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Evaluation of the control region of the mitochondrial genome is a common practice for forensic casework and research purposes. Since no kit is currently commercially available for the amplification of mitochondrial DNA (mtDNA), its sequencing procedure is time-consuming and laborious. Six steps are generally followed: DNA extraction, quantification and normalization, amplification of two regions (hypervariable regions 1 and 2), cycle sequencing, capillary electrophoresis and data analysis.

This project evaluated a mtDNA direct amplification kit by performing developmental and internal validations. The studies performed included sensitivity, stability, reproducibility, case- type samples, mixtures and accuracy. The mtDNA direct amplification kit successfully amplified reference samples used in each study without the need of extraction and quantification steps. In addition, mtDNA profiles were obtained from the sequenced amplification products. Using the validated direct amplification procedure in the laboratory will improve workflow, decrease operational cost and reduce the possibility of error by minimizing sample handling.

DEVELOPMENTAL AND INTERNAL VALIDATION

OF A MITOCHONDRIAL DNA DIRECT AMPLIFICATION KIT FOR FORENSIC

REFERENCE SAMPLES

Alessandra Alicea-Centeno, B.S.

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DEVELOPMENTAL AND INTERNAL VALIDATION OF A MITOCHONDRIAL DNA DIRECT AMPLIFICATION KIT FOR FORENSIC REFERENCE SAMPLES

THESIS

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas

Health Science Center at Fort Worth

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For the Degree of MASTER OF SCIENCE By

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Fort Worth, Texas

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CHAPTER 1

INTRODUCTION

Currently, about 90,000 missing persons records are active according to the National Missing and Unidentified Persons System (NamUs). Every year an estimated 4,400 unidentified human remains are reported in the United States, 23% (or 1,000) of those cases remain unidentified after a year (1). When working with unidentified human remains, the identification of the victim is very important. Identifying the remains will allow the family of the victim to stop questioning the whereabouts of their loved one and to properly bury them. In order to use DNA as a tool in the process of human identification, the collection of family reference samples is necessary to make comparisons with the victim's DNA recovered from the remains. The reference samples are obtained from the victim's available family members. They typically provide either buccal swabs obtained from the inside of their cheek or peripheral blood samples. The most appropriate family members to use as reference samples will vary depending on the victim's relationship to them. It is usually preferred to get the reference samples from close relatives such as the birth parents of the victim and the victim's siblings. In addition, if the victim has any biological children, the other biological parent of the child would be a good reference sample to use for DNA identification of the victim.

Once the reference samples have been processed, the data is uploaded into the National DNA Index System (NDIS). NDIS is a database containing DNA profiles divided in different categories known as indexes. One of these indexes stores the DNA profiles obtained from the family reference samples. Typically, two references from the reported missing person are attempted to be collected in order to create a family pedigree. Another index stores DNA

profiles generated from unidentified human remains (2). When a profile is acquired from an unidentified set of remains, it is uploaded into NDIS and is searched against the family reference index containing the pedigrees in order to make potential familial associations through DNA analysis.

In some forensic laboratories, such as the University of North Texas Center for Human Identification (Fort Worth, Texas), both nuclear and mitochondrial DNA analysis are attempted, on all unidentified human remains and potential reference samples. However, when processing human remains, the condition of the remains may not always be optimal for the recovery of nuclear DNA. Depending on the age of the remains and their exposure to different environmental conditions, nuclear DNA may be too degraded to obtain a full or even partial nuclear profile. Oftentimes a mitochondrial DNA (mtDNA) profile may be generated from these remains because of mtDNA's circular structure which makes it more resistant to degradation by nucleases (3). Mitochondrial DNA is found inside the mitochondria of the cell; the number of mitochondrial genomes (mtGenomes) per cell varies from hundreds to thousands of copies depending on the cell type (4-7). This higher copy number characteristic of mtDNA in contrast with only two copies of nuclear DNA per cell, can make amplification more successful even in the presence of inhibitors. Moreover, this characteristic also makes mtDNA more prone to contamination. The scientists performing experiments with mtDNA have to be more cautious than those working with nuclear DNA to avoid contaminating the samples with their own mtDNA. In addition, mtDNA is maternally inherited, which makes it useful to establish maternal familial relationships (8). Individuals that are maternally related will share the same mitochondrial haplotype, which is helpful in solving missing person's cases and mass disaster investigations. However, the fact that maternal relatives share their mtDNA can reduce the

power of discrimination of mtDNA testing but at the same time it increase the number of relatives that can be used to create the reference family pedigrees.

The mtGenome is approximately 16,569 base pairs (bp) and has both a coding and a non-coding region. A diagram of the entire genome can be observed in Figure 1 (9). The non-coding region is known as the control region (CR); more variation occurs in this region and it has no known deleterious effects on individuals (10). The CR contains sections known as hypervariable region 1 (HV1) and hypervariable region 2 (HV2) which are used for forensic identification purposes. The CR is approximately 1100bp in length, spanning from position 16,024 to position 576. HV1 covers from position 16,024 to 16,365, while HV2 covers from position 73 to 340. The DNA is amplified and then HV1 and HV2 are sequenced. The obtained mtDNA sequence is compared to a known reference sequence, the revised Cambridge Reference Sequence (rCRS) (11). Only the differences between the sequenced sample and the rCRS are reported. This differences are considered the mtDNA haplotype or profile of an individual. These haplotypes are uploaded into the NDIS database to make genetic associations of unidentified human remains and the family references through mtDNA.

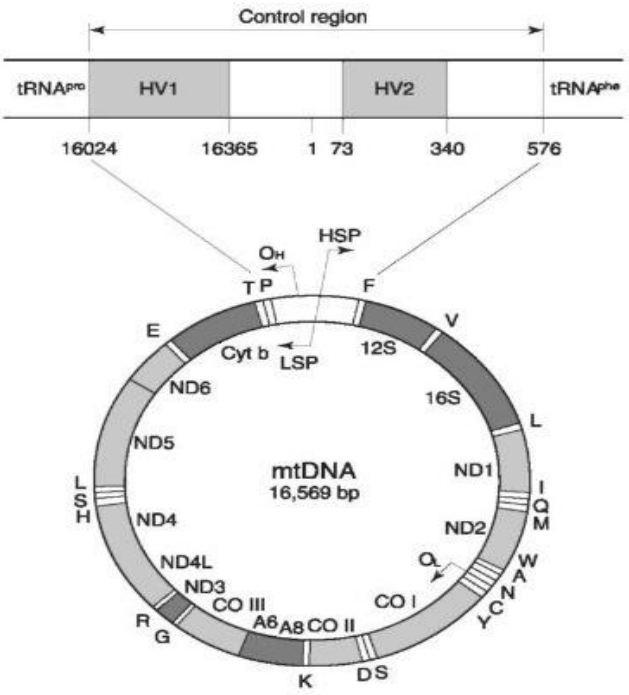

Figure 1. Mitochondrial DNA Genome. The entire mtDNA genome is represented on this diagram, from Holland and Parsons (9). It consists of 16,569 base pairs. The region of interest for forensic purposes is magnified on the top of the figure, the control region. Inside the control region is where HV1 and HV2 are positioned, spanning from 16024 to 16365 and 73 to 340 respectively. HV1 and HV2 are amplified and sequenced in order to obtain a mitochondrial DNA haplotype.

In comparison to nuclear DNA, processing mtDNA is more expensive and laborious. There are a lot of steps and reagents required for this procedure. Currently, the procedure consists of the following steps: DNA extraction (12), DNA quantification and normalization (13), amplification of HV1 and HV2 (14), cycle sequencing (15), capillary electrophoresis (16) and data analysis (17). Research at the University of North Texas Health Science Center (UNTHSC) has decreased the number of steps to make the whole procedure more amenable for automation (18, 19). In 2013, it was demonstrated that high quality mtDNA amplification and sequence data could be generated from reference samples without the need for extraction, quantification, and normalization (18). Using a direct amplification method that would omit these steps would

greatly reduce the amount of time spent on reference samples and would completely eliminate the need for extraction of such samples in laboratories that have validated a direct amplification procedure for nuclear DNA (19). This mtDNA direct amplification method is completed in three steps and only two reagents are needed. First, a punch is obtained from the sample. Second, the punch is incubated to lyse the cells and release the DNA using MitoReady Incubation Buffer (UNTHSC, Fort Worth, TX). Finally, MitoReady Amplification Master Mix (UNTHSC) is added directly to the incubated sample for DNA amplification. This technique will amplify HV1 and HV2 in a single amplification reaction using the mtDNA primers A1 and D1 (see Figure 2), which are already incorporated in the MitoReady reagents as well all the rest of the necessary reagents used for lysis and amplification of DNA.

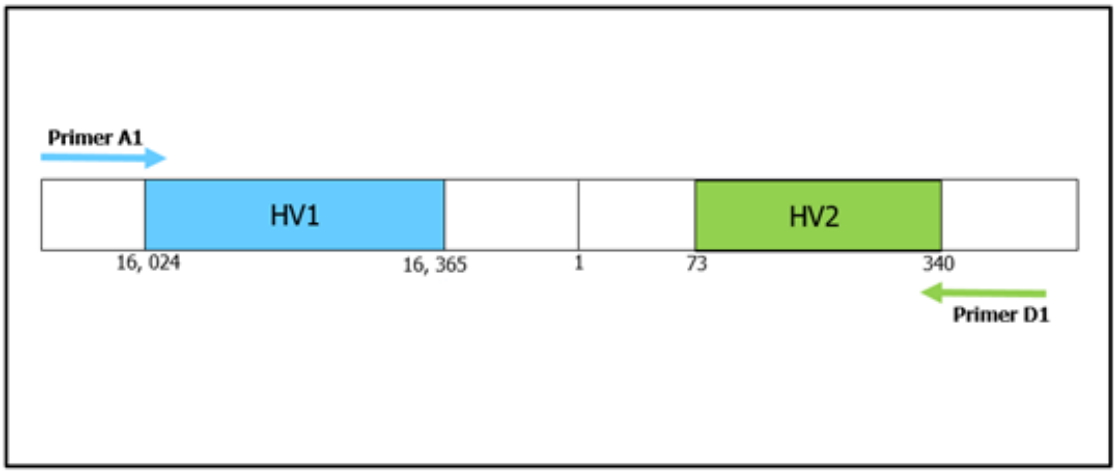

Figure 2. Mitochondrial DNA Amplification Primers. The mtDNA primers A1 and D1 cover the regions of interest, HV1 and HV2 inside the control region as shown in this picture. These primers are used to amplify the targeted DNA sequence in a single amplification reaction using the mitochondrial DNA direct amplification kit.

Currently, there are many commercially available kits that have been successfully developed for the direct amplification of nuclear DNA, and are being used in different forensic laboratories. These kits are: the AmpFLSTR® Identifiler® Direct PCR Amplification Kit (Applied Biosystems, Foster City, CA) (20), AmpFLSTR® NGM SElect™ Express PCR Amplification Kit (Applied Biosystems) (21), GlobalFiler™ STR Kit (Applied Biosystems) (22), PowerPlex® Fusion System (Promega Corp., Madison, WI) (23) and PowerPlex® 21 System (Promega Corp.) (24). Each of these kits reduce the time and money necessary to process samples by eliminating the need for extraction, quantification, and normalization. Nonetheless, laboratories like the UNTCHI that perform mtDNA analysis in addition to nuclear DNA analysis do not benefit from these technique. The reference samples still need to be extracted due to the lack of validation of a similar direct amplification procedure for mtDNA. The validation of a direct amplification procedure for mtDNA would be of great benefit to the forensic community and the UNTCHI service laboratory, because it would completely eliminate the need of extracting reference samples.

The following study aims to validate a mtDNA procedure for reference samples that incorporates direct amplification for reducing the overall time of the procedure, using the MitoReady Incubation Buffer and the MitoReady Amplification Master Mix.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: Using only two reagents, MitoReady Incubation Buffer and MitoReady Amplification Master Mix, the mtDNA from reference samples can be amplified without the need of extracting, quantifying and normalizing the DNA. All downstream procedures will remain the same, and quality sequence data will be obtained. This procedure will increase the throughput capabilities, and decrease the costs of mtDNA processing for reference samples.

Specific Aims:

Aim 1- To develop a mtDNA direct amplification procedure for reference samples using MitoReady Incubation Buffer and MitoReady Amplification Master Mix.

Aim 2- To validate the procedure according to the Quality Assurance Standards (QAS) for Forensic DNA Testing Laboratories and the QAS for DNA Databasing Laboratories in order to implement it in forensic laboratories including UNTCHI (25, 26).

Aim 3- To achieve 90% amplification efficiency on case-type samples.

CHAPTER 2

MATERIAL AND METHODS

Samples and Sample Preparation

Buccal swabs and blood samples were collected without identifying information from consenting adults. This project was approved by the Institutional Review Board (IRB) at UNTHSC (Protocol #2012-170: *Increasing the Efficiency of Mitochondrial DNA Processing of Reference Samples*). Each donor provided five buccal swabs using MacroPur™ Swab P (Solon Manufacturing Co., Rhinelander, WI) and one EDTA tube of approximately 4 mL of whole blood. The buccal swabs were Dacron swabs which are made of polyethylene terephthalate. A volume of 100 µL of blood was spotted onto a Whatman™ Human ID Bloodstain Card (GE Healthcare, Life Sciences, Piscataway, NJ), made of BFC180 paper.

Methodology

The collected samples were punched from their respective collection devices using a 1.2mm Harris MicroPunch (Ted Pella, Inc., Redding, CA), as shown on Figure 3, and the punches were placed in 0.2mL amplification strip tubes. 4 µL of MitoReady Incubation Buffer are added to each tube and the samples are incubated for 40 minutes at 70^{o}C using the AccuBlock™ Digital Dry Bath (Labnet International, Inc., Edison, NJ) This incubation period will result in the lysis of the epithelial or white blood cells releasing the DNA from the nucleus of those cells. Following the incubation step, the samples were amplified by polymerase chain reaction (PCR) in one reaction that covered HV1 and HV2 using 21 µL MitoReady Amplification Master Mix. The optimal PCR parameters for this procedure are shown in Table 1. The final volume of PCR the reaction is 25 µL. A 2% DNA Typing Grade® Agarose (Life Technologies,

Foster City, CA) yield gel was run to verify the presence of amplified mtDNA product using 2 µL of the amplification reaction and 1 µL of bromophenol blue dye. The yield gel was run for 20 minutes at 120 Volts. The gel was stained using ethidium bromide and viewed on an ultraviolet transilluminator. An image of the gel results was taken using an EC3 Imaging System and VisionWorks®LS Analysis Software (UVP BioImaging Systems, Upland, CA).

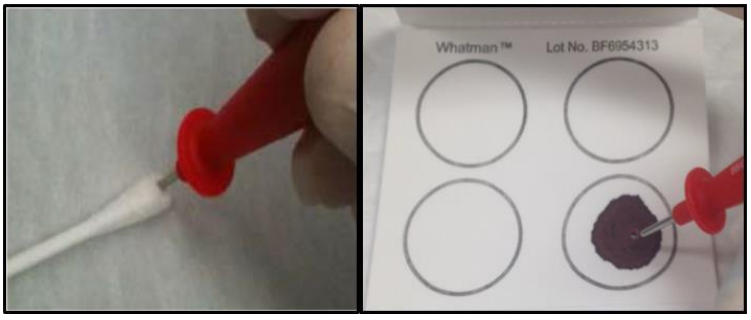

Figure 3. Direct Punch of Samples. Obtaining a punch from a buccal swab and a bloodstain card using a 1.2mm Harris MicroPunch as performed throughout this project.

Thermal Cycling Parameters		
HOLD	95°C	11:00
32 Cycles	95°C	0:10
	60°C	0:45
	72°C	1:00
HOLD	15°C	10:00
HOLD	4°C	∞

Table 1. Themal Cycling Parameters. Outlined thermal cycling parameters for the mtDNA direct amplification procedure of reference samples. All samples were subjected to the same parameters.

The amplified product was purified using 7 µL of ExoSAP-IT® (USB Corp., Cleveland, OH), which is an enzyme added to the PCR product with the purpose of eliminating the unused dNTPs and primer residues that can affect the downstream procedure. The cycle

sequencing reaction was performed using BigDye® Terminator v1.1 (Applied Biosystems), which contains fluorescently labeled ddNTPs and was designed for optimal basecalling adjacent to the primer as well as for the sequencing of short sequences. The cycle sequencing reaction is a total of 15 µL which include 1 µL of the BigDye Terminator v1.1, 5 µL of Better Buffer (The Gel Company, San Francisco, CA), 1.5 µL of the primer to be used and 6.5 or 5.5 µL of water depending on the amount of PCR product to be added. The amount of PCR product added to the reaction varied from 1 µL to 2 µL depending on the intensity of the amplification band observed on the yield gel. Finally a purification step using BigDye® XTerminator™ (Applied Biosystems) was performed to remove salts and unincorporated BigDye® residues. To each sample, 55 µL of the BigDye® XTerminator Premix were added, which consists of 5 µL of the XTerminator Solution, 22.5 µL of SAM Solution and 27.5 µL of water. The sequenced product was then subjected to capillary electrophoresis for analysis using a run voltage of 15.0 kV and ran for 2400 seconds. Each injection was 7 seconds long with a voltage of 1.2 kV and POP-6™ Polymer (Applied Biosystems) was used. The flow chart in Figure 4 conceptualizes all the steps in this procedure.

The mtDNA primers A1, B1, C1 and D1 were used for amplification and cycle sequencing throughout this project (see Table 2 for primer details). These primers are small synthetic pieces of DNA that range from 18 to 21 bps long and cover HV1 and HV2 regions. Normally, all four are used in the cycle sequencing procedure of casework samples. For the purpose of this project, not all samples were sequenced with the four primers. In most of the sequenced samples only the forward primer A1 was used in order to accommodate to the proposed expenses of the project.

The current casework procedure for mtDNA processing differs in several steps. All the samples have to be extracted, which is not the case in our procedure. The amplification procedure requires the separate addition of all the reagents needed for the amplification master mix (10X PCR Buffer, dNTPs, BSA, Mg_2Cl_2, AmpliTaq Gold, forward primer, reverse primer, water and extracted DNA). During the cycle sequencing step, the chemistry used currently in the casework laboratory at the UNTCHI is called dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems) which is known for its high accuracy of the base calls and longer read lengths in comparison to the BigDye® sequencing chemistry which was designed for sequencing shorter sequences.

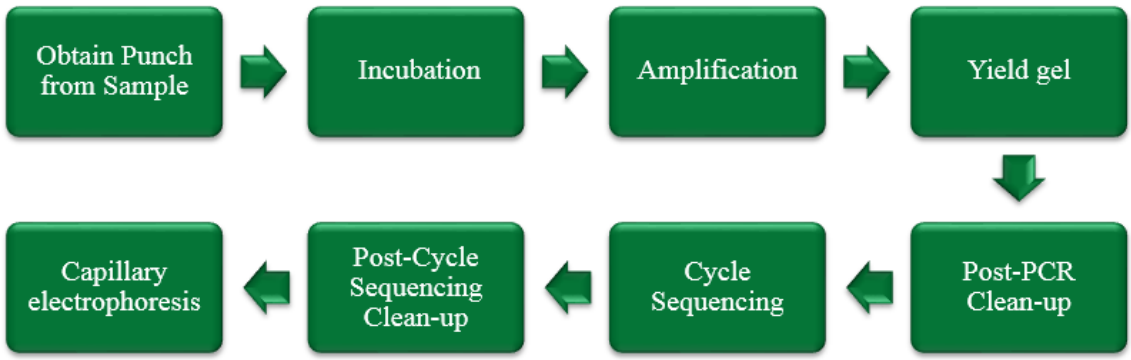

Figure 4. Methodology Flow Chart. Summary of all the steps to follow for the mtDNA direct amplification and sequencing procedure.

Primer	Direction	Region	Start Position	End Position	Sequence
A1	Forward	HV1	15,978	15,997	5' CAC CAT TAG CAC CCA AAG CT 3'
B1	Reverse	HV1	16,391	16,410	5' GAG GAT GGT GGT CAA GGG AC 3'
C1	Forward	HV2	30	48	5' TCA CGG GAG CTC TCC ATG C 3'
D1	Reverse	HV2	408	429	5' CAG TTA AAA GTG CAT ACC GCC A 3'

Table 2. Mitochondrial DNA Sequencing Primers. The mtDNA primers A1, B1, C1 and D1 cover the regions of interest, HV1 and HV2. All of these primers were used for sequencing some of the samples in this project and they are typically used in forensic casework for mitochondrial DNA sequencing. However, for the purposes of this project only A1 primer was used in most of the sequenced samples.

Instrumentation

Amplification and cycle sequencing reactions were performed on the GeneAmp® PCR System 9700 (Applied Biosystems) thermal cycler. Capillary electrophoresis was performed using a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing analysis was performed using the following computer software programs: Sequence Scanner Software (Applied Biosystems) and MTexpert™ Software (Mitotech™, Sante Fe, NM).

Experiments

Developmental and internal validation studies were performed according to the QAS for Forensic DNA Testing Laboratories and the QAS for DNA Databasing Laboratories which include: sensitivity, stability, reproducibility, case-type samples, mixture, and accuracy studies (25, 26). Characterization of the mtGenome was first discussed by Anderson *et al.* in 1981, where the whole genome was first sequenced and the genes encoded are introduced (9). A cross-reactivity study for the amplification primers A1 and D1 has been conducted by Wilson *et al.* 1995 (27). A compilation of population studies for the mtDNA CR including HV1 and HV2 was published in 1996 by Miller *et al.* (28). This study includes mtDNA sequences from over 65 populations for HV1 and from over 26 populations for HV2. Currently there is an online mtDNA database for forensic purposes which stores publically available mtDNA population data known as EMPOP (29). EMPOP has been widely used in the forensic community for population studies since it was first lunched in 2006. The characterization of the mtGenome, cross-reactivity and population studies have been previously conducted and will not be the subject of this validation project.

Sensitivity Study

The sensitivity study will help in understanding the strengths and limits of the proposed

procedure by determining the optimal amount of starting DNA material as well as the limitations in which no results would be expected. A sensitivity study on buccal swabs is challenging because the quantity of cells on the swab will vary depending on how each individual collects the buccal cells and how they perform the scraping on their inside cheek. Taking this into consideration, one donor's buccal swabs was used to create a punch series. The punch series consisted of: 2 punches, 1 punch, ½ punch and ¼ punch. The swabs were punched from the distal end of the head to avoid interference with the stick of the swab (Figure 3). This amplification was performed in triplicate for a total of 12 reactions and the amplified product was evaluated on a yield gel.

For the sensitivity study on blood, whole blood obtained from one donor was diluted with 1X phosphate buffered saline. The dilution series consisted of: neat (undiluted), 1:10; 1:100; 1:1,000 and1:10,000 dilutions. From each dilution, 100 µL were spotted onto a Human ID Bloodstain Card. A punch was taken from each dilution and direct amplification for mtDNA was performed in triplicate for a total of 15 reactions. The amplified product was evaluated on a yield gel. Since this sensitivity study is focused on evaluating the amplification capabilities of the proposed procedure with a varying range of mtDNA input, no sequencing was performed on the tested samples.

Stability Study

This study was performed to determine the stability of samples collected on two different substrates and stored for a certain period of time. In order to perform a stability study for buccal swabs, samples from individuals collected up to ten years ago were requested through the IRB protocol #2010-106: *Assembly of databank for development and validation of genetic assays*. The selected samples were collected specifically on the years: 2005, 2009, 2012 and 2013.

For the stability study of blood samples, bloodstains on Human ID Bloodstain Cards collected on the years 2009-2014 will be amplified using the proposed procedure. Two buccal swabs and bloodstain samples from each of year were amplified, for a total of 20 reactions. The amplified product was evaluated on a yield gel. Since this stability study is focused on evaluating the amplification capabilities of the proposed procedure when working with old samples, no sequencing was performed on the tested samples.

Reproducibility Study

Being able to obtain the same results when processing the same samples, makes a procedure reproducible. In this study we are testing the reproducibility of the procedure between different sample types. For this purpose, three different donors were used and from each donor a buccal swab and a blood samples were amplified and sequenced for HV1 and HV2. The previously mentioned sequencing technique was performed and the obtained haplotypes were compared to verify reproducibility of the sequence between samples types from the same donor.

Case-type Samples Study

Unlike the rest of the studies, the samples used for this study were adjudicated cases provided by the UNTCHI laboratory to be processed using the proposed procedure. A total of 15 buccal swabs were tested. These samples were amplified in duplicate. One set of the amplified samples was sequenced in our laboratory using BigDye® Terminator v1.1, as previously described while the other set of amplified samples was provided to the UNTCHI Missing Persons Laboratory to be tested with their sequencing procedure which uses another sequencing chemistry known as dRhodamine Terminator. The samples were sequenced with the A1 primer, to establish if sequencing of the direct amplification PCR product was possible, and to compare the reported results for concordance corroboration.

Mixture Study

It is important to test the capability of the proposed procedure to detect samples with mixed sequences because there is always the possibility that the reference samples provided for DNA testing are contaminated either by accident or on purpose with another person's DNA. A mixture study was performed for both saliva and blood. For the saliva mixture study, 1 mL of saliva was collected from two donors. The collected saliva was mixed at a 1:1 ratio and 100 µL were spotted on a buccal swab. For blood, a 1:1 mixture ratio from two donors was spotted on Human ID Bloodstain Cards. Only two amplification reactions were performed and the amplified product was sequenced with A1 primer to confirm detection of the mixed sequences.

Accuracy Study

Accuracy is defined in the QAS for Forensic DNA Testing Laboratories as "the degree of conformity of a measured quantity to its actual (true) value" (26). The accuracy of the sequencing results was determined by confirming the mtDNA haplotypes while using the Quality Values (QV) reported by the Sequence Scanner Software. QV is an indication of the probability of error of each base call and is calculated using the following equation: $QV = -10 \times \log_{10}(PE)$, where PE is the probability of error (30). A QV greater than 20 (QV>20) means the probability of error is 1% or less and it will be considered acceptable for our procedure. For this study the QVs reported from the Case-type Samples Study sequenced with primer A1, were summarized to demonstrate accurate base calls on the sequencing results using the proposed procedure. If the majority of the base calls display a QV of 20 or higher, it will be used as an indication that the mtDNA direct amplification method and the corresponding sequencing procedure for reference samples is sufficiently adequate for forensic purposes because it provides high quality sequence data with less amount of work invested.

CHAPTER 3

RESULTS

Sensitivity Study

A punch series for buccal swabs was created from donor AAC_13 using 2 punches, 1 punch, ½ punch and ¼ punch. The experiment was performed two months after the collection of the swabs. Each reaction on the punch series was performed in triplicate. The positive control used in this, and all the upcoming buccal swab amplification experiments, was 2 µL of HL-60 at a concentration of 0.03 pg/uL. No DNA was added to the negative control. Figure 5 illustrates the amplified product from the punching series on a 2% agarose yield gel. No visual difference in the band intensity was observed between the four experiments performed. As mentioned before, buccal swab results vary depending on each individual and their swabbing techniques. Using one punch is the most ideal approach for regular case working laboratories.

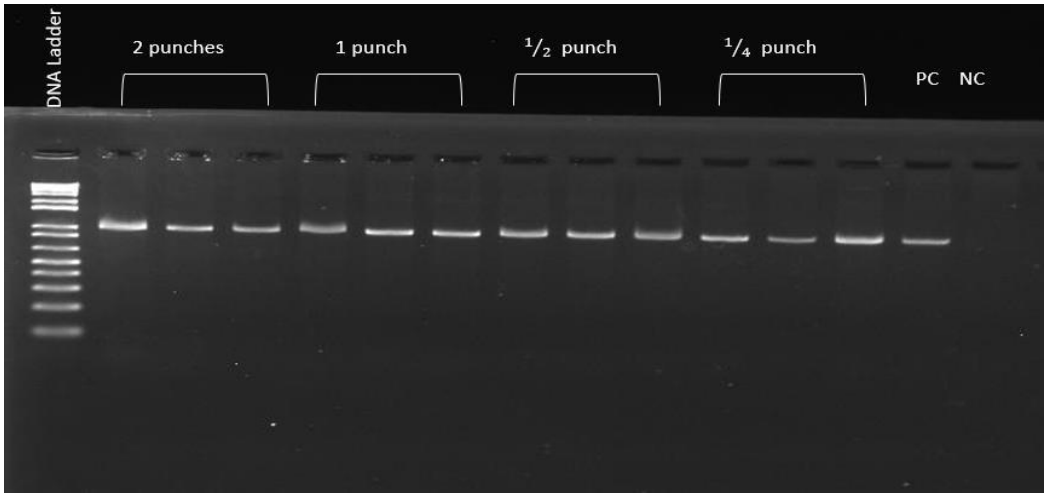

Figure 5. Sensitivity Study – Buccal Swabs. Gel image obtained after amplification of a punch series created to test the sensitivity of the mtDNA direct amplification procedure using buccal swabs. No visual difference in band intensity was observed between experiments.

A blood dilution series for a blood sample was generated consisting of neat (undiluted) blood, 1:10, 1:100, 1:1,000 and 1:10,000 blood dilutions. The experiment was performed two months after the collection of the blood from the donor. Each reaction on the punch series was performed in triplicate. The positive control used, in this and all upcoming experiments involving blood punches, is blood from a known donor spotted on a Human ID Bloodstain Card. This donor had been previously tested for mtDNA analysis and a haplotype was recorded in the laboratory. A punch of the positive control was taken and used the same way it was done with the tested samples. No DNA was added to the negative control. Figure 6 shows the amplified product from the dilution series on a 2% agarose yield gel. The results on the yield gel show no visible amplification product on dilutions greater than 1:100. This study demonstrates that at least a 1:100 dilution from neat blood is enough to yield an amplification DNA product using the mtDNA direct amplification method.

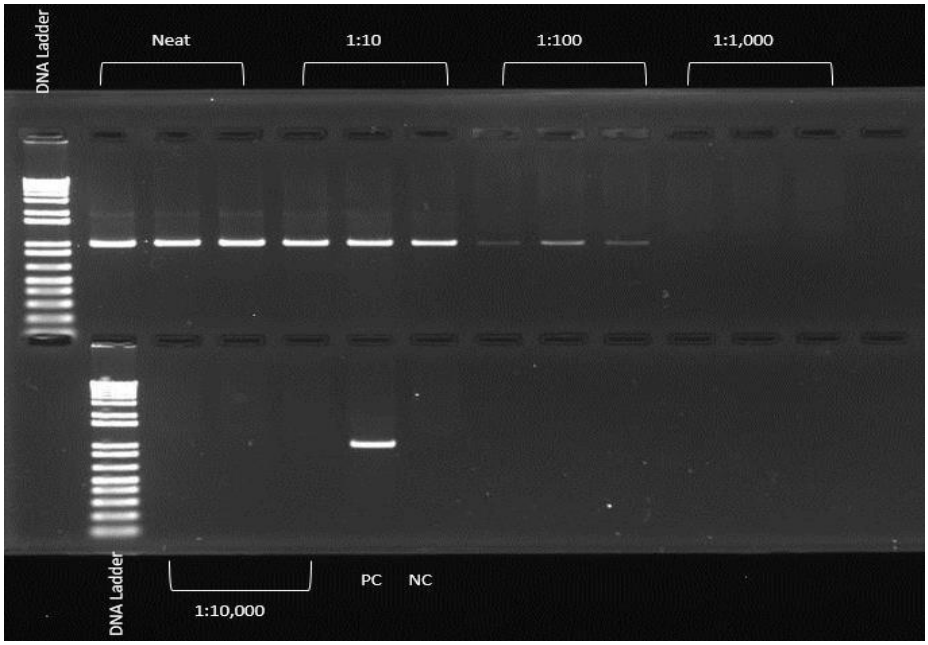

Figure 6. Sensitivity Study – Blood Samples. Gel image obtained after amplification of a blood dilution series generated to test the sensitivity of the mtDNA direct amplification procedure using blood samples. No visible amplification is observed beyond a 1:100 dilution.

Stability Study

In this study we tested the performance of the mtDNA direct amplification procedure on aged buccal swabs and blood samples that have been stored for several years. Buccal swabs collected in the years 2005 (Sta_17 and Sta_18), 2009 (Sta_19 and Sta_20), 2012 (Sta_21 and Sta_22) and 2013 (Sta_23 and Sta_24) were amplified. Two swabs from each time period were tested. Each sample corresponds to a different swab, from a different donor. Figure 7 shows the amplified product on a 2% agarose yield gel. This procedure was consistently successful with samples that are approximately one year old. However, the amplification of samples older than one year was not consistent in this experiment. The swabbing techniques of the donors could have resulted in reduced amounts of DNA, but also a long storage period may be inappropriate because nucleases degrade the DNA originally available in the swab especially if the storage conditions are not optimal to avoid degradation.

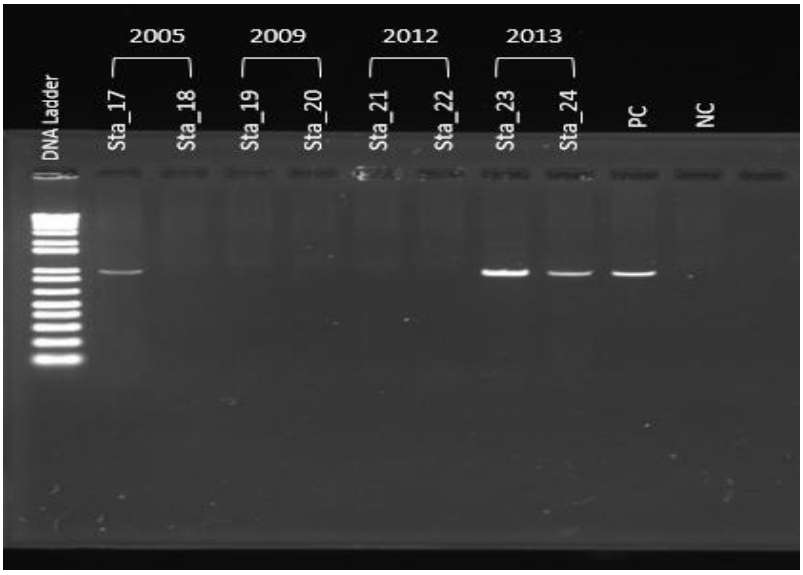

Figure 7. Stability Study – Buccal Swabs. Gel image obtained after amplification of old buccal swabs performed to test the stability of the mtDNA direct amplification procedure when testing old samples. Amplification of samples collected before 2013 was not consistent.

Aged blood samples manufactured in the years 2009, 2010, 2011, 2012, 2013 and 2014 were amplified. Two samples from each time period were tested. Each sample corresponds to a different donor. Figure 8 shows the amplified product on a 2% agarose yield gel. Amplified DNA product was obtained from all tested samples. The band intensity observed on samples Sta_06 to Sta_09, which correspond to one of the samples from the year 2011 through one of the samples from the year 2013, is not as strong as the rest of the samples on the yield gel. This abrupt change in band intensity coincided with a change in the manufacturing group of the samples used in this experiment. The manufacturer of the samples made in the second half of 2011 onward is different than that of the previous years. It is possible that an alteration in the manufacturing procedure, such as the type of anticoagulant on the collected blood, is causing inconsistency in the amplification product.

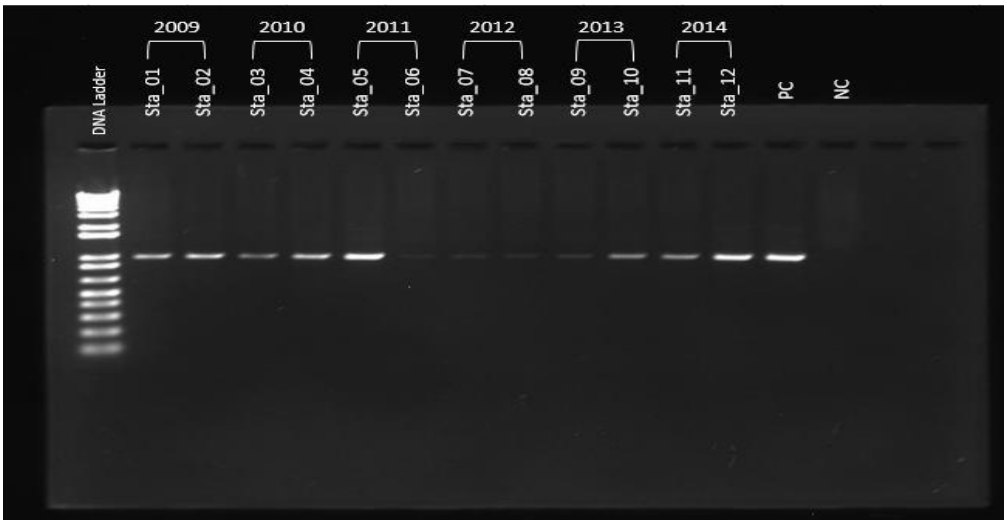

Figure 8. Stability Study – Blood Samples. Gel image obtained after amplification of old blood samples performed to test the stability of the mtDNA direct amplification procedure when testing old samples. A difference in band intensity can be observed in samples Sta_06 through Sta_09.

Reproducibility Study

Three different donors were used to test if the mtDNA profiles obtained using the mtDNA direct amplification method was reproducible when using two different samples types, buccal swabs and blood sample. From each donor both a buccal swab and a blood sample were amplified and sequenced for HV1 and HV2. The samples from donors AAC_04 and AAC_07 were collected 13 months prior to this experiment. The samples from donor AAC_13 were collected two months prior to this experiment. Figure 9 shows the amplification product on a 2% agarose yield gel and the resulting mtDNA profiles after the sequencing procedure are listed on Table 3.

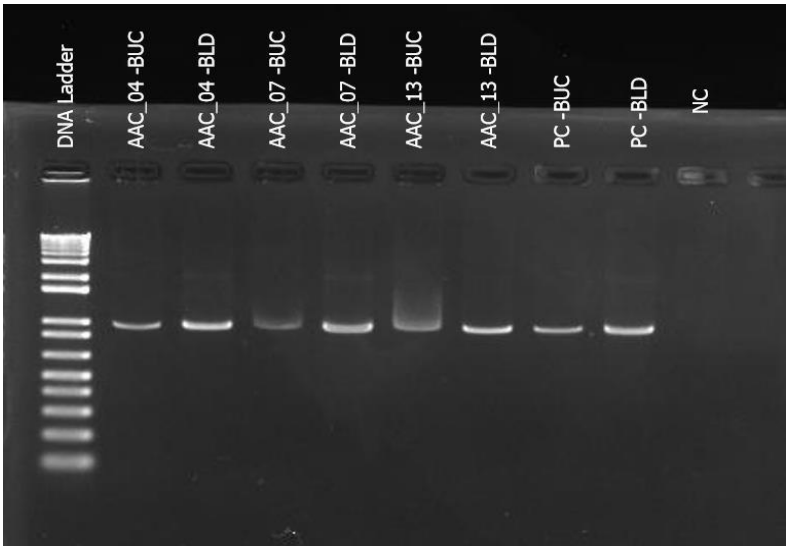

Figure 9. Reproducibility Study. Gel image obtained after amplification of buccal swabs and blood samples from the same donor to test the reproducibility of the mtDNA direct amplification procedure.

Sample type	Buccal Swabs		Blood Samples	
Region	HV1	HV2	HV1	HV2
Donor: AAC_04		263 G		263 G
		309.1 C		309.1 C
		315.1 C		315.1 C
Donor: AAC_07	16, 224 C	73 G	16, 224 C	73 G
	16, 270 T	150 T	16, 270 T	150 T
		263 G		263 G
		279 C		279 C
		315.1 C		315.1 C
Donor: AAC_13		152 C		152 C
		263 G		263 G
		315.1 C		315.1 C

Table 3. Reproducibility Study. One buccal swab and one blood sample were amplified and sequenced from each donor in order to test the reproducibility of the results between sample types. This table displays the mtDNA profiles obtained after sequencing each sample.

Case-type Samples

Adjudicated cases from the UNTCHI Missing Persons Unit were provided for the case-type samples study. A total of 15 buccal swab samples labeled as Family Reference Samples (FRS) were tested. The samples were amplified in duplicate using the proposed direct amplification procedure. One set was sequenced following the previously stated protocol, using BigDye® Terminator v1.1, and the other set was given to the UNTCHI casework laboratory to be tested with their sequencing procedure using dRhodamine Terminator Cycle Sequencing Kit. Figure 10 shows the amplified product that was sequenced using the BigDye® procedure and Figure 11 shows the amplified product that was provided to UNTCHI to sequence using dRhodamine. For only one sample, FRS_8, there was no visible amplification product observed on both yield gels. This means that a total of 93% of the case-type samples tested for this project showed amplified product using the developed direct amplification method for mtDNA.

When cycle sequenced with BigDye® using primer A1 most of the FRS samples resulted in good quality data, enough to allow for reading through the sequence. However, the quality of the sequence data of samples FRS_8 and FRS_11 was poor and could not be read. This was expected for samples FRS_8 because no amplified product was observed on the yield gel (see Figure 10). The amplified product for samples FRS_11 was a smear which can mean amplification of non-specific sequences hence the quality of the sequencing data decreases. Using Sequence Scanner Software, screen shots of the electropherograms obtained from four of the FRS samples and the positive control can be observed on Figure 12. These four samples are representative of the majority of the good quality sequence data obtained. Using dRhondamine there were also two samples which sequence could not be read, FRS_7 and FRS_8. The yield gel

(Figure 11) results for this samples suggests that there was very little amplification product for FRS_7 and no amplification at all for FRS_8, explaining why the sequencing data was poor. The quality of sequence data from other samples such as FRS_5 and FRS_12, was not high enough to be used in casework but for the purpose of this study the analyst was able to read through it and report the results. Screen shots from the electropherograms, of four samples and the positive control, that are representative of the good quality data obtained with dRhodamine are shown on Figure 13 using Sequence Scanner Software.

The sequencing results obtained from our laboratory were compared to those of the casework laboratory at UNTCHI for concordance. Table 4 shows a representation of five of the haplotypes obtained from both laboratories using the two different sequencing chemistries. Of the 13 samples (and positive control) that gave sequencing results in both laboratories, all 13 were concordant in the overlapping regions between laboratories. Table 5 compares the sequencing results obtained with both chemistries, BigDye® and dRhodamine. The major difference observed was on the range of covered bases, in the majority of the samples it was greater for dRhodamine than BigDye®. However, this difference is due to the fact that dRhodamine is design for longer read lengths whereas BigDye® is designed for shorter fragments. There was a particular sample, FRS_12 (marked with an asterisk on the *Concordance* column of Table 5), that was called an "N" at position 16,281 by the analyst using the dRhodamine chemistry at the casework laboratory. This is normal practice when the analyst is not able to call it any specific base due to the poor quality of the data. In our laboratory, using BigDye® sequencing chemistry, the quality of the data for that sample was better and the software was able to call that base. No difference from the rCRS was observed in that exact position.

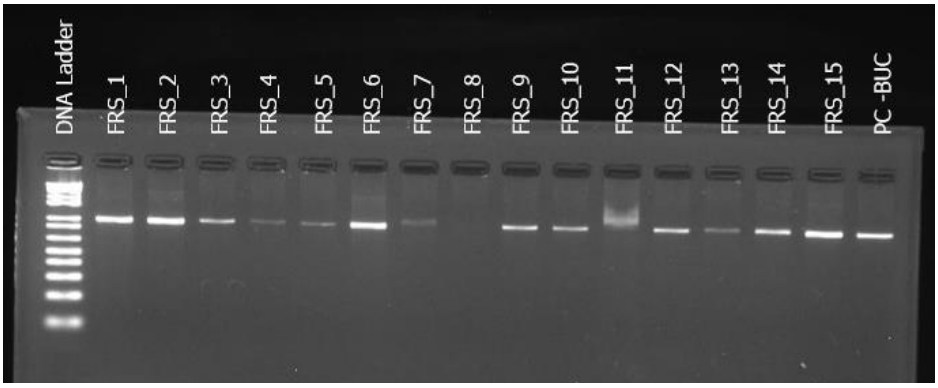

Figure 10. Case-type Samples Study – Amplification Results from Set 1. Gel image obtained after amplification of buccal swabs from adjudicated cases of the UNTCHI laboratory. These samples were sequenced in the Research & Development laboratory at UNTCHS for this study, using BigDye® Terminator.

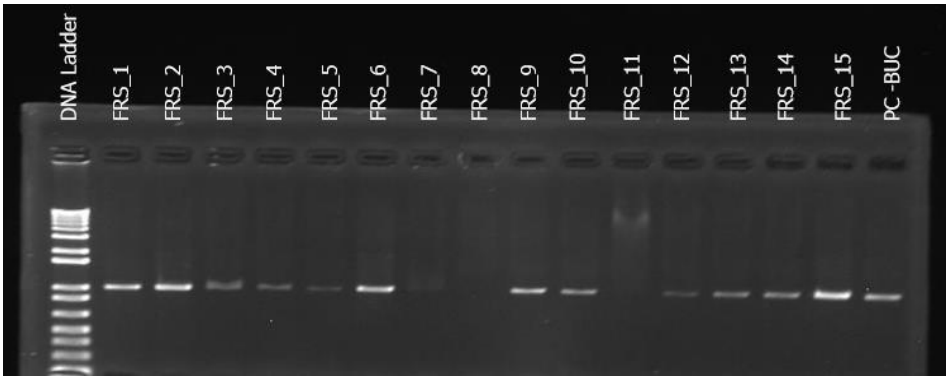

Figure 11. Case-type Samples Study – Amplification Results from Set 2. Gel image obtained after amplification of buccal swabs from adjudicated cases of the UNTCHI laboratory. These samples were sequenced in the Casework Laboratory at UNTCHI for this study, using dRhodamine Terminator.

Figure 12. Case-type Samples Study – Sequencing Results using BigDye®. Representative demonstration of the sequencing data obtained in the laboratory using BigDye® Terminator from four of the fifteen Family Reference Samples, and the positive control, used for this study. The range shown is 16,077 to 16,136, part of HV1. Data viewed on Sequence Scanner Software.

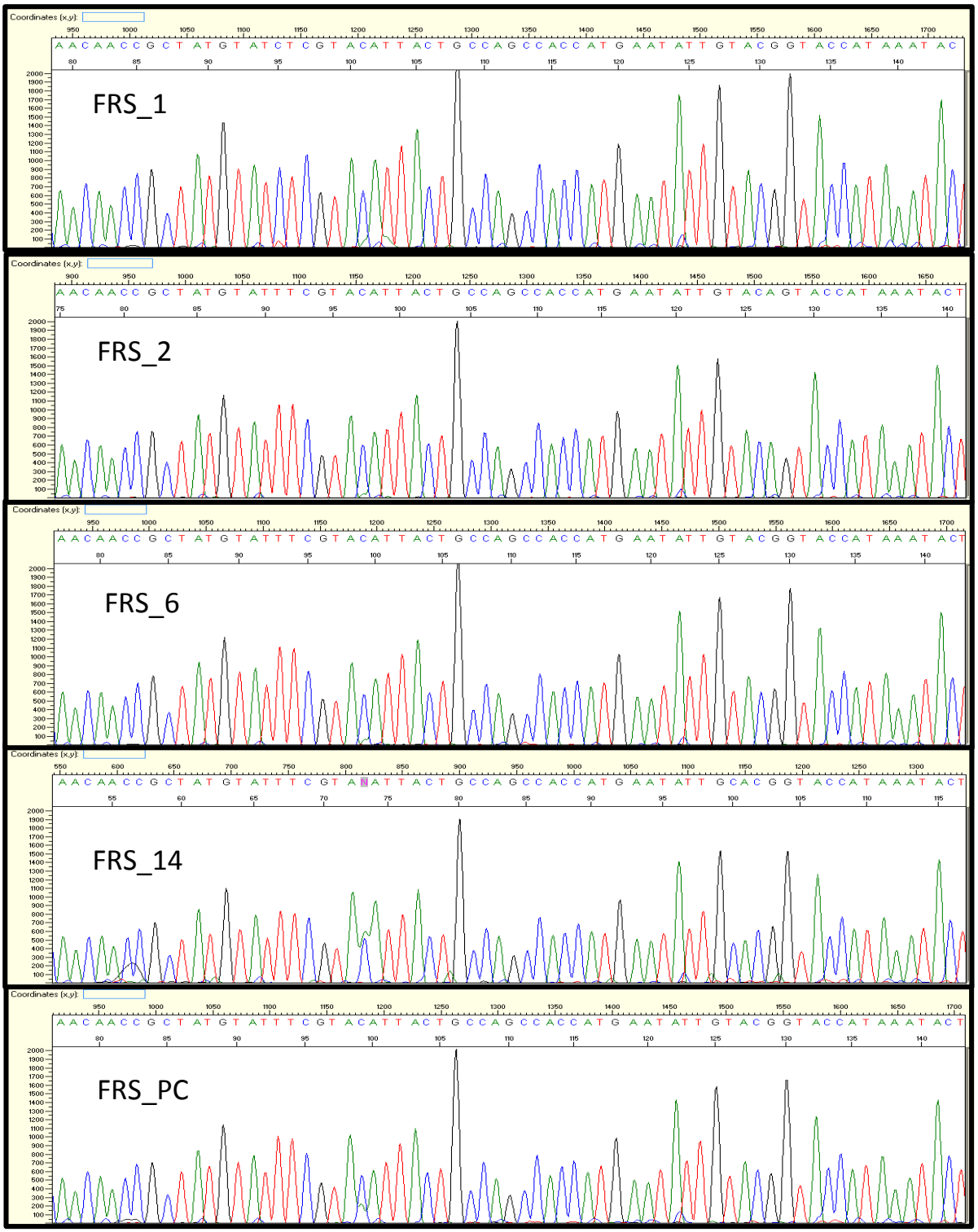

Figure 13. Case-type Samples Study – Sequencing Results using dRhodamine.
Representative demonstration of the sequencing data obtained using dRhodamine Terminator from four of the fifteen Family Reference Samples, and the positive control, used for this study. The range shown is 16,077 to 16,136, part of HV1. Data viewed on Sequence Scanner Software.

Sample Haplotype	Change of Base	dRhodamine	BigDye®
FRS_3			
16,298	T -> C	X	X
FRS_6			
No difference from rCRS	N/A	X	X
FRS_9			
16,126	T -> C	X	X
16,163	A -> G	X	X
16,186	C -> T	X	X
16,189	T -> C	X	X
16,294	C -> T	X	X
FRS_11			
16,235	A -> G	X	X
16,286	C -> T	X	X
16,291	C -> T	X	X
16,293	A -> G	X	X
FRS_13			
16,114	C -> A	X	X
16,129	G -> A	X	X
16,189	T -> C	X	X
16,193	C insertion	X	X

Table 4. Case-type Samples Study – Haplotypes. Demonstration of five mitochondrial DNA haplotypes obtained from the sequencing performed using the two different sequencing chemistries, BigDye® and dRhodamine. The "X" on the last two columns, denotes if that change of base was detected by each of the two different chemistries. The concordance on the haplotypes of these five samples is representative of the rest of the case-type samples.

Sample	Range dRhodamine	Range BigDye®	Covered bases dRhodamine	Covered bases BigDye®	Difference	Concordance
FRS 1	16001-16476	16017-16334	475	317	158	PASS
FRS 2	16003-16470	16001-16336	467	335	132	PASS
FRS 3	16001-16463	16016-16335	462	319	143	PASS
FRS 4	16132-16457	16031-16264	325	233	92	PASS
FRS 5	16107-16449	16035-16323	342	288	54	PASS
FRS 6	16036-16470	16017-16325	434	308	126	PASS
FRS 7		16010-16194		184		NR - dR
FRS 8						NR
FRS 9	16023-16457	16015-16320	434	305	129	PASS
FRS 10	16001-16493	16015-16323	492	308	184	PASS
FRS 11	16001-16463		462			NR - BD
FRS 12	16046-16389	16004-16292	343	288	55	PASS*
FRS 13	16045-16194	16028-16194	149	166	-17	PASS
FRS 14	16046-16463	16017-16318	417	301	116	PASS
FRS 15	16003-16463	16003-16316	460	313	147	PASS
FRS-Pos	16003-16463	16015-16306	460	291	169	PASS

Table 5. Case-type Samples Study – Sequencing Data Summary. Comparison of the sequencing results obtained at the Casework Laboratory using dRhodamine sequencing chemistry and the Research and Development Laboratory using BigDye® sequencing chemistry. The range of bases that was sequenced as well as the total number of covered bases are shown in the *Range* and *Covered Bases* columns respectively for each one of the two chemistries. When comparing this results between sequencing chemistries, dRhodamine was able to cover more bases than BigDye® in the majority of the samples. The *Difference* column helps to visualize this by showing the difference in the number of bases covered between sequencing chemistries. However, this difference is due to the fact that dRhodamine is design for longer read lengths whereas BigDye® is designed for shorter fragments. The concordance column establishes weather the obtained mitochondrial DNA haplotype was concordant between laboratories. The colored samples are the ones for which no results (NR) were obtained. (Blue- No result using dRhodamine; Green- No result using both chemistries; Orange- No result using BigDye®).

*Poor quality sequence data caused an extra call to be made in position 16,281 when using dRhodamine sequencing chemistry.

Mixture Study

Saliva and blood collected from two different donors was pulled together creating a 1:1 mixture ratio as described in Chapter 2. In the sequencing results of these mixed samples, point heteroplasmy is observed in the HV1 position 16239 which changes from a Cytosine (C) to a Thymine (T) in only one of the donors known profiles. Figure 14 shows the sequencing results obtained from the buccal swabs. In the top sequence shown on Figure 14 heteroplasmy, more than one nitrogenous base present at the same position, is observed in position 16239 (highlighted in a red square) even though it was not called by the software. In the positive control sequence, shown (bottom sequence of Figure 14) only one nitrogenous base, a C, is clearly observed and called by the software in the same position (highlighted by a red square).

Figure 15 shows the sequencing results obtained from the blood samples. In the top sequence of Figure 15 heteroplasmy is observed and it was called by the software in position 16239 (highlighted in a red square). In the positive control sequence, shown in bottom of Figure 15, only one nitrogenous base, a C, is clearly observed and called by the software in the same position (highlighted by a red square).

Figure 14. Mixture Study – Saliva. Alignment of sequence data obtained from the mixed saliva sample viewed on MTexpert. Inside the red rectangle, the same base position is highlighted. On the top image a mixture of two bases is observed while the bottom image (positive control) shows only one base being called.

Figure 15. Mixture Study – Blood Sample. Alignment of sequence data obtained from the mixed blood sample viewed on MTexpert. Inside the red rectangle, the same base position is highlighted. On the top image a mixture of two bases is observed while the bottom image (positive control) shows only one base being called.

Accuracy Study

The results obtained from the samples sequenced with BigDye® Terminator v1.1 for the Case-type Samples Study were analyzed further to determine the accuracy of the base calls. As mentioned before, QV is an indication of the probability of error of each base call and a QV greater than 20 (QV>20) means the probability of error is 1% or less. Table 6 summarizes the values obtained from Sequence Scanner Software for the Contiguous Read Length (CRL), which is calculated as the number of uninterrupted bases in the trace that have a QV greater than 20, and for the total number of base calls with a QV of 20 or more (QV20+). The percentage of base calls with a QV of 20 or higher was calculated for each sample, dividing the QV20+ values by the total number of called bases. The average percentage of QV values greater than or equal to 20 that were called on all the sequenced bases of this study was 88%. This means that for all the samples in this study that we obtained sequencing results, an average of 88% of the total base calls can be considered accurate.

Sample	Contiguous read length	QV20+	Total of called bases	Percentage of QV20+
FRS 1	309	303	317	96%
FRS 2	314	306	335	91%
FRS 3	193	217	319	68%
FRS 4	220	210	233	90%
FRS 5	264	260	288	90%
FRS 6	286	266	308	86%
FRS 7	158	139	184	76%
FRS 9	289	285	305	93%
FRS 10	284	272	308	88%
FRS 12	257	238	288	83%
FRS 14	290	278	301	92%
FRS 15	291	283	313	90%
FRS-Pos	284	286	291	98%
Average	264.54	257.15		88%
Standard deviation	46.63	46.25		8%

Table 6. Accuracy Study. Contiguous Read Length and QV20+ values obtained from Sequence Scanner Software. The final column shows the percentage of QVs greater than or equal to 20 obtained for each sample. Samples FRS_8 and FRS_11 not part of this study due to lack of sequencing results and sample FRS_13 due to a systematic error of the software.

Summary

The focus of this project was to validate a method for direct amplification of reference samples for mitochondrial DNA processing in a forensic environment. Table 7 summarizes the amplification success, meaning all the samples in which a band was visually detected on the yield gel, of the procedure by study and sample type. The most significant of all studies is the Case-type Samples study (highlighted in yellow on Table 7) because the samples on this study are more relevant to the majority of samples that are going to be encountered in casework than the rest of the studies. The Case-type Samples study had a 93% amplification success, which is higher than the 90% amplification success we targeted for in the specific aims of this project. When examining the amplification success on the rest of the studies on Table 7 we can see the majority of the studies had a 100% amplification success. Only two experiments resulted in lower amplification success. These were the extremely low blood dilutions from the sensitivity study (68% of amplification success) and the aged buccal swabs from the stability study (38% of amplification success), which are not representative of the majority of reference samples that would be encountered in a casework laboratory.

Study	Sample type	Amplification Success	Percentage
Sensitivity	Buccal Swab	12/12	100%
	Blood	9/15	60%
Stability	Buccal Swab	3/8	38%
	Blood	16/16	100%
Reproducubility	Buccal Swab	3/3	100%
	Blood	3/3	100%
Case-type Samples	**Buccal Swab**	**28/30**	**93%**
Mixture	Buccal Swab	1/1	100%
	Blood	1/1	100%

Table 7. Amplification Success. A summary of the amplification success from all the validation studies performed divided by buccal swabs and blood samples. The Case-type Samples studies (highlighted in yellow) is the most significant study because it is more representative of the majority of reference samples encountered in a casework laboratory. The amplification success for the Case-type Samples study was of 93% which is higher than the 90% that was set in the specific aims of this project.

CHAPTER 4

CONCLUSION

In forensic genetics, the ability to identify human remains in missing persons cases relies greatly on mtDNA. The director of the Center for Human Identification, Dr. Arthur Eisenberg, has stated publically on many occasions that laboratories that are not utilizing mtDNA analysis should not be doing the identification of human remains. Due to its unique characteristics, discussed in Chapter 1, mtDNA is often better preserved than nuclear DNA in old human remains. Some forensic laboratories, like the UNTCHI, depend on mtDNA analysis to solve cases on a regular basis. The development of the mtDNA direct amplification method for reference samples can greatly improve laboratory dynamics. Excluding the DNA extraction step, and using only two reagents, the MitoReady Incubation Buffer and the MitoReady Amplification Master Mix, can save the analyst significant time, which can be spent on other aspects of their job. The direct amplification procedure for mtDNA reference samples, can speed up the overall processing of the entire case making it possible for laboratories to be more efficient.

Throughout this study, the development of the mtDNA direct amplification procedure was validated using buccal swabs and bloodstain samples in the following aspects: sensitivity, stability, reproducibility, case-type samples, mixture and accuracy. For the sensitivity study, the amplified product obtained from the buccal swabs showed to be consistently stable, while using different size portions of the head of the swab; from the blood samples DNA product on a gel was visible on samples of up to a 1:100 dilution. In the stability study of buccal swabs, swabs collected in the past year show a consistent amplification product on gel. Amplified product

from blood samples was observed on samples that were 6 years old. This procedure demonstrated reproducibility of the mtDNA haplotypes obtained when processing different samples types from the same donor.

For the case-type samples study, 93% of the tested samples amplified using the proposed mtDNA direct amplification procedure. This number exceeds the minimum percentage of amplification success set as one of the specific aims of this project. These samples were sequenced by two laboratories independently, using two different sequencing chemistries, dRhodamine Terminator and BigDye® Terminator v1.1. The final mtDNA profiles were concordant on the overlapping regions between laboratories. In the mixture study, a known mixture of two donors' sequences was identified using both sample types, buccal swabs and blood stains. Finally, the accuracy study showed that, on average, 88% of the base calls on the sequencing results from the case-type samples amplified using the mtDNA direct amplification had a QV of 20 or greater, meaning that the probability of error is 1% or less on the majority of the base calls.

These studies show that good quality DNA can be obtained from reference samples without the need of a lengthy DNA extraction procedure. Furthermore, amplified product can be successfully sequenced as shown in this validation study. These results are consistent with our hypothesis which stated that amplifying and obtaining good quality sequence data from reference samples using only two reagents, MitoReady Incubation Buffer and MitoReady Amplification Master Mix, and thus streamlining the mtDNA processing for forensic purposes is possible. In view of the amplification success observed throughout these experiments, especially with buccal swabs which are the primary sample type used on reference samples, this procedure should be considered a necessary and worthy asset to be added to a casework laboratory that performs mtDNA work regularly.

Being able to perform mtDNA sequencing for casework purposes can be an advantage for forensic laboratories, especially when working with cases involving unidentified human remains or family relatives on the maternal lineage. In reality, the majority of forensic laboratories in the United States do not perform mtDNA work at all. The development and application of this mtDNA direct amplification procedure may lead towards the introduction of mtDNA work for forensic purposes in more laboratories around the country.

APPENDIX

LABORATORY PROTOCOL

UNT Center for Human Identification
Procedure Manual – Research & Development Laboratory

MtDNA Direct Amplification for Reference Samples

Purpose: To perform direct amplification of reference samples for HV1 and HV2 regions of the mtDNA control region without the need of DNA extraction and using only two reagents.

Equipment and Supplies

- Centrifuge, vortex
- Heat block
- Pipettors and pipette tips (aerosol barrier)
- 96-well plates (or 0.2mL strip tubes and caps) and base supports
- Reference samples (blood or buccal)
- 1.5mL or 2mL microcentrifuge tubes
- GeneAmp® PCR System 9700
- Strip caps

Safety

Gloves, lab coats and eye protection must be worn during this procedure.

Reagents

- MitoReady Incubation Buffer
- MitoReady Amplification Master Mix
- Positive Control: HL60 Control DNA (0.12 ng/µL) or a known blood sample

Procedure

A. Incubation

1. Add 4 µL of MitoReady Incubation Buffer to each sample well or tube.

   NOTE: Incubation Buffer is added first to help negate the effects of static electricity when working with punched discs.
2. Punch a 1.2mm disc from each sample and dispense into individual wells or tubes.
3. Visually confirm the presence of the disc in the MitoReady Incubation Buffer.

   NOTE: If necessary, use a clean pipette tip to submerge disc in MitoReady Incubation Buffer.
4. Seal columns of the 96-well plate with strip caps, or close lids of strip tubes.
5. Centrifuge the plate or tubes for 20 seconds.
6. Incubate in heat block at 70°C for 40 minutes.
7. Centrifuge the plate or tubes for 20 seconds.

B. Master Mix Addition

1. Vortex for 3 seconds and centrifuge briefly.
2. Dispense 21µL of MitoReady Amplification Master Mix into each well.
3. Seal plate/tubes.
4. Centrifuge for 20 seconds.
5. Place the tubes in the retainer on the thermal cycler or the plate directly on the thermal cycler; record the thermal cycler number on the worksheet.

C. Thermal Cycling Parameters

1. Select the appropriate program.
2. Select START.
3. Enter 25µL for the total reaction volume.
4. After the run is complete, samples processed further or stored 14 days at 4°C.

mtDNA Direct Amplification		
Number of cycles	**Temperature**	**Time (min:sec)**
HOLD	95°C	11:00
32 CYCLES	95°C	0:10
	60°C	0:45
	72°C	1:00
HOLD	15°C	10:00
HOLD	4°C	∞

Revisions

Date of revision	Revised by	Description of changes made

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