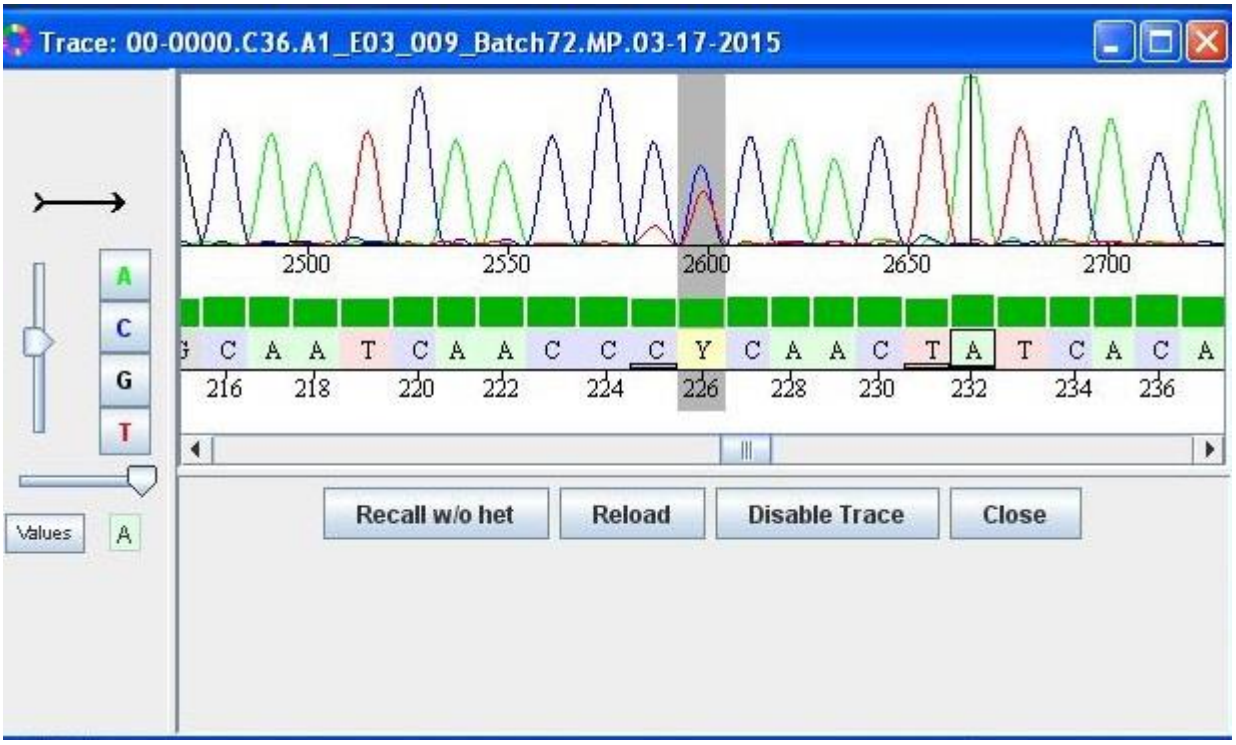


Figure 3: Heteroplasmy seen within Section C3.6. *At position 16224, a C was substituted for the T (as seen in the Cambridge Reference Sequence), and heteroplasmy was seen with C as the major base, and T as the minor base. This example is from the transcript produced by the forward primer for the HVI region, A1.*

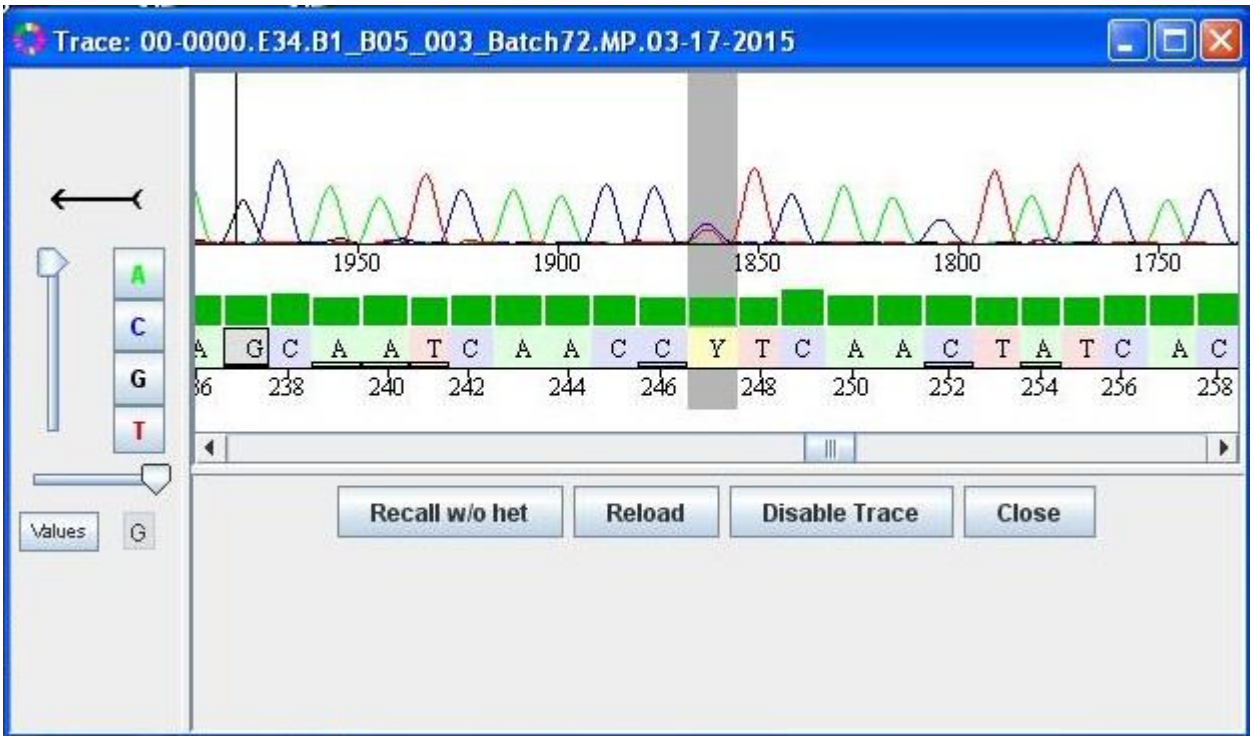


Figure 4: Heteroplasmy seen within Section E3.4. *At position 16223, heteroplasmy was seen with C as the major base, and T as the minor base. This example is from the transcript produced by the reverse primer for the HVI region, B1.*

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