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MITOCHONDRIAL DNA ANALYSIS BY PYROSEQUENCING

by

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B.S. University of Central Florida, 1998
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A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Chemistry
in the College of Arts and Sciences
at the University of Central Florida
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ABSTRACT

Mitochondrial DNA (deoxyribo nucleic acid) is typically used in forensic casework when small quantities of high molecular weight quality DNA is not expected to be present thus negating the chances of obtaining usable nuclear DNA. Typical samples that utilized mitochondrial DNA analysis are: hair, bones, teeth, ancient remains (samples or remains that are at least 100 years old) or very old samples (samples that are less than 100 but greater than 10 years old). The current method used to evaluate mitochondrial DNA is Sanger sequencing. Although robust, it is also time consuming and labor intensive, on the other hand pyrosequencing is a nonelectrophoretic, rapid, reliable, and sensitive sequencing method which can be easily automated. Therefore pyrosequencing could enable the widespread use of mitochondrial DNA in forensic casework and reduce the amount of time spent on each sample without compromising quality.

The aim of this study is to evaluate the efficacy of pyrosequencing for forensic DNA applications, in particular mitochondrial DNA. Two dispensation orders, cyclic and directed, were examined to determine if there is any effect on the sequence generated. The accuracy of pyrosequencing was evaluated by sequencing samples of known sequence provided by the FBI. The sensitivity of pyrosequencing was evaluated by sequencing samples at different DNA concentrations and inputs. Experiments were conducted to determine the ability of pyrosequencing to detect mixtures and heteroplasmy. Additionally, the ability of pyrosequencing to sequence damaged/degraded DNA was evaluated using blood, semen, and saliva samples that were subjected to three different environmental conditions. A blind study will be conducted to

confirm the accuracy of pyrosequencing. Finally, a comparison study will be conducted in which pyrosequencing will be compared to Sanger sequencing.

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TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS	iv
LIST OF FIGURES	vii
LIST OF TABLES.....	ix
LIST OF TABLES.....	ix
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 MATERIALS AND METHODS	12
2.1 Sample Preparation	12
2.2 DNA Extraction	12
2.4 DNA Quantification (yield gel)	14
2.5 Primers	14
2.6 PCR Amplification.....	16
2.7 Product Gel	16
2.8 Sequencing Dispensation Order.....	17
2.9 Pyrosequencing.....	17
2.10 Sensitivity	18
2.11 Mixtures	19
2.12 Fingerprints.....	19
2.13 Case Study	20
CHAPTER 3 RESULTS.....	21
3.1 Dispensation Order	21

3.2 DNA Sensitivity.....	23
3.3 Mixtures	26
3.4 True Heteroplasmic Sample.....	35
3.5 Environmentally Compromised Samples	39
3.6 Lampshade	68
CHAPTER 4 DISCUSSION.....	70

LIST OF FIGURES

Figure 1 Overview of the pyrosequencing reaction.....	7
Figure 2 Diagram of mitochondrial DNA.....	10
Figure 3 Diagram of primer position	10
Figure 4 Cycle dispensation vs. directed dispensation	22
Figure 5 Pyrograms of varying DNA inputs.....	24
Figure 6 Pyrograms of mixtures	30
Figure 7 Graph of peak heights in mixture	32
Figure 8 Pyrogram of a true heteroplasmic sample (HRM).	36
Figure 9 Graph of peak heights for the heteroplasmic sample	38
Figure 10 Pyrograms of FS1 after 1 day treatment 1	41
Figure 11 Pyrograms of FS1 after 3 months of treatment 1	42
Figure 12 Pyrograms of FS1 after 1 day of treatment 2	44
Figure 13 Pyrograms of FS1 after 1 week of treatment 2.....	45
Figure 14 Pyrograms of FS1 saliva after 1 day of treatment 3	47
Figure 15 Pyrograms of a FS1 saliva sample after 1 week of treatment 3	48
Figure 16 Pyrograms of FS1 blood after 1 day of treatment 1	50
Figure 17 Pyrograms of FS1 blood after 6 months of treatment 1	51
Figure 18 Pyrograms of FS1 blood after 1 day of treatment 2	53
Figure 19 Pyrograms of FS1 blood after 1 month of treatment 2.....	54
Figure 20 Pyrograms of FS1 after 1 day of treatment 3	56

Figure 21 Pyrograms of FS1 blood after 1 month of treatment 3	57
Figure 22 Pyrograms of J semen after 1 day of treatment 1	59
Figure 23 Pyrograms of J semen after 12 days of treatment 1.....	60
Figure 24 Pyrograms of J semen after 1 day of treatment 2	62
Figure 25 Pyrograms of J semen after 12 days of treatment 2.....	63
Figure 26 Pyrograms of J semen after 1 day of treatment 3	65
Figure 27 Pyrograms of J semen after 12 days of treatment 3.....	66

LIST OF TABLES

Table 1 Pyrosequencing primers.....	15
Table 2 Mixture table (20 pg DNA)	28
Table 3 Mixture table (20 pg DNA) F2 major component	29
Table 4 Mixture table (1pg DNA)	34
Table 5 Table of the different elements that are present in the different treatments.	39
Table 6 The table shows the length of time that DNA could be recovered from the environmental samples.....	68
Table 7. Mitotypes of lampshade samples and researchers	69

CHAPTER 1 INTRODUCTION

Mitochondrial DNA is found in the mitochondria (a double membrane organelle in the cell's cytosol) of eucaryotic organisms. Mitochondrial DNA replicates autonomously from nuclear DNA (Lutz et al 1999). Each mitochondrion carries several copies of mitochondrial DNA that is useful to forensic science casework when nuclear DNA is not available. In humans the copy number of mitochondrial DNA ranges from approximately 100-10000 copies per cell. Mitochondrial DNA is a closed circle of double stranded DNA approximately 16569 base pairs in length. The two strands can be distinguished from each other based on their weight in a denaturing caesium chloride gradient (Taanman 1999). Due to their differing weight the strands have been designated the heavy (purine rich) and a light (pyrimidine rich) strand (Budowle et al 2000). Mitochondrial DNA is extremely compact and only encodes a few RNAs and proteins. The heavy strand carries most of the information and codes for two ribosomal RNAs, fourteen transfer RNAs, and twelve proteins, whereas the light strand codes for eight transfer RNAs and one protein (Budowle et al 2000; Taanman 1999). The remaining 1100 bases are non-coding and make up what is called the displacement loop (D-loop) or the control region (Budowle et al 2000). The genes in mitochondrial DNA lack introns and with the exception of one regulatory region, intergenic sequences are missing or are only a few bases in length (Taanman 1999; Wolstenholme 1992).

Mitochondrial DNA is strictly maternally inherited due to the degradation of mitochondrial DNA in sperm shortly after fertilization. The degradation of mitochondrial DNA from sperm occurs during embryogenesis by selective destruction, inactivation or dilution

(Budowle 2003; Bender et al 2000). Additionally there is no recombination in mitochondrial DNA; therefore all maternal relatives should carry the same mitochondrial DNA barring any mutation. The mode of inheritance makes mitochondrial DNA useful in missing person cases and identification of persons for which there is no reference sample. Due to the small size of mitochondrial DNA and its enclosure in the mitochondrion, mitochondrial DNA is more resistant to degradation compared to nuclear DNA. This in addition to mitochondrial DNA's high copy number, enables mitochondrial DNA to be used in cases in which there may not be much DNA present or the DNA may be highly degraded, such as in bones, teeth, hair and samples that have been exposed to the environment for long periods of time, or remains that have been subjected to extreme temperatures such as those found in burn victims. Additionally to recover nuclear DNA from hair the follicular tag must be present; however the use of mitochondrial DNA can enable recovery from as little as 1-2 cm of the hair shaft.

The region of mitochondrial DNA currently used for forensics is in the displacement loop (D-loop), specifically hypervariable region I (HVI), which spans 16024-16365 and hypervariable region II (HVII), which spans 73-340 (Budowle 2003). The D-loop region of mitochondrial DNA does not encode any genes and has a significantly higher rate of mutation compared to the rest of the mitochondrial DNA (Malik et al 2002). This elevated rate of mutation makes the control region an excellent tool for distinguishing individuals that are not maternally related.

A phenomenon exists in mitochondrial DNA where a single individual carries two different populations of mitochondrial DNA either in a tissue, cell, or mitochondrion, which is referred to as heteroplasmy. Heteroplasmy probably exists in all individuals due to accumulations of mutations throughout a lifetime, however these mutations are at such low

levels that they cannot be detected by current methods (Salas et al 2001). In order to distinguish the mutation from the background noise it must be present at 20% in height of a normal peak (Salas et al 2001). Documented cases of individuals with heteroplasmic tissues or regions of the body display different levels and proportions of the mutations (Jazin et al 1996). The different levels of heteroplasmy in a single tissue, for example a single hair, suggest that there is a narrow bottleneck in mitochondrial DNA (Salas et al 2001). The occurrence of heteroplasmy in some cases can increase the significance of a match if the proportion of the heteroplasmy in the questioned and known sample is similar (Salas et al 2001).

Conversely, to the power of mitochondrial DNA in forensic science casework some drawbacks exist, both biologically and functionally. Due to the high copy number of mitochondrial DNA, it is fairly easy to contaminate the sample being examined. However, the contamination can be avoided using simple precautions such as dedicated sterile hoods that are UVC (254nm) irradiated after each use, employment of clean lab coats, frequent glove changes, sterile solutions and the use of 10% bleach to wipe the instruments, reagent tubes, and hood allows contamination to be avoided. The biological drawback is due to a high substitution rate in mitochondrial DNA especially in the control region due to an increased transition rate between pyrimidines on the L-strand of the D-loop (Tumura 2000). These transitions create the possibility of maternal relatives differing at one or more base positions (Parsons et al 1997). The rate of this substitution has been estimated to be one in every thirty-three generations (Parsons et al 1997). This rate can be used to aid in the comparison of sequences that are suspected to be of the same maternal lineage but may differ by one base.

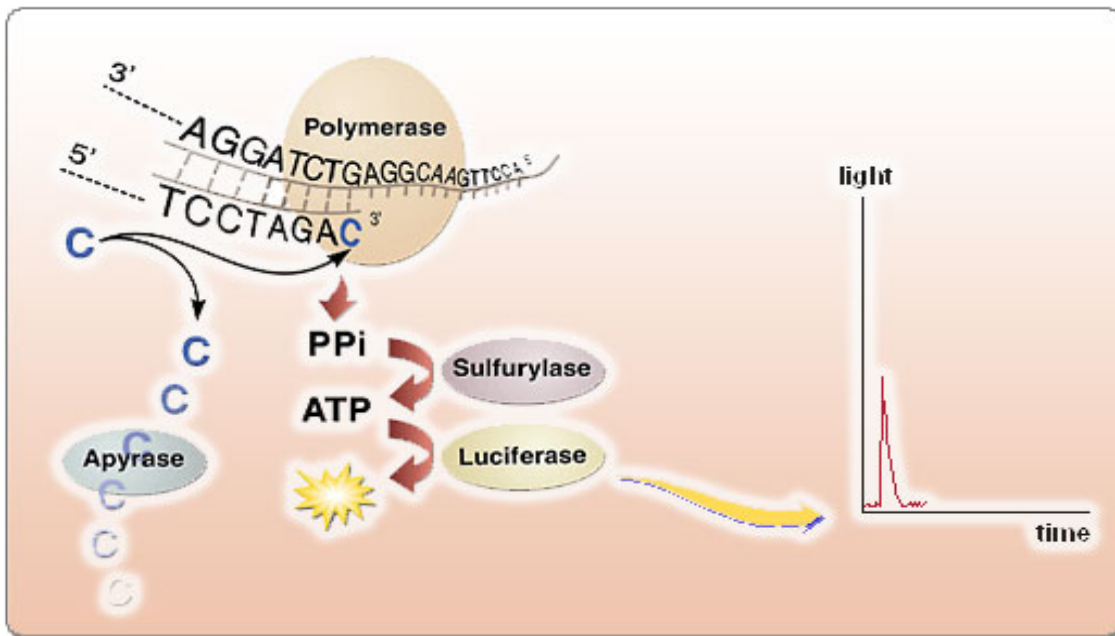
The standard method currently employed for DNA sequencing is Sanger sequencing. This method has proven to be reliable, robust, and accurate. In this method single stranded DNA is synthesized *in vitro* using a primer and DNA polymerase (Eriksson 2004; Budowle et al 2000). In addition to an excess of deoxy nucleotide triphosphates (dNTPs), fluorescent-labeled dideoxy nucleotide triphosphates (ddNTPs) are also included (Eriksson 2004; Budowle et al 2000). These ddNTPs will incorporate randomly into the newly forming DNA chain and prevent further extension of the chain by DNA polymerase as ddNTPs lack the a free 3' hydroxyl group (Eriksson 2004; Budowle et al 2000). As a result DNA fragments of all different sizes will be generated. The DNA templates of varying lengths will then pass through an electric current on an instrument such as ABI genetic analyzer which will separate the DNA fragments by size as smaller pieces of DNA migrate faster than larger pieces of DNA through the matrix (Eriksson 2004). The DNA fragments will then pass through a laser beam, which excites the different color ddNTPs, and the instrument will record the color and corresponding ddNTP and generate the sequence information (Eriksson 2004). Problems are however associated with this method, that it is time consuming and labor intensive (Ronaghi et al 1999). In addition band compressions may take place in electrophoresis, which causes larger pieces of DNA to migrate faster than normal, and may lead to difficulty in interpretation of the sequence (Ronaghi 1999).

Due to its small size mitochondrial DNA is an excellent template for pyrosequencing. Pyrosequencing is a rapid, robust and sensitive method of sequencing by synthesis. In addition, the problems associated with electrophoresis are not associated with pyrosequencing. It uses pyrophosphate (PPi) that is generated in DNA synthesis to drive an enzyme cascade that produces light that can be measured, recorded and converted into base incorporations. Nucleic

acid incorporation driven by a polymerase results in the release of inorganic pyrophosphate, which is converted to adenosine triphosphate (ATP) by sulfurylase, derived from a recombinant version of *Saccharomyces cerevisiae* (Ronaghi 2001). The ATP generated provides energy needed by luciferase (derived from the American firefly *Photinus pyralis*) to oxidize luciferin, which creates light (Ronaghi 2001). The sequence of the template can be determined because the nucleotide that was added was known (Ronaghi 2001). If light was not generated it means that the nucleotide was not incorporated. In the pyrosequencing reaction, one pico mole of DNA produces 6×10^{11} ATP molecules, which is used to create more than 6×10^9 photons of light at 560 nanometers (Ronaghi 2001). The light generated is then detected by a charge-coupled device (CCD) camera, measured and converted into a number of nucleotide incorporations by the PSQ 96 software. After each addition of a nucleotide, apyrase was added to degrade any unincorporated nucleotides and ATP. This prevents nucleotides from a previous dispensation from being incorporated later in the reaction, allowing the sequencing to take place in a single well without washing in between the nucleotide dispensations. The enzyme cascade is shown in Figure 1A. To prevent ATP dispensed from being used by luciferase to generate light and hence false signals a modified ATP, ATP α S is used in pyrosequencing (Ronaghi 2001). A generic pyrogram is shown in Figure 1B. The nucleotide dispensation is shown on the x-axis and the peak height (measure of fluorescence) is on the y-axis. The 'E' under the pyrogram represents the enzyme mix that is the first of the reagents to be dispensed. Next is the 'S', which represents the substrate, a peak should always be seen here as it indicates that the reaction is taking place. A 'T' nucleotide is the first to be dispensed, however it is not incorporated and no peak is produced. The second nucleotide dispensed is 'C'; the height of the peak indicates that two C's

were incorporated. This double 'C' peak is followed by a triple 'G' peak then a 'T', 'G', 'T', and a 'A' peak. The sequence reads CCGGGTGTA.

(A)



(B)

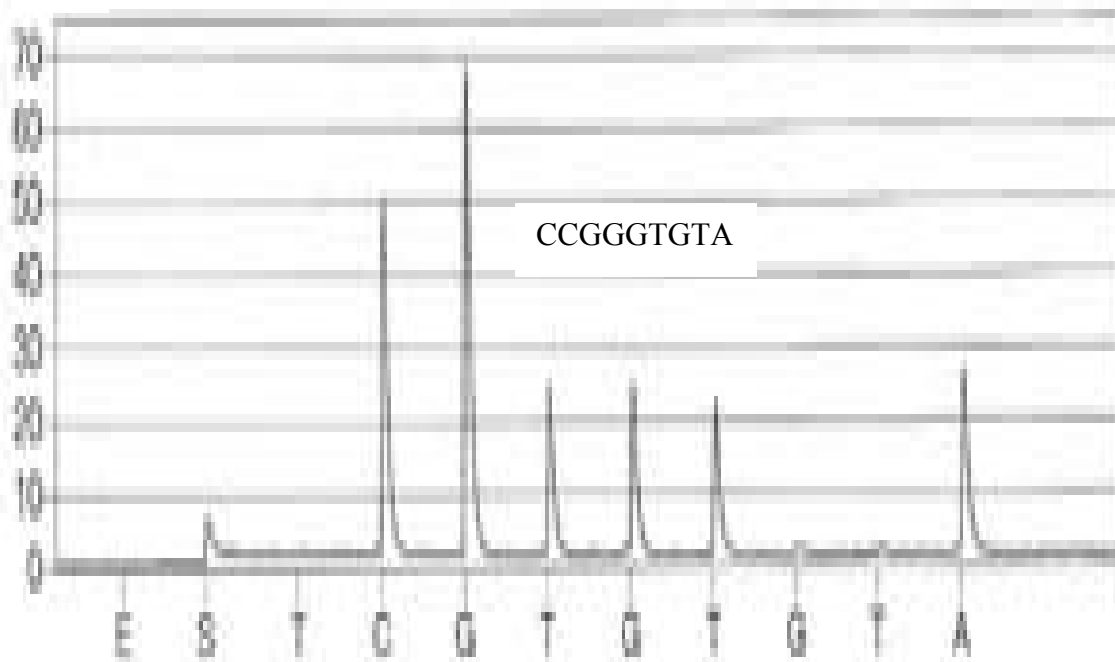


Figure 1 Overview of the pyrosequencing reaction.

The advantages of pyrosequencing over traditional Sanger sequencing is that pyrosequencing is rapid, easily automated, and has high throughput. Sanger sequencing although robust, is laborious, time consuming, and requires significantly more DNA than pyrosequencing. Additionally with Sanger sequencing, band compressions may occur during electrophoresis (Ronaghi et al 1999). This causes a particular DNA fragment to migrate faster than expected resulting in overlap with an adjacent fragment which may create problems with interpretation of the sequence (Ronaghi et al 1999). Since there is no electrophoresis involved in pyrosequencing the problems associated with migration are eliminated. Samples can be analyzed in days as compared to weeks with Sanger sequencing. The low amounts of DNA needed for pyrosequencing is of vast importance to forensic samples where human DNA is in limited supply. The reliability and robustness of pyrosequencing was tested successfully in house using femtogram quantities of human mitochondrial DNA.

A problem that may occur during pyrosequencing, when longer templates are used or in GC-rich regions, is the presence of secondary structures in the DNA. This problem may be alleviated by the addition of single-stranded DNA binding protein (SSB) (Ronaghi 2000). SSB consists of four identical sub-units which bind with high affinity to single stranded DNA but does not bind well to double stranded DNA (product insert Amersham). The SSB binds to the DNA preventing it from forming secondary structures in addition to stabilizing the DNA preventing it from degradation (Ehn et al 2002). The use of SSB increases the read length for PCR templates of differing lengths, though the effect is more dramatic for longer templates (Ehn et al 2002). It has been shown that SSB increases the efficiency of the polymerase; enabling faster nucleotide incorporation, which results in more accurate base calls in homopolymer

regions in addition to longer read lengths (Ehn et al 2002; Ronaghi 2000). DNA templates that are longer reduce the effectiveness of apyrase to degrade unincorporated nucleotides. However, by the addition of SSB to the reaction well the negative effect on the enzyme is negated (Ehn et al 2002; Ronaghi 2000). Additionally, SSB has been shown to reduce mispriming of the DNA template by dislodging the nonspecifically binding primers from the template, which then increases the signal intensity by protecting the DNA template from degradation (Ronaghi 2000).

In this study the sensitivity and specificity of pyrosequencing was evaluated by testing the varying DNA inputs into PCR and into the sequencing reaction. The ability of pyrosequencing to analyze damaged DNA was tested by subjecting blood, semen, and saliva samples to three different environmental conditions, extracting, amplifying, and sequencing the DNA. Mixtures and heteroplasmic samples were analyzed to determine the ability of pyrosequencing to detect them. A case study was conducted in which DNA was extracted from a lampshade that was made of human skin (provided by the University of Florida). The region of the mitochondrial DNA examined is diagramed in Figure 2. The primers that were used in this study are shown in Figure 3 as they occur in relation to each other on the mitochondrial DNA.

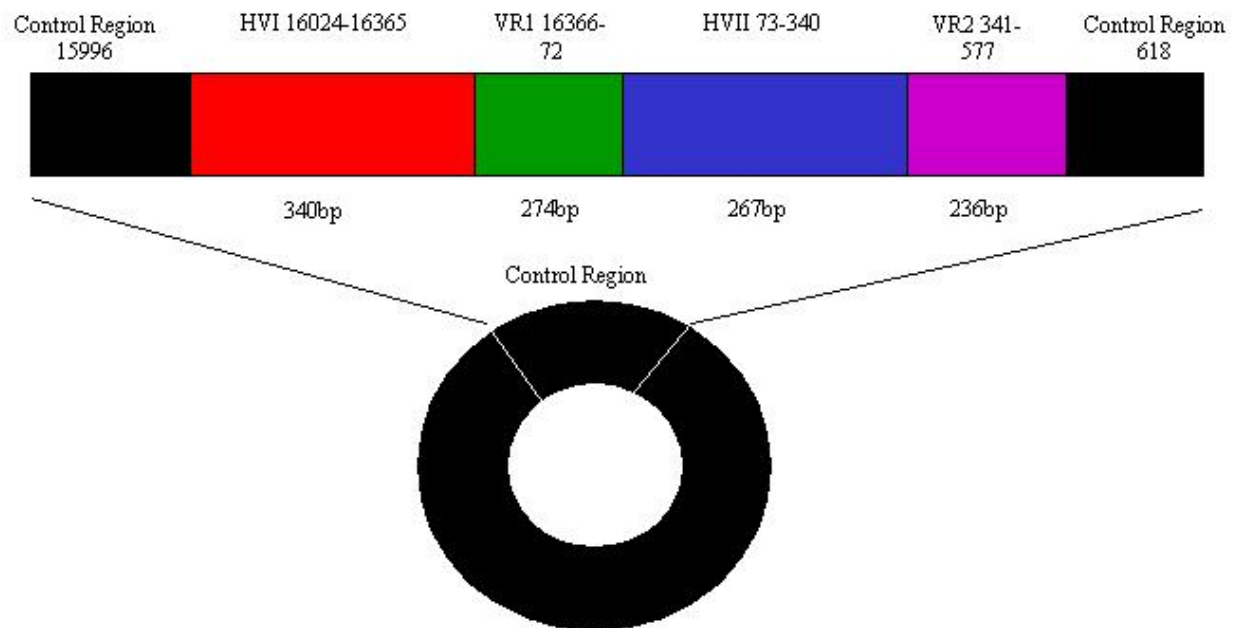


Figure 2 Diagram of mitochondrial DNA

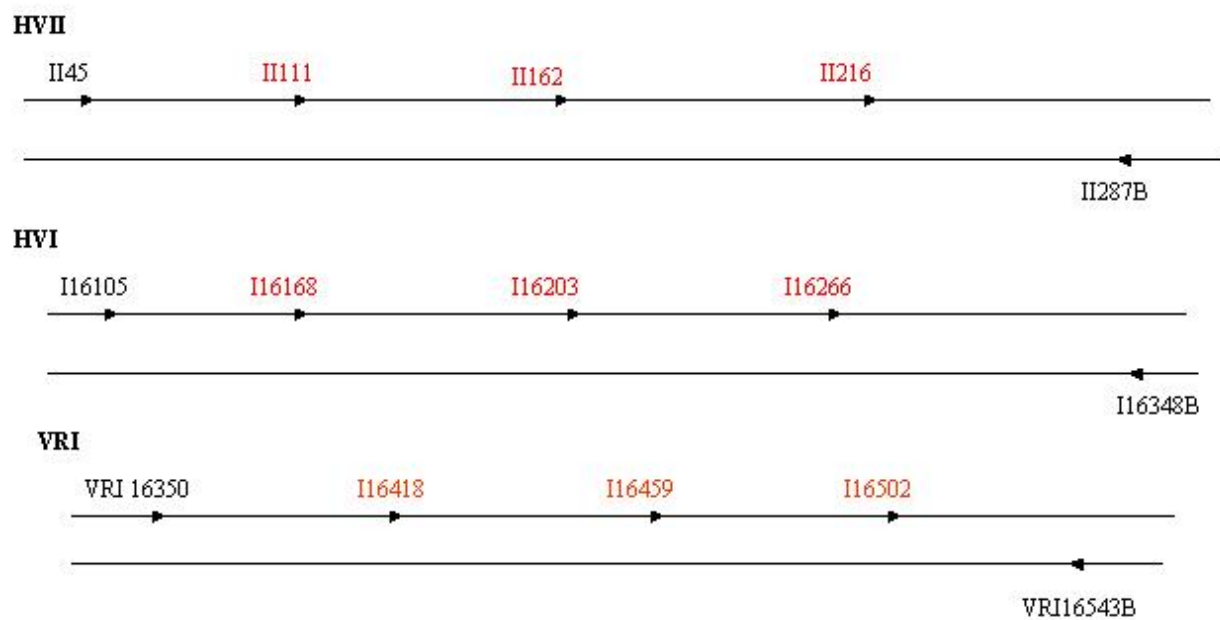


Figure 3 Diagram of primer position

Pyrosequencing has been shown to be a rapid, robust, and reliable method of *de novo* DNA sequencing. It is much faster, less labor intensive, and less time consuming than traditional Sanger sequencing. This could potentially allow the widespread use of mitochondrial DNA sequencing especially since the cost associated with sequencing would decrease. Additionally, pyrosequencing is successful at sequencing tremendous ranges of DNA inputs (1 fg to above 450 ng), which makes it very appealing to forensic casework where DNA quantification may not be conducted in an attempt to conserve DNA samples.

CHAPTER 2 MATERIALS AND METHODS

2.1 Sample Preparation

Buccal and vaginal swabs were collected and allowed to dry. After drying, the swab were either used or returned to individual envelopes and placed into a plastic bag to be stored at 4°C.

Sterile cotton swatches were made for the environmental samples by boiling the swatches for at least one hour in distilled water and allowing the swatches to dry under a hood. Blood, semen and saliva were spotted onto the cotton swatches in 50µl volumes. Blood obtained for the environmental study was collected into Vacutainer ® Plus tubes, containing K₂EDTA 7.2mg. Semen was collected over a period of a few days and stored frozen until enough sample was obtained. Saliva was collected from a subject throughout one day. All samples were allowed to dry overnight under a hood and then placed in their respective environment.

2.2 DNA Extraction

All DNA samples were extracted under a sterile hood. When the samples had to be removed from the sterile hood (for centrifugation and water bath incubation) the tubes containing the samples were sealed with parafilm to prevent contamination from the environment. The

Microcon ® YM-100 regenerated cellulose 100,000 MWCO tubes were used for all extractions (Millipore, Bedford, MA).

The organic extraction procedures used were modified from Comey 1994. The prepared samples were cut into small pieces using sterile razor blades (for swabs) or sterile scissors and tweezers (for swatches) and placed into Safelock ® extraction tubes (Gibco-BRL, Grand Island, NY). The samples were then mixed with 400µl of DNA stain extraction buffer (TRIS, NaCl, 20% SDS, 0.5M EDTA), 13µl proteinase K (0.1mg/ml). For samples containing semen 40µl of DTT (0.39M) was also added. The samples were incubated overnight in a 56°C water bath. The following day the cut pieces were removed and placed into a Spin Ease Basket® using tweezers that had been flamed with 95% ethanol. The spin ease baskets were placed into the Safelock ® tubes, sealed with parafilm and centrifuged at 14000 rpm for 5 minutes to remove the liquid in the substrate. After the centrifugation the basket was removed and discarded leaving only the crude extract. To the crude extract in the Safelock® tubes 400µl of phenol/chloroform/isoamyl alcohol (25:24:1, Fisher, Norcross GA) was added and mixed gently by inversion. The samples were centrifuged at 14000 rpm for 5 minutes to separate the phases. Microcon ® tubes were spun rinsed with 50µl of sterile Millipore water for 2 minutes at 2475 rpm. The DNA contained in the upper aqueous layer was removed (ensuring not to disturb the interface of the aqueous and organic layer which may contain proteins) and placed into the Microcon ® reservoir. The samples were centrifuged for 9 minutes at 2475 rpm (additional spins were conducted as needed). To the Microcon ® reservoir 400µl of TE⁻⁴, pH 7.5 was added and the samples were centrifuged at 2475 rpm for 9 minutes. Additional spins were conducted as needed and the flow thru was discarded. The 400µl of TE⁻⁴, pH 7.5 wash was repeated for a total of 3 times and the

samples were centrifuged at 2475 rpm for 9 minutes. Additional spins were conducted as needed and the flow thru was discarded. After the final wash the reservoirs were inverted into new Microcon ® tubes and centrifuged at 2475 rpm for 5 minutes in order to collect the clean DNA. If insufficient sample volume was obtained additional TE⁻⁴, pH 7.5 was added to the surface of the Microcon ® filter and the reservoir inverted over the collection tube and centrifuged for an additional 5 minutes at 2475 rpm.

2.4 DNA Quantification (yield gel)

DNA samples were quantified by yield gel as described by Kline. A 1% agarose gel stained with 10% ethidium bromide (5mg/ml) was cast with two rows of wells, and 5µl of sample and 1µl of 6X loading buffer was added to the bottom wells. The top wells contained DNA quantitation standards used to visually estimate the quantity of DNA in the sample. The gel was run for fifteen minutes at 200V, after which the gel was placed on a trans illuminator and a picture taken. A visual comparison of the DNA quantitation standard to the samples was conducted in order to estimate the amount of the DNA present in the sample.

2.5 Primers

Primers designed by Andreasson, H et al 2002 were used, which included primers II45, II111, II162, II216, II287B, II6105, II6168, II6203, II6266 and II6348B. Additional in house

polymerase chain reaction (PCR) primers were designed using Primer 3 (<http://frodo.wi.mit.edu>). These PCR primers were evaluated for primer dimers, cross dimers, palindromes with netprimer launch (<http://www.premierbiosoft.com>). The PCR primers were each given a score based on the lack of previous conditions and the highest scoring primers were chosen. After the PCR primers were chosen they were BLASTed using <http://ncbi.com> to ensure that they were specific to human mitochondrial DNA. Pyrosequencing primers were designed using technical support program AB version 1.0.1 available at <http://www.biotage.com>.

Table 1 Pyrosequencing primers. The table shows the primers utilized in this study. Primers in red serve both as PCR and pyrosequencing primers. Primers in blue serve as reverse PCR primers and are labeled with biotin. Primers in black are used only as pyrosequencing primers.

Primer	Region	Sequence	Reference
I145	HVII	ATGCATTTGGTATTTTCGTCTG	Andreasson, H. et al
II111	HVII	ACCCTATGTCGCAGTATCT	Andreasson, H. et al
II162	HVII	CGCACCTACGTTCAATATTACA	Andreasson, H. et al
II216	HVII	TTAATGCTTGTAGGACATAATAA	Andreasson, H. et al
II287B	HVII	TTGTTATGATGTCTGTGTGGAAAG	Andreasson, H. et al
I16105	HVI	TGCCAGCCACCATGAATA	Andreasson, H. et al
I16168	HVI	CCAATCCACATCAAAACC	Andreasson, H. et al
I16203	HVI	AGCAAGTACAGCAATCAA	Andreasson, H. et al
I16266	HVI	CCCACTAGGATACCAACA	Andreasson, H. et al
I16348B	HVI	GACTGTAATGTGCTATGTACGGTAAA	Andreasson, H. et al
VRI16350	VRI	AATCCCTTCTCGTCCCAT	In house design
VRI16418	VRI	CAATATCCCGCACAAG	In house design
VRI16459	VRI	CCATAACACTTGGGGG	In house design
VRI16502	VRI	TGGTTCCTACTTCAGGG	In house design
VRI16543B	VRI	CGTGTGGGCTATTTAGGCTT	In house design

2.6 PCR Amplification

The components of the PCR amplification totaled 50 μ l which was composed of 5 μ l of 10X PCR buffer II, 4 μ l of MgCl₂ (25mM), 2.5 μ l of dNTPs (10mM), 20 pmoles each of forward and reverse primer, 0.3 μ l of Ampli Taq Gold (5U/ μ l), 20 pg of input DNA and sterile Millipore® water to make up the reaction volume to 50 μ l. The samples were amplified on a GeneAmp PCR system 9700 under the following conditions:

95°C – 11 minutes

95°C – 30 seconds

60°C – 45 seconds

72°C – 1 minute

72°C – 7 minutes

4°C – hold

40 Cycles



2.7 Product Gel

After the PCR amplification 10 μ l of each sample and 1.5 μ l of 6X loading buffer III was run on a 1% agarose gel stained with 10% ethidium bromide (5mg/ml), along with a molecular ladder to ensure the correct size product was produced, at 200V for 1 hour and 20 minutes to ensure that the samples were amplified and that there was no contamination. If the samples were determined to be contaminated they would be discarded and the amplification repeated.

2.8 Sequencing Dispensation Order

Two different dispensation methods were initially used: cycle dispensation and directed dispensation. For cycle dispensation the nucleotides G, C, A and T were dispensed in order twenty-five times. Directed dispensation was based on the Revised Cambridge Reference Sequence (CRS) and the mutations documented by MITOMAP. Therefore, the nucleotides would be dispensed in order of the revised CRS until a base was reached where there was a documented mutation at a particular position, the base was then dispensed in addition to all possible mutations that could occur at that position. The dispensation would then be continued according to the revised CRS until another base was reached where there was another documented mutation. This process was then repeated for a total of 100 bases.

2.9 Pyrosequencing

The pyrosequencing procedure begins with adding 24µl of Streptavidin SepharoseTM High performance -binding buffer pH 7.6 (10mM Tris-HCl, 2M NaCl, 1mM EDTA, 0.1% Tween 20) mix (1:5 mixture). 20µl of biotin labeled PCR product was added to each well of a MultiscreenTM-HV filter plate and mixed on a shaker for ten minutes at speed seven. While the samples were incubating the pyrosequencer was programmed for the run. Next the MultiscreenTM-HV filter plate was removed and a vacuum applied to remove all liquid. After the removal of the liquid from the wells 50µl of denaturing solution (0.2M NaOH) was added to denature the samples, separating the DNA strands. This reaction was allowed to take place for

one minute before a vacuum was applied to the plate. The samples were washed with 160µl of wash buffer pH 7.6 (10mM Tris-Acetate) followed by the removal of the wash buffer by vacuum. The wash was repeated. The samples were transferred to the sequencing plate by addition of 40µl of annealing buffer (1X pH 7.6 20mM Tris-Acetate, 2mM MgAc₂). The buffer was added and pipetted up and down 6-8 times and transferred to the corresponding wells on the PSQ 96 plate low. Sequencing primer (20 pmoles) was added to the respective wells. The plate was heated to 80°C for 2 minutes. After heating the plate single stranded binding protein (0.9µg) was added to prevent the formation of secondary structures, which may interfere with the sequencing reaction. The appropriate amount of nucleotides, enzyme and substrate (specified by the instrument) was added to the cartridge. Finally, the plate and the cartridge were placed into the instrument and the run started. After the run was completed, automatic analyses of the samples were conducted and the data entered into a spreadsheet manually. If any discrepancies were noticed between the CRS and the known mutations the pyrograms were examined manually.

2.10 Sensitivity

Varying the amount of the DNA that was placed into PCR tested the sensitivity of pyrosequencing. Samples (F2, F3, AH) were amplified using 450 ng, 112 ng, 1 ng, 20 pg, 10 fg, and 1 fg of total genomic DNA, after which the samples were sequenced. In addition varying the amount of amplified DNA placed into the sequencing reaction tested the amount of amplified

DNA that could be successfully sequenced. A total of 20µl (standard sequencing volume), 15µl, 10µl, and 5µl of DNA that was amplified using 20 pg input was sequenced to test the minimum volume of DNA that could be used to obtain good sequences.

2.11 Mixtures

Mixtures consisting of two different mitochondrial DNA samples (F5 and F2) were made as a preliminary examination of heteroplasmy. The mixtures were made in ratios of 1:1, 1:6, 1:10, 1:12, 1:20 and 1:30. The samples were amplified and sequenced, first with F5 as the major component with the total input DNA into the PCR being 20pg and then with F2 as the major component with the total input DNA into the PCR being 20pg. The experiment was repeated with F5 as the major component and the total input DNA into the PCR being 1pg. The pyrograms were evaluated manually at the sites where the mixture would be evident.

2.12 Fingerprints

In order to determine if usable DNA could be obtained from fingerprints, two subjects held two sterile Falcon® tubes in their hands for 10 seconds. The entire area of the first tube was swabbed with a dry swab and then with a swab that had been moistened with sterile Millipore water. The entire area of the second tube was swabbed only with a swab that was moistened with sterile Millipore water. In addition to the first two tubes, subjects also held a third tube between their thumb and index finger for ten seconds. The area of each print was marked and each print

was swabbed separately with a swab moistened with sterile Millipore water. The samples were extracted, amplified and sequenced as described above.

2.13 Case Study

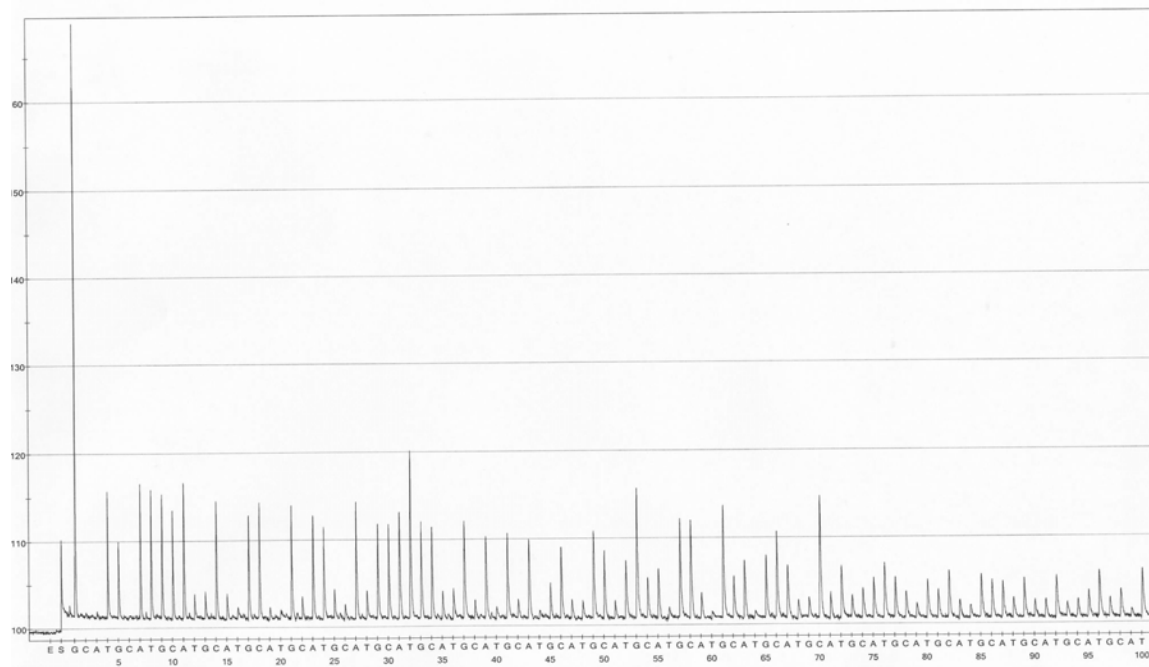
Samples from three panels of a lampshade obtained from a holocaust museum by researchers at the Maples Center at the University of Florida were received. These samples were extracted, amplified, and sequenced. The sequences generated were then BLASTed to determine if the DNA was human in origin. The sequences were then compared to the sequences of the researchers that came in contact with the samples to ensure the DNA obtained was not that of the researchers.

CHAPTER 3 RESULTS

3.1 Dispensation Order

The experiment of comparing cycle dispensation versus directed dispensation was conducted in order to find the most efficient method of sequencing the DNA with regards to read length, accuracy, and speed. Samples F1, F2, F3, F4, and F5 obtained from the FBI were used to test the dispensation order at 20 pg and 1ng total genomic DNA inputs into the PCR reaction. When cycle dispensation was compared to directed dispensation, a longer read length was typically obtained with directed dispensation. This was because more dispensations resulted in base incorporation as can be seen in Figure 4. In addition, directed dispensation was less wasteful of reagents and therefore more economic than cyclic dispensation. However, if the sequence contains a novel mutation the read may be thrown off when directed dispensation was used, which may shorten the read length of the DNA being analyzed. This is unlikely to occur with mitochondrial DNA because it has been sequenced numerous times and the mutations are well documented in MITOMAP. Therefore the chances of a mutation that would throw off the read would be very rare. Due to the longer read length directed dispensation was used for the rest of the study.

(A)



(B)

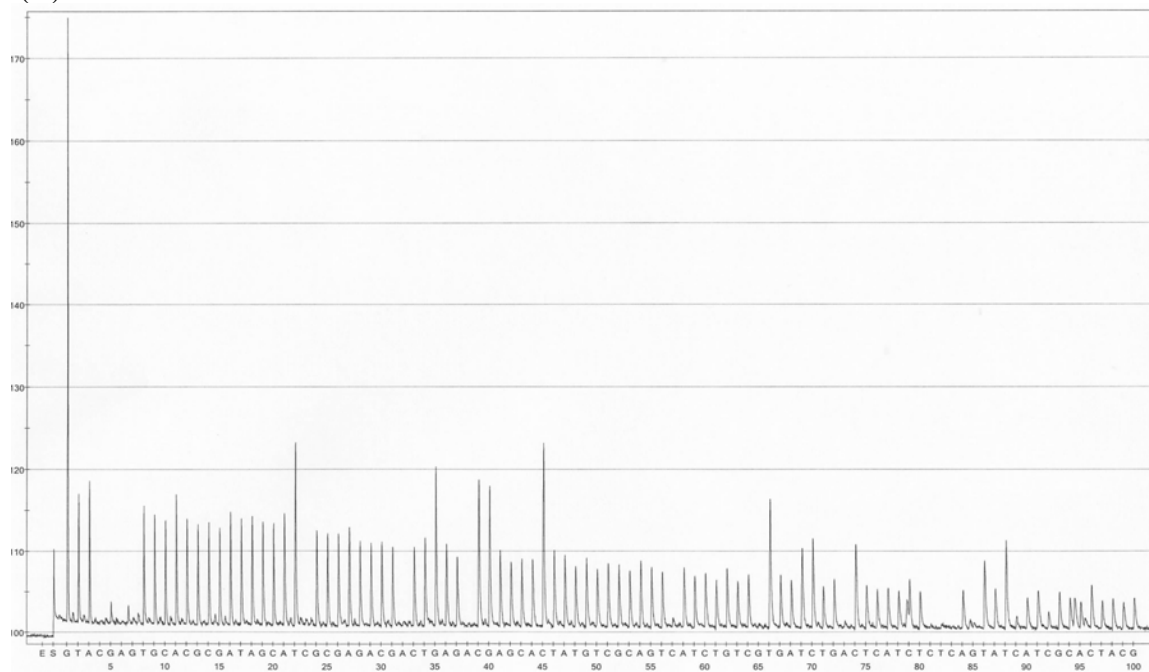
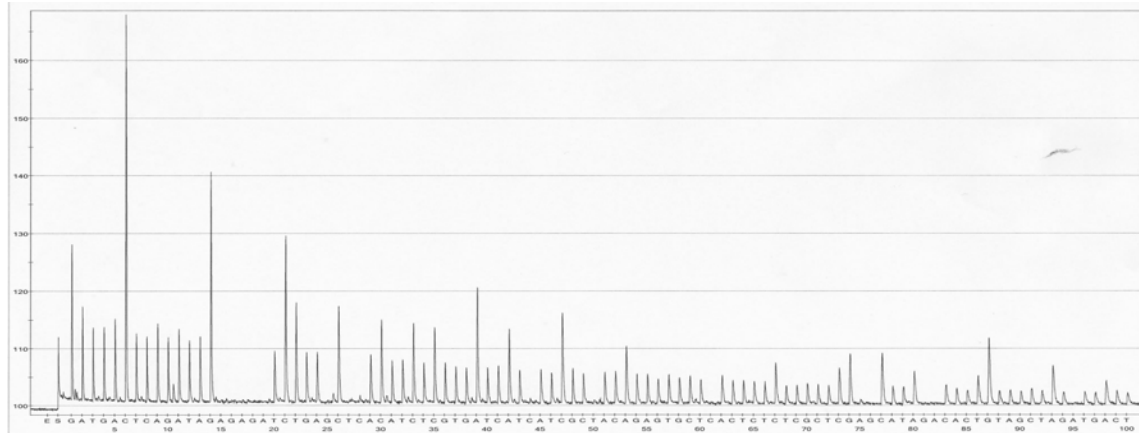


Figure 4. Cycle dispensation vs. directed dispensation. The figure illustrates the difference between cycle dispensation (A) and directed dispensation (B) when primer II45 was used.

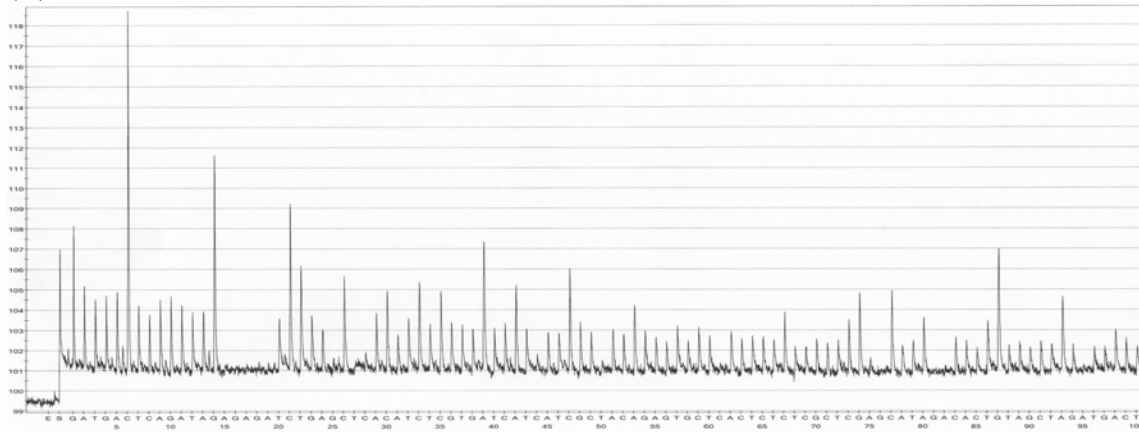
3.2 DNA Sensitivity

In forensic casework, the DNA sample is sometimes not quantified in an attempt to conserve the amount of DNA that is consumed in the non-identifying portion of the examination. This could result in a wide range of input DNA into the PCR reaction. The sensitivity of pyrosequencing was tested to ensure that this method would be able to sequence a wide range of DNA concentrations. Samples F2, F3 (obtained from the FBI), and AH (in house) were amplified using 450 ng, 112 ng, 1 ng, 20 pg, 10 fg, and 1 fg of total genomic DNA and 20 µl of this amplified DNA was placed into the sequencing reaction. The amplified DNA was sequenced using all the primers included in the study listed in Table 1 on page 14. Accurate sequences were generated with as little as 10 fg of input DNA for primer sets II45/II287B and I16105/I16348B and 1 fg of input DNA for primer set VRI16305/VRI16543B as shown in Figure 5. It can be seen that the quality of the pyrogram decreases as the DNA input decreases. For the other primer sets a sequence was not generated when 1 fg of input DNA was used. Additionally the quality of the pyrograms generated for the other primer sets using 10 fg of input DNA were similar to the pyrogram generated when primer set VRI16350/VRI16543B was used with 1 fg of input DNA. This suggests that primer set VRI16350/VRI16543B is a better primer for amplifying DNA. This could be attributed to the high G/C content present in the VRI16350 and VRI16543B primers (66.7% and 50% respectively). The justification for the 'G' and 'C' content being significant is because the 'G' and 'C' nucleotides form triple bonds while the 'A' and 'T' nucleotides can only form double bonds, which are weaker. This high G/C content primers allows them to bind more strongly at the annealing temperature to the DNA and amplify more successfully. Throughout the study primer set VRI16350/VRI16543B was more successful at amplifying samples.

(A)



(B)



(C)

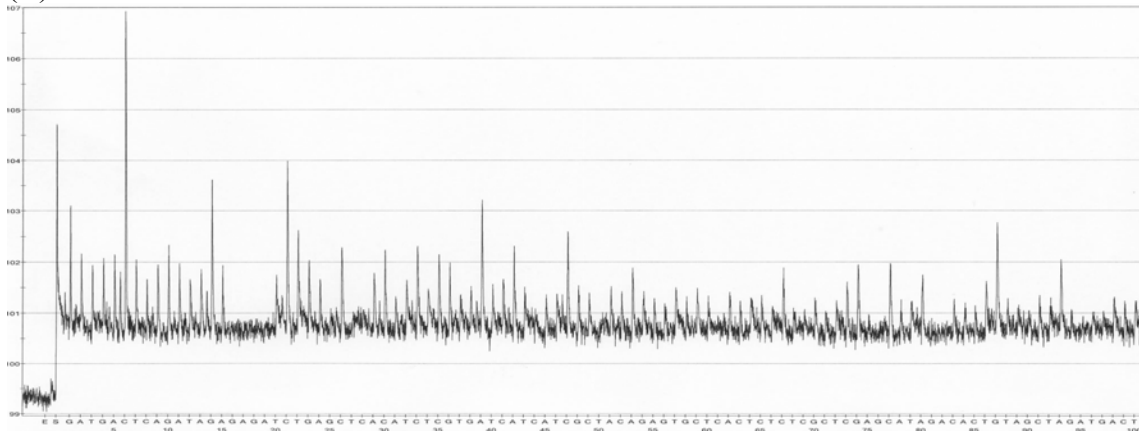


Figure 5 Pyrograms of varying DNA inputs. The figure illustrates the difference in the quality of pyrograms obtained when varying amounts of input DNA was used. (A)-20pg, (B)-10fg, (C)-1fg. Primer VRI16350 and sample F2 was used.

Pyrosequencing was successful in sequencing DNA from sample F2 when 20 pg of DNA was amplified and 20 μ l, 15 μ l, and 10 μ l the amplified DNA used in the sequencing reaction. However pyrosequencing was unsuccessful when 5 μ l of amplified DNA was used. This could be because there is simply not enough DNA present to generate sufficient light detectable to the CCD camera.

3.3 Mixtures

Occasionally, heteroplasmy and mixtures are encountered in forensic casework. A system is needed that can on a regular basis distinguish a heteroplasmic sample or a mixture. In order to examine the ability of pyrosequencing to detect mixtures and heteroplasmy (initially mimicked by making a mixture of two samples that differ at certain locations) a mixture of sample F2 and F5 (samples obtained from the FBI) was made at ratios of 1:1, 1:6, 1:10, 1:12, 1:20, and 1:30 with initially with F5 as the major component and after F2 as the major component with the total DNA input in the PCR reaction being held at 20 pg. A third set of mixtures was made using the same ratios and F2 as the major component but this time the total DNA input into the sequencing reaction was 1 pg. The ability of pyrosequencing to detect mixtures varied according to the primer used. Primers that were successful in detecting the mixture automatically with F5 as the major component and a total of 20 pg of DNA was added to the PCR amplification were II111 with a 1:1 mixture, II120 with a 1:1 mixture, II6168 in the 1:1 mixture, II6203 in the 1:1 mixture and II6266 in the 1:1 mixture. After manual examination, primer II6266 was the most successful in detecting the mixture. In addition, the pyrosequencer was most successful in detecting mixtures when the ratio was 1:1. When the instrument was not successful in detecting the mixture the sequence of the major component was observed. The results of sequencing the mixtures using 20 pg of input DNA into PCR amplification and sample F5 as the major component is displayed in Table 2. After the mixtures were analyzed it became apparent that sample F5 had more mitochondrial copies than sample F2. This is because when the 1:1 mixture was sequenced, if the mixture was not detected, the sequence that was observed was consistently

that of sample F5. Therefore, to test this deduction, the mixtures were remade at the same ratios but with F2 as the major component and the samples amplified and sequenced.

Table 2 Mixture table (20 pg DNA). The table shows the results when a mixture of sample F5 and sample F2 were mixed in different ratios keeping sample F5 as the major component and the total input DNA into PCR at 20 pg. The distance from the end of the sequencing primer in bases is indicated in the last column.

Primer	Region detected	Ratio Detected	Distance
II45	Not detected	N/A	N/A
II111	152 (F5/C, F2/T), 182 (F5/T, F2/C)	1:1	23, 53
II120	152 (F5/C, F2/T), 182 (F5/T, F2/C), 185 (F5/C, F2/A)	1:1	14, 44, 47
II162	Not detected	N/A	N/A
II216	Not detected	N/A	N/A
I16105	Not detected	N/A	N/A
I16168	16187 (F5/T, F2/C), 16233(F5/T, F2/C)	1:1	2, 38
I16203	16233 (F5/T, F2/C)	1:1	3
I16266	16293 (F5/G, F2/A)	1:1, 1:6, 1:10, 1:12, 1:20, 1:30	10

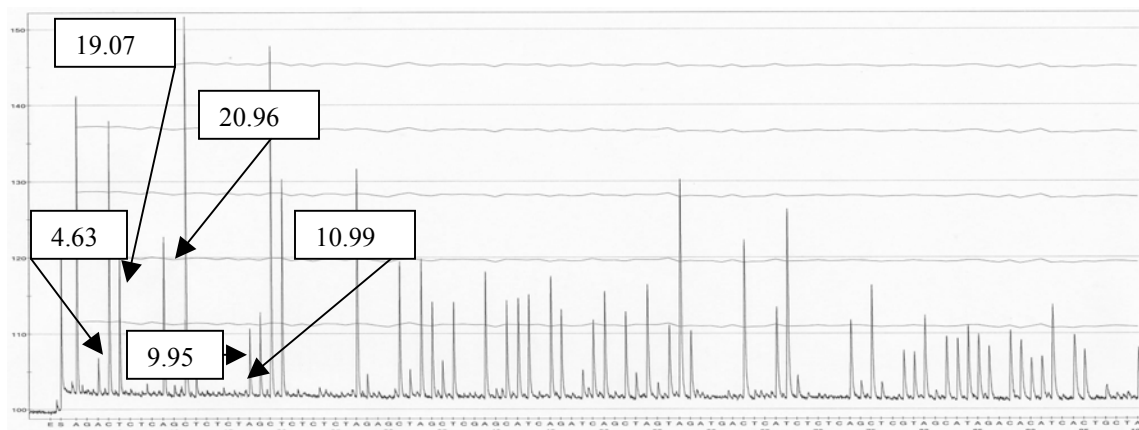
The ability of pyrosequencing to detect the mixtures varied from primer to primer when 20 pg of DNA was input into the PCR amplification and sample F2 was the major component. The primers successful in detecting the mixtures were: II45, II111, II216, I16168, I16203 and I16266. In addition, the pyrosequencer was more successful in detecting the mixture when the ratio was greater than 1:1; this further suggests that sample F5 contained more mitochondrial DNA copies than sample F2. The results of sequencing the mixtures using 20 pg of input DNA into the PCR amplification and sample F2 as the major component is displayed in Table 3. The pyrograms for the different mixtures using 20 pg of input DNA and sample F2 as the major component is displayed in Figure 6. The peak heights and the expected peak heights are

indicated on the pyrograms. It can be seen from the pyrograms that as the concentration of sample F2 increases the 'A' peak increases as the 'G' peak contributed by sample F5 decreases.

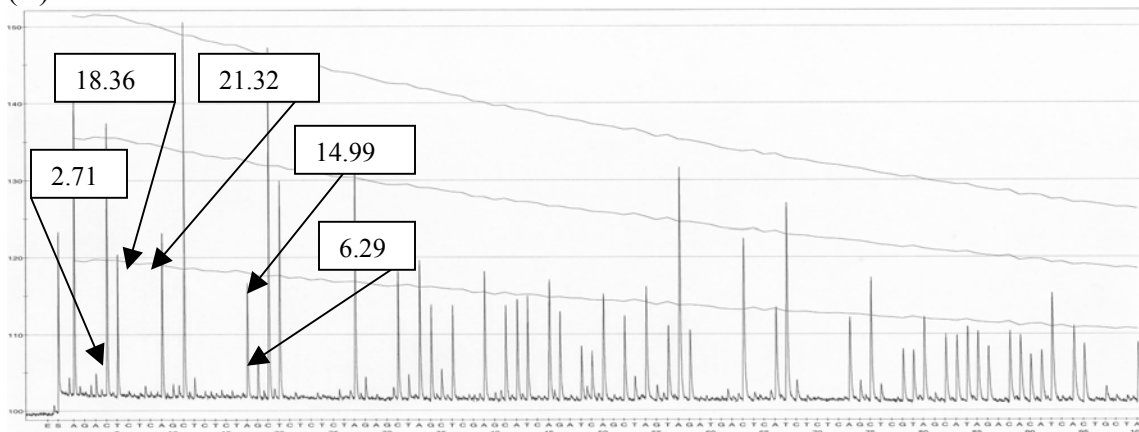
Table 3 Mixture table (20 pg DNA) F2 major component. The table shows the results when a mixture of sample F5 and sample F2 were mixed in different ratios keeping sample F2 as the major component and the total input DNA in the PCR amplification at 20 pg. The distance from the end of the sequencing primer in bases is indicated in the last column.

Primer	Region detected	Ratio Detected	Distance
II45	152 (F5/C, F2/T)	1:12, 1:20	86
II111	152 (F5/C, F2/T), 182 (F5/T, F2/C) 195 (F5/C, F2/T)	1:10 – positions 152, 182 1:1 – position 195	23, 53, 66
II162	Not detected	N/A	N/A
II216	247 (F5/A, F2/G)	1:6, 1:10	9
II6105	Not detected	N/A	N/A
II6168	16187 (F5/T, F2/C), 16233(F5/T, F2/C)	1:1, 1:6, 1:10	2, 38
II6203	16233 (F5/T, F2/C)	1:6, 1:10, 1:12, 1:20, 1:30	3
II6266	16293 (F5/G, F2/A), 16293 (F5/G, F2/A),	1:1 – position 16293 1:6 – position 16311	10, 28

(A)



(B)



(C)

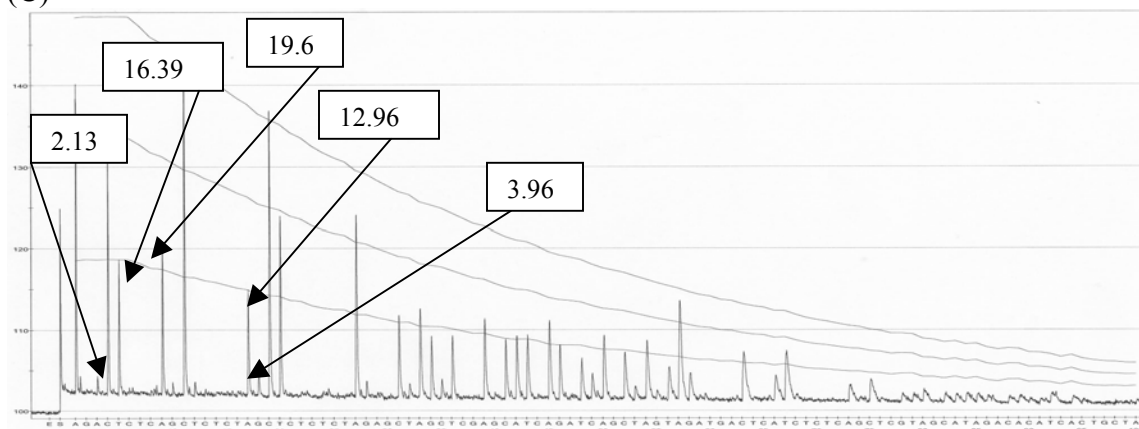
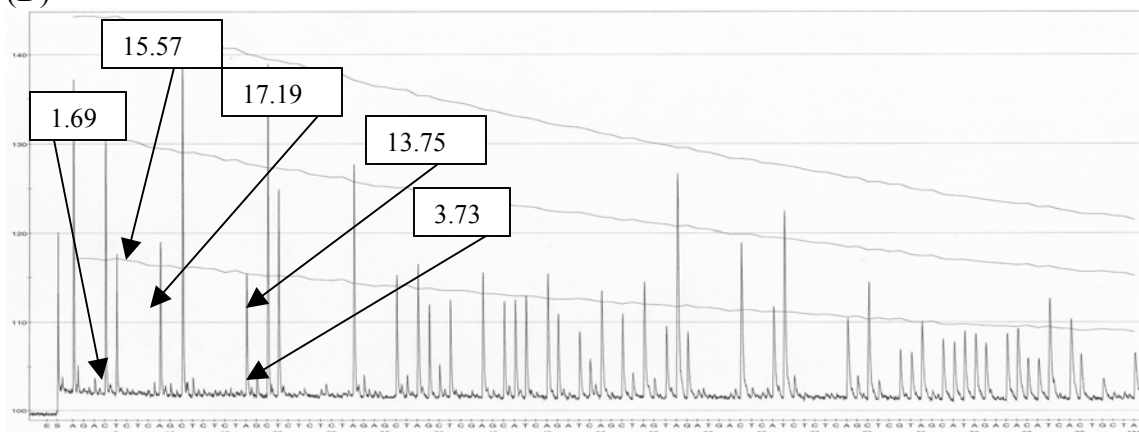
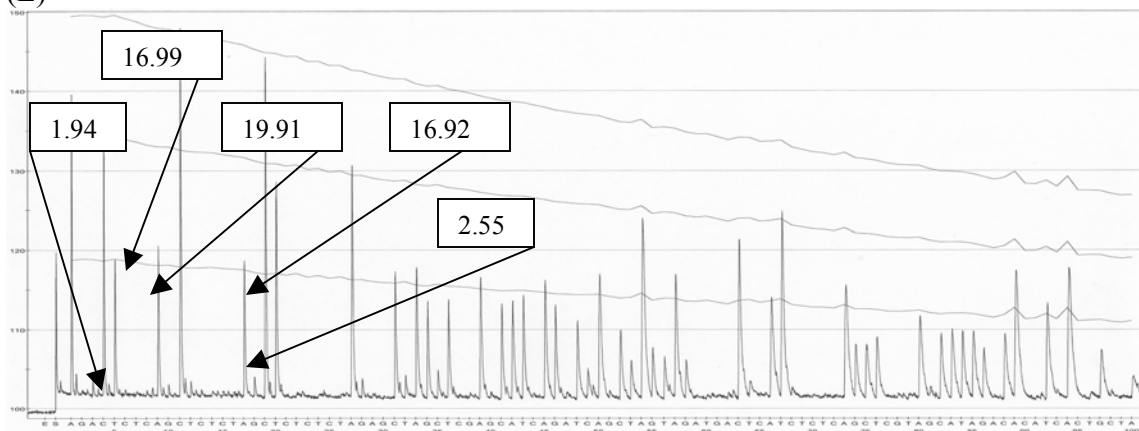


Figure 6 Pyrograms of mixtures. The pyrograms show the results when a mixture of mtDNA is sequenced. Figure (A) – 1:1, (B) – 1:6, (C) – 1:10, (D) – 1:12, (E) – 1:20, (F) – 1:30. Peak heights are indicated in the boxes.

(D)



(E)



(F)

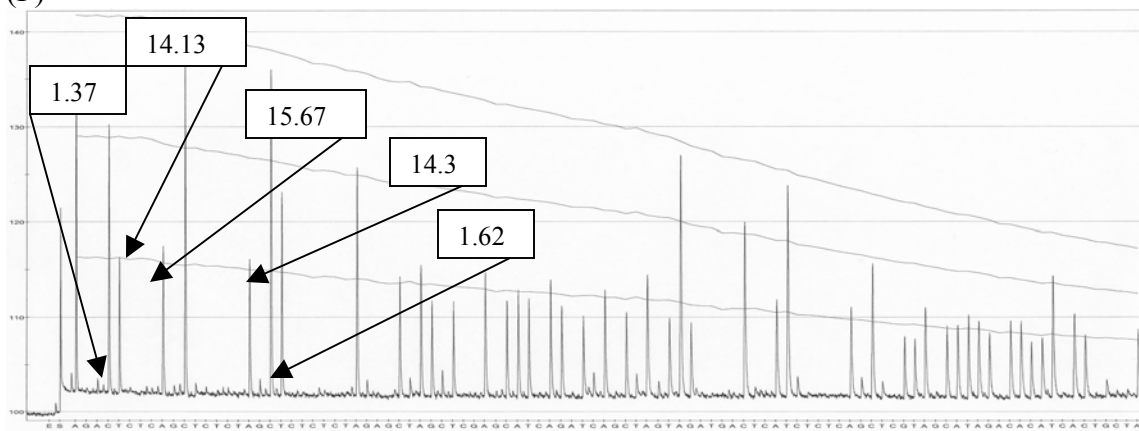


Figure 6 continued.

Figure 7 demonstrates that as the major component, sample F2, increases the 'A' peak increases while the minor component, sample F5, decreases the 'G' peak decreases. The R^2 value for both the minor component and the major component suggests a linear relationship. That is, as the amount of DNA contributed by sample F2 increases the peak height of the 'A' nucleotide increases and that as the amount of DNA contributed by sample F5 decreases the peak height of the 'G' nucleotide decreases.

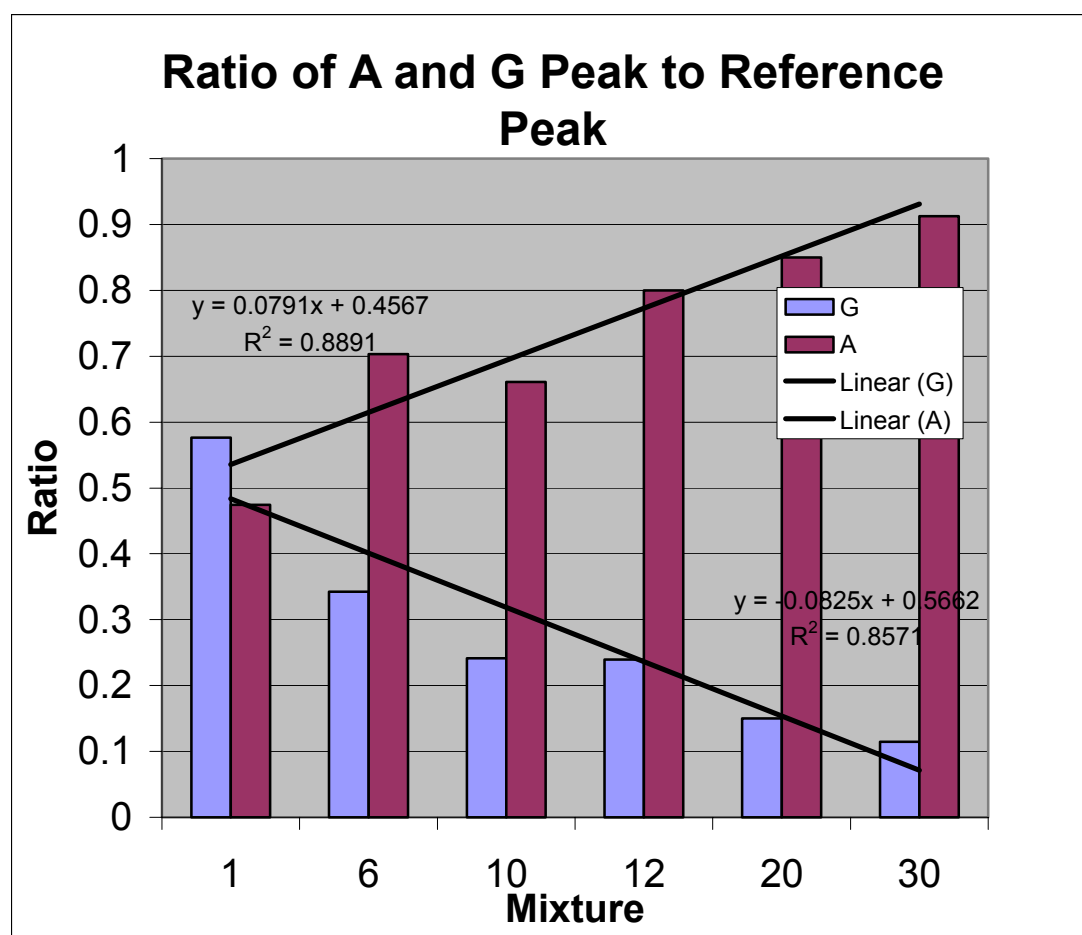


Figure 7 Graph of peak heights in mixture. The graph shows that there is a linear relationship between the quantity of DNA placed in the PCR reaction and the peak heights of the bases that differ between the two samples.

The ability of pyrosequencing to detect the mixtures when 1 pg of input DNA and sample F2 as the major component, varied from primer to primer. The primers successful in detecting the mixtures were: II45, II111, I16168, I16203 and I16266. In addition the pyrosequencer was more successful in detecting the mixture when the ratio was greater than 1:1 (F2 in the greater concentration), this further suggests that sample F5 contained more mitochondrial DNA copies than sample F2. On the other hand pyrosequencing was slightly less successful in detecting the mixture when 1 pg of DNA was placed into the PCR amplification as compared to when 20 pg of input DNA was used. Additionally, the read length was slightly reduced when a lower quantity of DNA was used (data not shown). The results of sequencing the mixtures using 1 pg of input DNA into PCR and sample F2 as the major component are displayed in Table 4.

Table 4 Mixture table (1pg DNA). The table shows the results when a mixture of sample F5 and sample F2 were mixed in different ratios keeping sample F2 as the major component and the total input DNA in the PCR amplification at 1 pg. The distance from the end of the sequencing primer in bases is indicated in the last column.

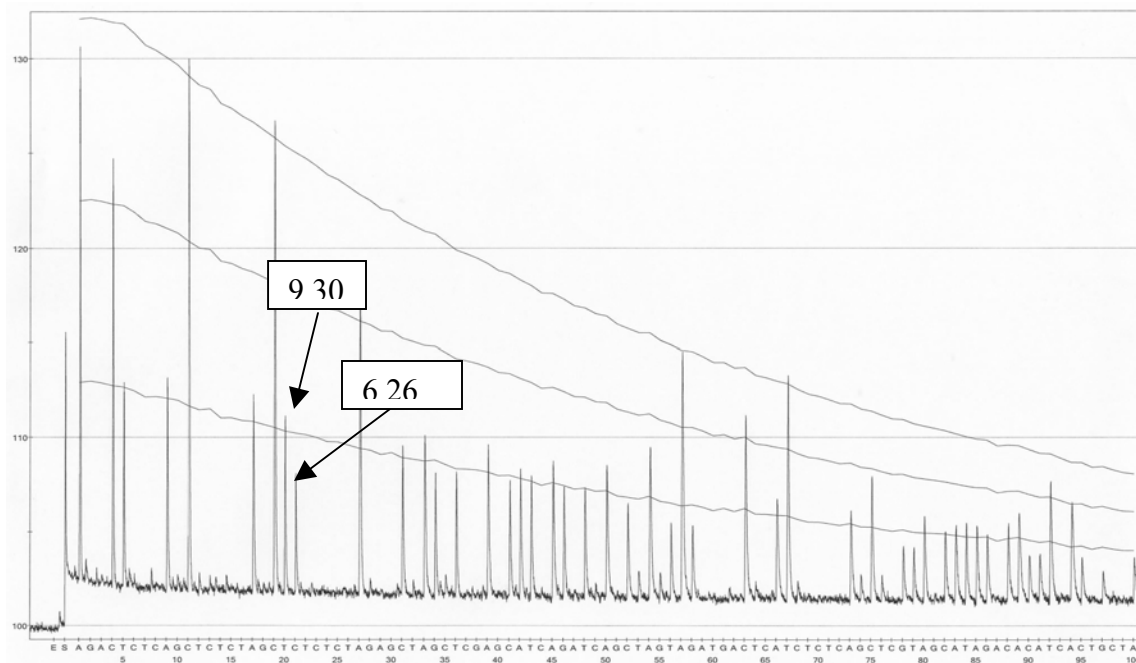
Primer	Region detected	Ratio Detected	Distance
II45	152 (F5/C, F2/T)	1:12	86
II111	152 (F5/C, F2/T), 195 (F5/C, F2/T)	1:6	23, 66
II162	Not detected	N/A	N/A
II216	Not detected	N/A	N/A
I16105	Not detected	N/A	N/A
I16168	16187 (F5/T, F2/C), 16233(F5/T, F2/C)	1:1, 1:6, 1:10, 1:12	2, 38
I16203	16233 (F5/T, F2/C), 16270 (F5/T, F2/C)	1:1, 1:6, 1:10 – position 16233 1:6, 1:10 – position 16270	3, 50
I16266	16293 (F5/G, F2/A), 16293 (F5/G, F2/A),	1:1, 1:6 – position 16293 1:1, 1:6,1:10 – position 16311	10, 28

The ability of pyrosequencing to detect mixtures varied depending on the primer and the concentration of DNA used in the amplification reaction. Pyrosequencing was able to successfully detect mixtures with increased accuracy when 20 pg of input DNA was used compared to 1 pg of input DNA. Pyrosequencing does not always automatically detect the mixture, however, manual examination of the pyrogram increases the detection rate. In some cases upon manual examination it was not clear that there was a mixture present but the pattern of the peak heights suggested that there was something different with the sample at a particular location, which may warrant re-sequencing those samples.

3.4 True Heteroplasmic Sample

The heteroplasmic sample HRM, obtained from the FBI, contains both of the following sequences: ACCCTTAA and ACCCTCA (heteroplasmic site is indicated in red). That is at nucleotide 16298 either a 'T' or a 'C' is present. The heteroplasmic sample (HRM) was amplified and sequenced a total of four times. However, pyrosequencing did not automatically detect the heteroplasmic site. After manual examination of the pyrograms (Figure 8), it became evident that there was an inconsistency in the 'C' peak height at position 16298 in that it was reduced compared to a single 'C' peak for that region. The heteroplasmic 'C' peak was approximately 20-30% shorter than that of a normal 'C' peak of that region as can be seen in Figure 9. The ratio of a single 'C' nucleotide to a single 'T' nucleotide in the sample could not be determined as there were no single 'T' and 'C' peaks present in the proximity of the heteroplasmic site. This could account for the same discrepancy in peak height not seen in the 'T' peak present at the heteroplasmic site. Additionally, the discrepancy could be due to the difficulty the instrument occasionally has with repeated bases.

(A)



(B)

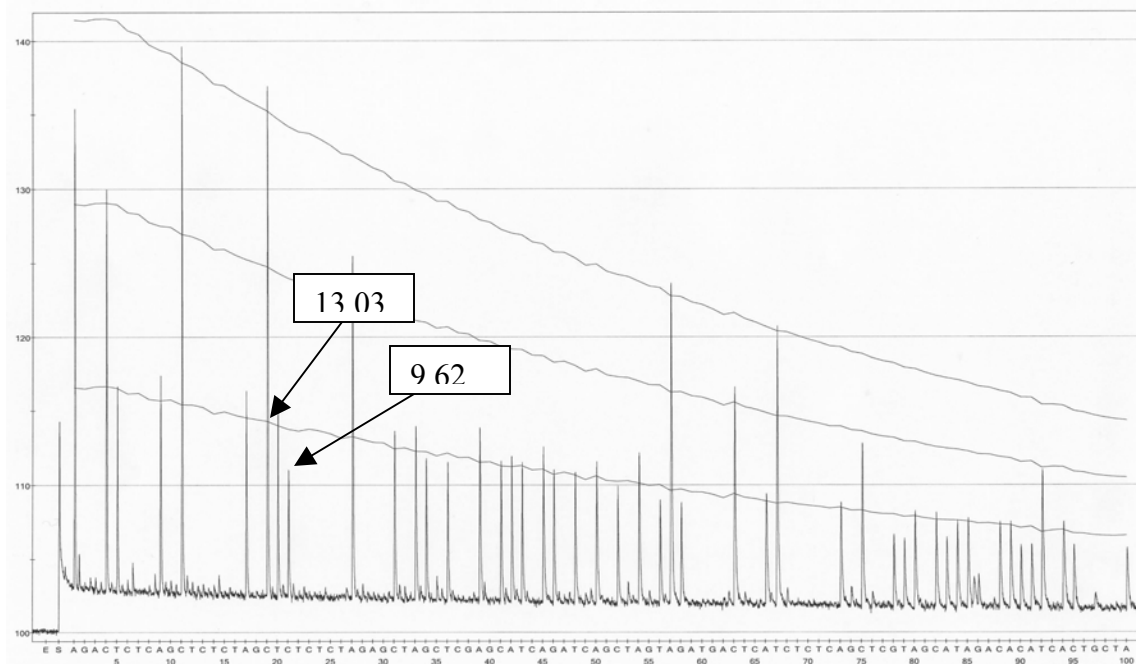
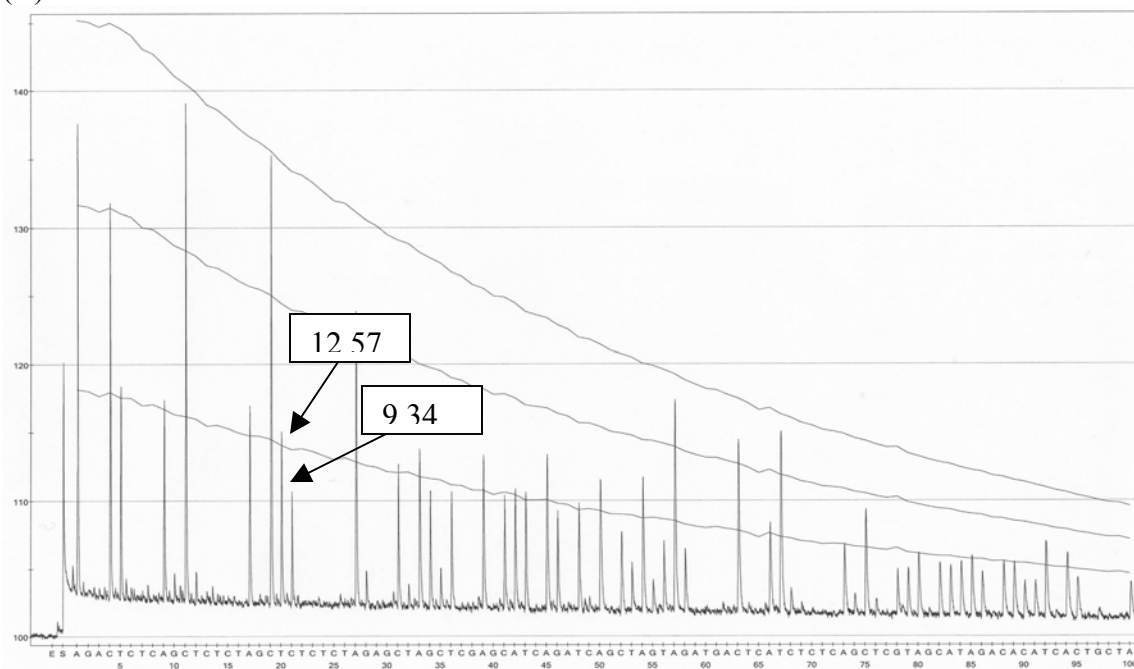


Figure 8 Pyrogram of a true heteroplasmic sample (HRM).

(C)



(D)

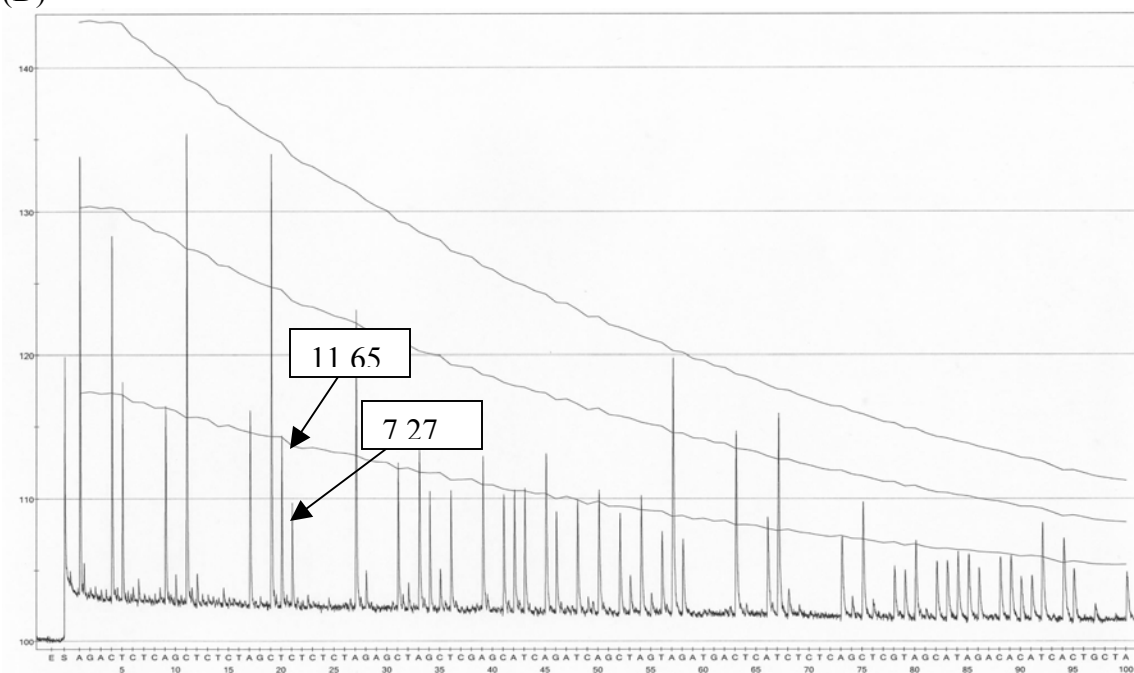


Figure 8 continued.

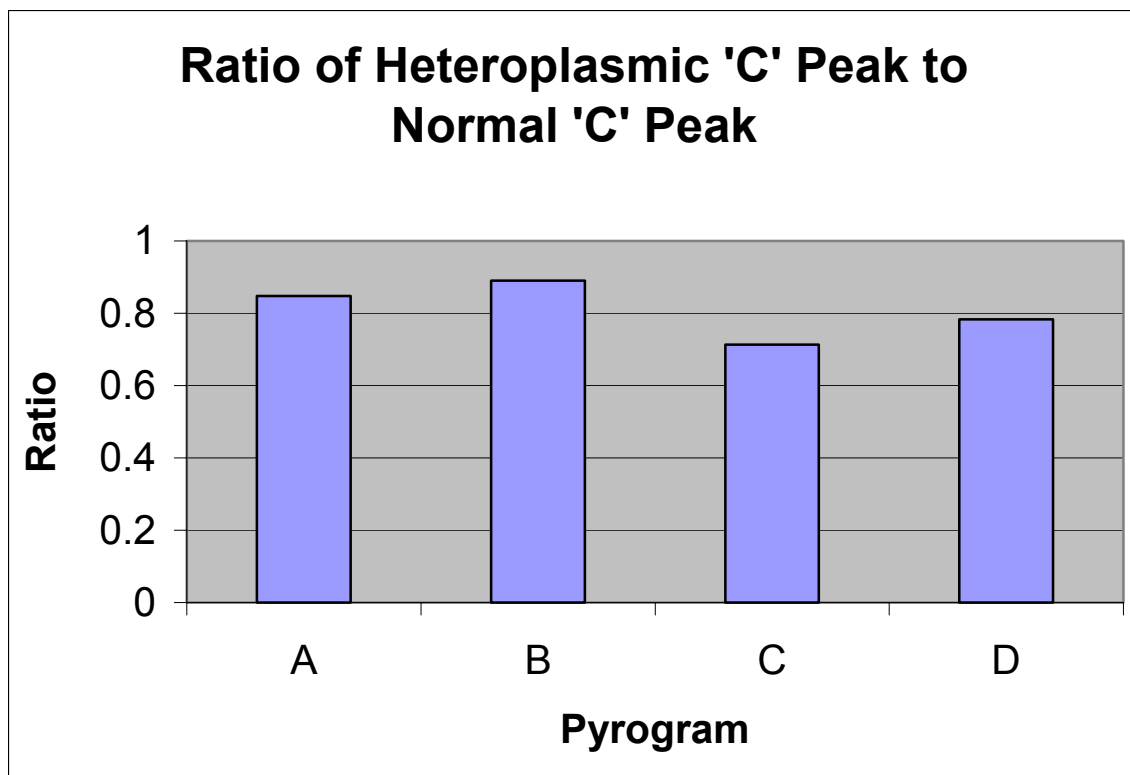


Figure 9 Graph of peak heights for the heteroplasmic sample. The graph shows the ratio of the 'C' peak present at the heteroplasmic site compared to the peak height of a normal 'C' peak in the same region.

3.5 Environmentally Compromised Samples

The different elements included for each treatment of the study are shown in table 5. A (+) indicates that this element was incorporated in the treatment while a (-) indicated that the element was not used in the treatment.

Table 5 Table of the different elements that are present in the different treatments.

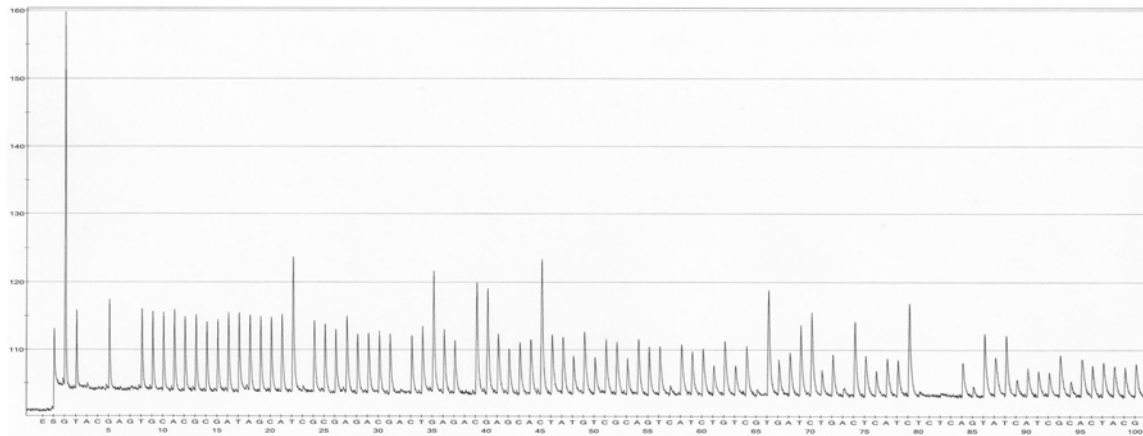
	Heat	Humidity	Sunlight	Rain
Treatment 1	+	+	+	-
Treatment 2	+	+	+	+
Treatment 3	+	+	-	+

The FS1 saliva sample was exposed to sunlight, heat, and humidity for one day and sequenced with primers II45, I16105 and VRI16350. The results are shown in Figure 10. The pyrograms are consistent with that of the control sample of FS1 that was not subjected to the elements. The pyrograms have a flat baseline with very little background. In addition, pyrograms generated with the other primers were of similar quality. Similar read lengths were obtained with these samples as compared to the control samples.

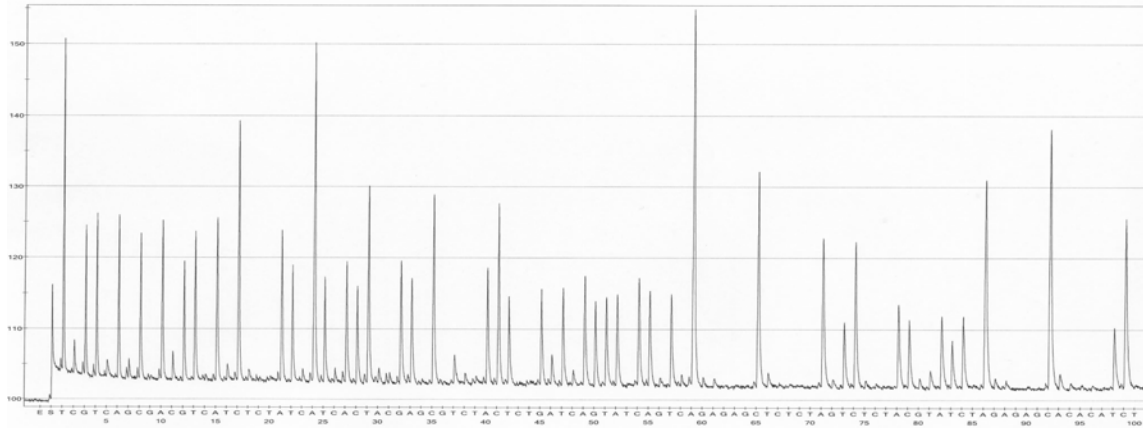
The FS1 saliva sample, which was exposed to sunlight, heat, and humidity for three months was sequenced with primers II45, I16105 and VRI16350 respectively, produced the pyrogram in Figure 11. The pyrograms have considerable background. However, the instrument was still able to make base calls, but the read length was significantly reduced when compared to the read length of the control sample. In addition, a base line drift was observed when primer I16105 was used. This shows that the DNA obtained was significantly damaged as compared to

the control sample. Samples collected after three months could not be amplified due to the severity of the damage sustained by the DNA.

(A)



(B)



(C)

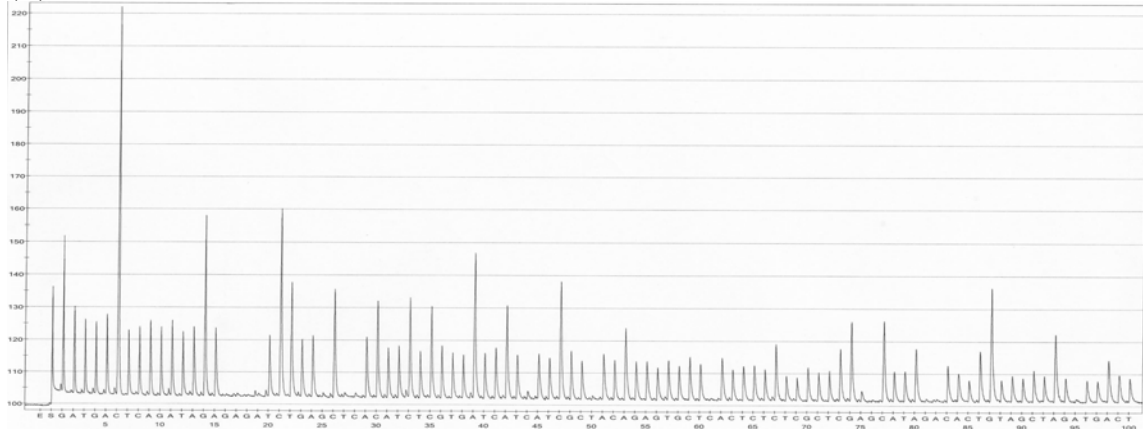


Figure 10 Pyrograms of FS1 after 1 day treatment 1. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from FS1 saliva sample after being exposed to sunlight, heat and humidity (treatment 1) for 1 day.

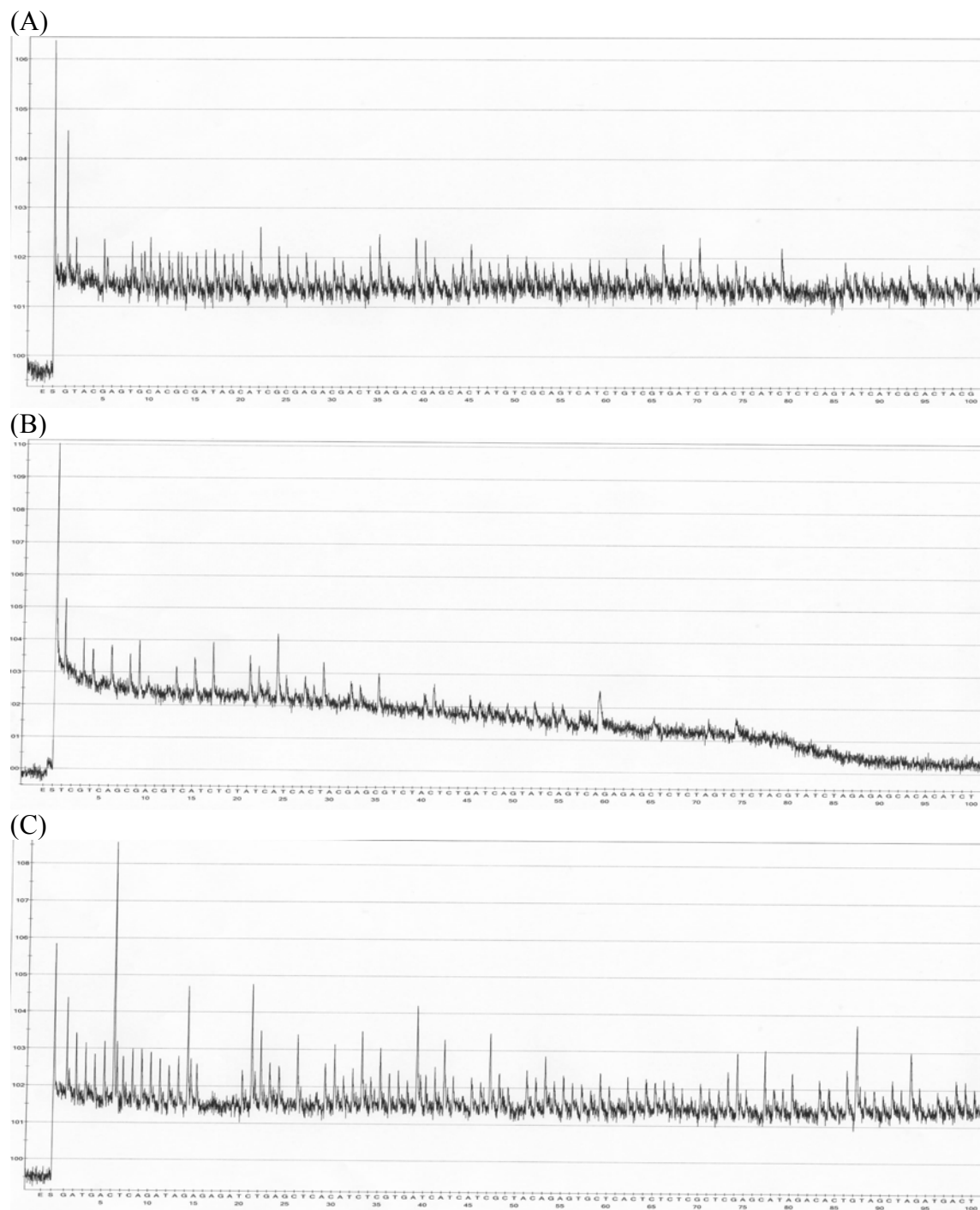
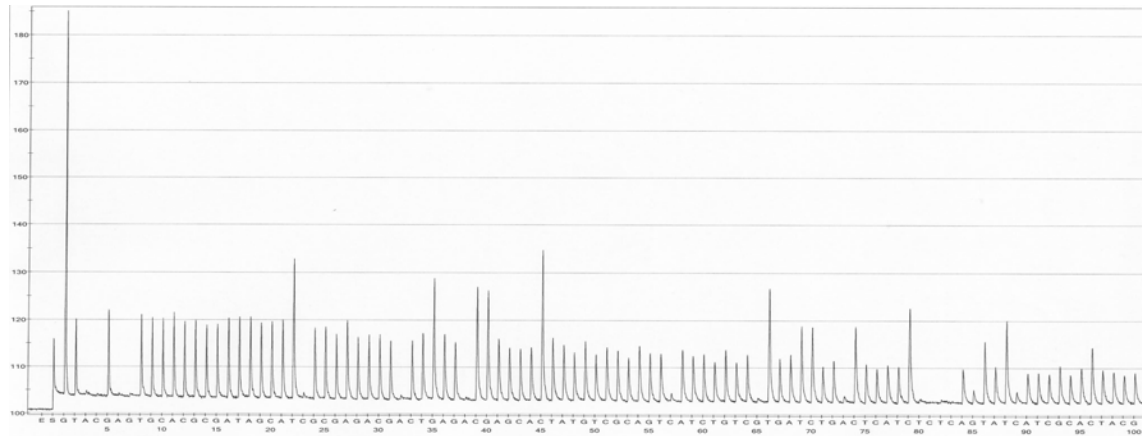


Figure 11 Pyrograms of FS1 after 3 months of treatment 1. The pyrograms show parts of the HVII (A), HVI (B) 2 months, and VRI region of DNA extracted from FS1 saliva of sample FS1 after being exposed to sunlight, heat, and humidity (treatment 1) for 3 months.

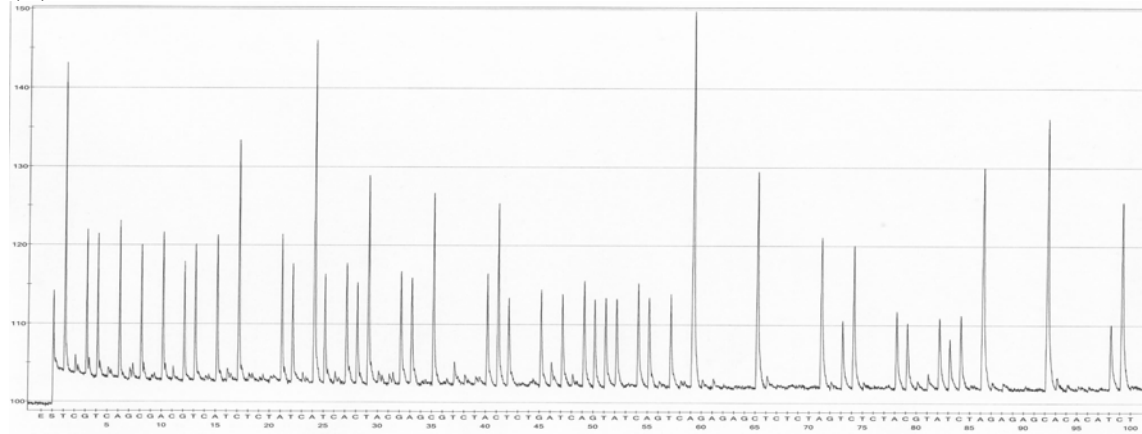
The pyrogram in Figure 12 is of DNA from a FS1 saliva sample that was exposed to sunlight, heat, humidity, and rain for one day and sequenced using primers II45, I16105, and VRI16350 respectively. The pyrograms are consistent with that of the control sample of FS1 that was not exposed to any treatment. The pyrograms are of good quality, that is, they have a flat baseline with very little background. Pyrograms generated with the other primers were of similar quality. Similar pyrogram quality and read lengths were obtained with the control samples.

The pyrograms in Figure 13 is of DNA from a FS1 saliva sample that was exposed to sunlight, heat, humidity, and rain for one week and sequenced using primers II45, I16105, and VRI16350 respectively. The pyrograms obtained display slightly more background noise than the observed for the one-day samples of the same treatment, but the overall quality of the pyrograms were still good and the read length was still comparable with the control. However, samples collected at the one month time period and beyond were not successfully amplified and therefore could not be sequenced. The yield gel of these samples (which was used to quantify the amount of DNA present in the sample) suggested that there was sufficient DNA present to be amplified however this DNA must not have been human in origin. Mold and bacteria were seen growing on the samples and must have been the contributors of the DNA present in the yield gel.

(A)



(B)



(C)

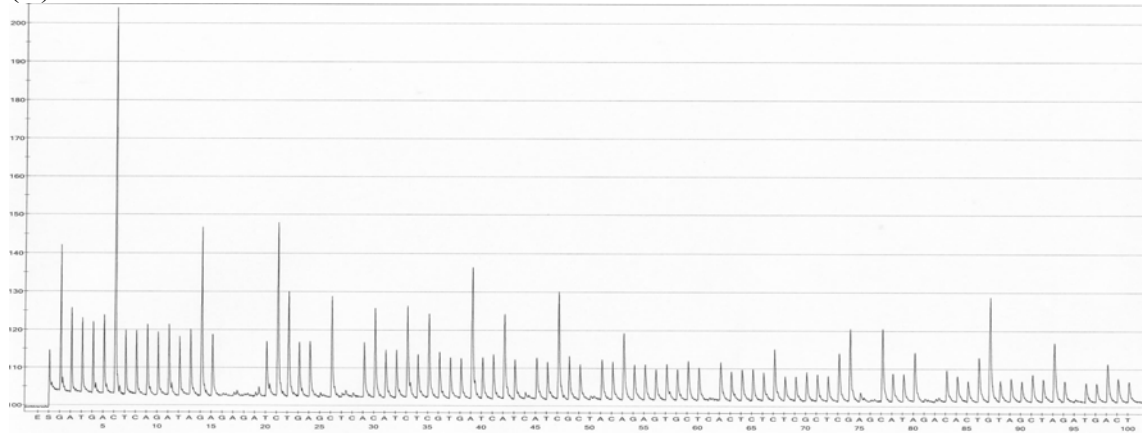


Figure 12 Pyrograms of FS1 after 1 day of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 saliva sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 1 day.

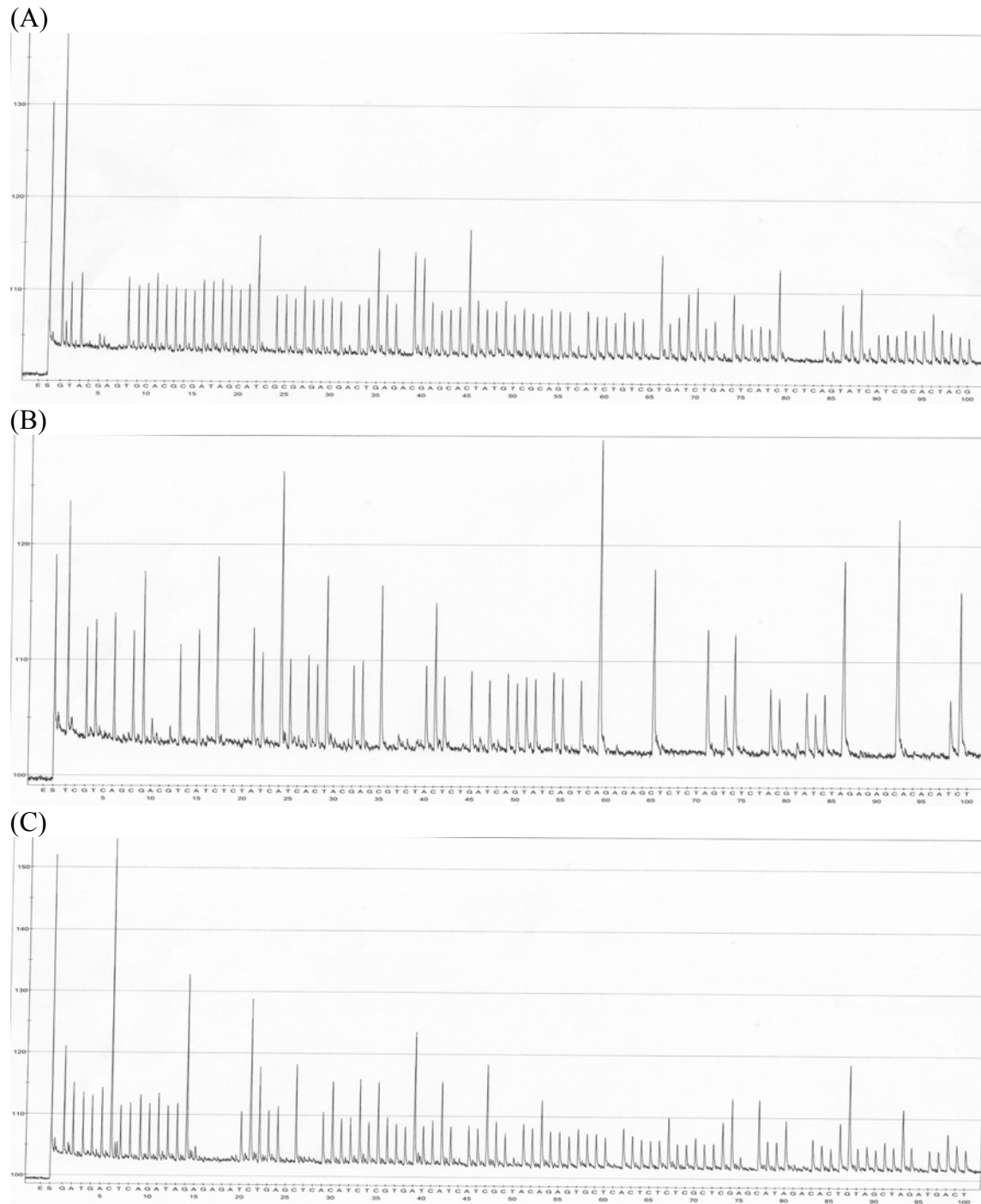
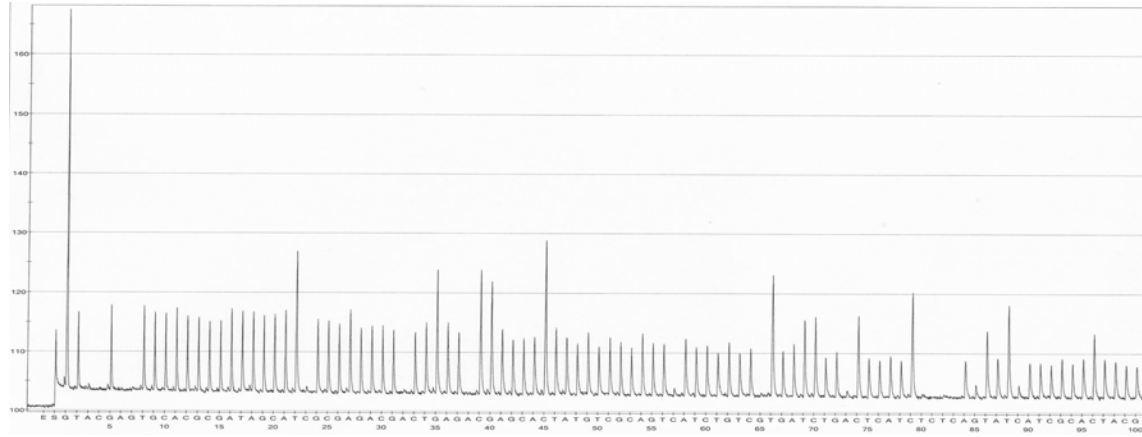


Figure 13 Pyrograms of FS1 after 1 week of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 saliva sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 1 week.

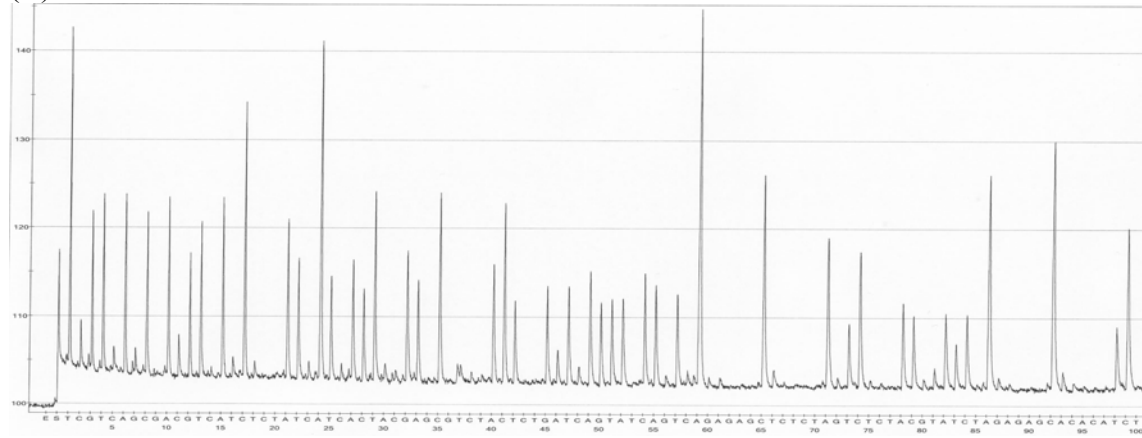
The pyrograms produced when DNA of sample FS1 saliva that was exposed to heat, humidity, and rain for one day and was sequenced with primers II45, I16105, and VRI16350 respectively are shown in Figure 14. The pyrograms are consistent with that of the control sample of FS1 which was not subjected to any treatment. The pyrograms have a flat baseline with very little background noise. Pyrograms generated with the other primers were of similar quality. The samples also produced read lengths of similar quality to that of the control.

The pyrograms produced when DNA of sample FS1 saliva was exposed to heat, humidity, and rain for one week were sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 15. The pyrograms are still of very good quality and the samples produced fairly long read lengths. However, when the samples were collected at one month and beyond the samples did not amplify. This could be due to the distribution of human DNA by microbes, which were seen growing on the sample. The yield gel of the sample suggested that there was sufficient DNA present on the sample.

(A)



(B)



(C)

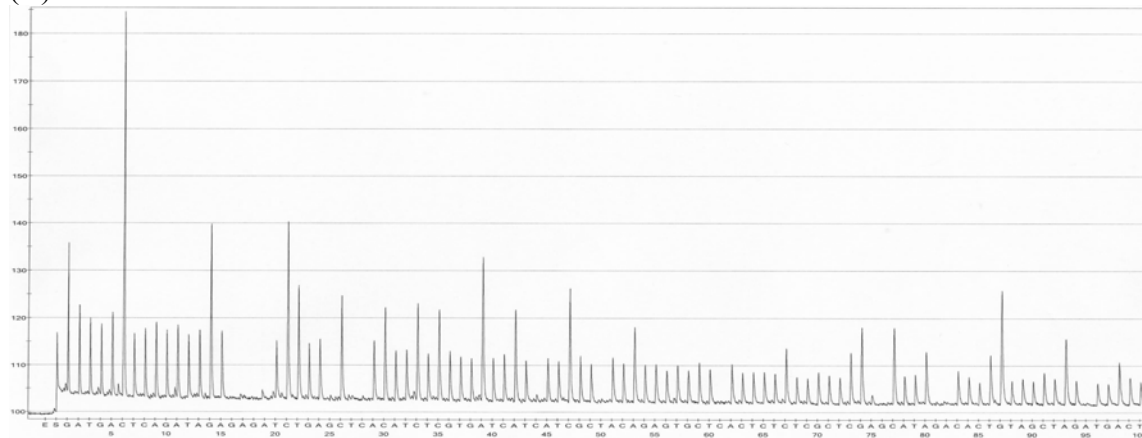


Figure 14 Pyrograms of FS1 saliva after 1 day of treatment 3. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 saliva sample after being exposed to heat, humidity, and rain (treatment 3) for 1 day.

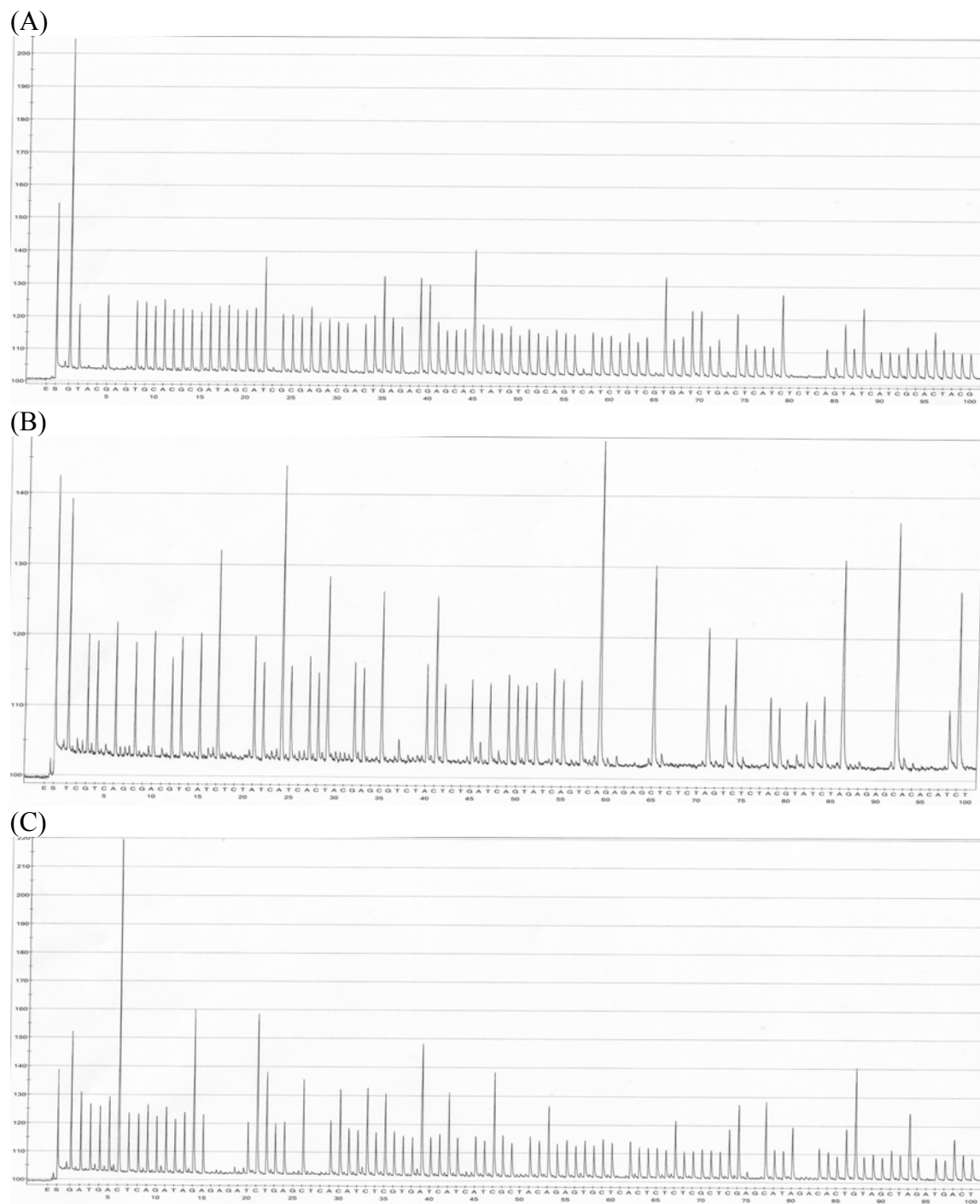
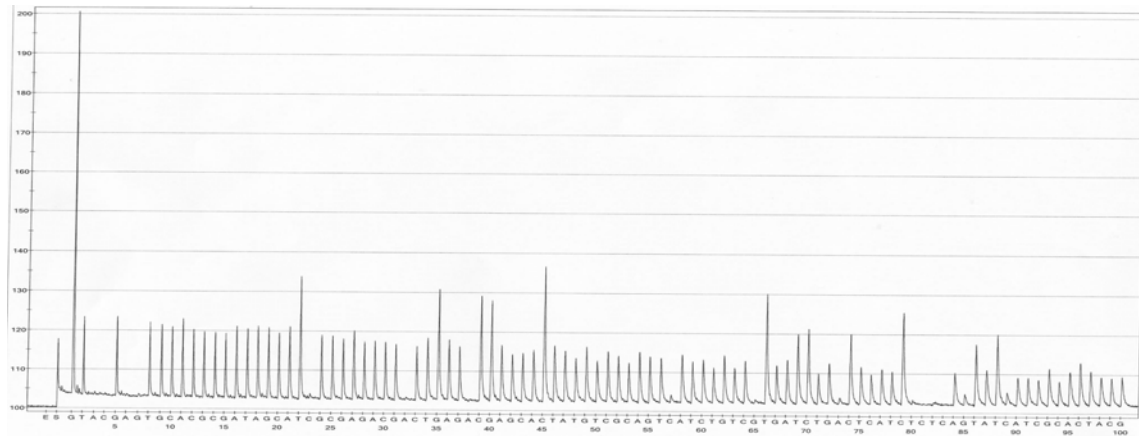


Figure 15 Pyrograms of a FS1 saliva sample after 1 week of treatment 3. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 saliva sample after being exposed to heat, humidity, and rain (treatment 3) for 1 week.

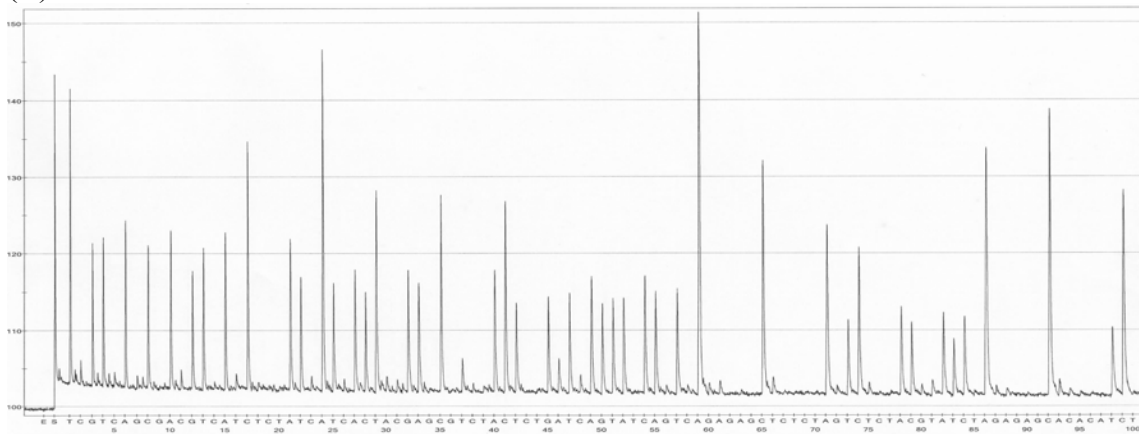
The pyrograms presented in Figure 16 is of DNA from a FS1 blood sample that was exposed to sunlight, heat, and humidity for one day and sequenced using primers II45, I16105, and VRI16350 respectively. The pyrograms are consistent with that of the control sample of FS1 that was not exposed to any treatments. The pyrograms are of good quality, that is, they have a flat baseline with little background noise. Pyrograms generated with the other primers were of similar quality. In addition similar pyrogram quality and read lengths were obtained with the control samples.

The pyrograms in Figure 17 is of DNA from a FS1 blood sample that was exposed to sunlight, heat, and humidity for six months and sequenced using primers II45, I16105, and VRI16350 respectively. These pyrograms are consistent with that of the control samples of FS1 that were not exposed to any treatment. The pyrograms are still of good quality, that is, they have little background and a flat baseline. The samples also produced long read lengths and there was no evidence of microbial growth. Pyrograms generated with other primers are of similar quality and read lengths as the control sample.

(A)



(B)



(C)

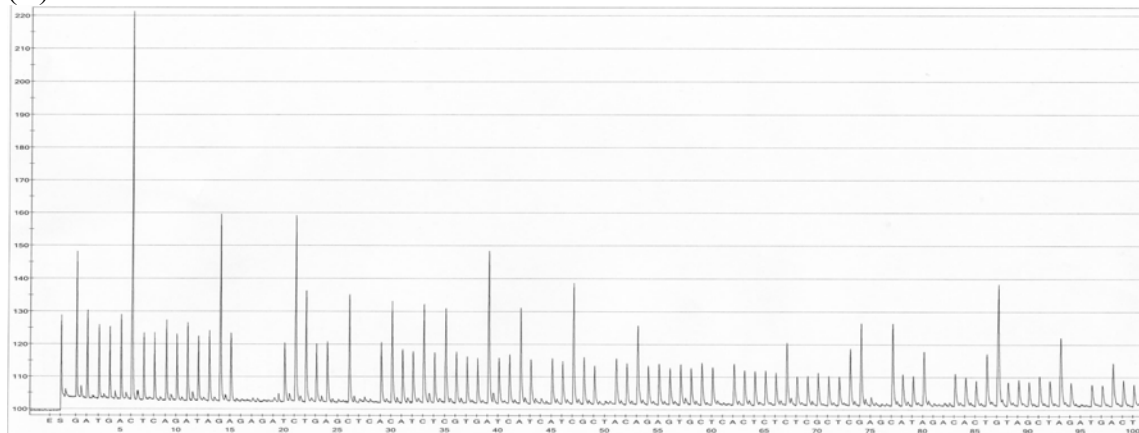


Figure 16 Pyrograms of FS1 blood after 1 day of treatment 1. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 blood sample after being exposed to sunlight, heat, and humidity (treatment 1) for 1 day.

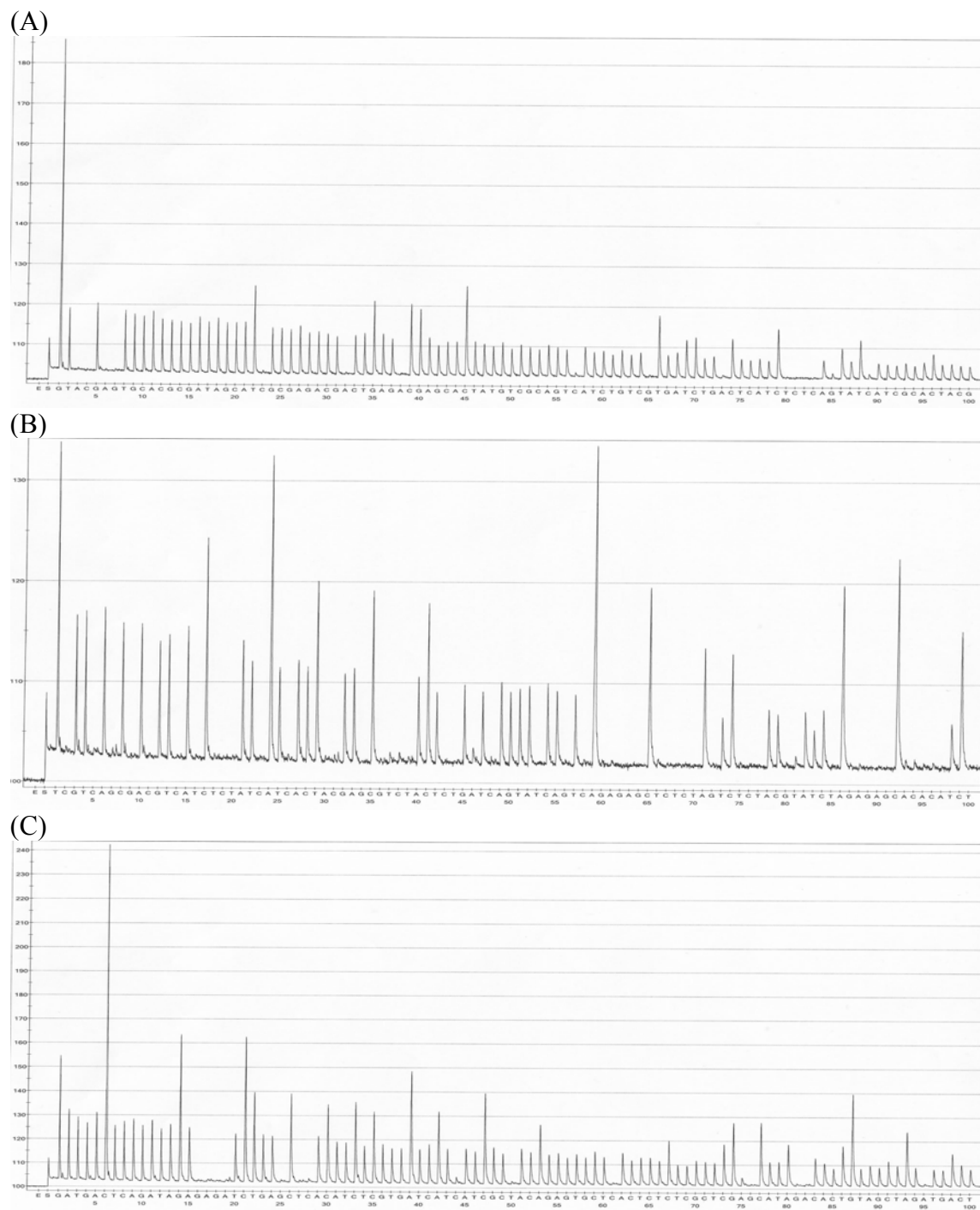
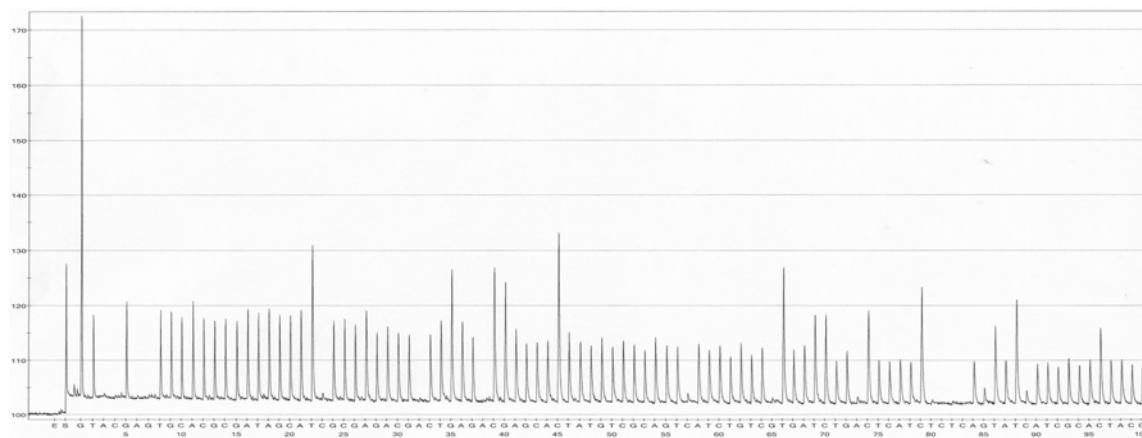


Figure 17 Pyrograms of FS1 blood after 6 months of treatment 1. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 blood sample after being exposed to sunlight, heat, and humidity (treatment 1) for 6 months.

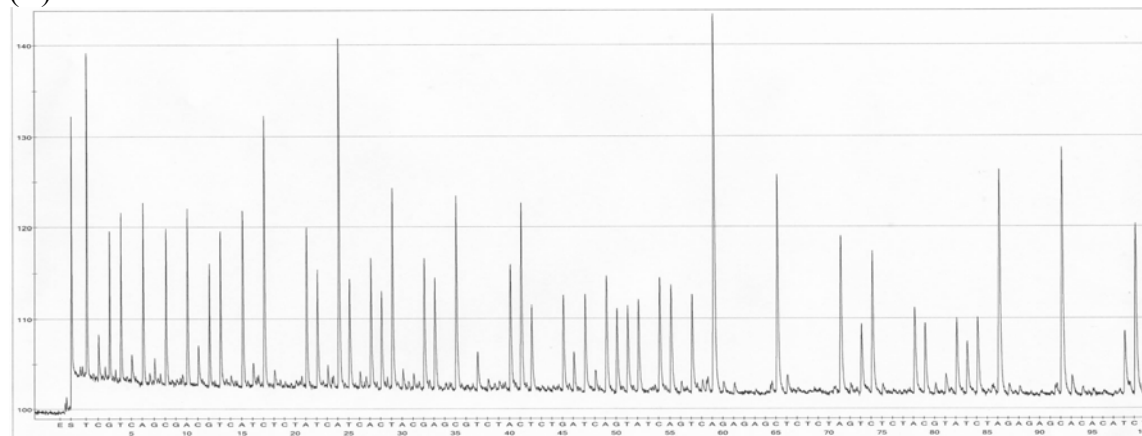
The pyrograms produced when DNA of the sample FS1 that was exposed to sunlight, heat, humidity, and rain for one day was sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 18. The pyrograms are consistent with that of the control sample of FS1 that was not exposed to any treatment. The pyrograms have a flat baseline with little background noise. Pyrograms generated with the other primers were of similar quality. The samples also produced similar read lengths to that of the control sample.

The pyrograms produced when DNA of sample FS1 that was exposed to sunlight, heat, humidity, and rain for one month was sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 19. The pyrograms produced are fairly noisy and have a slight baseline drift. However, the instrument was still able to make base calls. These pyrograms suggests that the DNA was damaged. Samples collected at two months and after did not amplify and therefore could not be sequenced. There was evidence of microbial growth on the samples. These microbes could have caused /contributed to the degradation of the DNA. The yield gel of the samples showed large amounts of high molecular weight DNA that must have been contributed by the microbes.

(A)



(B)



(C)

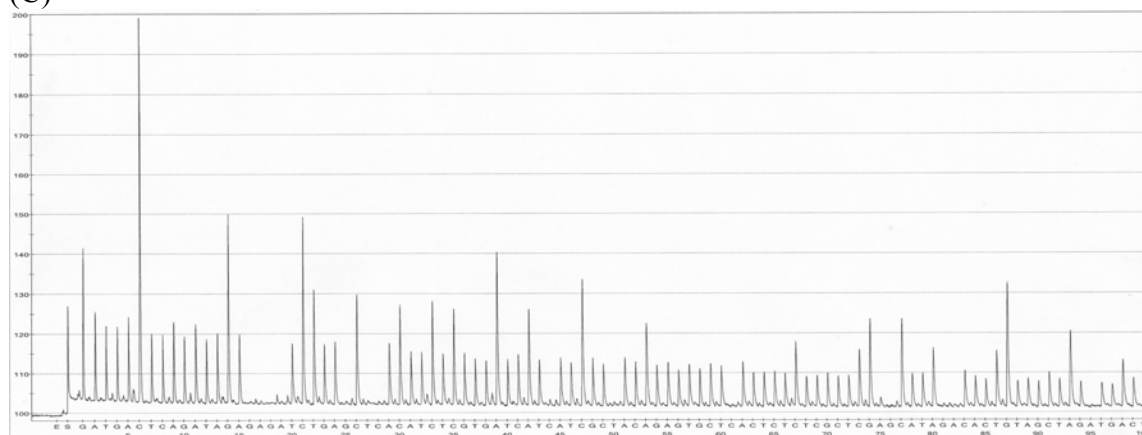
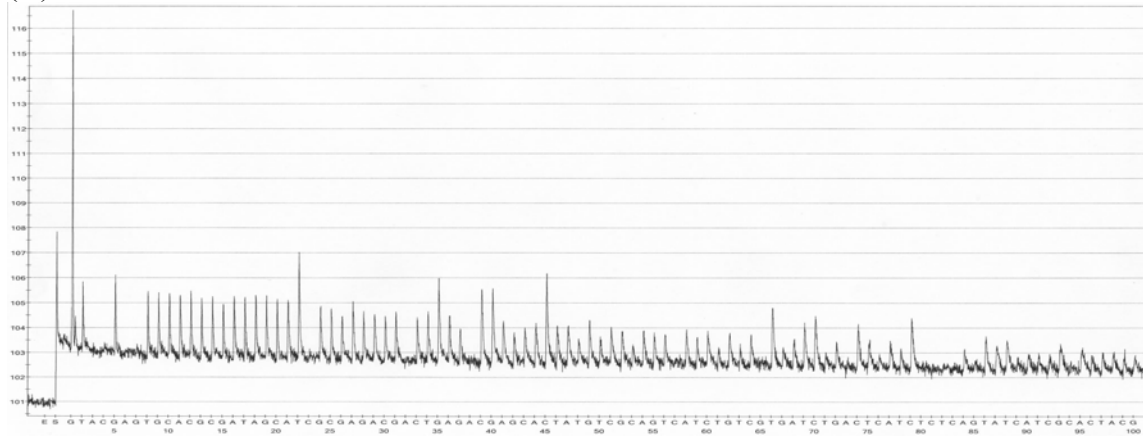
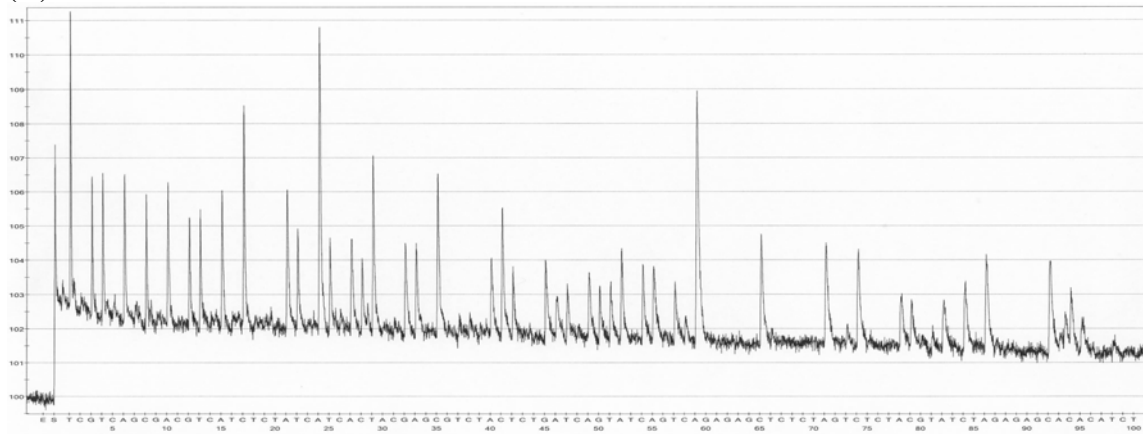


Figure 18 Pyrograms of FS1 blood after 1 day of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 blood sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 1 day.

(A)



(B)



(C)

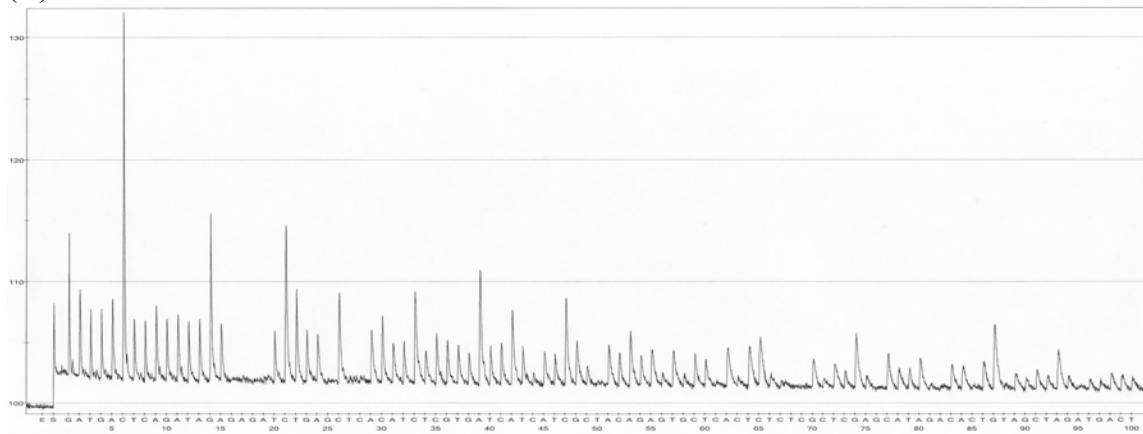
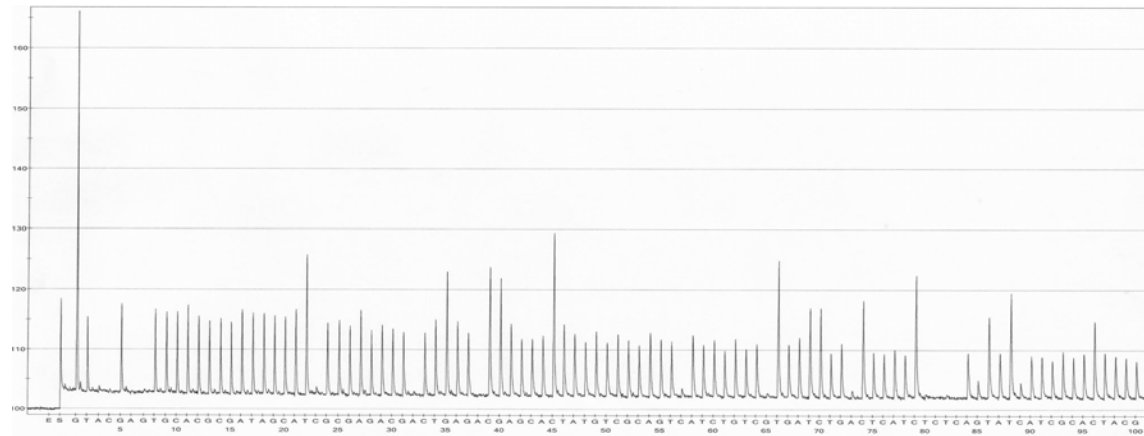


Figure 19 Pyrograms of FS1 blood after 1 month of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 blood sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 1 month.

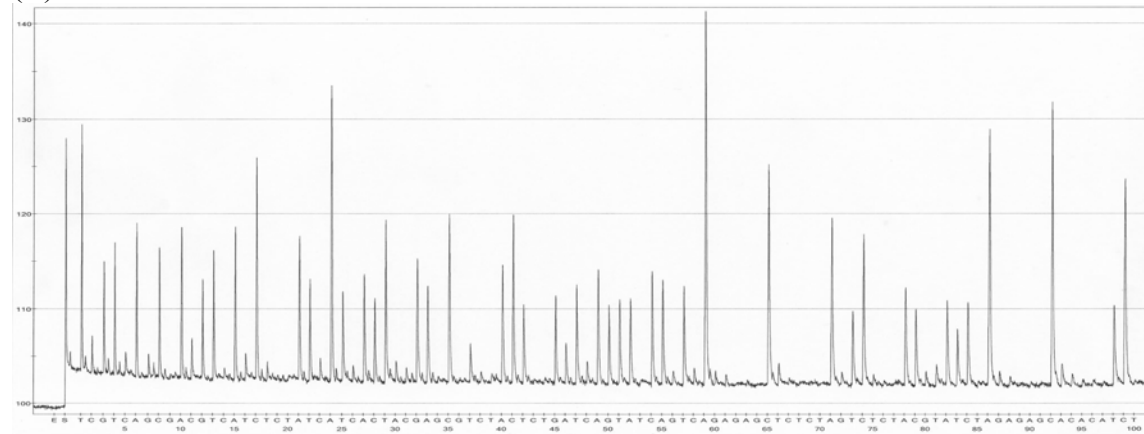
The pyrogram in Figure 20 is of DNA from a FS1 blood sample that was exposed to heat, humidity, and rain for one day and sequenced with primers II45, I16105, and VRI16350 respectively. These pyrograms are consistent with that of the control samples of FS1 that were not exposed to any treatment. The pyrograms are of good quality, that is, they have a flat baseline with little background noise. Pyrograms generated with other primers were of similar quality and produced similar read lengths as that of the control sample of FS1.

The pyrogram in the Figure 21 is of DNA from a FS1 blood sample that was exposed to heat, humidity, and rain for two months and sequenced using primers II45, I16105, and VRI16350 respectively. These pyrograms display high amounts of background noise. They also have elevated baselines. The overall quality of the pyrograms produced was reduced when compared to that of the control. Samples collected at three months and after were not successfully amplified and therefore not sequenced. The yield gel of these samples suggested that there was sufficient high molecular weight DNA present to be amplified, however this DNA must have originated from the microbes seen growing on the samples. Microbial DNA would not amplify with the primers used as they were designed to be specific to human mitochondrial DNA.

(A)



(B)



(C)

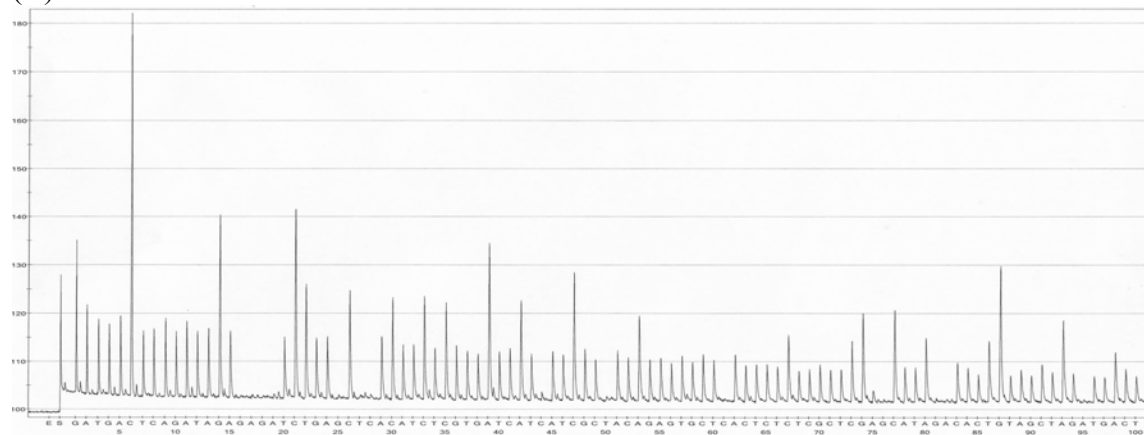
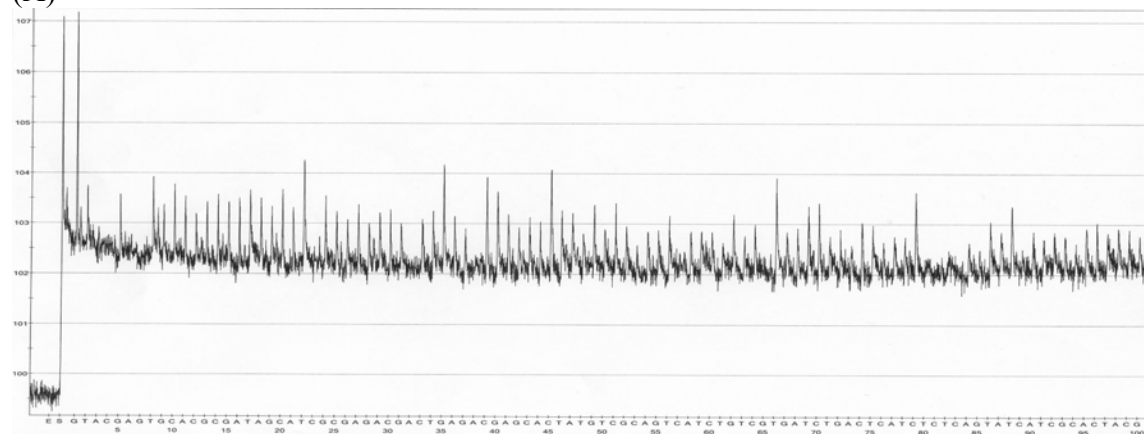
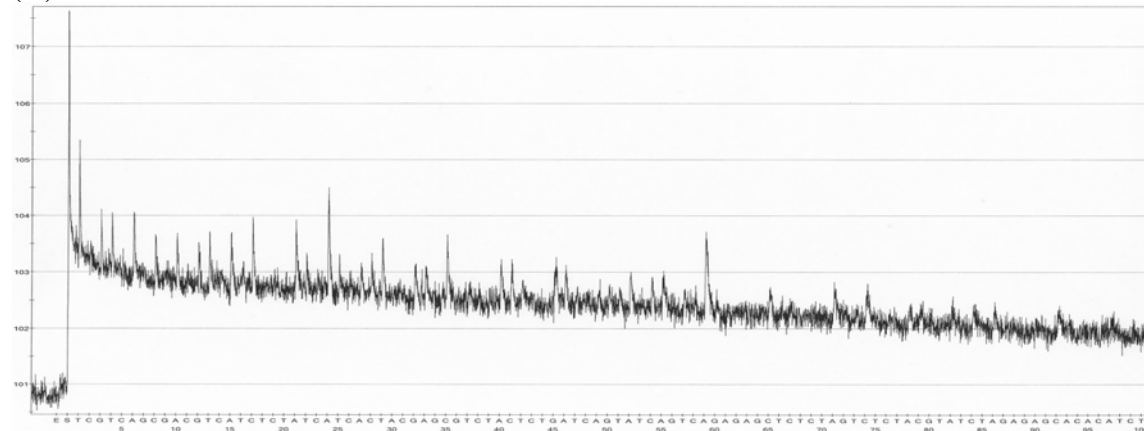


Figure 20 Pyrograms of FS1 after 1 day of treatment 3. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a FS1 blood sample after being exposed to heat, humidity, and rain (treatment 3) for 1 day.

(A)



(B)



(C)

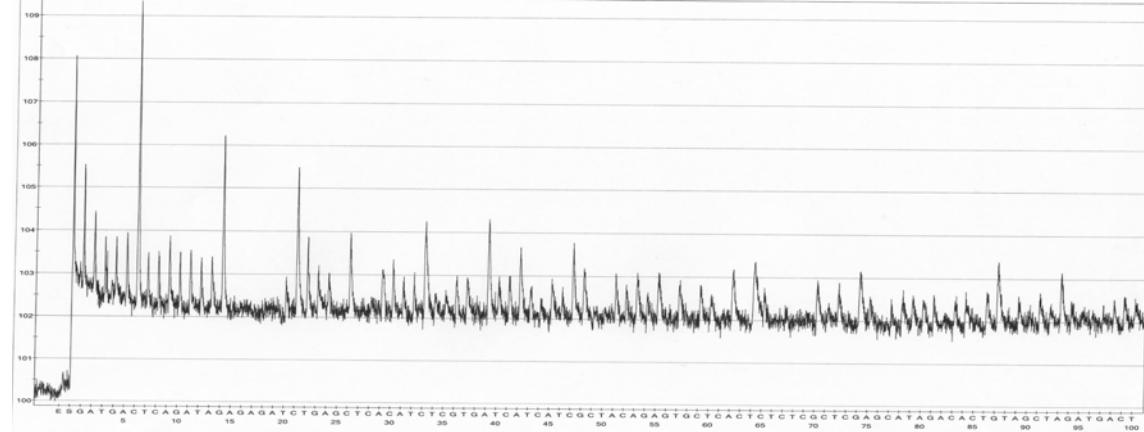
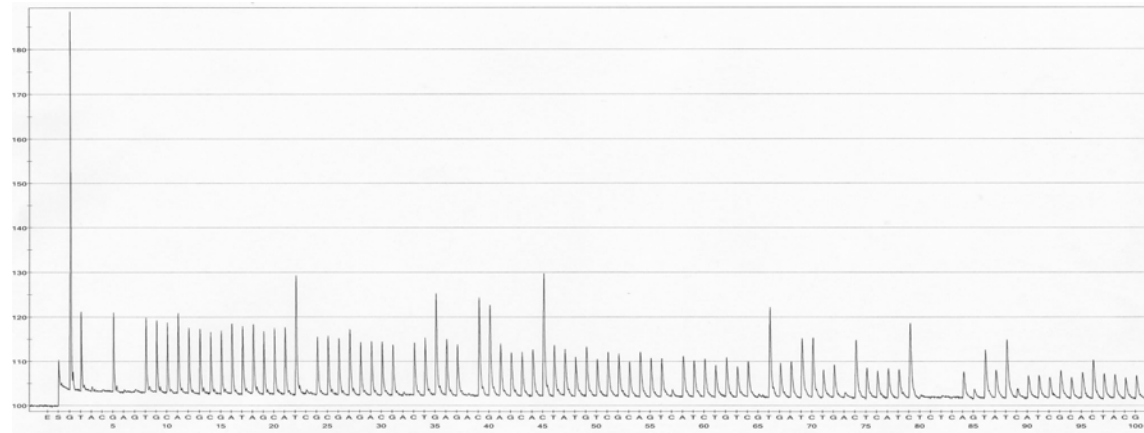


Figure 21 Pyrograms of FS1 blood after 1 month of treatment 3. The pyrograms show parts of the HVII (A) 1 month, HVI (B), and VRI (C) 1 month region of DNA extracted from a FS1 blood sample after being exposed to heat, humidity, and rain (treatment 3) for 2 months.

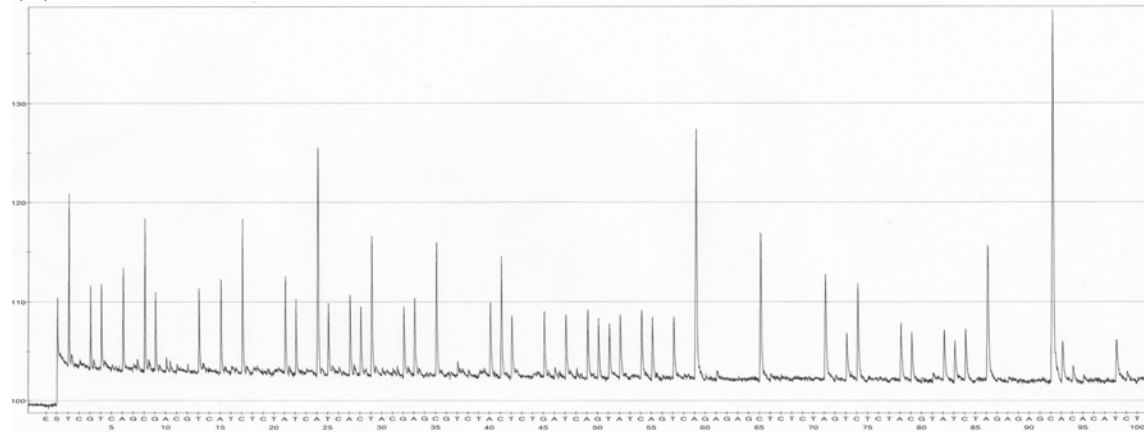
The pyrograms produced when DNA of sample J semen was exposed to sunlight, heat, and humidity for one day was sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 22. The pyrograms are consistent with that of the control sample of J that was not exposed to any treatment. The pyrograms have a flat baseline with little background noise. Pyrograms generated with other primers were of similar quality. The read lengths obtained from all the sequences were comparable to that of the control.

The pyrograms produced when DNA of sample T semen was exposed to sunlight, heat, and humidity for twelve days was sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 23. The pyrograms are still of good quality and still generated good read lengths comparable to that of the control sample. However, when samples were collected after one month and beyond, the samples were not successfully amplified. This suggests that there was insufficient DNA of good quality to amplify. The DNA must have been degraded.

(A)



(B)



(C)

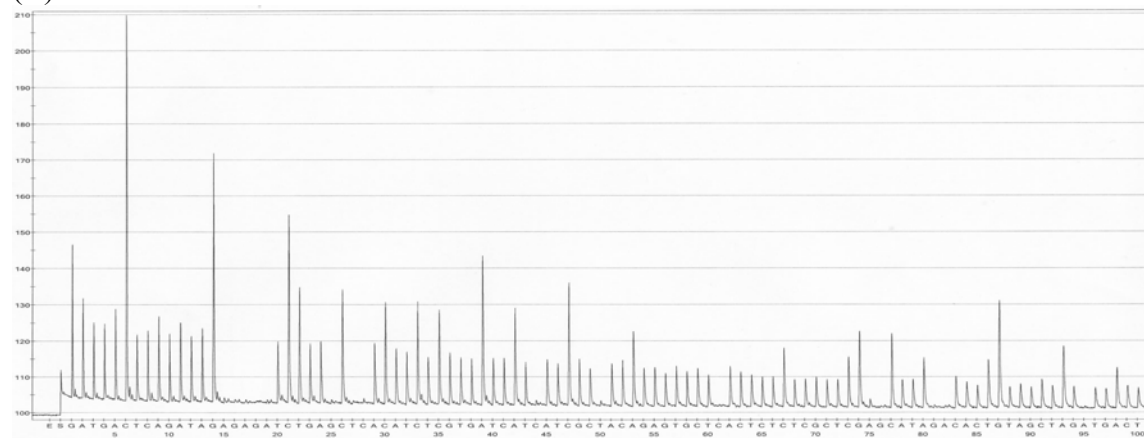
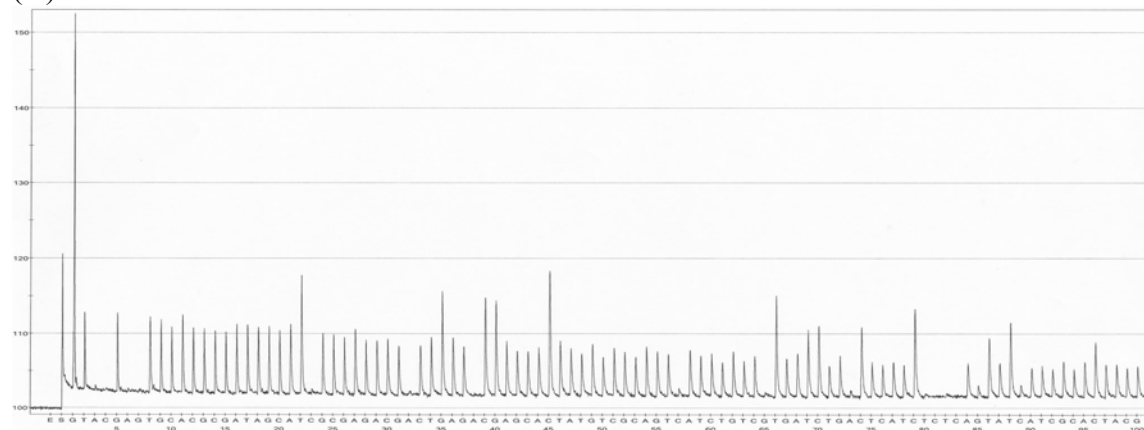
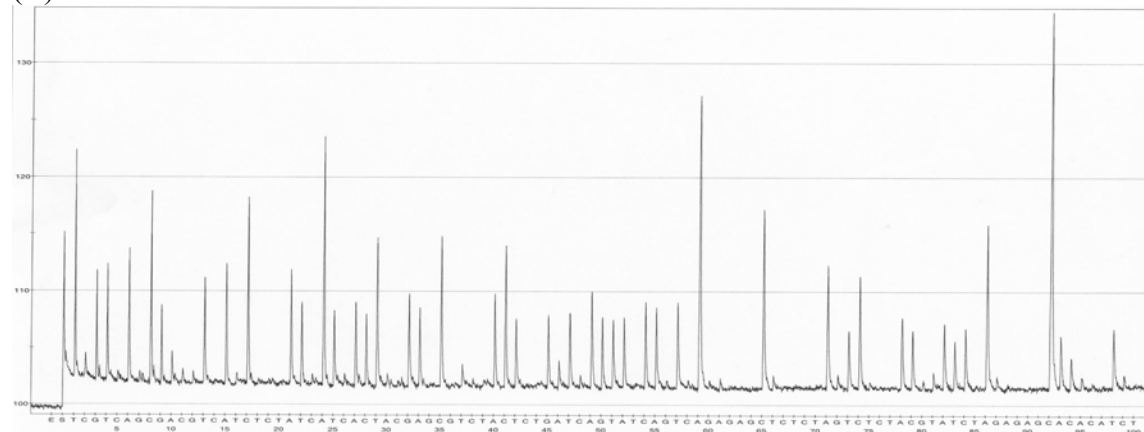


Figure 22 Pyrograms of J semen after 1 day of treatment 1. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to sunlight, heat, and humidity (treatment 1) for 1 day.

(A)



(B)



(C)

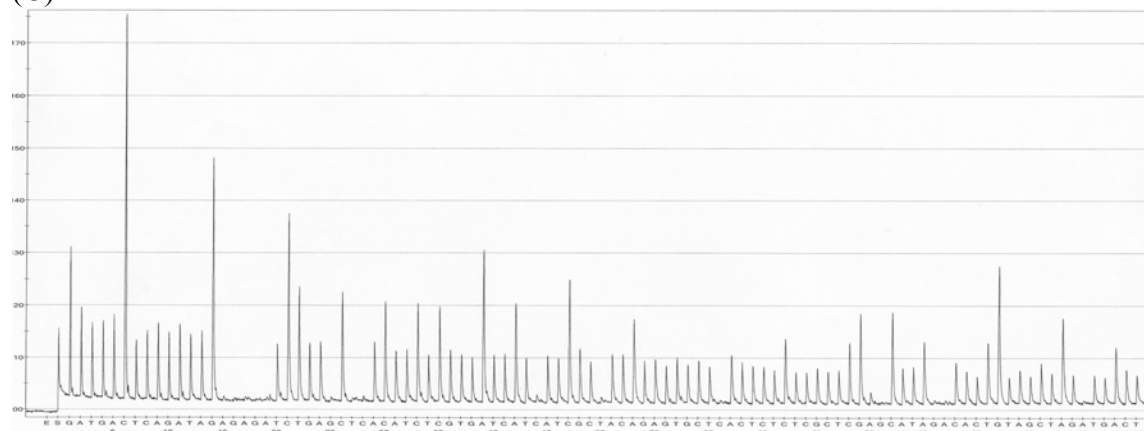
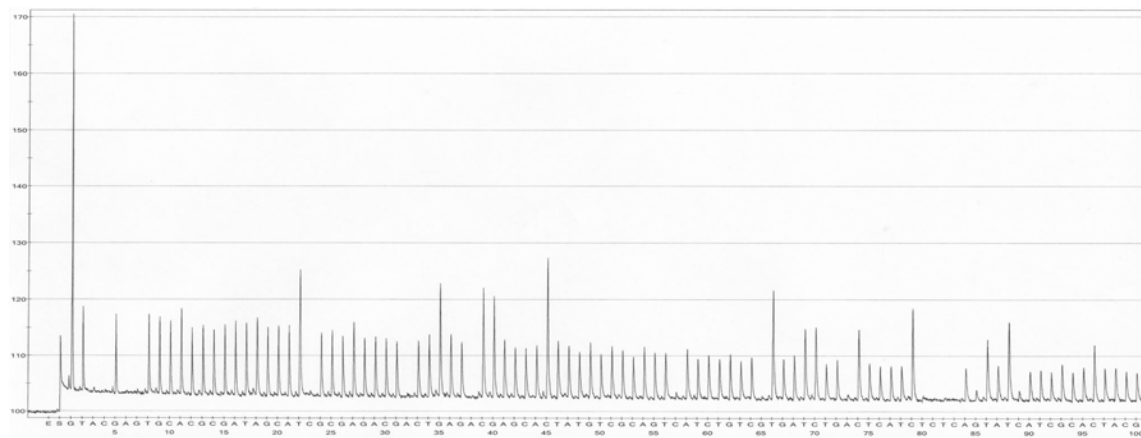


Figure 23 Pyrograms of J semen after 12 days of treatment 1. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to sunlight, heat, and humidity (treatment 1) for 12 days.

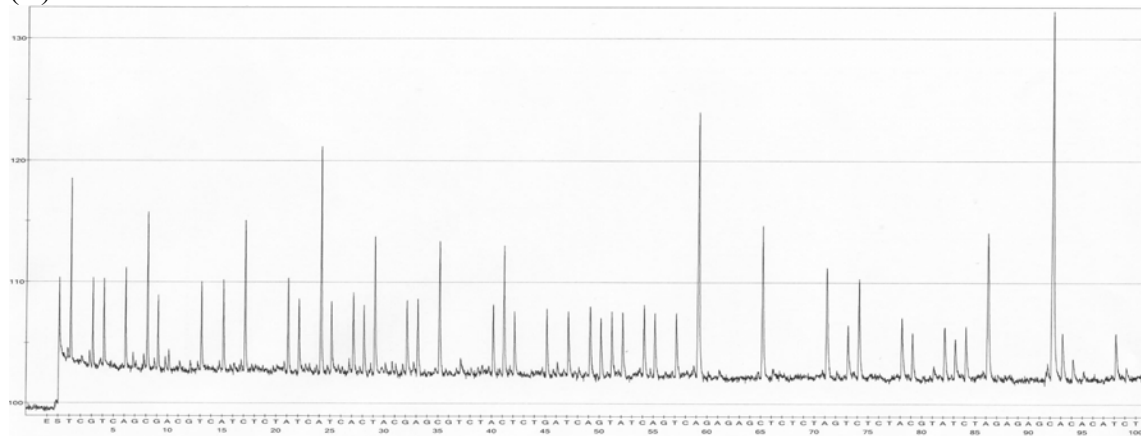
The pyrograms in Figure 24 is of DNA from a J semen sample that was exposed to sunlight, heat, humidity and rain for one day and sequenced using primers II45, I16015, and VRI16350 respectively. The pyrograms are consistent with that of the control sample of J, which was not exposed to any treatment. The pyrograms are of good quality, that is, they have a flat baseline with little background noise. Additionally the pyrograms generated with the other primers were of similar quality and read lengths. These samples were also comparable to the control with regards to quality and read length.

The pyrograms in Figure 25 is of DNA from a J semen sample that was exposed to sunlight, heat, humidity and rain for twelve days and sequenced using primers II45, I16105, and VRI16350 respectively. These pyrograms are noisier than those observed for the one day samples especially for the pyrogram generated with primer I16105. The read length was reduced though not as much for the VRI region. The samples collected at one month and after were not successfully amplified and therefore could not be sequenced. The yield gel of the samples suggested that there was sufficient DNA present to be amplified, however the DNA must have been extracted from the microbes seen growing on the samples.

(A)



(B)



(C)

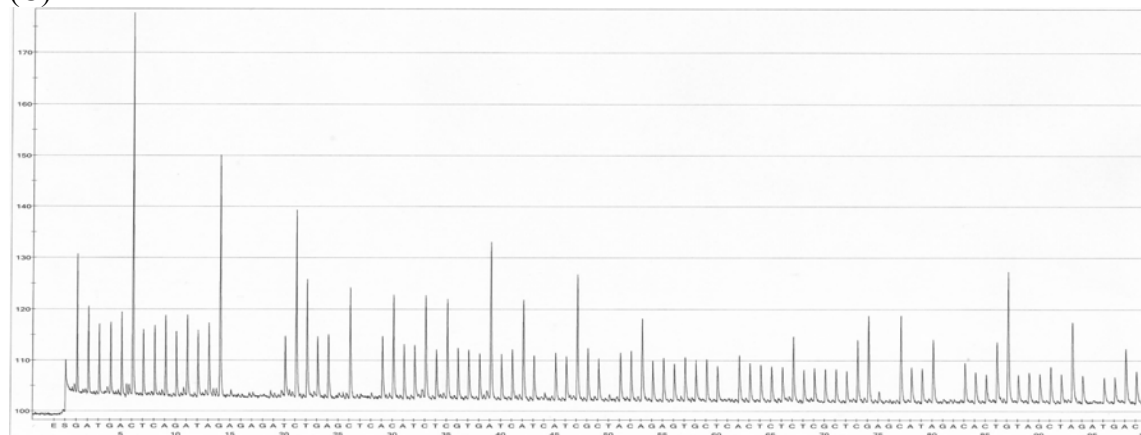
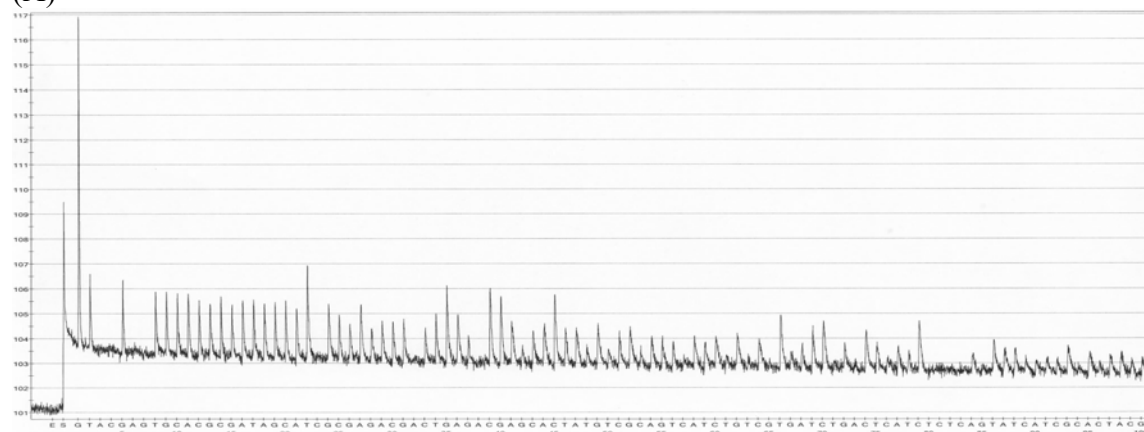
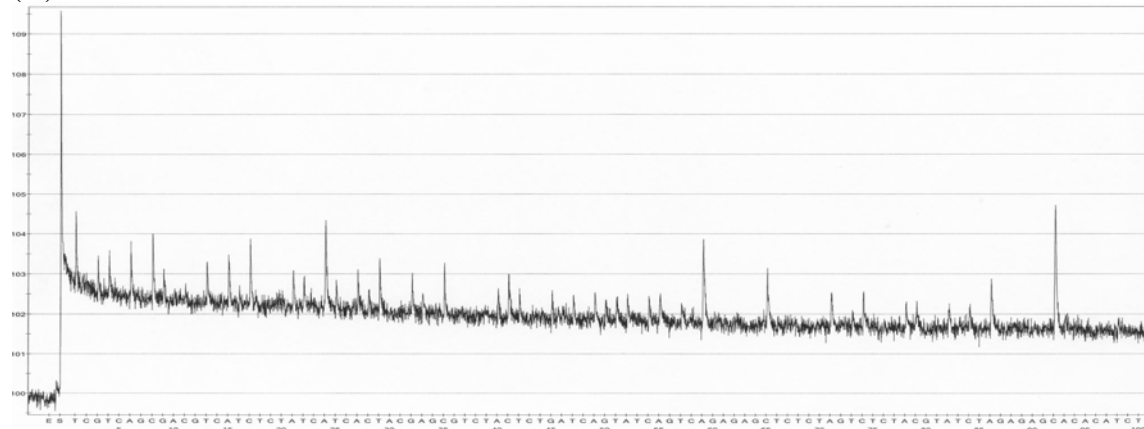


Figure 24 Pyrograms of J semen after 1 day of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 1 day.

(A)



(B)



(C)

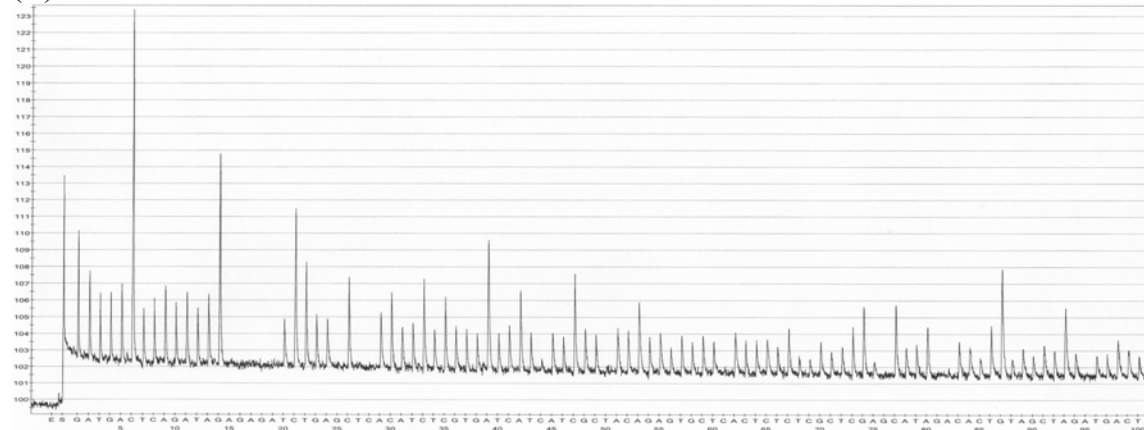
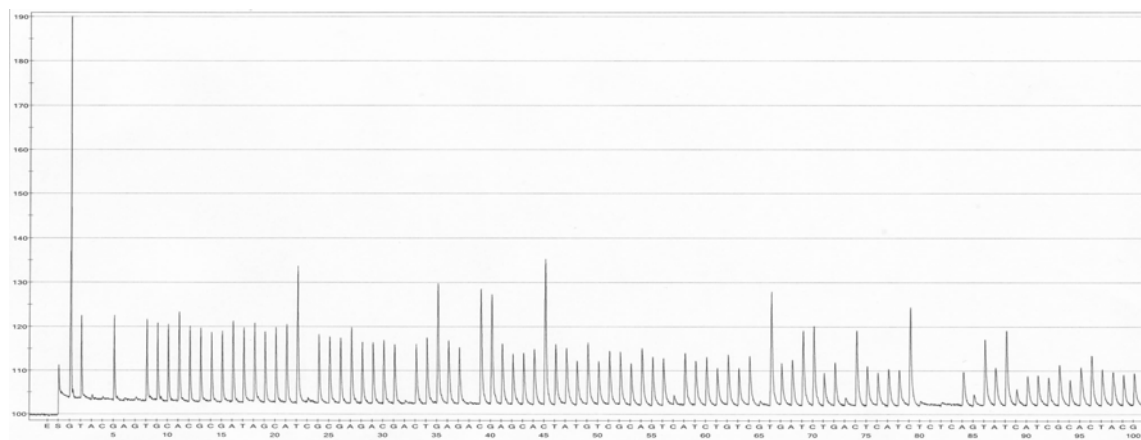


Figure 25 Pyrograms of J semen after 12 days of treatment 2. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to sunlight, heat, humidity, and rain (treatment 2) for 12 days.

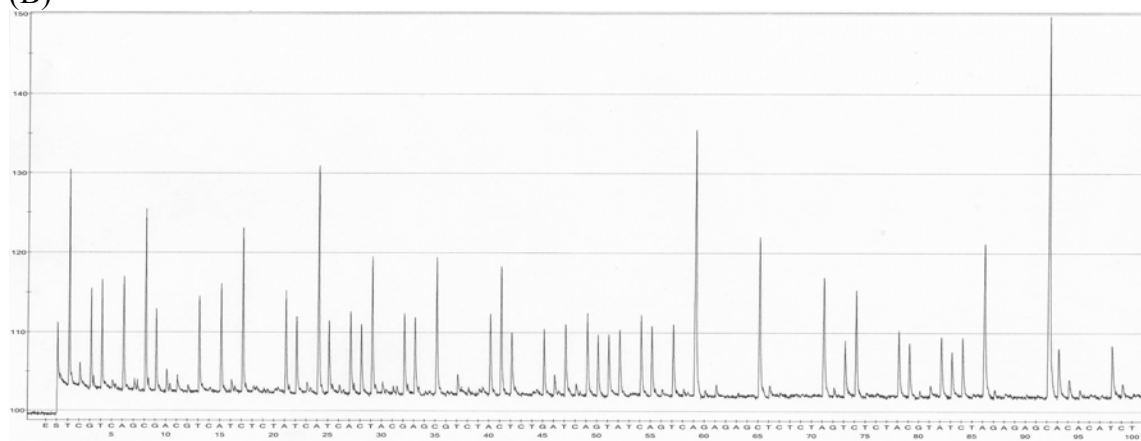
The pyrograms produced when DNA sample J semen was exposed to heat, humidity, and rain for one day and sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 26. These pyrograms are consistent with that of the control samples of J, which were not subjected to any treatment. The pyrograms have a flat baseline with very little background. Pyrograms generated with the other primers were similar quality. The sample had similar read length when compared to the control.

The pyrograms produced when DNA of sample J semen that was exposed to heat, humidity and rain for twelve days and sequenced with primers II45, I16105, and VRI16350 respectively are displayed in Figure 27. The pyrograms are noisy and with the exception of the VRI region had a baseline drift. The instrument was still able to make base calls but the read length was reduced when compared to the control sample. This shows that the DNA is being degraded. Additionally samples collected at one month and after did not amplify even though the yield gel suggested there was sufficient DNA in the sample. The DNA seen on the yield gel must have been that of the microbes growing on the sample.

(A)



(B)



(C)

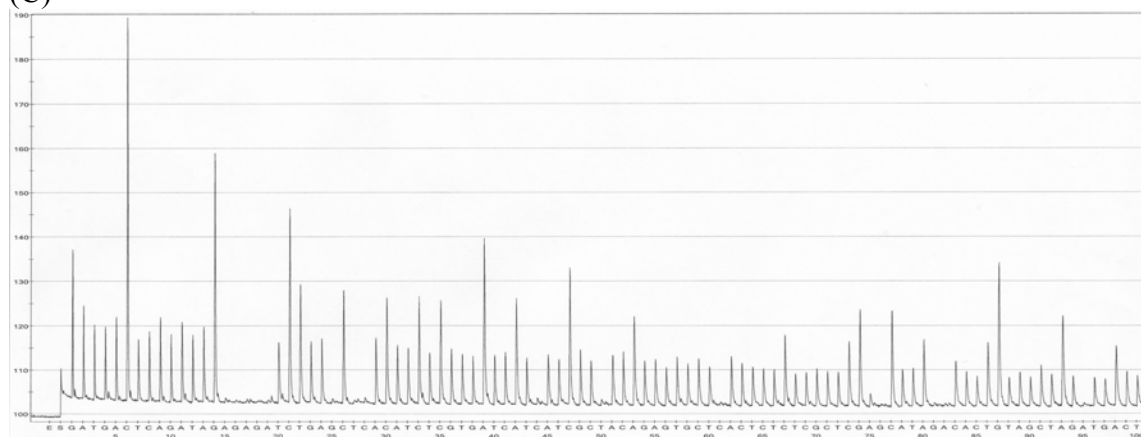
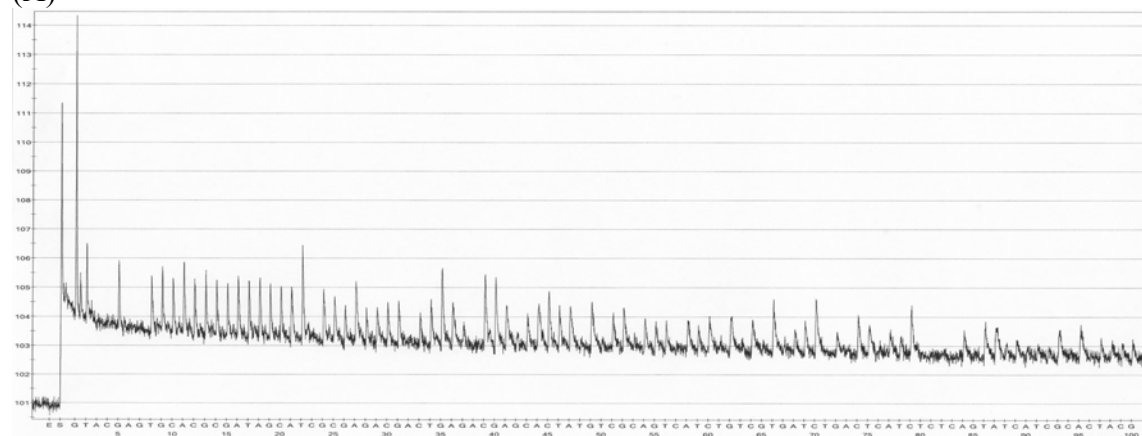
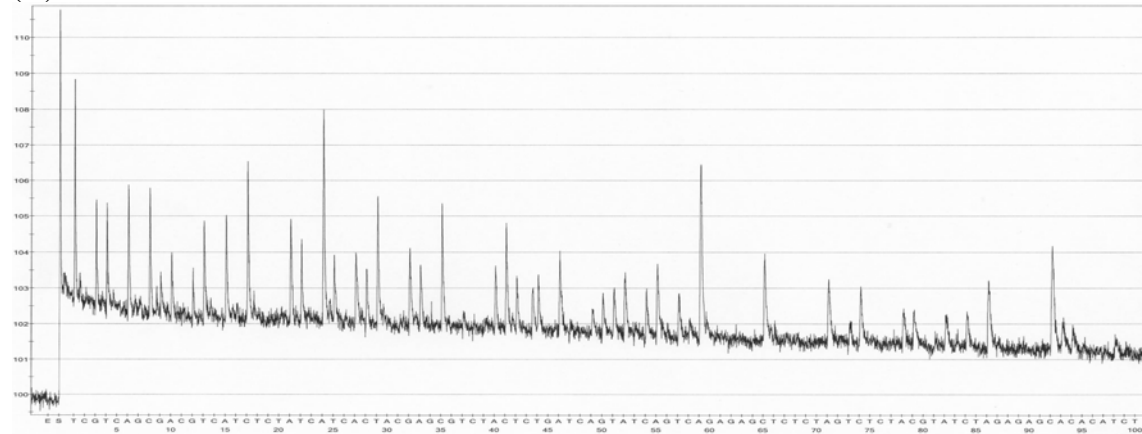


Figure 26 Pyrograms of J semen after 1 day of treatment 3. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to heat, humidity, and rain (treatment 3) for 1 day.

(A)



(B)



(C)

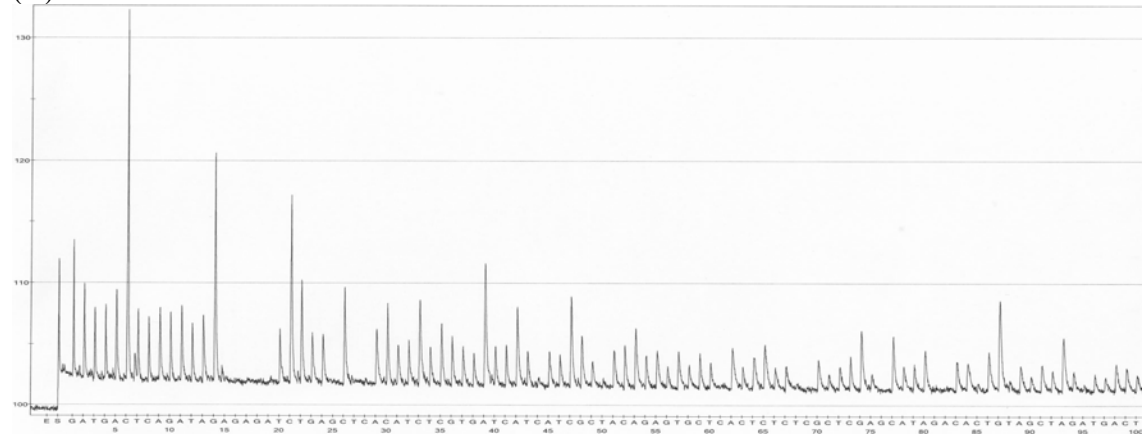


Figure 27 Pyrograms of J semen after 12 days of treatment 3. The pyrograms show parts of the HVII (A), HVI (B), and VRI region of DNA extracted from a J semen sample after being exposed to heat, humidity, and rain (treatment 3) for 12 days.

The results for the environmental study are summarized in Table 6. When saliva was subjected to the three different treatments the samples that could be analyzed for a longer period of time were samples subjected to treatment 1, three months as opposed to one week for treatment 2 and 3. This suggests that the major factor contributing to the degradation of the samples was rain. This could be due to the ‘washing out’ of the DNA by the rain or by the microbes that were able to grow and feed on the sample after the samples were soaked by the rain.

When blood was subjected to the three different treatments the samples that could be analyzed for the longest period of time were samples subjected to treatment 1, which was six months (when the study was ended), compared to one month for treatment 2 and two months for treatment 3. The results of this study again suggest that the major factor contributing to the degradation of the samples was rain. This study also shows that sunlight may also play a smaller role in the degradation of the samples after longer periods because samples that were subjected to treatment 2 degraded a month before samples subjected to treatment 3. The blood samples may have endured the treatments longer than saliva samples because there may have been more cells to start with or clotting may have held the cells present more tightly to the cotton substrate. Additionally, there were preservatives present in the tubes that were used to collect the blood, which may have aided in the preservation of the DNA.

Semen samples were the fastest to degrade out of the three samples tested. No sequence was obtained from the semen samples after twelve days. However if Figures 23, 25 and 27 are compared it can be seen that the pyrograms seen in Figure 23 (treatment 1) was of much better quality than that of pyrograms produced for samples subjected to treatments 2 and 3. The

pyrograms produced for samples subjected to treatment 1 show no indication of degradation while the pyrograms for samples subjected to treatments 2 and 3 have increased background and baseline drift. These signs of degradation are not as prominent when primer VRI16350 is used. One possible reason for the faster degradation of semen is the nature of the sample. Semen is a nutrient rich medium that may encourage microbial growth. Another reason that the semen samples did not fair as well as the other samples could be due to the collection/storage method. The semen was frozen before use; this could have resulted in cell lysis. Thus causing the DNA to lose the protection of the cell membrane allowing it to be more susceptible to the elements.

Table 6 The table shows the length of time that DNA could be recovered from the environmental samples.

Sample	Heat	Humidity	Sun	Rain	1d	2d	1w	1m	2m	3m	4m	5m	6m
Saliva	+	+	+	-	+	+	+	+	+	+	-	-	-
Saliva	+	+	+	+	+	+	+	-	-	-	-	-	-
Saliva	+	+	-	+	+	+	+	-	-	-	-	-	-
Blood	+	+	+	-	+	+	+	+	+	+	+	+	+
Blood	+	+	+	+	+	+	+	+	-	-	-	-	-
Blood	+	+	-	+	+	+	+	+	+	-	-	-	-
Semen	+	+	+	-	+	+	+	-	-	-	-	-	-
Semen	+	+	+	+	+	+	+	-	-	-	-	-	-
Semen	+	+	-	+	+	+	+	-	-	-	-	-	-

3.6 Lampshade

Three panels from a lampshade (obtained from a holocaust museum by Goldberger and Algera from the Maples Center at the University of Florida) were extracted, amplified, and

sequenced. The sequences obtained from the three panels of the lampshade were BLASTed and proven to be human in origin. These sequences were compared to the sequences of the researchers that came in contact with the samples to ensure that the DNA sequenced did not originate from the researchers. The sequences generated for panel 1 and panel 2 proved to be different from the mitotype of the researchers and different from each other. Not enough sequence data was observed for panel 3 to distinguish it from Glodberger or from the other panels. The mitotypes of the lampshade panels and the researchers that handled them are shown in Table.

Table 7. Mitotypes of lampshade samples and researchers

Region	Nucleotide	Sample1	Sample 2	Sample 3	Algera	Goldberger	Susan
HVII	73	G	G		A	A	G
HVII	146		T		T	T	T
HVII	150		C		C	C	T
HVII	195	T	T		T	T	C
HVII	198		C		C	C	T
HVI	16129	A	G		G	G	A
HVI	16213	G	G		G		A
VRI	16390	G	G	G	G	G	A
VRI	16482	A	A	A	G	A	A
VRI	16519	C/T	C	C	T	C	T

CHAPTER 4 DISCUSSION

Sequencing of the mitochondrial DNA control region for forensic comparison of samples is typically conducted when there is insufficient nuclear DNA, degraded DNA, telogen hairs, or when there is a lack of a reference sample from the subject but maternal relatives are available as seen in missing person cases (Calloway et al 2000; Hühne et al 1999; Higuchi et al 1998; Ivanov et al 1997; Wilson et al 1995; Holland et al 1993; Hopgood et al 1992). Due to the maternal inheritance and lack of recombination maternal relatives several generations away from the subject/evidence may serve as a reference sample, where nuclear DNA, with the exception of the nonrecombinant portion of the Y-chromosome, cannot serve as a reference (Budowle et al 2003). Also in mass disaster situations where small samples of tissues are recovered from many individuals, as seen in the September 11, 2001 attack, mitochondrial DNA can prove to be very helpful (Calloway et al 2000). Although other methods such as STR typing may be more discriminating than mitochondrial DNA, the use of mitochondrial DNA may be valuable because of the higher sensitivity attainable with mitochondrial DNA (Szibor et al 2000). Mitochondrial DNA has been used in casework such as: recovery of mitochondrial DNA from paper that was used to deliver a false suicide note - the DNA found on the note was not that of the victim, from the trigger, grip, hammer, magazine and cartridge of pistols, telephone and electrical cable used in strangulations and many other cases (Szibor et al 2000).

For mitochondrial DNA to become more widely used a large mitochondrial DNA database detailing the frequency of mutations needs to be established in order to determine the mitochondrial DNA profile's significance (Budowle et al 2003). Additionally, the rate of

nucleotide substitution in the control region should be determined (Tamura 2000). This would be helpful in cases where the reference maternal relative is generations away from the subject or much older or younger than the subject because the samples may be slightly different due to mutation. Studies have been conducted to determine if there is a higher occurrence of mutation and or heteroplasmy with age and if there is a difference in tissue types (Calloway et al 2000; Liu et al 1998; Melov et al 1995; Zhang et al 1993; Corral-Debrinski et al 1992; Cortopassi et al 1992; Hattori et al 1991; Cortopassi and Arnheim 1990). Two independent studies conducted by Calloway 1998 and Jazin et al 1996 found there to be a higher point mutation heteroplasmy in the control region with age while others (Pallotti et al 1996) have suggested that there is no correlation with mutation rate and age. More studies need to be conducted in this area before conclusions can be drawn.

Since occasional mutations and varying levels of heteroplasmy occur, there is a need to establish what would be considered an inclusion, inconclusive, or exclusion in the courtroom. Budowle et al 2003 describes an inclusion as the mitochondrial DNA sequences that are the same at the same locations. In the case of heteroplasmic samples, if the sequences are heteroplasmic at the same site, in addition to the sequence in the remaining portion of the DNA being the same, it is said that the sample is an inclusion. If one sample is heteroplasmic and the other homoplasmic and they have the same bases at the site of the heteroplasmy, the result is failure to exclude the samples as having the same origin because they share at least one mitochondrial species (Budowle et al 2003). If the samples are different at one site and there is no evidence of heteroplasmy the evidence is inconclusive because it could be a result of

mutation, however, if the samples differ by two or more bases the samples are excluded from being from the same source (Budowle et al 2003).

From the experiments conducted in this study it can be concluded that pyrosequencing is a fast and accurate method of sequencing mitochondrial DNA. Typically longer read lengths are obtained when directed dispensation is used as opposed to cycle dispensation though the accuracy is not affected. The pyrosequencer is capable of sequencing DNA from blood, semen, saliva, vaginal secretions, and epithelial cells. Minute quantities of mitochondrial DNA can be used to attain accurate sequences as little as 10 fg of total DNA or DNA obtained from a single fingerprint. The system is not easily overloaded because only DNA that binds to the streptavidin beads are in the reaction, unbound DNA which may otherwise interfere with the sequencing reaction sequence are washed away. Pyrosequencing is able to sequence damaged DNA though the read length may be shorter. The ability to detect heteroplasmy and mixtures varies depending on the primer used, the surrounding sequences, and the ratios of the different types of mitochondrial DNA. From the sequence results obtained in this study it can be concluded that the lampshade obtained from the Holocaust Museum was made from human skin that was obtained from at least two different individuals.

Currently a blind study is being conducted in which samples of unknown mitotypes supplied by the FBI were extracted, amplified, and sequenced. Upon completion the results will be sent to the FBI who will then determine the accuracy of the sequences obtained. Additionally, studies are underway to compare the ability of pyrosequencing to standard Sanger sequencing. In this study extractions of three buccal swabs (HRM, FS1 and AH) were extracted and diluted to 1 ng/ μ l, 100 pg/ μ l, 10 pg/ μ l, 1 pg/ μ l, 500 fg/ μ l, and 100 fg/ μ l. The extractions were then divided

equally. One set of the samples were sent to the FBI where they will undergo Sanger sequencing and the other half was retained and will be pyrosequenced. In addition the FBI extracted DNA from hairs of three individuals (DH, LO, and BK). The hair extracts were divided and one set was retained in the FBI laboratory to undergo Sanger sequencing and the other set was sent to undergo pyrosequencing. Comparisons will be made and hopefully conclusions can be drawn as to the advantages and disadvantages of pyrosequencing as compared to Sanger sequencing.

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