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# Increasing the Efficiency of Mitochondrial DNA processing of Reference Samples

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Mitochondrial DNA (mtDNA) analysis in forensic testing is especially useful for human skeletal remains and hair samples. When a profile is generated for unidentified human remains or hair samples, comparison to a reference sample is critical to the case. The following steps are involved in the processing of family reference samples for mtDNA: DNA extraction, HV1 and HV2 amplification, cycle sequencing, electrophoresis, and analysis. Some procedures require DNA quantification and normalization. Analysis of mtDNA is expensive and time-consuming. A direct lysis and amplification method was previously shown to eliminate the need for DNA extraction, quantification, and normalization of reference samples. This study was performed to further develop, optimize, and validate its use for future implementation in routine casework of reference samples. The results have shown that quality mtDNA sequencing data can be obtained using a direct amplification method from blood and buccal samples from a variety of collection devices.

INCREASING THE EFFICIENCY OF

MITOCHONDRIAL DNA PROCESSING

OF REFERENCE SAMPLES

Katherine Morgan, B.S.

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REFERENCE SAMPLES


THESIS


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Katherine Morgan, B.S.

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CHAPTER 1


INTRODUCTION


The National Institute of Justice (NIJ) is a division of the Unites States Department of

Justice.  In 2004, through the President's DNA Initiative, NIJ awarded the University of North

Texas Health Science Center (UNTHSC) a grant for the "Anthropological, STR and mtDNA

Testing of Unidentified Remains and Family Reference Samples."  Under this grant, UNTHSC

established a Missing Persons Program to process unidentified human remains and reference

samples from family members with a missing loved one for both nuclear DNA and

mitochondrial DNA (mtDNA).  DNA profiles from these samples can then be uploaded to the

COmbined DNA Index System (CODIS) to assist in the identification of these unknown

decedents (1).  The identification of the decedent can potentially help families and assist law

enforcement agencies with an investigative lead.  The efficiency of DNA analysis is extremely

important in this process, and a significant amount of work has been done to improve it.

Research at UNTHSC has focused on the development of more efficient protocols that will

reduce the time and/or reagents required for DNA extraction, amplification (2), software

development, and data analysis (3, 4).

Currently available to forensic laboratories are commercial kits for direct amplification of

nuclear DNA from single-source blood and buccal samples: the AmpFLSTR® Identifiler®

Direct PCR Amplification Kit (Applied Biosystems, Foster City, CA) (5), PowerPlex® 18D

System (Promega Corp., Madison, WI) (6), PowerPlex® 21 System (Promega Corp.) (7),

AmpFLSTR® NGM SElect™ Express PCR Amplification Kit (Applied Biosystems) (8), and

GlobalFiler™ STR Kit (Applied Biosystems) (9).  These kits reduce the time and money

necessary to process samples by eliminating the need for extraction, quantification, and normalization.  As a result, profiles can be uploaded to CODIS more quickly, accelerating the identification process.

Similar strides have been made to increase the efficiency of mtDNA amplification methods at UNTHSC using the direct amplification concept presented by the nuclear kits.  The application of mtDNA analysis in forensic testing is especially useful in the identification of human remains and in the processing of hair samples.  The circular structure and high copy number of mtDNA make it much less prone to degradation than nuclear DNA.  Each autosomal cell contains only two copies of nuclear DNA, but there are on average 500 copies of mtDNA present per cell (10).  Since mtDNA is only passed from a mother to her children, it can also be very useful in establishing maternal relationships. The processing of mtDNA samples requires a number of time-consuming and costly steps as well as numerous sample transfers, which increase the risk of introducing contamination to the sample.  The implementation of a direct mtDNA amplification method in crime laboratories would allow analysts to eliminate the extraction and quantification steps and reduce the number of sample transfers, as is currently possible with direct nuclear assays.

UNTHSC developed a method and reagents to lyse the cells and directly amplify mtDNA without the need for extraction or multiple sample transfers.  This was accomplished with only two buffers, one for incubation and one for amplification.  The incubation step lyses the cells to release the DNA.  The amplification buffer interrogates the entire control region.  In the UNTHSC casework laboratory, two amplifications are performed for each reference sample, an amplification for Hypervariable Region 1 (HV1) and an amplification for Hypervariable Region 2 (HV2).  Blood and buccal samples were amplified, sequenced, and analyzed with this high

throughput method and produced excellent results.  This protocol was developed for bloodstains, buccal swabs, and anagen hairs (2).

The Missing Persons Program at UNTHSC has validated Identifiler® Direct (Applied Biosystems) for the processing of nuclear DNA from reference samples.  A direct amplification method for mtDNA has yet to be validated.  As a result, there is currently a bottleneck for cases that require both nuclear and mtDNA typing.  Nuclear DNA typing can be performed much more quickly because it is not necessary to extract or quantify the samples.  Validation of the previously discussed mtDNA method designed at UNTHSC would greatly reduce the time involved in sample processing.  The current assay requires the analyst to mix a number of reagents prior to the addition of extracted DNA.  The proposed method employs two pre-mixed buffers, significantly reducing the time required for the quality control and preparation of reagents (Table 1).  The mtDNA direct method also interrogates the entire control region with the use of only two primers: R1 and R2.  Amplification of mtDNA is currently accomplished with the use of four primers: A1, B1, C1, and D1.  Figure 1 displays a comparison of the current method to the proposed method adopting direct amplification for mtDNA.
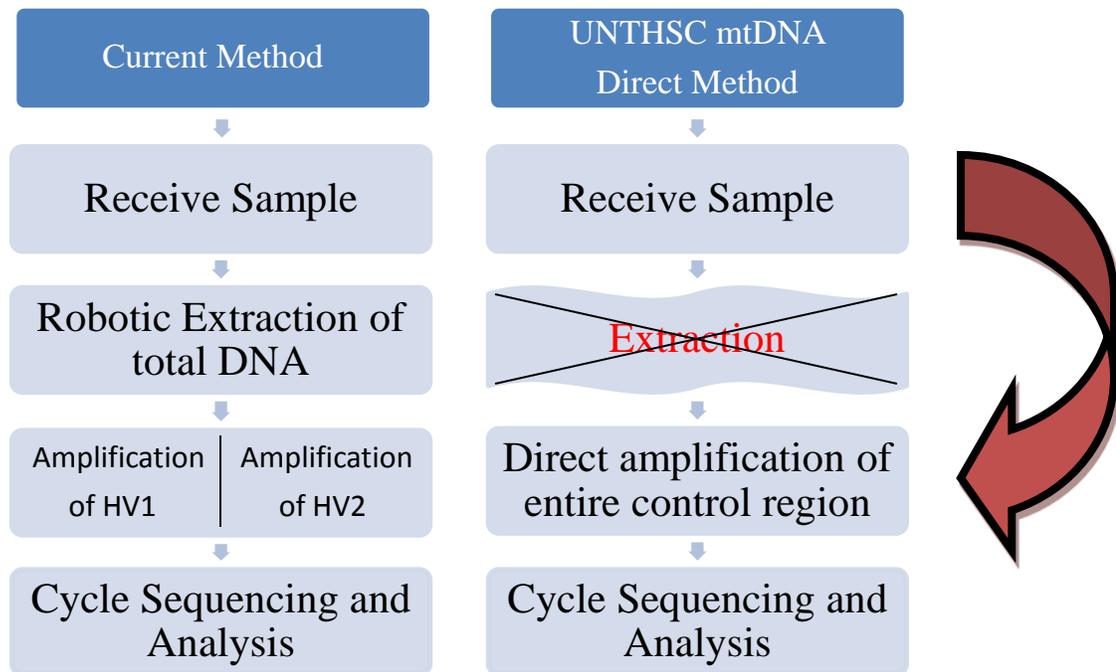
**Figure 1. Comparison of Current Method Versus the UNTHSC mtDNA Direct Method**. The proposed UNTHSC mtDNA direct method would improve the cost and efficiency of mtDNA analysis by eliminating the need for the robotic extraction step. In addition, the proposed method uses a single amplification rather than two amplifications for the entire control region.

| Reagents Required for Current Method | Volume per Sample (µL) | Reagents Required for UNTHSC mtDNA Direct Method | Volume per Sample (µL) |
|---|---|---|---|
| ddH$_2$O | 5 | | |
| 10X PCR Buffer II | 2.5 | | |
| BSA (1.6µg/µL) | 2.5 | | |
| 25mM MgCl$_2$ | 2.0 | UNTHSC Amplification Master Mix | 13 |
| dNTP mix (10mM) | 1.0 | | |
| Primer 1 (10µM) | 0.5 | | |
| Primer 2 (10µM) | 0.5 | | |
| AmpliTaq Gold (5U/µL) | 0.5 | | |

**Table 1. Comparison of Reagent Requirements for the Current Versus Proposed mtDNA Amplification Protocols.** The current method for mtDNA amplification requires the analyst to mix numerous reagents. In addition, two amplification reactions are necessary to amplify the entire control region. In the proposed method, the UNTHSC Amplification Master Mix can be directly added to the incubated sample. A similar comparison could be made for the DNA IQ™ Reference Sample Kit (Promega) versus the UNTHSC Incubation Buffer. The current extraction procedure is laborious and time-consuming; the proposed lysing method is inexpensive and fast.

**Hypothesis:**

Quality mtDNA sequence data can be obtained from reference samples without the need for

costly DNA extraction, quantification, and normalization by replacing these steps with a simple

incubation step using the UNTHSC Incubation Buffer and direct amplification using the

UNTHSC Amplification Master Mix.

**Specific Aims:**

*Aim 1A-* Optimize the direct amplification method using the UNTHSC Incubation Buffer and the

UNTHSC Amplification Master Mix.

*Aim 1B-* Evaluate the benefits and limitations of the UNTHSC direct mtDNA amplification

method for reference samples.

*Aim 2-* Test the developed procedure with different substrates: the Buccal DNA Collector™,

MacroPur™ Swab P applicators, and 4N6 FLOQSwabs™ using buccal cells and blood.  In

addition, whole blood will be tested.

CHAPTER 2


MATERIALS AND METHODS


*Sample Preparation and Collection Devices*

The subjects used for this study were recruited and tested following protocols approved

by the Office of the Protection of Human Subjects-Institutional Review Board (Project #2012-

170: Increasing the Efficiency of Mitochondrial DNA Processing of Reference Samples; UNT

Health Science Center, Fort Worth, TX). All experiments were conducted in laboratories at

UNTHSC.

Blood and buccal samples were collected with no identifying information from

consenting adults. Initially, all experiments were performed with samples from two individuals.

The Buccal DNA Collector™ (Bode Technology, Lorton, VA) (referred to as Bode),

MacroPur™ Swab P applicators (Solon Manufacturing Co., Rhinelander, WI) (referred to as

Dacron), and 4N6 FLOQSwabs™ (Copan Flock Technologies, Brescia, Italy) (referred to as

FLOQSwab) were all used as collection devices (Figure 2). Each individual provided a 7mL

EDTA tube of peripheral blood and five buccal samples deposited on each collection device by

self- collection. Blood samples were then created by depositing 25µL of whole blood on five

collection devices of each type. Once a protocol was developed, eight additional volunteers were

recruited to contribute blood by fingerstick and buccal samples. Two blood and buccal samples

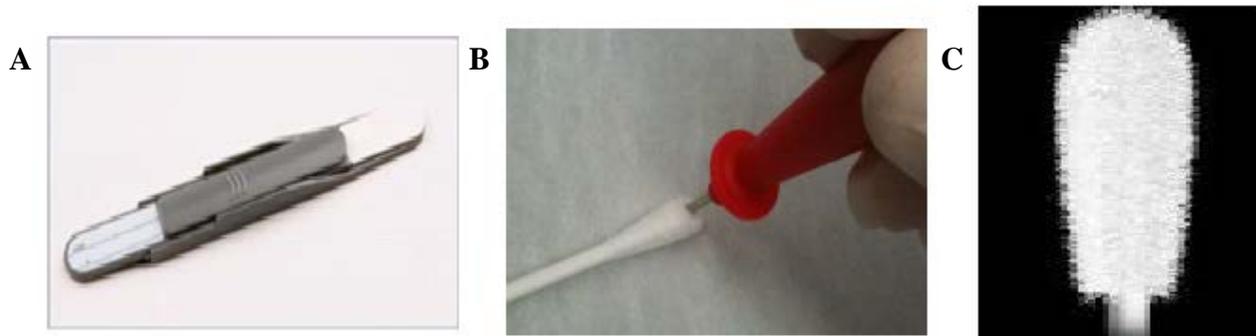from each collection device were amplified, sequenced, and analyzed.

**Figure 2. Sample Collection Devices. A.** Buccal DNA Collector™ (bodetech.com/collection-products/buccal-dna-collector-line/buccal-dna-collector/) **B.** Sampling of a MacroPur™ Swab P Applicator using a 1.2 mm Harris Micro-Punch™ (Ted Pella, Inc., Redding, CA) (2) **C.** 4N6 FLOQSwab™ (copanusa.com/index.php/products/4n6/).

*Processing of Bode and Dacron Samples*

Samples from the Bode and Dacron collection devices were processed according to the UNTHSC "mtDNA Amplification for Reference Samples" protocol (11). Samples were punched using a 1.2mm Harris Micro-Punch™ and placed in 2µL of UNTHSC Incubation Buffer (UNTHSC, Fort Worth, TX) in a 0.2mL tube. They were then incubated at 70°C for 40 minutes.

*Processing of FLOQSwab Samples*

Initially, an assay for the FLOQSwabs was evaluated using two buccal samples. A solution of 15mL Isoton III (Beckman Coulter, Inc., Brea, CA) and 150µL of Coomassie Brilliant Blue R (Sigma-Aldrich, Saint Louis, MO) was made in a 50mL conical tube. Buccal cells were eluted from the swabs by placing them in 400µL of the Isoton III/Coomassie Blue solution in a 1.5mL microcentrifuge tube. Each swab was swirled ten times in the solution. Excess liquid was removed by pressing the swab against the side of the tube three times. The samples were then centrifuged in a Fisher Scientific Marathon 16KM Centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) at 3000 RPM for five minutes. The supernatant was completely

removed and the pellet was re-suspended in 2µL of UNTHSC Incubation Buffer. The re-suspended pellet was moved to a 0.2mL tube and incubated for 40 minutes at 70$^\circ$C.

The FLOQSwab assay was slightly modified for a second experiment using ten buccal samples. Buccal cells were eluted from the swabs in 400µL of Isoton III in a 1.5mL tube (Coomassie Blue was not added; Coomassie Blue dyes the cell pellet for visualization only). The tubes were then centrifuged for five minutes in a Fisher Scientific Marathon 16KM Centrifuge at 3000rpm. Following centrifugation, 380µL of the supernatant was removed. The pellet was re-suspended in the remaining supernatant. To lyse the cells, 2µL of the re-suspended pellet was moved to a 0.2mL tube containing 2µL of the UNTHSC Incubation Buffer. The samples were incubated at 70°C for 40 minutes. This assay was then repeated to process ten FLOQSwab blood samples.

*Amplification*

Following the incubation step, 11µL of the UNTHSC Amplification Master Mix (UNTHSC, Fort Worth, TX) was added to all samples. This proprietary amplification master mix contains all of the components necessary for amplification. This master mix amplifies the entire control region. See schema in Figure 3 for amplification of the large amplicon. Water was then added to reach a total reaction volume of 15µL. Samples were amplified in a GeneAmp® PCR System 9700 (Applied Biosystem using the thermal cycling parameters listed in Table 1.
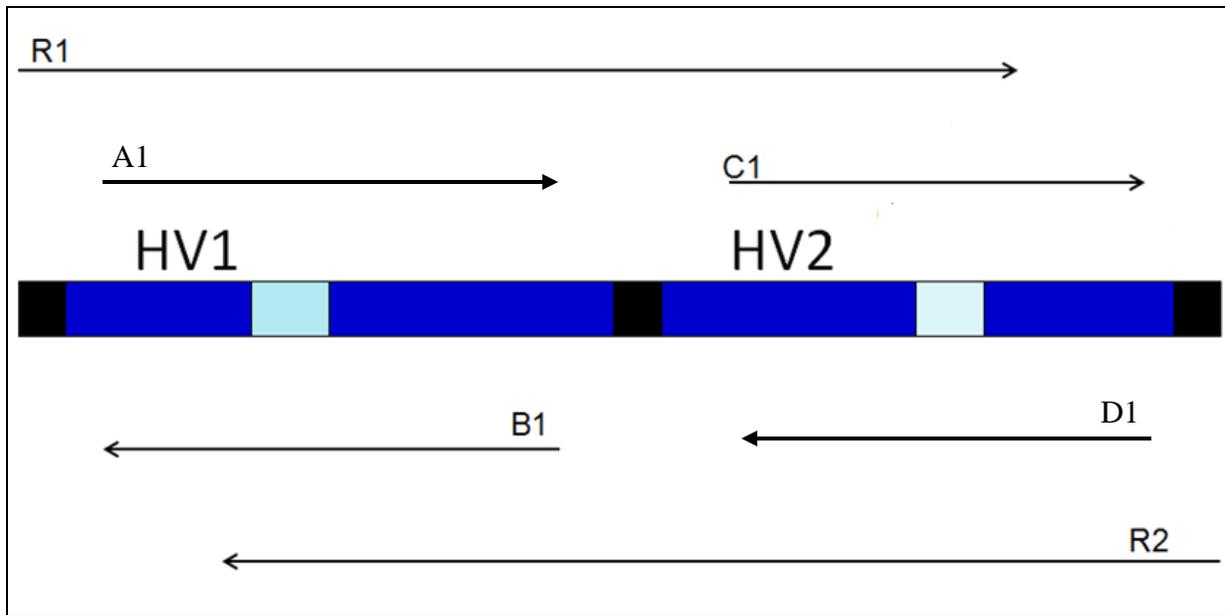
**Figure 3. Control Region of mtDNA and Amplification Primers.** The UNTHSC forensic laboratory currently uses four primers for mtDNA amplification. Primers A1 and B1 are used to amplify HV1. Primers C1 and D1 are used to amplify HV2. The UNTHSC direct amplification method only uses two primers, R1 and R2, to amplify the entire control region.

| Number of Cycles | Temperature | Time (min:sec) |
|:---:|:---:|:---:|
| **HOLD** | 95°C | 11:00 |
| **BLOOD-28** | 95°C | 0:10 |
| | 60°C | 0:45 |
| **BUCCAL-32** | 72°C | 1:00 |
| **HOLD** | 15°C | 10:00 |
| **HOLD** | 4°C | ∞ |

**Table 2. Amplification Parameters for mtDNA Direct Amplification Method.** Thermal cycling parameters as outlined in the UNTHSC mtDNA Amplification for Reference Samples protocol.

*Visualization of PCR Product*

The presence of PCR product was visualized using agarose gel electrophoresis. A 2% gel was created by dissolving 4g of Certified™ Molecular Biology Grade Agarose (Bio-Rad Laboratories, Hercules, CA) in 200mL of 1X tris-acetate EDTA (TAE) buffer. The 1X TAE buffer was diluted with double distilled water (ddH$_2$O) from a stock of UltraPure™ 10X TAE Buffer (Life Technologies, Grand Island, NY). The solution was then heated in a microwave for 3 minutes. Once slightly cooled, 16mL of the agarose solution was poured into a casting tray and allowed to firm for 30 minutes. The firm gel was placed in a RunOne™ Electrophoresis System (Embi Tec) and covered with 1X TAE buffer. Samples were prepared for loading by mixing 2µL of PCR product with 1µL of 1X GelPilot Loading Dye (QIAGEN, Gaithersburg, MD). A 1 Kb Plus DNA Ladder (Life Technologies) was diluted with double distilled water to a final concentration of 0.25 Kb, and 2µL of the ladder was loaded into the first well of each gel. Samples were electrophoresed at 100V for 30 minutes. The gel was then stained in 5µL of Ethidium Bromide Solution (10mg/mL) (Bio-Rad Laboratories) and 60mL of 1X TAE for 30 minutes. The gel image was captured using an AlphaImager Mini System (ProteinSimple, Santa Clara, CA).

*Sequencing of mtDNA*

Sequencing was performed according to the UNTHSC "Post-PCR mtDNA Processing" protocol (12). Post-PCR mtDNA purification was performed using 5µL of ExoSAP-IT® (USB Corp., Cleveland, OH) per sample to reach a total reaction volume of 18µL (2µL was consumed for product visualization) on the GeneAmp® PCR System 9700 thermal cycler using the conditions listed in Table 2.

| Temperature | Time (min:sec) |
| --- | --- |
| 37°C | 15:00 |
| 80°C | 15:00 |
| 4°C | ∞ |

**Table 3. Post-PCR Purification Using ExoSAP-IT®.** Incubation parameters as outlined in the UNTHSC "Post-PCR mtDNA Processing" protocol.

Following purification, samples were cycle sequenced using the BigDye® Terminator™ v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing master mix was made according to Table 4.

| Reagent | Volume per Sample (µL) |
| --- | --- |
| **BetterBuffer** (Gel Company, San Francisco, CA) | 5.0 |
| **BigDye Terminator v1.1** | 1.0 |
| **Primer (3.3µL) R1, B1, C1, or R2** | 1.5 |
| **PCR Product** | 0.5-7.5 |
| **ddH$_2$O** | QS to 15 |
| **TOTAL** | **15** |

QS=*quantum sufficit*

**Table 4. Master Mix for mtDNA Cycle Sequencing.** Volume per sample of cycle sequencing reagents as outlined in the UNTHSC Post-PCR mtDNA Processing protocol. Primers used for each cycle sequencing reaction were: R1, B1, C1, and R2.

The volume of PCR product used per sequencing reaction was determined by evaluating the intensity of the gel band for each sample. For Bode and Dacron samples, 3µL of PCR product was added. For samples from the FLOQSwabs, 4µL of PCR product was added to each

reaction.  All samples were adjusted to reach a total reaction volume of 15µL.  Samples were

placed in the GeneAmp® PCR System 9700 thermal cycler and cycle sequenced using the

parameters listed in Table 5.

| Number of Cycles | Temperature | Time (min:sec) |
| :---: | :---: | :---: |
| HOLD | 96°C | 3:00 |
| 25 | 96°C<br>50°C<br>60°C | 0:15<br>0:10<br>3:00 |
| HOLD | 4°C | ∞ |

**Table 5.  Cycle Sequencing Parameters.**  Sequencing parameters as outlined in the UNTHSC
Post-PCR mtDNA Processing protocol.

Samples were then purified with Performa® Spin Columns (Edge BioSystems,

Gaithersburg, MD).  The Performa Gel Filtration Cartridge was first centrifuged in the Fisher

Scientific Marathon 16KM Centrifuge at 3500rpm for 3 minutes.  The cartridge was then moved

to a 1.5mL microcentrifuge tube.  Samples were added drop-wise to the center of the packed gel

column and centrifuged at 3500rpm for 3 minutes.  The eluate was subjected to capillary

electrophoresis using a 3130*xl* Genetic Analyzer (Applied Biosystems).  Samples were loaded on

the CE by electrokinetic injection at 1200 V for 18 seconds.

*Sequence Data Analysis*

Sequence data analysis was performed using Sequence Scanner v1.0 (Applied

Biosystems), eFAST™ Software (UNTHSC, Fort Worth, TX) and MTexpert™ (Mitotech™,

Sante Fe, NM).  Trace scores (TS) and contiguous read length (CRL) values were tabulated for

all sequenced samples.  A two-way ANOVA with TS and CRL values was conducted using SPSS software (IBM, Armonk, NY) to examine the effect of the collection device and cell type on sequence data.

*HL-60 PCR and Sequencing Control DNA*

Control mtDNA from the HL-60 cell line was used as a positive control.  A 0.24pg/µL concentration was used for all blood samples.  A 0.12pg/µL concentration was initially used for buccal samples.  In order to determine the optimal HL-60 concentration for buccal sample processing, decreasing concentrations of HL-60 (*i.e.,* 0.12pg/µL, 0.09pg/µL, 0.06pg/µL, and 0.03pg/µL) were amplified at 32 cycles alongside buccal samples from the Bode (n=4).  PCR product was visualized using agarose gel electrophoresis.  Buccal samples and HL-60 samples were arranged on the gel in an alternating fashion to assist in comparison of band intensities.

*Quality Control and Stability of the UNTHSC Incubation Buffer*

Initial experiments were performed with Lot A of the UNTHSC Incubation Buffer.  To test the quality control of the buffer, a new lot, Lot B, was manufactured.  Blood (n=2) and buccal (n=2) samples punched from the Bode collectors were processed using Lot B.  Control mtDNA from the HL-60 cell line (0.24pg/µL) was also amplified.  A punch from a blank Bode collector was used as a negative control.  To test the stability of the UNTHSC Incubation Buffer, an aliquot of Lot B was removed from storage at 25°C and at 4°C after 28 days and again after 61 days.  Blood (n=2) and buccal (n=2) samples from the Bode collectors were processed using both the 28 and 61 day aliquots for both storage conditions.  Control mtDNA from the HL-60 cell line (0.24pg/µL) was processed using both aliquots.

CHAPTER 3


RESULTS


*Amplification of Blood Samples from Ten Donors Using the Bode Collector*

Blood samples were collected from ten donors using the Bode collectors.  Samples were

processed as described.  All samples generated amplified product, with some variation of band

intensity (Figure 4).    The positive control amplified as expected.  These results indicate that the

use of the Bode collector for blood sample processing is extremely compatible with the direct

lysis and amplification method described.



**Figure 4.  Amplification of Blood Samples from Ten Donors: Bode Collector.**  Gel image obtained following amplification of blood samples from ten donors.  Samples were punched from the Bode collector.  All samples generated PCR product.

*Amplification of Buccal Samples from Ten Donors Using the Bode Collector*

 Buccal samples were collected from ten donors using the Bode collectors. Samples were processed per developed protocol. Eight of the samples generated amplified products. All eight bands were of similar intensity. Two of the samples did not amplify, which may be due to the variability of self-collection of buccal samples (Figure 5). Samples were punched from Region I or II of the Bode collector. Although these areas have been determined through previous studies to be the most ideal regions for buccal cell recovery, these samples can still be less optimal in comparison to a blood sample since the buccal cells cannot be visualized on the collector (13). The positive control amplified as expected. Overall, the use of the Bode collector for buccal sample processing is compatible with the direct lysis and amplification method described. A non-specific amplification product was present in several of the gels for Donor 9. The band was observed at approximately 300bp. Future studies should consider sequencing this band (Figures 5, 6, and 7).
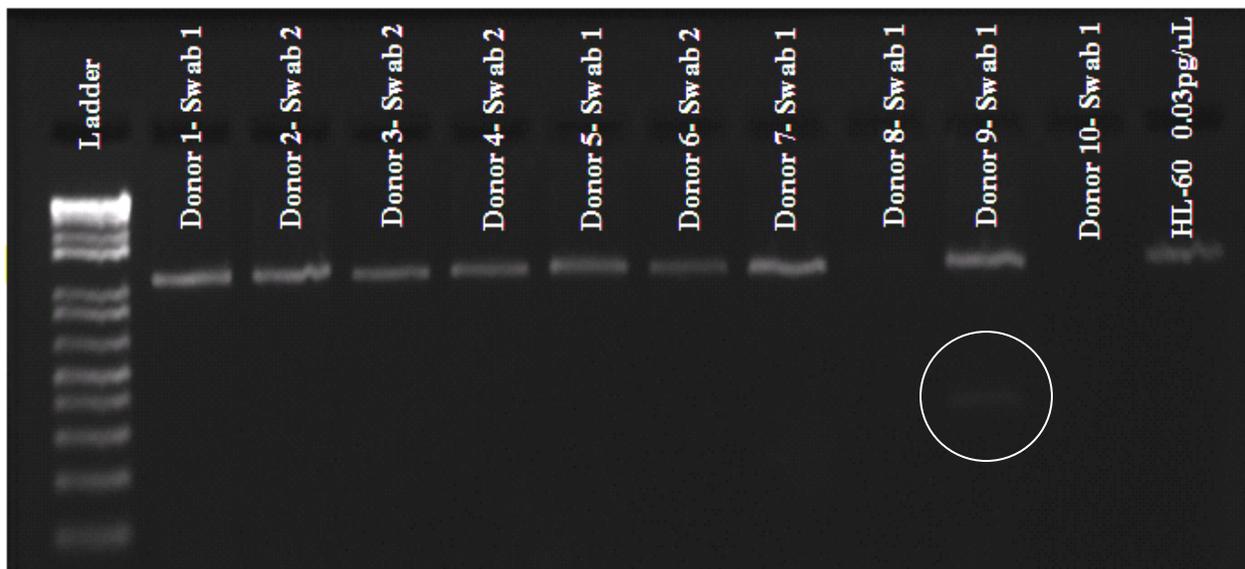


**Figure 5. Amplification of Buccal Samples from Ten Donors: Bode Collector.** Gel image obtained following amplification of buccal samples from ten donors. Samples were punched from the Bode collector from Region I or II. Eight of the ten samples generated PCR product. The circle signifies the non-specific amplification product observed for Donor 9.

*Amplification of Blood Samples from Ten Donors Using the Dacron Swabs*

Blood samples were collected from ten donors using the Dacron swabs. Samples were processed per the developed protocol. Gel bands were of varied intensity, but all samples generated amplified product (Figure 6). The positive control amplified as expected. It was most efficient to punch the swab in a location in which the blood had caused the Dacron to stiffen. The use of the Dacron swabs for blood sample processing proved to be compatible with the direct lysis and amplification method described.
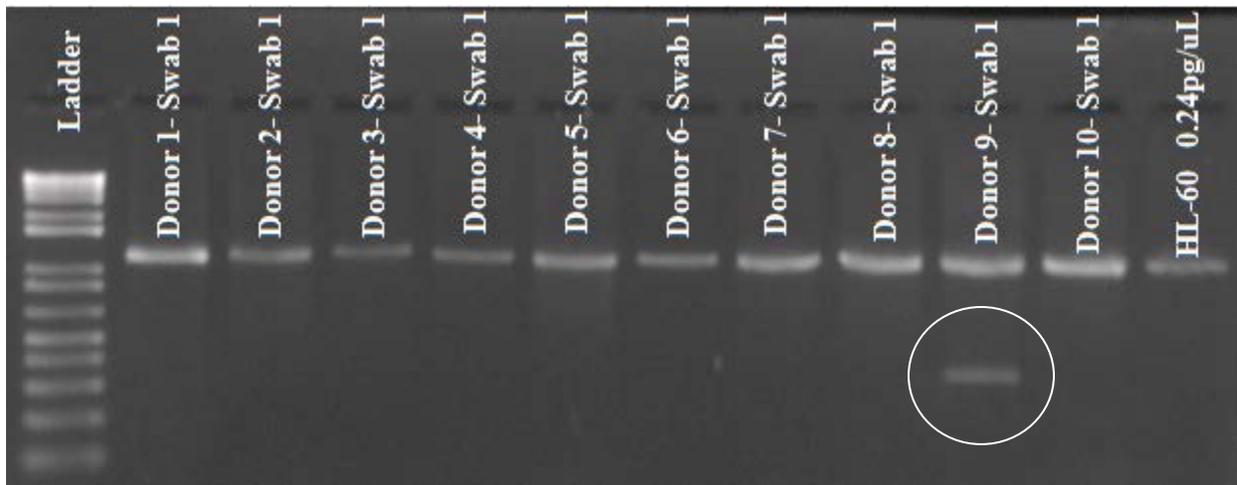


**Figure 6. Amplification of Blood Samples from Ten Donors: Dacron Swabs.** Gel image obtained following amplification of blood samples from ten donors. Samples were punched from the Dacron swabs. All samples generated PCR product. The circle signifies the non-specific amplification product observed for Donor 9.

*Amplification of Buccal Samples from Ten Donors Using the Dacron Swabs*

Buccal samples were collected from ten donors using the Dacron swabs. Samples were processed per the developed protocol. All samples generated amplified product of similar intensity (Figure 7). The positive control amplified as expected. The use of the Dacron swabs for buccal sample processing proved to be compatible with the direct lysis and amplification method described.
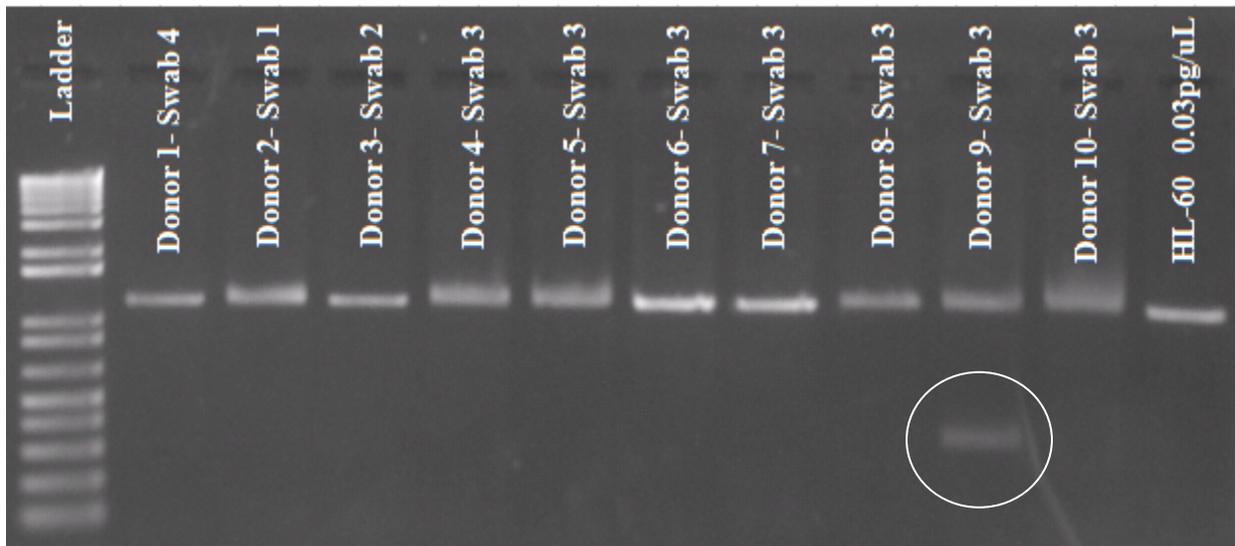


**Figure 7. Amplification of Buccal Samples from Ten Donors: Dacron Swabs.** Gel image obtained following amplification of buccal samples from ten donors. Samples were punched from the Dacron swabs. All samples generated PCR product. The circle signifies the non-specific amplification product observed for Donor 9.

*Buccal Samples from the 4N6 FLOQSwabs™*

An assay for the Nylon swabs was evaluated using two buccal samples. Buccal cells were eluted from the swabs using an Isoton III/Coomassie Blue solution. Following centrifugation, the supernatant was removed and the pellet was re-suspended in UNTHSC Incubation Buffer, moved to a 0.2 mL tube, and incubated for 40 minutes at $70^{\circ}$C. The UNTHSC Amplification Master Mix was then added and the samples were amplified. Both samples generated amplified product (Figure 8). However, this protocol used additional steps that were not needed in the processing of samples punched from the Bode and Dacron collectors. Further experiments were performed to assess the feasibility of eliminating several of these steps and reducing the reaction to only one tube.
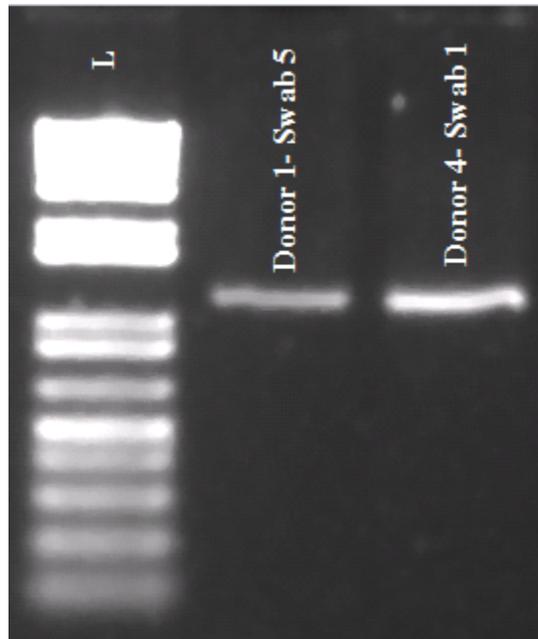


**Figure 8. Amplification of Buccal Samples: 4N6 FLOQSwabs™.** Gel image obtained following amplification of buccal samples from the 4N6 FLOQSwabs™. Both samples generated PCR product. Further experiments were performed to reduce the number of steps involved.

The FLOQSwab assay was slightly modified for a second experiment.  Buccal cells were eluted from the swabs in 400µL of Isoton III in a 1.5mL tube.  Following centrifugation, 380µL of the supernatant was removed and the pellet was re-suspended in the remaining supernatant. To lyse the cells, 2µL of the re-suspended pellet was moved to a 0.2mL tube containing 2µL of the UNTHSC Incubation Buffer.  The samples were then incubated and amplified per the developed protocol.  Nine of the ten samples generated amplified product with varying intensity (Figure 9).
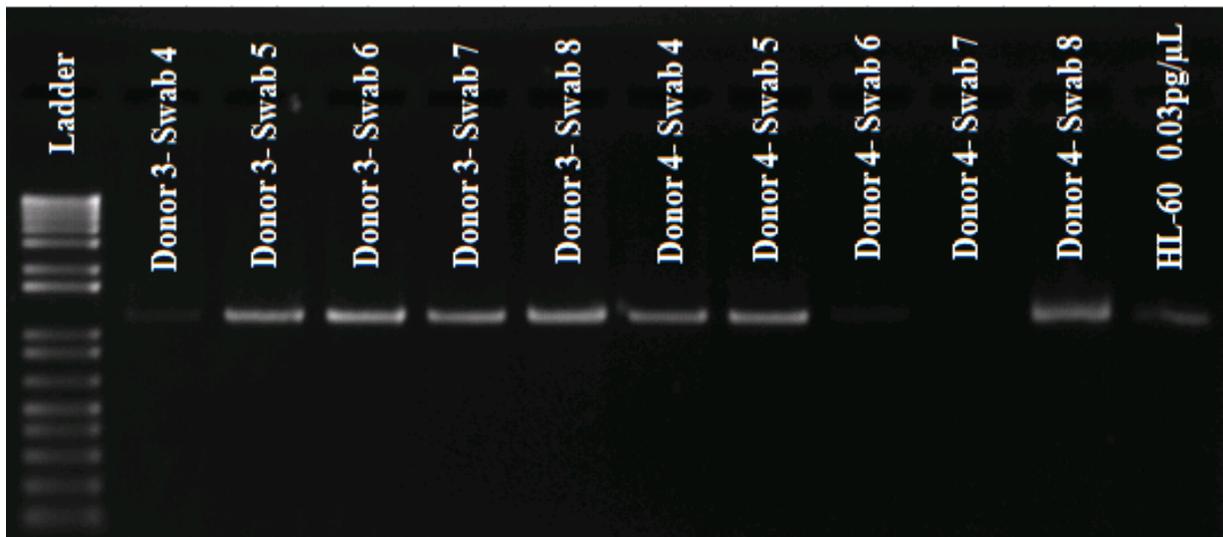


**Figure 9.  Modification to 4N6 FLOQSwab™ Protocol for Buccal Samples.**   Gel image obtained following amplification of buccal samples from two donors using a modification to the protocol for FLOQSwabs.  Nine of the samples generated PCR product.

*Blood Samples from the 4N6 FLOQSwabs™*

 Blood samples were processed according to the protocol modified for buccal sample

processing.  The samples were then incubated and amplified per the developed direct

amplification protocol.  Six of the ten samples generated amplified product (Figure 10).  It is

possible that inhibition prevented some of these blood samples from amplifying.  Centrifugation

of the sample and subsequent re-suspension in a lesser amount of supernatant could have

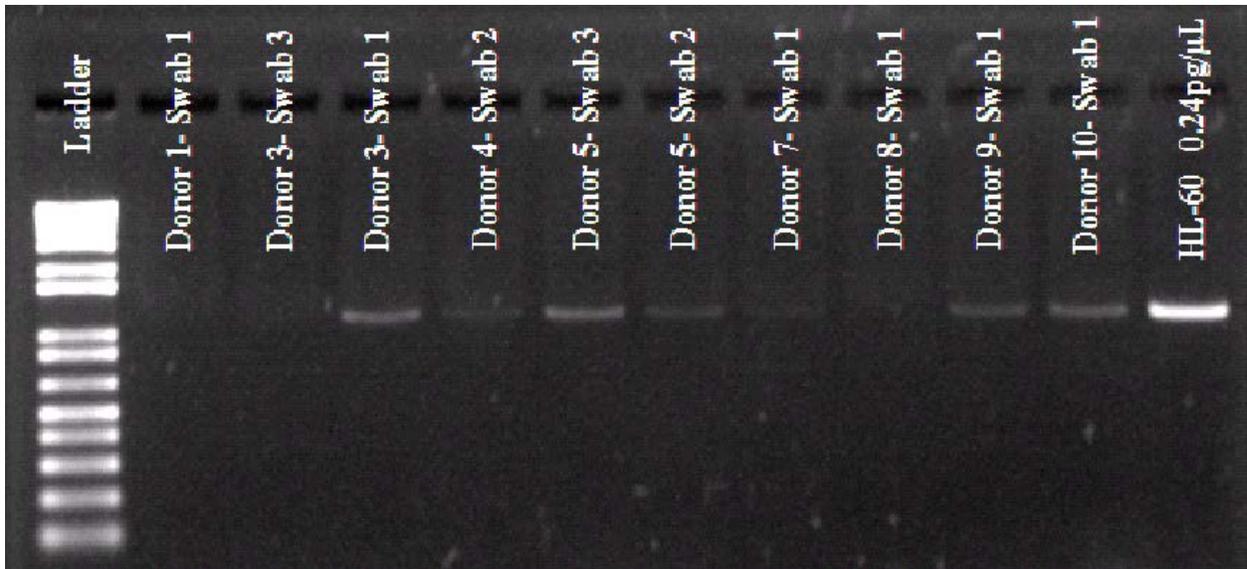introduced enough heme to inhibit the amplification reaction (14).



**Figure 10. Amplification of Blood Samples: 4N6 FLOQSwabs™.** Gel image obtained
following amplification of ten blood samples from eight donors.  Seven of the samples generated
PCR product.

*Determination of Optimal HL-60 Concentration for Buccal Samples*

Decreasing concentrations of HL-60 were amplified at 32 cycles alongside buccal samples from the Bode collectors.  This experiment was performed to determine which concentration of HL-60 would be most appropriate for use as a positive control for buccal sample analysis.  The band intensity of the 0.03 pg/µL HL-60 was most similar to that of the buccal samples and was therefore determined to be the optimal concentration for a control when amplifying buccal samples (Figure 10) using the thermal cycling parameters described in Table 2.
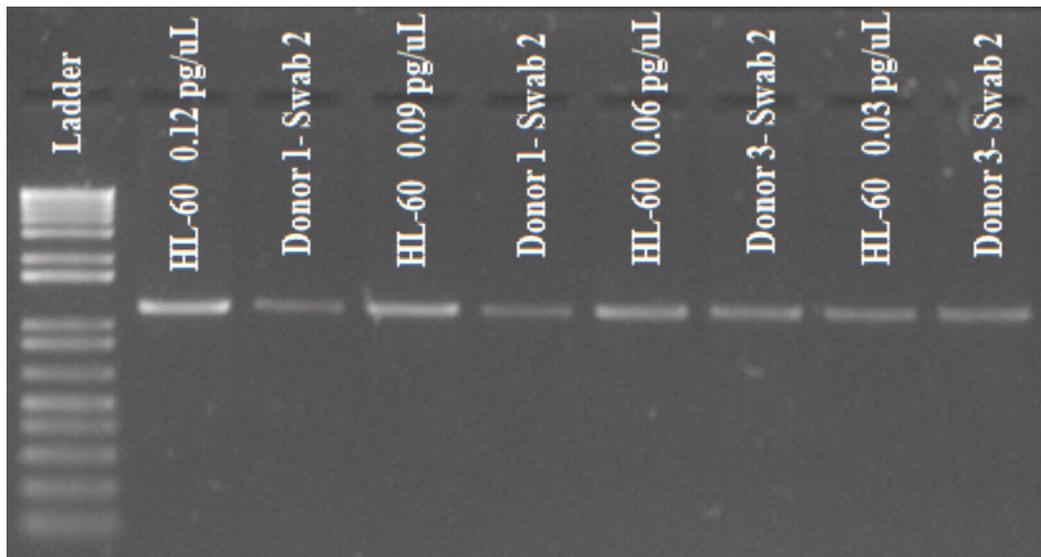


**Figure 11.  Determination of Optimal HL-60 Concentration for Buccal Samples.**  Gel image obtained following the amplification of buccal samples with decreasing concentrations of HL-60.  The 0.03 pg/µL HL-60 was determined to be the optimal concentration for buccal sample analysis using 32 cycles for amplification.

*Quality Control of the UNTHSC Incubation Buffer*

Blood and buccal samples from the Bode collectors were processed using Lot B of

UNTHSC Incubation Buffer.  All samples generated amplified product similar to Lot A buffer.

The positive control performed as expected.  There was no evidence of contamination in the

negative control (labeled 'Blank') (Figure 12).  The results of this experiment indicate that the

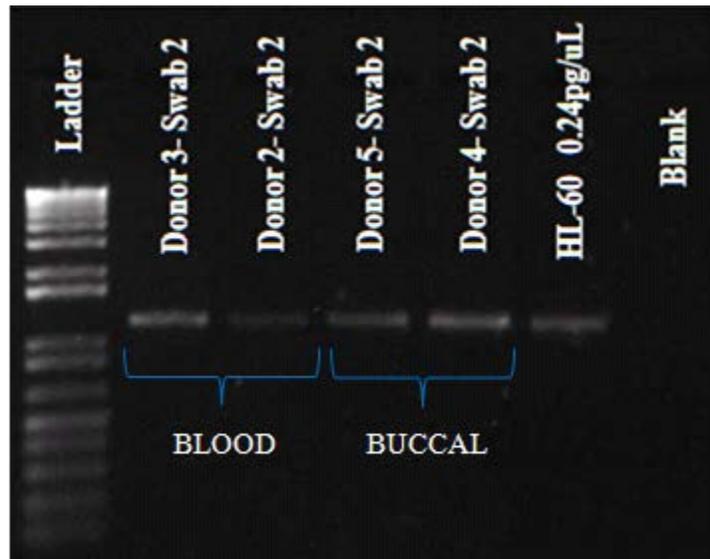UNTHSC Incubation Buffer is easy to manufacture and is reproducible.



**Figure 12.  Reproducibility of the UNTHSC Incubation Buffer.**  Gel image obtained following amplification of blood and buccal samples using Lot B of the UNTHSC Incubation Buffer.  All reference samples generated PCR product.

*Stability of the UNTHSC Incubation Buffer at 25°C and 4°C*

An aliquot of Lot B of the UNTHSC Incubation Buffer was removed after storage at 25°C and at 4°C for 28 days and 61 days. No precipitation was observed in either aliquot. Blood and buccal samples from the Bode collectors were processed using both the 28 and 61 day aliquots for both storage conditions. Control mtDNA from the HL-60 cell line (0.24pg/µL) was processed using both aliquots. All samples generated PCR product (Figures 12 and 13). There was some variation in the intensity of the bands, which is most probably due to variation in the sample and not in the UNTHSC Incubation Buffer.
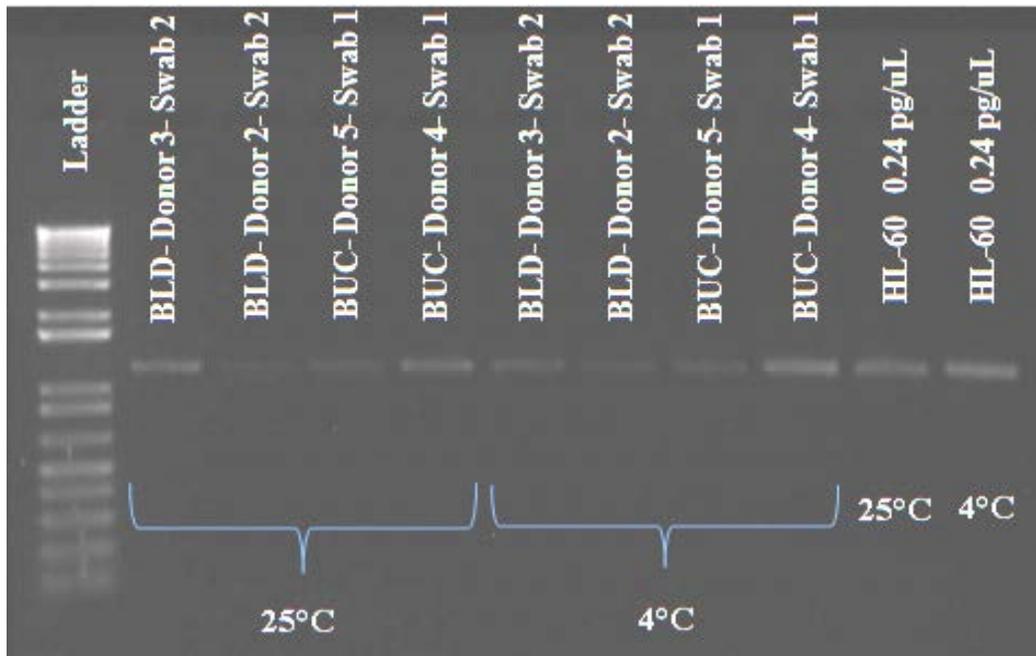


**Figure 13. Stability of the UNTHSC Incubation Buffer (28 Days).** Gel image obtained following the amplification of blood and buccal samples using UNTHSC Incubation Buffer stored at 25°C and 4°C for 28 days. All reference samples and both positive controls generated PCR product.
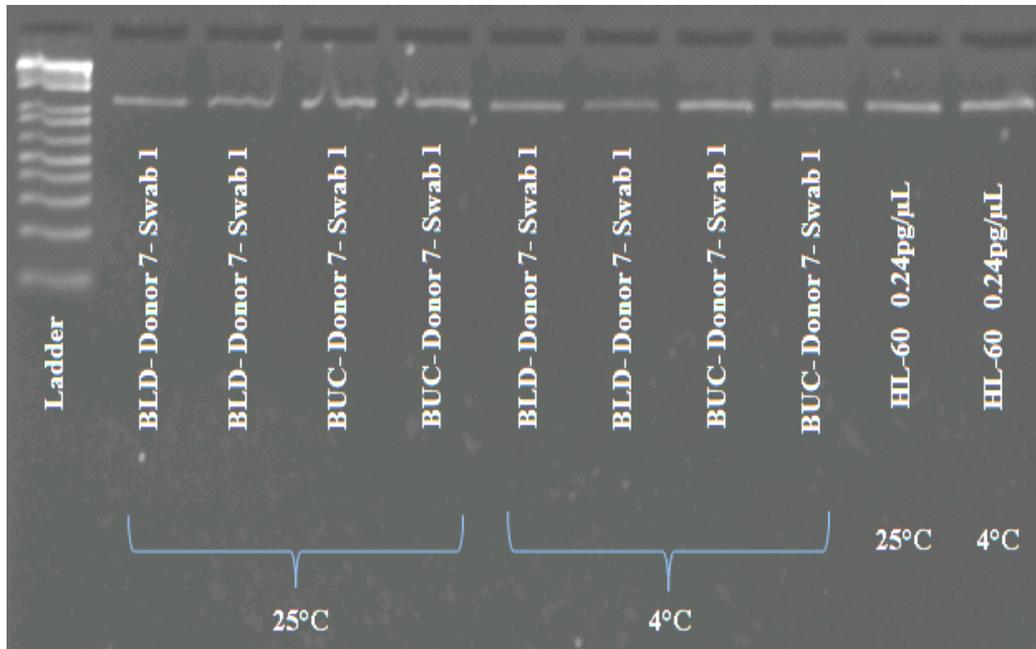
**Figure 14. Stability of the UNTHSC Incubation Buffer (61 Days).** Gel image obtained following the amplification of blood and buccal samples using UNTHSC Incubation Buffer stored at 25°C and 4°C for 61 days. All reference samples and both positive controls generated PCR product. This gel should have been electrophoresed for a longer time period.

*Sequence Data Quality*

The sequence data for all Bode and Dacron samples appeared uniform and of excellent quality. The sequence data from both blood and buccal samples for both collection devices produced high quality trace scores. Buccal samples from the FLOQSwab also generated high quality sequence data. The only sample to generate a low quality trace score was the FLOQSwab blood sample from Donor 5.



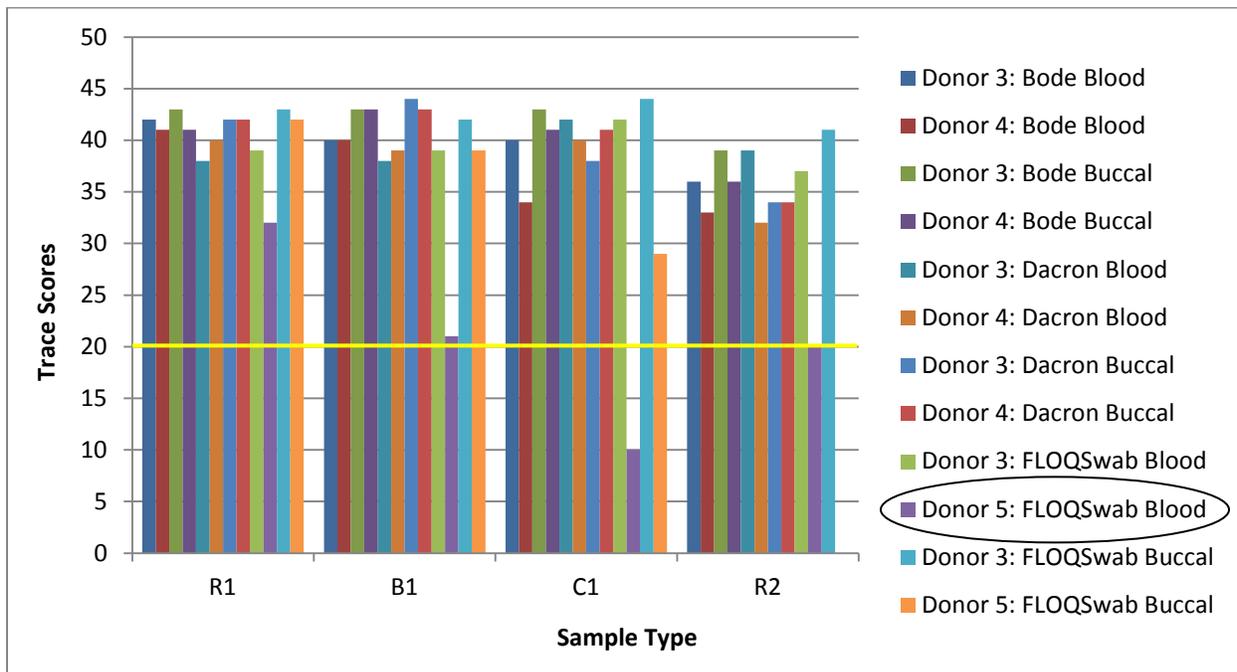**Figure 15. Trace Scores from Sequence Data.** Trace score values generated using Sequence Scanner v1.0. Chart depicting the range of quality scores assigned to each sequence trace. Trace scores greater than 20 indicate high quality sequence data. Most sequence traces had trace scores well above 20 (as shown by the yellow line). The sequence data from the FLOQSwab blood sample from Donor 5 had a low quality trace score.

*Statistical Analysis of Trace Score Data*

A two-way ANOVA was conducted to determine whether the collection device used or the cell type had an influence on the trace scores. The collection device (p=0.030) and the cell type (p=0.015) both had a significant effect on trace scores. However, the interaction effects were insignificant (p=0.169).



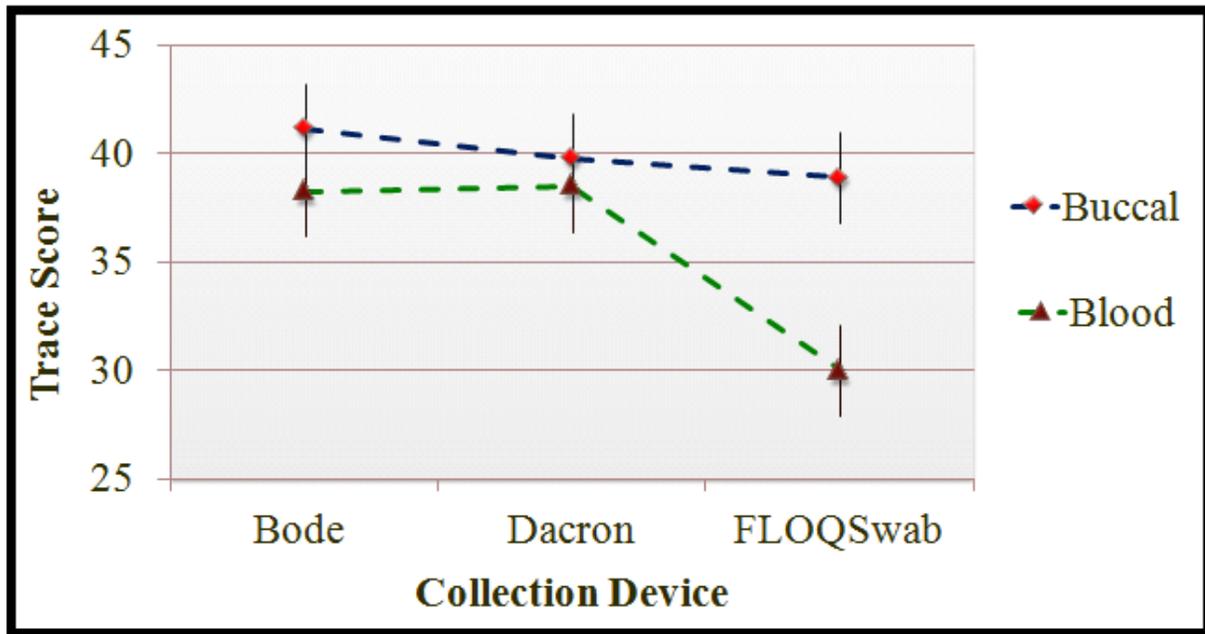**Figure 16. Mean Trace Scores for Three Collection Devices with Two Cell Types.** Two-way ANOVA data for all three collection devices and both cell types. The standard error is 2.086 and is displayed (standard deviations are as follows: $Bode_{Blood} = 3.412$; $Dacron_{Blood} = 2.928$; $FLOQSwab_{Blood} = 11.588$; $Bode_{Buccal} = 2.532$; $Dacron_{Buccal} = 3.955$; $FLOQSwab_{Buccal} = 5.693$).

*Sequencing: Contiguous Read Length*

CRL values were collected for the sequence data generated. CRL values are assigned as follows: long (400 bases or greater), medium (200-400 bases), and short (200 bases or less). Donor 3 blood sample from the Dacron swab produced a medium CRL for R1 primer, whereas all other samples generated long CRL values for that primer. Donor 3 blood sample for the Dacron swab produced a short read length for B1 primer. Donor 4 blood sample from the Bode collector resulted in a medium CRL value for C1 primer. CRL values for R2 were generally medium, with the exception of the Donor 4 buccal sample from the Bode collector, which produced an exceptionally long CRL value. The Donor 5 FLOQSwab blood sample generated short CRL values for primers B1, C1, and R2.
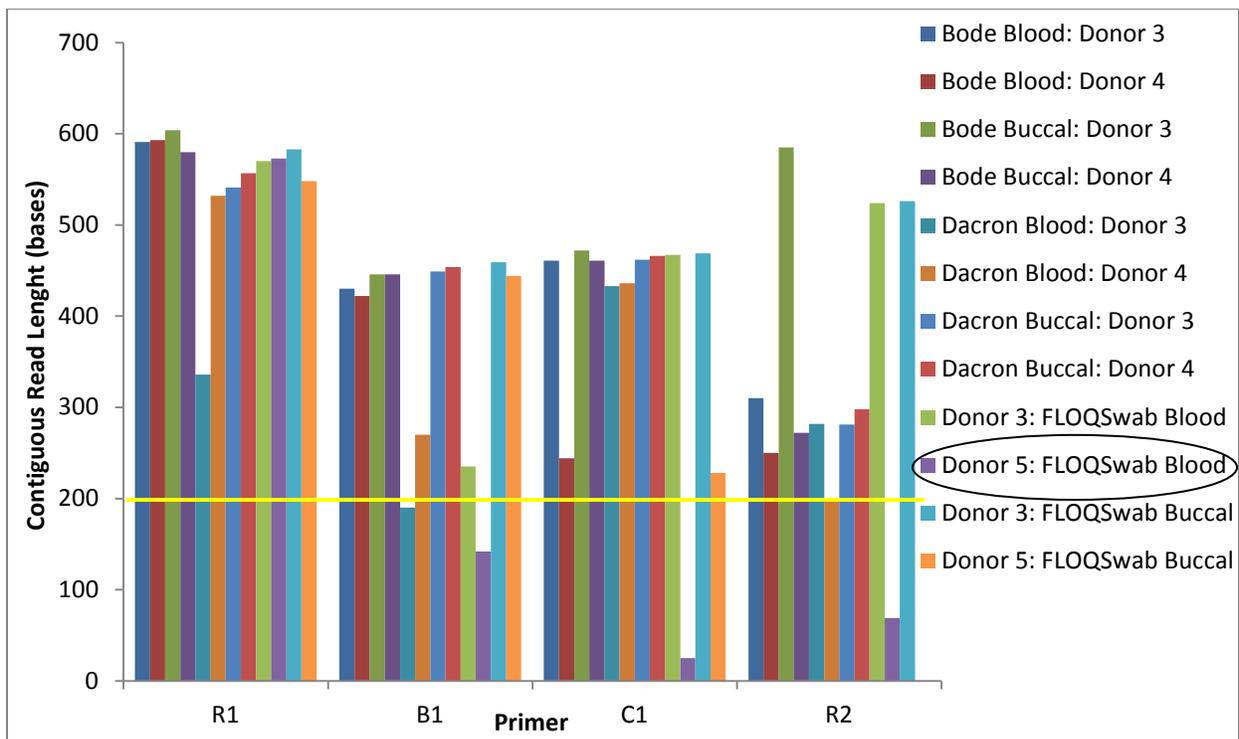


**Figure 17. Contiguous Read Lengths from Sequence Data.** CRL values generated using Sequence Scanner v1.0. Chart depicting the range of CRL values assigned to each sequence trace. There was some variation noted between the different collection devices. The FLOQSwab blood sample from Donor 5 had the lowest CRL values.

A two-way ANOVA was conducted to determine whether the collection device used or the cell type had an influence on the CRL values. The collection device (p=0.365) did not have a significant effect on the CRL values. However, the cell type (p=0.030) had a significant effect. The interaction effects were insignificant (p=0.918).



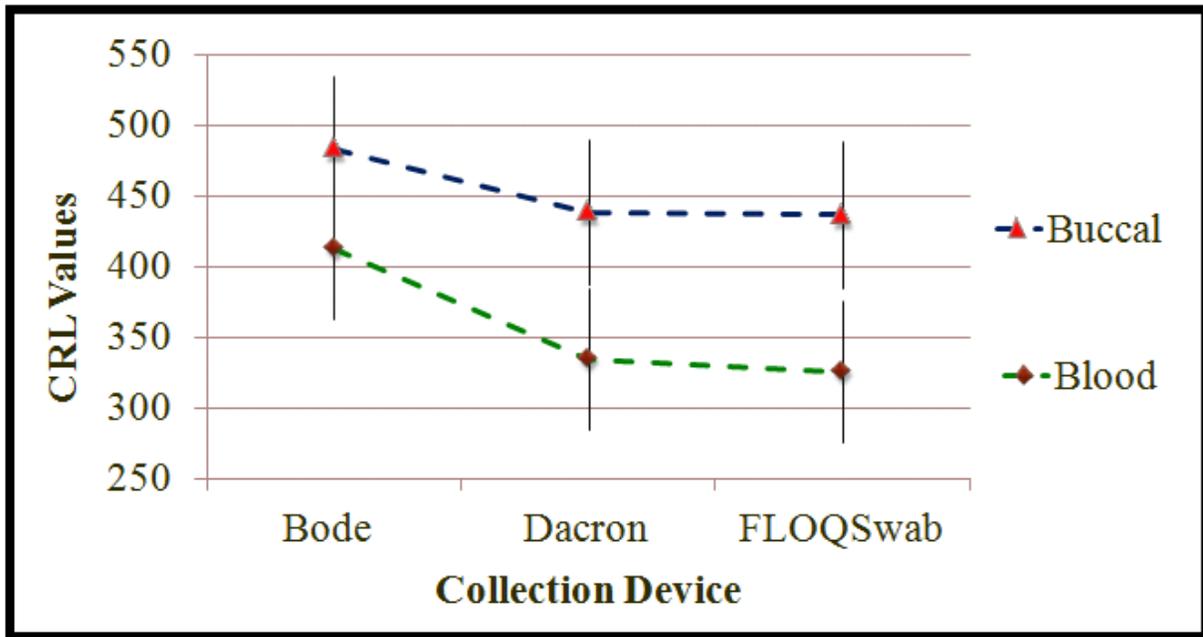**Figure 18. Mean CRL Values for Three Collection Devices with Two Cell Types.** Two-way ANOVA data for all three collection devices and both cell types. The standard error is 51.681 and is displayed (standard deviations are as follows: $Bode_{Blood}$ = 137.579; $Dacron_{Blood}$ = 122.732; $FLOQSwab_{Blood}$ = 232.507; $Bode_{Buccal}$ = 108.442; $Dacron_{Buccal}$ = 100.537; $FLOQSwab_{Buccal}$ = 135.226).

Two donors of each sample type were sequenced using four primers: R1, B1, C1, and R2. Blood and buccal samples from the Bode collectors and the Dacron swabs all generated sequence data of similar quality. All primers produced quality sequence data with little or no baseline noise.
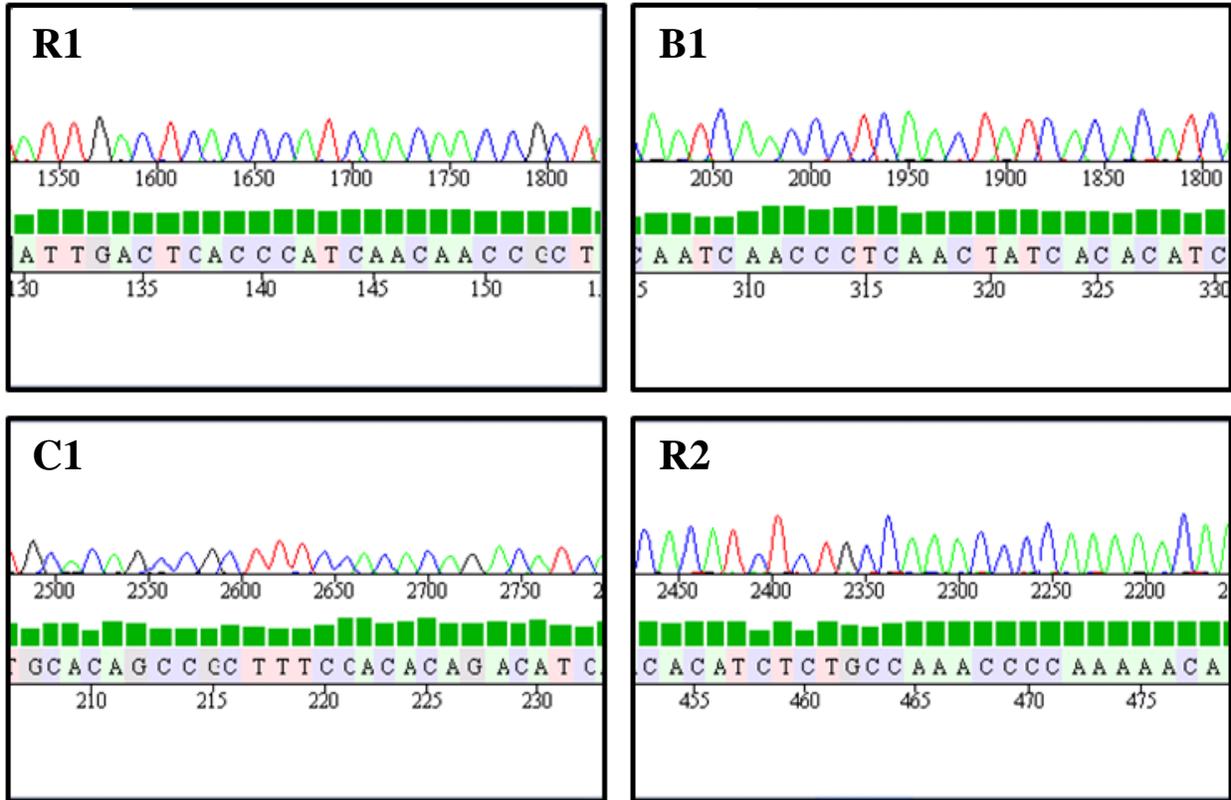


**Figure 19. Evalution of Sequence Data for Each Primer.** Sequence data obtained from a blood sample as seen using MTexpert™ software. All primers performed well and generated quality sequence data.

The quality of sequence data was compared across all sample types for two donors.  The majority of the samples generated high quality sequence data with little or no baseline noise.  All sample types were aligned with the revised Cambridge Reference Sequence (rCRS) (15).  All samples from the Bode and Dacron collectors, as well as buccal samples from the FLOQSwabs, generated high quality sequence data.  Blood samples from the FLOQSwabs produced mixed results.  Figure 20 displays sequence data from all sample types for Donor 3.

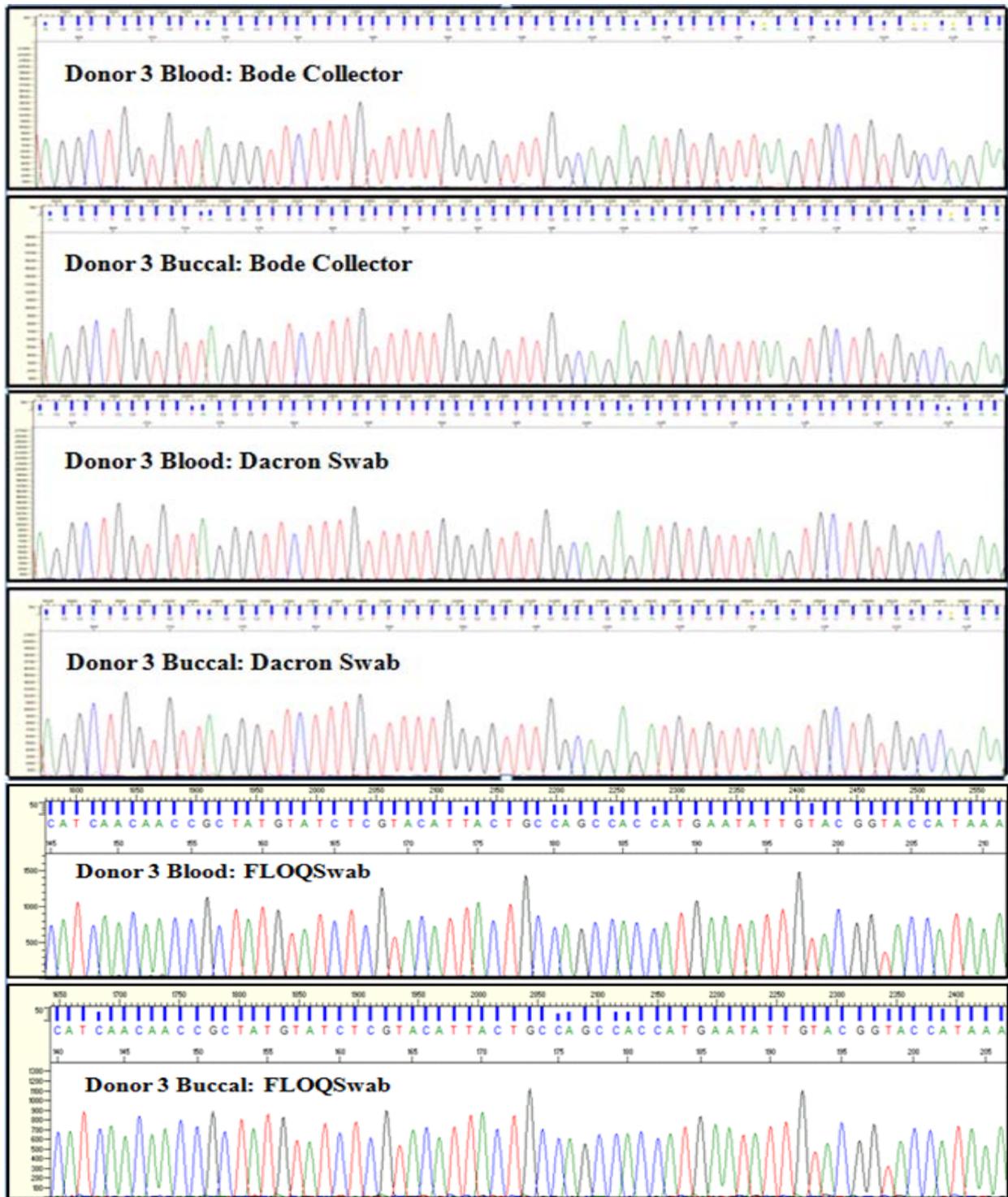**Figure 20. Sequence Data from One Donor for All Collection Devices and Both Cell Types.** Sequence data generated from R1 primer obtained from one donor using Sequence Scanner v1.0. All collection devices and cell types produced quality sequence data.

CONCLUSIONS

Mitochondrial DNA (mtDNA) analysis in forensic testing is especially useful for human skeletal remains and hair samples (16). Just as with any unknown sample, it is necessary to compare the profile generated from these samples to a reference. The immediate need for the University of North Texas Center for Human Identification is to improve the efficiency in which reference samples are processed for mtDNA. Currently, mtDNA analysis involves a series of time-consuming and costly steps. Several of these steps are not necessary. Because of recent advances in nuclear DNA testing, many laboratories have adopted direct amplification methods for reference samples, eliminating the need for extraction, quantification, and normalization. Since it is still necessary to extract the DNA for mtDNA testing, the bottleneck is shifted to extraction, which in turn slows the entire process for casework that requires both nuclear and mtDNA typing. A direct lysis and amplification method for mtDNA would prove to be of great value by removing this bottleneck. In this study, we validated a direct amplification assay for mtDNA. This method uses two buffers, the UNTHSC Incubation Buffer and the UNTHSC Amplification Master Mix, to directly lyse and amplify mtDNA from blood and buccal samples in a single tube.

The developed procedure was tested using the following media: Buccal DNA Collectors™ (Bode), MacroPur™ Swab P Applicators (Dacron), and 4N6 FLOQSwabs™ (FLOQSwab). Blood and buccal samples from the Bode and Dacron collectors were used to successfully amplify mtDNA from a total of ten donors. Both collection devices proved to be extremely compatible for use with the UNTHSC direct amplification method for mtDNA. Blood samples from both collection devices and buccal samples from the Dacron swabs, all from different donors, produced 100% positive results. Buccal samples from the Bode collectors from

different donors produced 80% positive results, indicating that it may be necessary at times to take a second punch from the Bode collector for reamplification. Buccal samples often present an additional challenge because the biological material is not visible on the collector, whereas blood samples are visible. Under the current method, the technician must take a second sampling from the collector approximately once in every batch of forty reference samples (personal communication with Patricia Gibson and Barbara Frankovich, UNT Center for Human Identification, Fort Worth, TX, April 18, 2013).

Ten buccal samples from two donors were amplified from the FLOQSwabs with 90% success using the direct mtDNA amplification method. Ten blood samples from eight donors were also amplified from the FLOQSwabs with 70% success. While the success rate was high for buccal samples, the FLOQSwabs proved to be less compatible with the direct amplification assay than the Bode and Dacron collectors. The processing of FLOQSwabs requires the use of an additional reagent, Isoton III, to elute the cells prior to lysis. The elution and incubation steps of the protocol must be performed in separate tubes, introducing a sample transfer and increasing the possibility for contamination and sample mix-up. The FLOQSwab protocol may allow reamplification from buccal cells if necessary. The buccal cell pellet is re-suspended in 20µL of Isoton III, only 2µL of which is needed for the incubation step. The remaining pellet could potentially be stored at 4°C and used for future laboratory studies. As for blood samples from the FLOQSwabs, inhibition may have played a role in the low success rate. It is possible that centrifugation followed by re-suspension of the blood cell pellet may have introduced heme into the amplification reaction.

Sequencing was performed for all collection devices using blood and buccal samples. The presence of the swab punch in the Bode and Dacron samples often presented difficulty when

preparing sequencing reactions. The swab punch absorbed most of the reaction mix in the sample tube, making it difficult to add the necessary 3µL of PCR product for the cycle sequencing reaction. Samples from the FLOQSwab offered a benefit in that there was no sample punch present, so it was possible to add 4µL of PCR product to each sequencing reaction.

Despite the differences in ease of preparation, samples from all three collectors were successful in generating sequence data of excellent quality using four primers: R1, B1, C1, and R2. Initially, one concern when developing a direct amplification method was the absence of a purification step could result in low quality sequence data. In this study, the cleanliness of the data was not an issue. All primers produced quality sequence data with little or no baseline noise for blood and buccal samples from all collection devices. Haplotypes were confirmed for the donors sequenced with the collection device and cell type reported by comparison to the revised Cambridge Reference Sequence (15). Trace score and CRL values were also evaluated for all sequencing traces. Statistical analysis of the data indicated that the collection device used has a significant effect on trace scores, but not on CRL values. The cell type has a significant effect on both trace score and CRL values. The interaction effects between the cell type and the collection devices were insignificant. Whereas parametric ANOVA tests were conducted on the data, it might have been preferable to use a non-parametric test if the assumption of normality of the data could not be met. Regardless, the measured significance would most likely have been similar.

This project has successfully demonstrated that quality mtDNA sequence data can be generated from reference samples without the need for extraction, quantification, and normalization. Future studies should consider optimizing the protocol for each collection device, by increasing the reaction volume for Bode and Dacron sample processing and eliminating the

sample transfer step from the FLOQSwab protocol.  A modification to the FLOQSwab protocol

for blood samples should also be evaluated.

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