
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

ABSTRACT

The introduction of a new instrument into an accredited laboratory requires a documented internal validation. Validations typically include sensitivity, precision, and mixture studies. These tests assess the reliability and efficiency of the instrument and allow for interpretation guidelines to be established. This project consisted of a validation of Applied Biosystems' 3130*xl* Genetic Analyzer and an evaluation of three mitochondrial DNA amplification primer sets for the control region.

The validation was designed to evaluate the efficacy, robustness, and working limitations of the 3130*xl* instrument by performing a sensitivity study. A sensitivity study was performed using DNA sample dilutions, which were quantified using ABIs Quantifiler™ system to measure the amount of total nuclear DNA content in the samples. The samples were amplified on the GeneAmp® PCR System 9700 in triplicate to evaluate stochastic activity. Three different primer sets were utilized which allowed for the amplification of different regions of the human mitochondrial genome control region. After amplification, the quality and quantity of the DNA in all the samples was assessed using the Agilent 2100 BioAnalyzer, and subsequent sequence analysis was performed on the 3130*xl* Genetic Analyzer. Preliminary work was begun on a mixture study, but due to lack of time and reagents, this study was not completed and will have to be performed at a later date. All sequence data from the sensitivity study was evaluated using Sequencher™ version 4.1.4Fb19.

VALIDATION OF APPLIED BIOSYSTEMS 3130*xl* GENETIC ANALYZER FOR HUMAN mtDNA: AN EVALUATION OF THREE AMPLIFICATION SERIES

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INTERNSHIP PRACTICUM

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Jamalia Junelle David, B.S.

Fort Worth, Texas

August 2006

ACKNOWLEDGMENTS

I would like to express my deepest thanks and sincerest appreciation to Allison Wright, BS, Rhonda Roby, MPH, and Xavier Aranda, MS, for their continued support and assistance during this project; I could have not accomplished this feat without them. I would also like to extend my gratitude to John Planz, PhD and Arthur Eisenberg, PhD for their guidance throughout this project. Lastly, I would like to thank my major professor Joseph Warren, PhD, for his consistent support, guidance, and encouragement throughout this project.

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ABBREVIATIONS

A, Adenine

C, Cytosine

DNA, deoxyribonucleic acid

dNTP, deoxyribonucleoside triphosphate

F, forward

G, Guanine

Identifiler, AmpF*l*STR® Identifiler™ typing kit

mtDNA, mitochondrial DNA

ng, nanogram

PCR, polymerase chain reaction

QC, quality control

R, reverse

RFU, relative fluorescent unit

STR, short tandem repeat

T, Thymine

µL, microliter

CHAPTER I

INTRODUCTION

Upon receiving a forensic sample in the lab, the quality and quantity of the nuclear DNA template is unknown until after a portion of the DNA extract has been used for analysis. In cases where the sample is highly degraded and allele/locus drop-out is prevalent, mitochondrial DNA testing is attempted; however, this may not be feasible if the sample has been consumed. A validation of all new instruments and methodologies in the lab must be performed prior to incorporating forensic casework samples. Validations typically include a sensitivity study, a mixture study, and a reproducibility and precision study.

The DNA Advisory Board proposed guidelines and standards for all forensic DNA testing laboratories that were adopted by the director of the Federal Bureau of Investigation (FBI). These national standards range from performing proper evidence handling and documentation to the execution of an internal validation of all new methods, procedures, and instrumentation within the lab. Standard 8.1.3 states, "[An] internal validation shall be performed and documented by the laboratory" [1]. For proper use and optimal results, extensive tests should be employed to avoid any unwanted occurrences

and to elucidate the most advantageous conditions under which the instrument or technique should be operated.

Currently, the University of North Texas Health Science Center (UNTHSC) DNA Identity Laboratory utilizes modifications of the FBI protocol for mitochondrial amplification. The technical leader of the lab has been considering switching to other means of amplification but first wants to ensure that the quality of the sequences are the same, if not better, to current methodologies, and that the efficiency of the amplification is within acceptable limits. The goal of this internship project was to conduct a sensitivity and mixture study utilizing human mitochondrial DNA and evaluate the sequences on a new AB1 3130*xl* instrument. The purpose of these tests was to validate the 3130*xl* instrument and evaluate the efficiency of three amplification strategies.

CHAPTER II

BACKGROUND

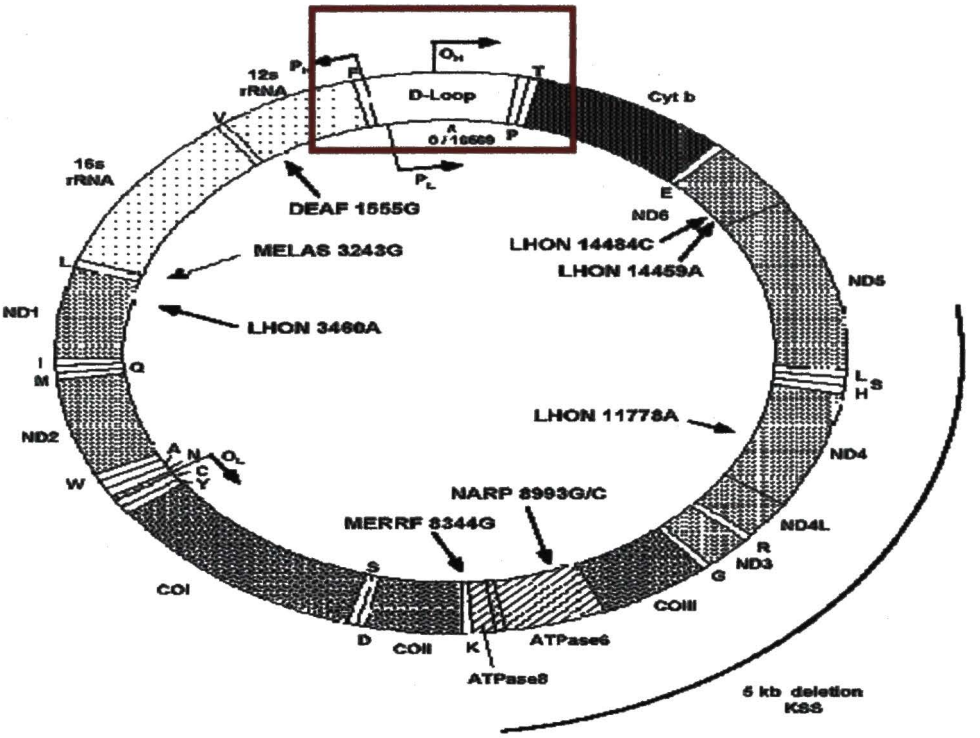
Biological samples typically encountered at crime scenes, such as blood, semen, hair, bones, and teeth, are subjected to STR analysis to identify the contributor to the stain. STR analysis is preferentially performed due to the high discriminating power of the autosomal markers. These autosomal markers are unlinked on their respective chromosomes resulting in the ability to multiply the frequencies of the alleles across all loci, thereby increasing the discriminating power of the results [2]. However in cases of degraded samples or low genomic DNA copy number, STR analysis can yield partial profiles or completely successful STR (short tandem repeat) typing. In such instances, analysis of mitochondrial DNA has been considered useful for identifying human remains [2, 3, 4].

Mitochondrial DNA

Mitochondria are organelles that supply energy for cells and contain an extrachromosomal genome separate from the nuclear genome. The mtDNA genome contains 16,569 base pairs (bp) of circular DNA and has been completely sequenced [3]. The areas of interest for forensic applications are in the non-coding segments of the mitochondrial control region (Figure 1). Two specific non-coding segments of DNA,

hypervariable region I (HVI) and hypervariable region 2 (HV2), have a high mutation rate and offers an abundant amount of information as to differences between individuals [5, 6].

Figure 1: Illustration of mitochondrial genome



http://www.mitomap.org/mitomapgenome.pdf

Compared to the nuclear genome, some regions of the mtDNA genome evolve at rates 5-10 times faster, making mtDNA highly polymorphic when compared to the nuclear genome [3]. The genome is maternally inherited, because mtDNA present in sperm deteriorates at or immediately after fertilization [6], and due to lack of recombination, all maternal relatives will have the same mtDNA sequence, barring

mutation. This is especially advantageous in cases of mass disasters where the only reference sample may come from a maternal relative.

A mitochondrion can contain anywhere from 2 to 10 copies of the mtDNA sequence and there may be thousands of mitochondria present in a single cell [3, 6]. This high copy number of mtDNA molecules allows for sequencing mtDNA from very limited samples. The double membrane structure of mitochondria acts as a barrier from harsh conditions and the circular structure of mtDNA protects from exonuclease activity, allowing for the analysis of mtDNA from highly degraded DNA samples [7]. Having the instrumentation and appropriate protocol for mitochondrial testing is beneficial to labs that routinely encounter low copy or highly degraded DNA.

Capillary Electrophoresis

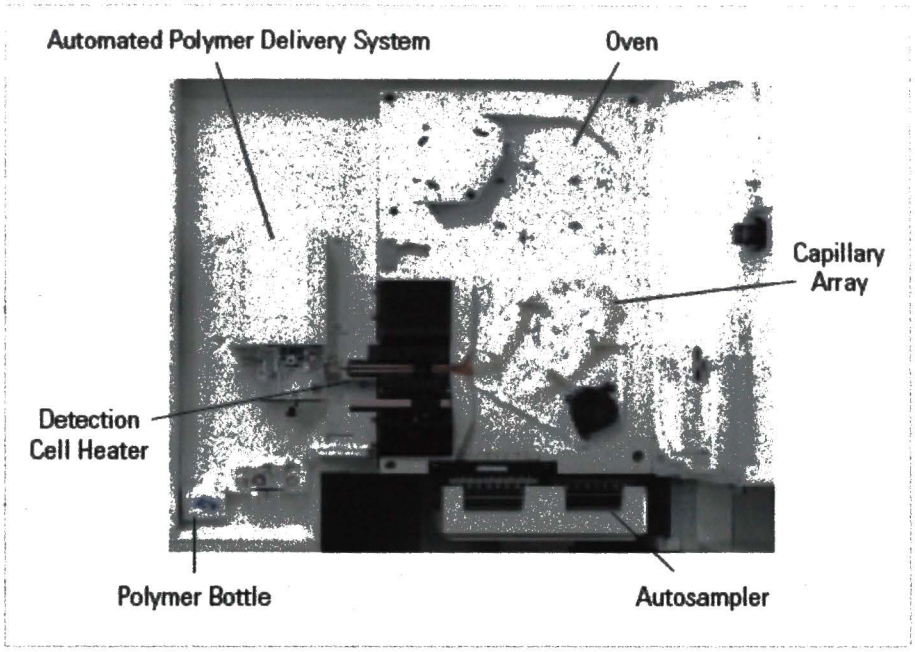
Capillary electrophoresis is the process of detecting and separating DNA fragments for STR analysis or sequencing analysis. The samples are drawn into the capillary via electro-kinetic injection and they move through a polymer that operates as a sieving medium. During the cycle sequencing step labeled bases fluoresce as it passes a laser detection window in the capillary instrument; these samples are detected by a charged coupled device and computer software generates raw data that is then analyzed by the analyst [8].

The evolution of ABI capillary instruments begins with single capillary systems such as Applied Biosystems 310 Genetic Analyzer. This instrument is excellent for growing labs with minimal throughput [9]. The drawbacks to this particular instrument

are the length of time to process several samples and the amount of analyst set-up and preparation of the instrument. With a one sample per 36 minute rate, 96 samples would take approximately 2 days to complete; this would make relieving any backlog very difficult. In addition, pump block cleaning and instrument set-up makes automation not as feasible as with other instruments. Large labs that demonstrate high throughput of samples are better equipped with a multicapillary instrument that contains a 16 or 96 capillary array [9]. Ninety-six (96) capillary array systems process hundreds of samples quickly and may be too high of a throughput instrument for even the busiest lab, so the 16 capillary array systems are more beneficial in terms of the amount of usage and money.

The 3130*xl* Genetic Analyzer (Figure 2) is a 16-capillary instrument that can be used to process STR and mitochondrial DNA samples. This semi-automated instrument can sequence mtDNA samples utilizing the 16 capillary array that operates in parallel. The 3130*xl* series has an automated polymer delivery system that eliminates polymer loading and clean up and automates sample injection, separation and detection, and data analysis [10]. The fluorescently-labeled PCR amplicons, created during the dRhodamine Dye Termination cycle sequencing reaction step, emit signals that range from 525nm to 680nm and are detected by a charged-coupled camera detection system [9].

Figure 2: Picture of 3130*xl* instrument



Applied Biosystems. System Profile: Applied Biosystems 3130 and 3130*xl* Genetic Analyzers

This instrument can process a 96 well plate in approximately 4.5 hours and allows for minimal analyst intervention.

Switching from a 3100 platform, which is currently used in the lab, to the 3130*xl* platform should not prove to be a large feat since both systems are very similar. The ABI 3130*xl* differs most from the ABI 3100 in polymer delivery. The ABI 3100 instrument requires manual loading of polymer into syringes while the ABI 3130*xl* has a mechanical pump block system that supplies the polymer directly from the bottle. Other minor differences that do not explicitly effect how the instrument performs are upgrades in the software package. The software contains several wizards that are user friendly and, if used, offer optimal instrument maintenance. If running duplicate plates, the system

allows the user to easily duplicate the plate template instead of re-typing 96 samples which can be very time-consuming. The similarity of the instruments and the improvements to software should make for a smooth transition from the ABI 3100 to the ABI 3130*xl*.

CHAPTER III

RESEARCH DESIGN AND MATERIALS

Sample Preparation

Pre-quantified, human genomic DNA extracts were obtained from Promega Corporation (female known DNA 9947A and male known DNA 9948), and were used as PCR samples for the sensitivity study (Madison, WI). Samples from a single source were pooled together to provide ample material. Pre-quantified DNA extracts obtained from Applied Biosystems (human genomic DNA standards from the Quantifiler™ kits) were used to generate the standard curves for the Quantifiler™ system (Foster City, CA). Forty (40) whole blood sample extracts were obtained from University of North Texas Health Science Center (UNTHSC) DNA Identity Laboratory and 21 buccal swabs were obtained from seven UNTHSC DNA Identity Laboratory volunteers (three swabs per person).

DNA extracts from volunteer samples were obtained using the UNTHSC DNA Identity Laboratory organic extraction protocol that consists of SDS and proteinase K digestion, phenol-chloroform extraction, and a Microcon 100 concentration (Millipore, Billerica, MA) clean up.

A dilution series was prepared using three DNA samples: the known DNA extracts from Promega Corporation (9947A and 9948) and the organically-extracted

buccal swabs from one volunteer (3A-3.2). The dilution series provided the following input DNA quantities for STR and mtDNA amplification: 10 ng, 2.0 ng, 1.0 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.062 ng, 0.032 ng, 0.015ng, and 0.007 ng. The dilution series was quantified in duplicate.

Out of the 40 whole blood sample extracts, four samples were analyzed in preparation for the mixture study. All of these samples have been previously sequenced and analyzed, so an examination of the differences between each sample allowed for the determination of the two best samples to mix.

Quantifiler™ qPCR Quantification

The Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) was used to quantify human DNA on an Applied Biosystems 7000 Prism® Sequence Detection System qPCR instrument and 7500 Real-Time PCR Sequence Detection System. The Quantifiler kits contain sufficient reagents for 400 reactions and the reaction volume is 25 µl: 23 µl of reaction mix and 2 µl of sample. Data was collected using the 7000 SDS Collection Software, V 1 and 7500 SDS Collection Software, V 1.2.

DNA Quantification using UV Spectroscopy

UV spectroscopy was performed using Spectronic BioMate 3 (Thermo Electron Corporation, Waltham, MA) for quantifying the DNA in the sensitivity study. A 1: 40 dilution was performed on all the samples except the blank and absorbance readings were

taken for 260 nm and 280 nm. Results were calculated using the conversion of 1 double-stranded DNA copy equals 50 μg/ mL.

Amplification of mtDNA

Sensitivity

mtDNA was amplified with three primer sets, in triplicate, using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA).

1. Modified FBI Laboratory Mitochondrial DNA Analysis Protocol, DNA Analysis Unit II, or the UNTHSC protocol
2. LINEAR ARRAY mtDNA HVI/HVII Region-Sequencing Typing Kit (Roche Applied Science, Indianapolis, IN), or the Roche protocol
3. Celera Genomics Large Mitochondrial DNA Amplicon for Amplification and Sequencing, or the Roby* protocol

*Rhonda Roby, Director of Forensic Program at Celera Genomics

The primers used in each respective mode of amplification cover different regions of the mitochondrial genome (Table 1). The UNTHSC amplification protocol is a modified version of the standard FBI mitochondrial sequencing protocol where HV1 and HV2 are amplified separately. The Roche Applied Sciences amplification methodology amplifies both HV1 and HV2 regions in a duplex amplification, while the Roby technique amplifies the entire control region capturing HV1 and HV2 in the process.

Table 1: Position of the primers and the sequences used in the amplification strategies.

	Primer	Sequence
UNTHSC	F15978	5' CAC CAT TAG CAC CCA AAG CT 3'
	R16410	5' GAG GAT GGT GGT CAA GGG AC 3'
	F29	5' CTC ACG GGA GCT CTC CAT GC 3'
	R429	5' CTG TTA AAA GTG CAT ACC GCC A 3'
Roche	F15975	5' CTC CAC CAT TAG CAC CCA A 3'
	R16418	5' ATT TCA CGG AGG ATG GTG 3'
	F15	5' CAC CCT ATT AAC CAC TCA CG 3'
	R429	5' CTG TTA AAA GTG CAT ACC GC
Roby	F15910	5' CAC CAG TCT TGT AAA CCG GAG A 3'
	R564	5' CTT TGG GGT TTG GTT GGT TC 3'

The UNTHSC amplification protocol called for the addition of 10 µL of DNA. The positive control samples required 2 µL of HL60 and 8 µL of water, and the negative control required 10 µL of sterile water. Twenty-five µL of PCR product for both HV1 and HV2 samples were produced with this protocol. The input quantity of DNA was: 0.5 ng, 0.25 ng, 0.125ng, 0.062ng, 0.031ng, 0.015ng, and 0.007ng.

The PCR cycling parameters were:

HOLD:	95°C / 11 minutes	
CYCLE:	95°C / 10 seconds 61°C / 30 seconds 72°C / 30 seconds	For 36 cycles
HOLD	70°C / 10 minutes	
HOLD	4°C / Forever	

The Roche amplification protocol suggested up to 20 µL of DNA with the remaining volume being sterile water, but for purposes of this project, 20 µL of DNA extract was added to the reaction mix with no sterile water. For the positive control,

20 µL of HL60 was added, and for the negative control, 20 µL of sterile water was added to the reaction mix. Because 20 µL of DNA was added to the reaction, the input quantities of DNA were double when compared to the UNTHSC protocol. The values were as follows: 1 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.062 ng, 0.031 ng, and 0.015 ng. Fifty µL of PCR product was produced with this protocol.

The PCR cycling parameters were as follows:

HOLD	94°C / 14 minutes	
CYCLE	92°C / 15 seconds	
	59°C / 30 seconds	For 36 cycles
	72°C / 30 seconds	
HOLD	72°C / 10 minutes	
HOLD	4°C / Forever	

The Roby amplification protocol contained the most variability between the three amplification methodologies; 5 µL of DNA was added to 5 µL of master mix. For the positive and negative controls, 5 µL of HL60 and sterile water, respectively, were added. Because 5 µL of DNA was added to the reaction, the quantity of input DNA was halved when compared to the input quantity of DNA for the UNTHSC protocol. The values were as follows: 0.25 ng, 0.125 ng, 0.062 ng, 0.031 ng, 0.015 ng, 0.007 ng, and 0.0035 ng. A total of 10 µL of PCR product was obtained with this reaction.

The PCR cycling parameters were:

HOLD	96°C / 5 minutes	
CYCLE	95°C / 10 seconds	
	60°C / 45 seconds	For 36 cycles
	72°C / 1 minute	
HOLD	15°C / 10 minutes	

HOLD 4°C / Forever

Because the approach to amplifying the control region of the human mitochondrial genome was different between the three primer sets, it was important to assess the quality and efficiency of the amplifications by analyzing the results from the sequencing electropherograms (results presented in the Electrophoresis and Analysis section of Chapter 4). The specific amplification protocols, per reaction, are compared against each other in Appendix A.

The mixture study amplifications were not completed due to lack of time; however, all the samples were labeled and stored so that further analysis can be completed at a later date.

Post Amplification Quantification

Select samples from each replicate and primer set were analyzed using the Agilent 2100 BioAnaylzer (Agilent Technologies, Palo Alto, CA) to assess the quality and quantity of the PCR amplicons. One (1) μL of amplified product was added to the Agilent chips.

ExoSAP-IT®

To remove unconsumed primers and dNTPs in the PCR product, ExoSAP-IT® (USB Corporation, Cleveland, OH) was added to each sample and incubated in the GeneAmp® PCR System 9700 for 15 minutes at 37°C and at 80°C for 15 minutes to inactivate the enzymes.

Currently, the UNTHSC DNA Identity Laboratory uses 5 µL of the enzyme to the 25 µL of PCR product for a final reaction volume of 30 µL. It was important to perform all tasks just as the forensic analysts would on a daily basis. This consistency allows for an easier transition into new instrumentation and methodology. For the Roche samples, since the PCR volume was 50 µL, the amount of enzyme was doubled to account for the larger volume; 10 µL of ExoSAP-IT® was added for a total reaction volume of 60 µL. The Roby amplification protocol yielded 10 µL of PCR product; so 3 µL of enzyme was added. The samples were placed in the GeneAmp® PCR System 9700 for 15 minutes at 37° C and for 15 minutes at 80° C concluding with a 4° C hold.

The Roche amplification yielding 50 µL of PCR product, poses a slight concern when performing ExoSAP-IT® downstream. The addition of 10 µL of ExoSAP-IT® cocktail makes for a total volume of 60 µL of reaction mix. The highest quantity allowed to be input in the set-up stage of the GeneAmp 9700 is 50 µL, so one has to wonder if the higher volume of the reaction mix affects the thorough heating and cooling of the sample. The instrument ramps for a longer period of time when the volume of sample is higher, so if the instrument only accommodates 50 µL of sample, but in actuality the total volume is 60 µL, would this difference have an affect on the efficiency of the enzyme cocktail? This question was not examined in this project, but it is a valid concern.

Cycle Sequencing and Column Clean-Up

Sample preparation for electrophoresis included a cycle sequencing reaction step using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit. Subsequent to

cycle sequencing, all samples were filtered using the Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD). All samples were denatured at 95°C for four minutes and snap-cooled in an ice bath for four minutes prior to loading on the instrument.

Electrophoresis and Analysis Using ABI 3130*xl*

Conditions for the electrophoretic run on the ABI 3130*xl* were as follows: Usage of the 36cm uncoated 16 capillary array, separation medium POP™ 6, a 10 second electro-kinetic injection at 1.5 kV, oven temperature set at 55°C, and data collection in the Sequencing Analysis V 5 software (Applied Biosystems). All analysis was performed with Sequencher™ program version 4.1.4Fb19 (GeneCodes, Ann Arbor, MI).

Mixture Studies

All initial mixture studies were performed using the pre-sequenced database samples from the lab where the mtDNA profiles were already known. Four samples were considered: 92H, 175H, 176H, and 179H. Determination of the final two samples to mix was decided upon examining the differences between each sample. The goal of the mixture study was to acquire a wide range of polymorphisms in the HV1 and HV2 regions when the samples were mixed. Sample 92H and 175H were chosen to be mixed together for further mixture studies (See Table 2).

Table 2: Polymorphisms observed in samples 92H, 175H, 176H, and 179H for HV1 and HV2 regions.

		Sample			
Position	**Sequence***	**92H**	**175H**	**176H**	**179H**
HV1					
16092	**T**	C	--	--	--
16111	**C**	T	T	--	--
16183	**A**	C	--	C	--
16189	**T**	C	--	C	--
16193.1	**--**	N	--	N	--
16217	**T**	C	--	C	--
16223	**C**	--	T	--	T
16278	**C**	--	--	T	--
16290	**C**	--	T	--	--
16298	**T**	--	--	--	C
16311	**T**	--	--	C	C
16319	**G**	--	A	--	--
16325	**T**	C	--	--	C
16327	**C**	--	--	--	T
16362	**T**	--	C	--	--
HV2					
73	**A**	G	G	G	G
146	**T**	--	C	--	--
153	**A**	--	G	--	--
228	**G**	A	--	--	--
234	**A**	G	--	--	--
235	**A**	--	G	--	--
263	**A**	G	G	G	G
309.1	**--**	C ins.	C ins.	--	--
315.1	**--**	C ins.	C ins.	C ins.	--
333	**T**	--	--	--	C
337	**A**	--	--	T	--

*As compared to the revised Cambridge reference sequence (Andrews et. al. Nature Genetics 1999)

CHAPTER IV

RESULTS AND DISCUSSION

Sample Preparation

Buccal swabs were taken from all participating volunteers and extracted organically (phenol-chloroform). This proved to be an effective method for the extraction of total DNA from the buccal swab samples. Difficulties, however, were observed when the samples were quantified using UV spectroscopy. Samples 9947A, 9948, 1A-1.1, 1A-1.2, 3A-3.1, 3A-3.2, and the reagent blank were all evaluated using the UV spectrometer. Extremely high levels of DNA were detected in many of the samples where the value expected was zero, for the reagent blank, or ten times less than what was observed. This was attributed to poor removal of phenol during the extraction phase. The reagent blank was the last sample measured, and upon making the appropriate calculations, a reading of 1432 ng per one μL was obtained. The presence of contamination in the reagent blank prompted an immediate amplification of the samples using the Identifiler® kit and a 3100 Genetic Analyzer run to ensure the DNA samples were clean. The DNA samples were clean so a decision was made to move forward but to eliminate the reagent blank from further testing.

Real-Time Quantification

Real-time quantification of the known DNA samples from Promega Corporation yielded results that were approximately two-fold greater than expected. It was noted that Quantifiler kits, with specific lot numbers, contained DNA standards that were off two-fold. It was suggested that a calculated adjustment be made for all values before performing the serial dilution or account for the discrepancy in downstream analysis (for STR studies) by doubling the amount of input PCR product. Because the quantification of samples was based on nuclear DNA and not mitochondrial DNA copies, it was presumed that the skewed results would not affect the mitochondrial DNA sensitivity study as it would for STR sensitivity studies. All values were taken at face value and no adjustments were made.

Organic DNA extraction yielded sufficient quantities of DNA for each sample and the quality of extracted DNA was deemed suitable for further analysis. Sample 3A-3.2 was selected as the third sample because the STR and mitochondrial profile was already on file, and the extraction produced ample DNA. The final three samples utilized for all subsequent analysis were 9947A, 9948, and 3A-3.2. Serial dilutions were performed based on the Quantifiler results with values of: 10ng, 2.0ng, 1.0ng, 0.5ng, 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.015ng, and 0.007ng input DNA into all reactions. The dilutions were quantified twice to ensure the same trends were being observed. The lower values of the serial dilution failed to be detected using Quantifiler but this was expected since they fell outside the range of the standard curve.

PCR Amplification

Only the values ranging from 0.5ng to .007ng were utilized for mitochondrial amplification. Every sample (9947A, 9948, and 3A-3.2) dilution series was run with a positive control and a negative control and they were amplified in triplicate (refer to Table 3 for set-up) using the PCR amplification tube strips.

Table 3: Organization of the PCR amplification samples

9947A-0.5ng	**9947A**-(-) Control	**9948**-(+) Control	**3A-3.2**-0.007ng
9947A-0.25ng	**9948**-0.5ng	**9948**-(-) Control	**3A-3.2**-(+) Control
9947A-0.125ng	**9948**-0.25ng	**3A-3.2**-0.5ng	**3A-3.2**-(-) Control
9947A-0.062ng	**9948**-0.125ng	**3A-3.2**-0.25ng	
9947A-0.031ng	**9948**-0.062ng	**3A-3.2**-0.125ng	
9947A-0.015ng	**9948**-0.031ng	**3A-3.2**-0.062ng	
9947A-0.007ng	**9948**-0.015ng	**3A-3.2**-0.031ng	
9947A-(+) Control	**9948**-0.007ng	**3A-3.2**-0.015ng	

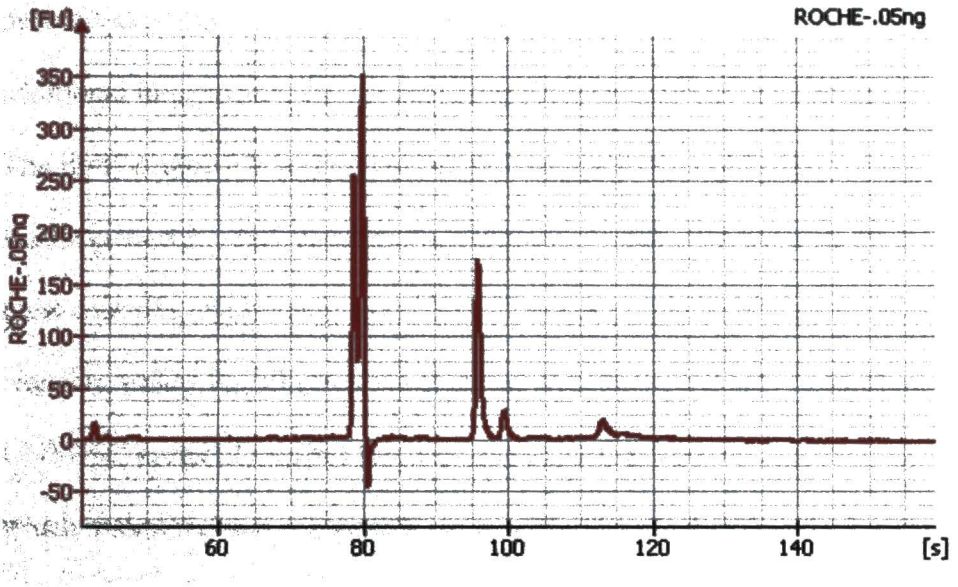
The three primer sets used in the sensitivity study were labeled alphabetically with the UNTHSC DNA Identity Laboratory designated A through D; the Roche Applied Science technology designated E through H, and the Roby primers designated letters I through L. Each sample set was also labeled with the appropriate replication number, 1, 2 or 3. All the amplifications were successful as demonstrated by the Agilent 2100 results.

Agilent 2100 BioAnalyzer

Upon completing all the amplifications a total of 324 samples needed to be processed. The UNTHSC protocol contained 162 total samples, 81 for the HV1 region and 81 for the HV2 region, and both the Roche and Roby amplifications produced 81 samples. To run 324 samples on the Agilent would have been extremely costly in terms of time and money so a decision was made to only evaluate the quantity and quality of DNA in a selection of the samples. Nine samples, from each amplification, were analyzed on the Agilent for a total of 36 samples. Referring back to Table 3, all the 9947A samples (0.5ng through the negative control) were processed.

In visualizing the gels, the 36 samples processed produced expected results; the UNTHSC HV1 and HV2 samples showed one band in the gels indicating that the amplification of that specific region was successful. The Roche samples produced two bands demonstrating that both regions were detected and amplified in the duplex reaction. The Roby samples produced a single band which suggests the large single amplicon reaction performed efficiently (Appendix B). In several of the electropherograms several peaks were observed indicating that the sample contained a polycytosine stretch (Figure 3).

Figure 3: Agilent 2100 BioAnalyzer electropherogram of Roche sample 9947A 0.5ng.



Note: Sample label value on electropherogram is [DNA]/1 μL.

Cycle Sequencing and Column Clean-up

All 324 amplified samples could not be electrophoresed on the 3130*xl* due to lack of reagents. Ultimately, all the UNTHSC samples were processed, along with the entire first replicate for the Roche and Roby samples, for a total of 216 samples.

Cycle sequencing was performed using the primers presently used in the DNA Identity Laboratory. For the HV1 region, the forward primer used was A1 (F15978) and the reverse primer was B1 (R16410). For the HV2 region, the forward primer utilized was C1 (F29) and the reverse primer used was D1 (R429). Some concern was voiced that using these primers versus the specific primers from the Roby amplification (RF1-F15910 and RR2-R564) was to the detriment of the Roby amplification protocol. Being a large mitochondrial DNA amplicon, there is valuable information that can be detected

that the current primers will not detect due to where they are positioned. If it could be demonstrated that the RF1 and RR2 primers produced quality sequence while offering additional information, the lab could benefit greatly in terms of information and money. The Roby protocol is less expensive than the current amplification protocol and than the Roche protocol, which requires the purchase of a kit. Per reaction, the Roby protocol is most cost efficient because more than 50 reactions can be performed with all the reagents, unlike the 50 reactions obtained with the Roche kit. In addition, since the UNTHSC protocol requires two separate amplifications, double the reagents are used which makes the cost of reagents per amplification higher than the Roby protocol (Appendix C).

While it has been demonstrated that BigDye™ version 1.1 may produce more consistent, clean results in terms of balanced peak heights and minimal baseline noise [11], ABI Prisim® dRhodamine is very effective, and in this project produced promising results (data shown in following section).

Electrophoresis and Analysis

Overall, the sequences were of good quality and there was no contamination which was a concern due to the sensitivity of mitochondrial testing. Quality of the sequences was assessed by observing the number of ambiguous bases, or N's due to bases which could not be designated (Figure 4A); observing the number of spacing ambiguous bases, or N's due to spacing issues (Figure 4B), and lastly errors, which were

the bases that were called incorrectly. In all cases, once the sequences were edited, the correct polymorphisms were detected, so no errors were observed.

Figure 4A: Ambiguous bases- N's due to bases that could not be designated

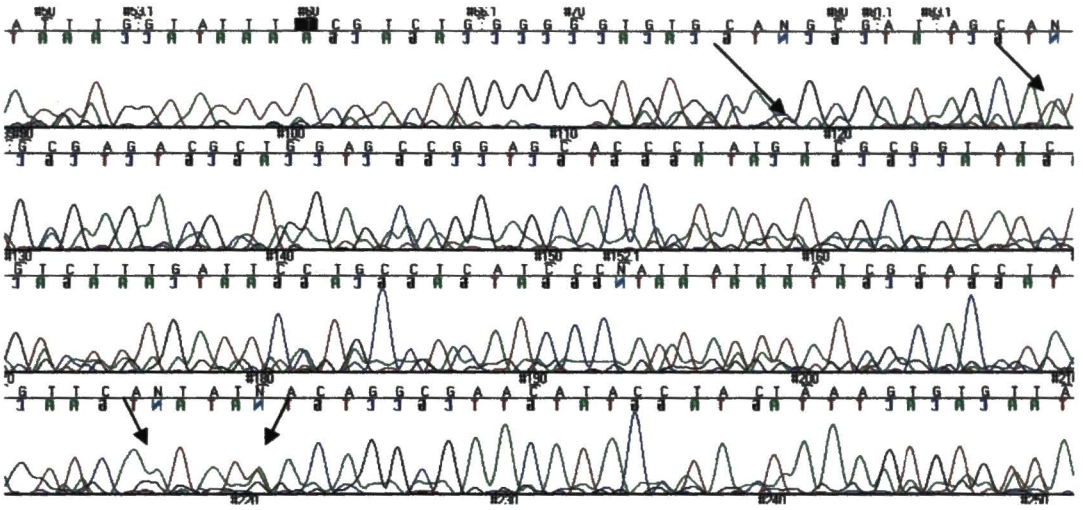
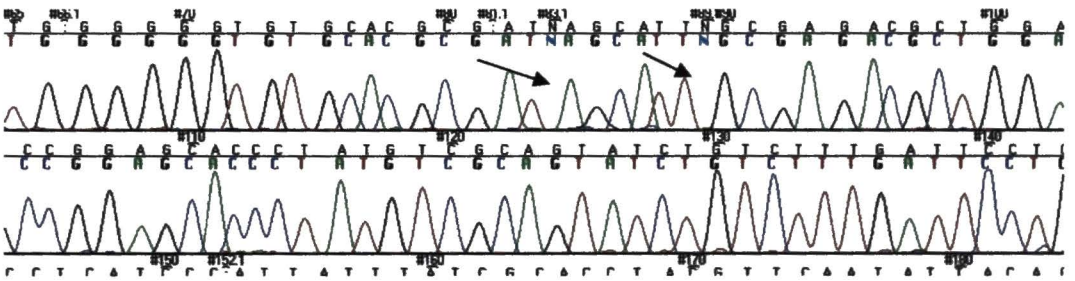


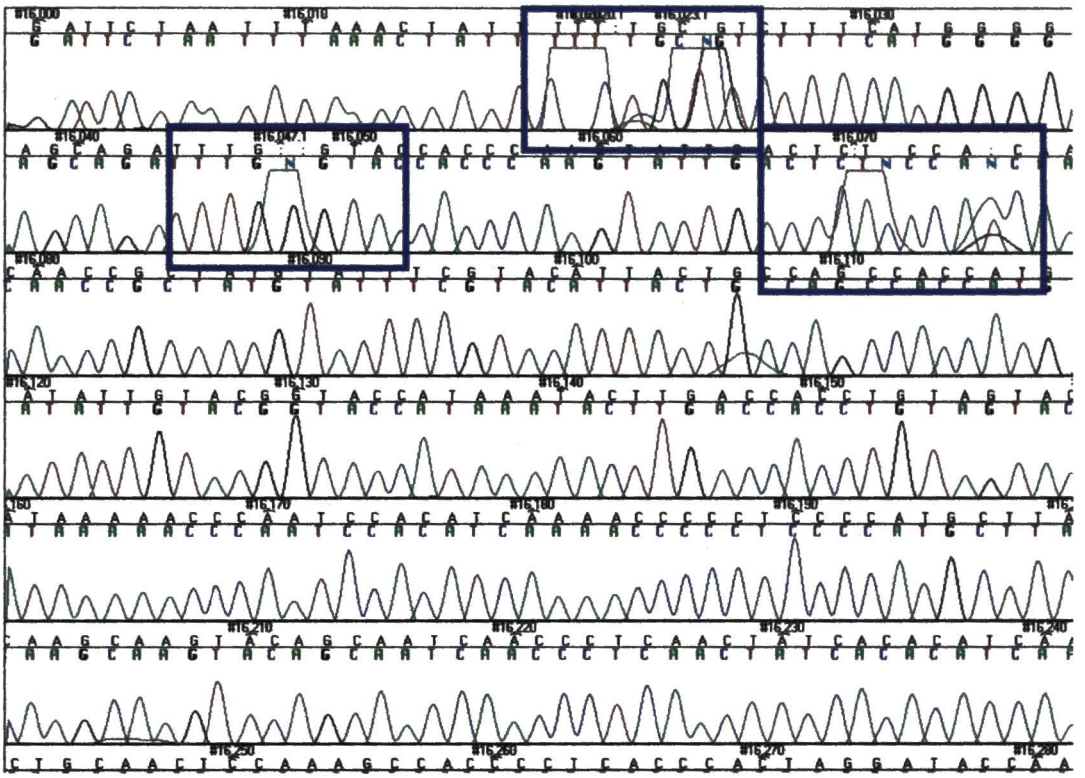
Figure 4B: Spacing ambiguous bases- N's due to spacing issues



The only anomaly observed was in the first run. What was thought to be spikes were detected in the forward direction of HV1 sample 9947A (Figure 5). However, the presence of these irregular peaks in subsequent samples, in the exact same position (data

not shown) indicates that there was poor clean up of the sample causing dye blobs. This phenomenon was not detected in later runs.

Figure 5: Electropherogram of sample 9947A using the UNTHSC protocol (0.5ng HV1F)



Once all the samples had been processed on the instrument, and the appropriate contigs formed, it was important to assess the quality of the sequences between each amplification technique. Currently, the protocol utilized by the DNA identity laboratory is the modified FBI procedure, where two amplifications are performed, one for the HV1 region and one for the HV2 region. This is very laborious and time consuming for the analysts [12], so the possibility of switching over to a quality single amplification

protocol is highly favored by those working in the lab. Trimming all sequences to match that of the Cambridge Reference, sequences from the 0.25ng, 0.125ng, and 0.031ng dilutions were placed in a project for each primer set in the forward and reverse direction. Sample 9947A in the HV1 region for quantities 0.25ng and 0.031ng looked comparable between all three primer sets (Figure 6 and 7).

Figure 6: Three primer set comparison for sample 9947A (0.25ng HV1)

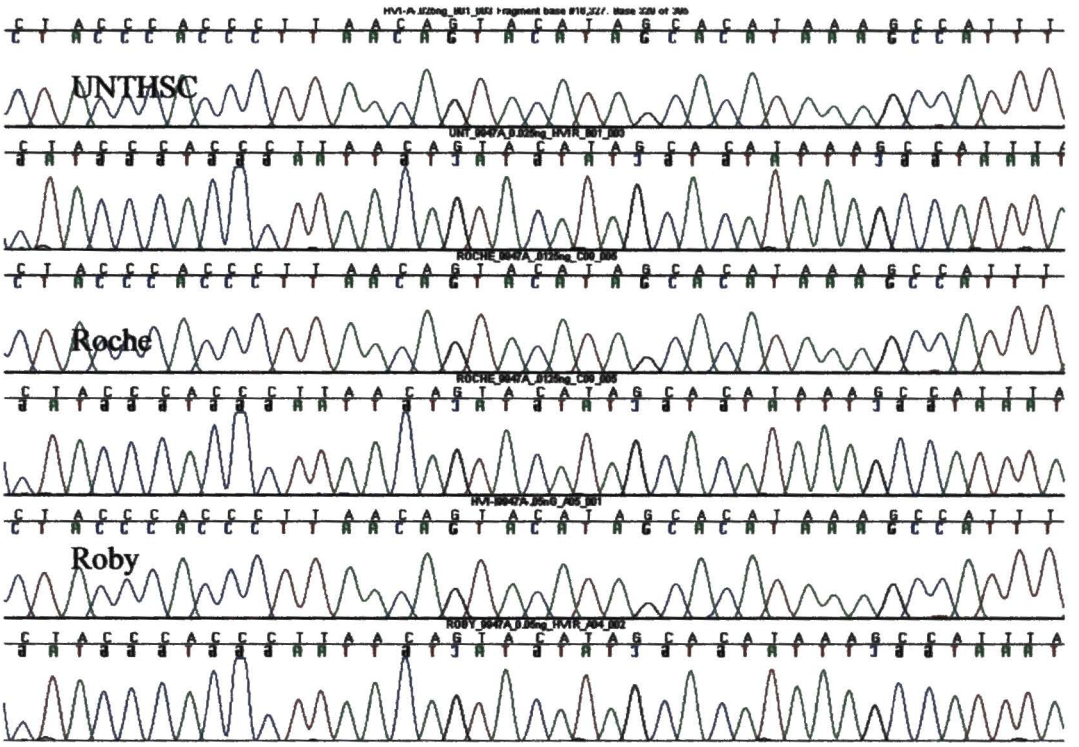


Figure 6 represents the sequences for the UNTHSC amplification in the forward and reverse direction, the Roche amplification in the forward and reverse direction, and the sequences for the Roby amplification in the forward and reverse direction.

Figure 7: Three primer set comparison for sample 9947A (0.031ng HV1)

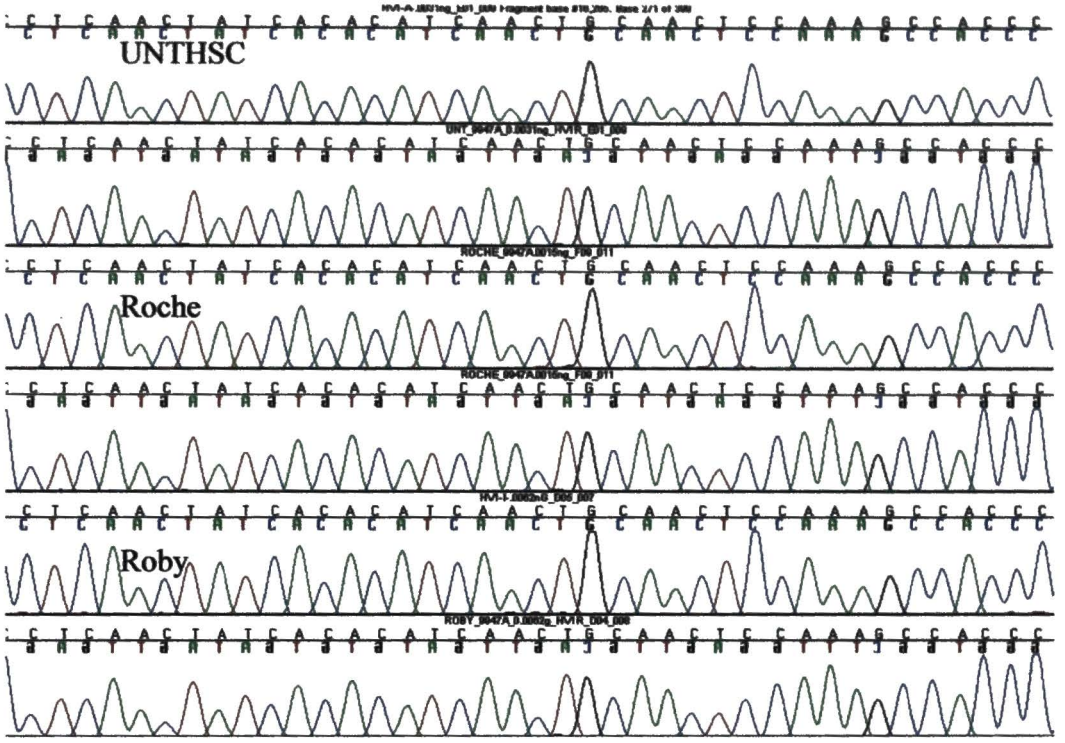


Figure 7 represents the sequences for the UNTHSC amplification in the forward and reverse direction, the Roche amplification in the forward and reverse direction, and for the Roby amplification in the forward and reverse direction.

Both the relative upper and lower ends of the dilution series, 0.25ng and 0.031ng, respectively, were analogous between the three primer sets (See Figures 6 and 7). The resolution of the sequences was of good quality and there were not any major discrepancies that would cause for apprehension in using either of the 3 primer sets. The other 2 samples, 9948 and 3A-3.2, produced similar results for the HV1 region (data not shown).

The sequences in the HV2 amplifications (Figures 9 and 10) were not of the same quality as that observed in the HV1 sequences; many ambiguous base calls were detected

and the resolution of the sequences was poor in some cases. Length heteroplasmy (Figure 8) was observed between nucleotide positions (nt) 303 and 315 for all three samples (9947A, 9948, and 3A-3.2) causing the downstream sequence to loose resolution. Length heteroplasmy occurs when more than one mtDNA type exists with in an individual and is represented by a stretch of cytosines [13]. In many cases, the presence of heteroplasmy requires additional amplification using a subset of primers that are positioned with in the polycytosine stretch so that the sequence can be analyzed.

Figure 8: Length Heteroplasmy at nt 303

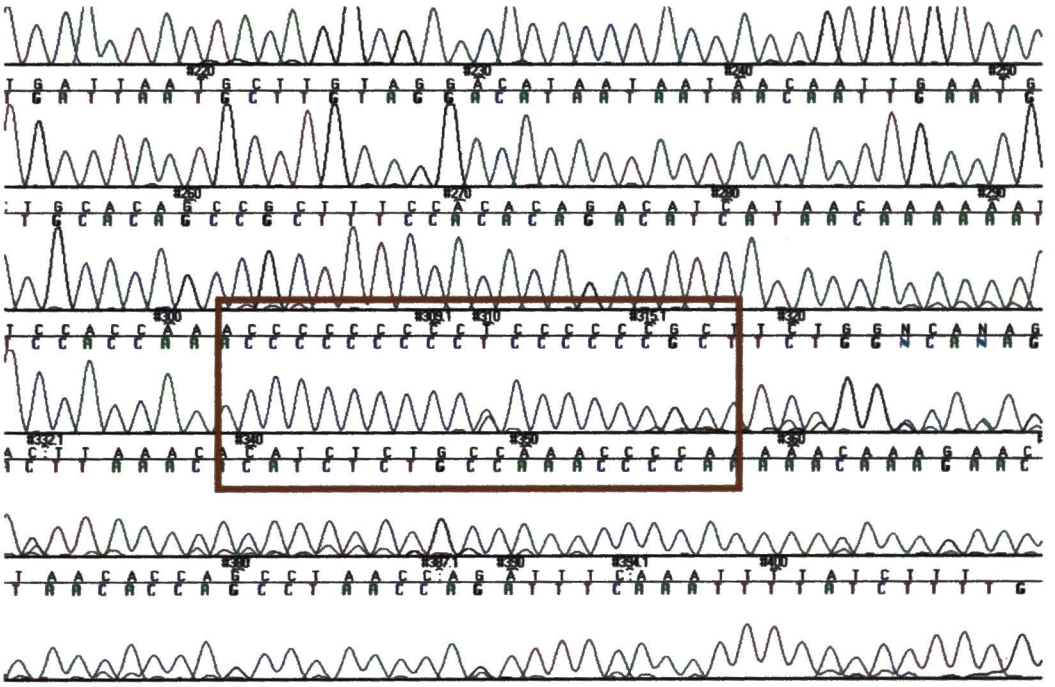


Figure 9: Three primer set comparison for sample 9947A (0.25ng HV2)

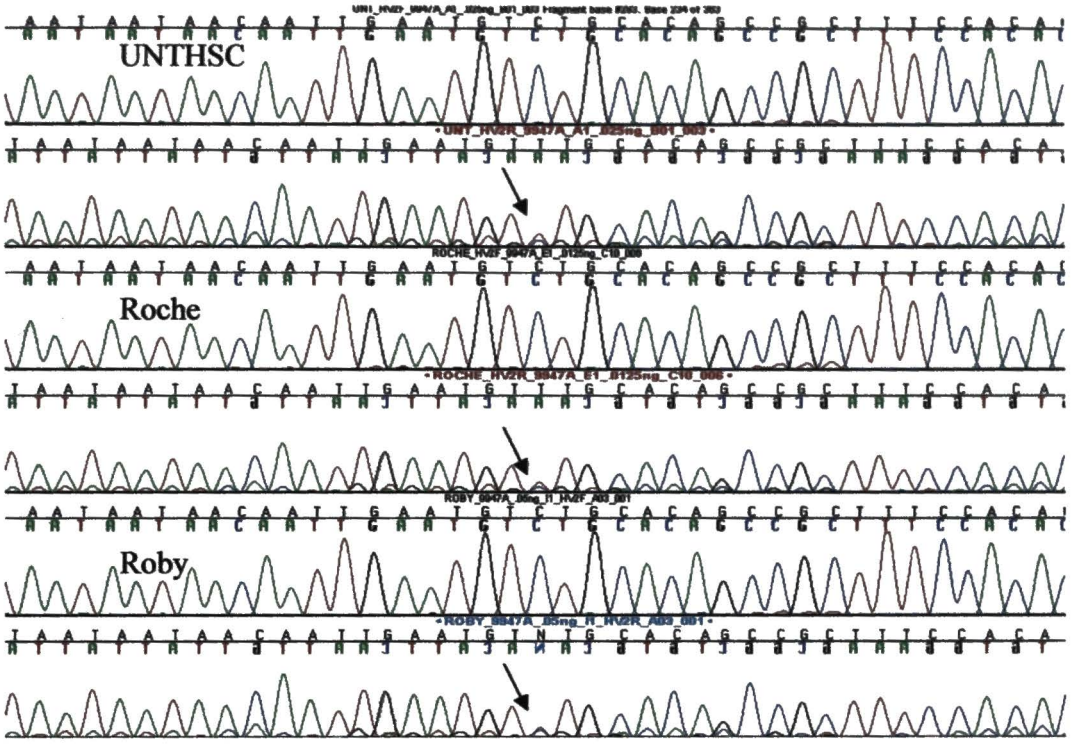


Figure 9 represents the sequences for the UNTHSC amplification in the forward and reverse direction, the Roche amplification in the forward and reverse direction, and the Roby amplification in the forward and reverse direction.

Figure 10: Three primer set comparison for sample 9947A (0.031ng HV2)

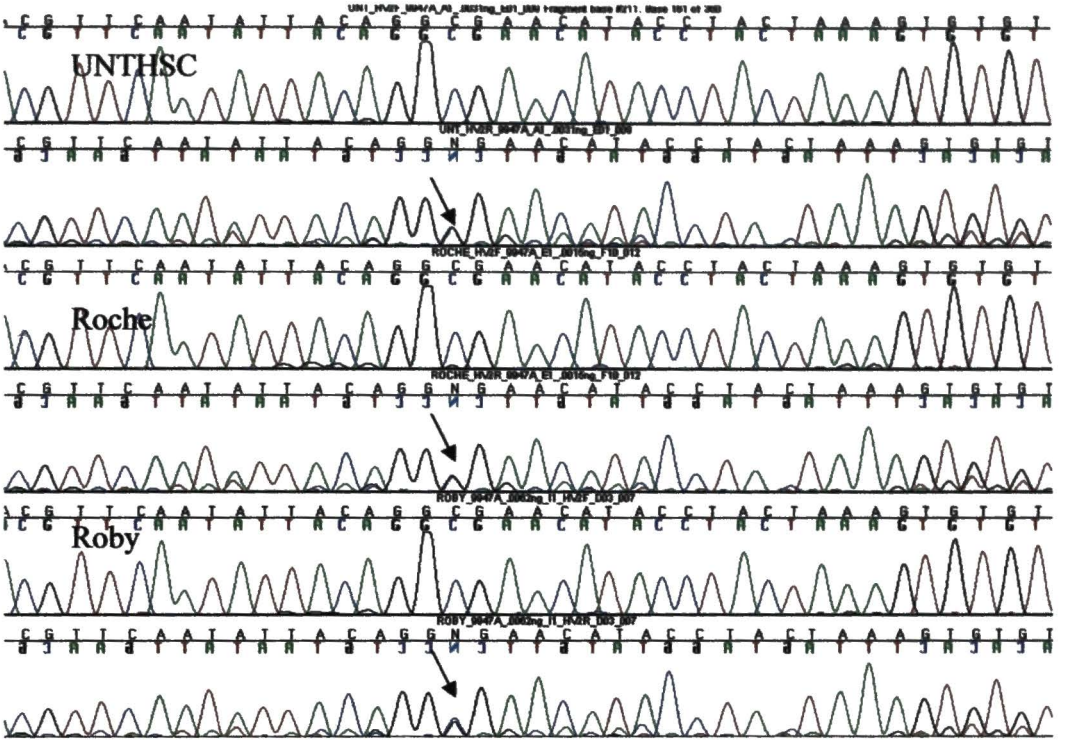


Figure 10 represents the sequences for the UNTHSC amplification in the forward and reverse direction, the Roche amplification in the forward and reverse direction, and the Roby amplification in the forward and reverse direction.

In general, the HV2 sequences were of lesser quality, but this was most apparent with the Roby amplification sequences. The baseline was noisy, to the extent that some bases could not be distinguished, and the sequences were not as concise, but still interpretable (Figure 11 and 12, respectively).

Figure 11: Roby protocol, sample 9948 (.031ng HV2R)

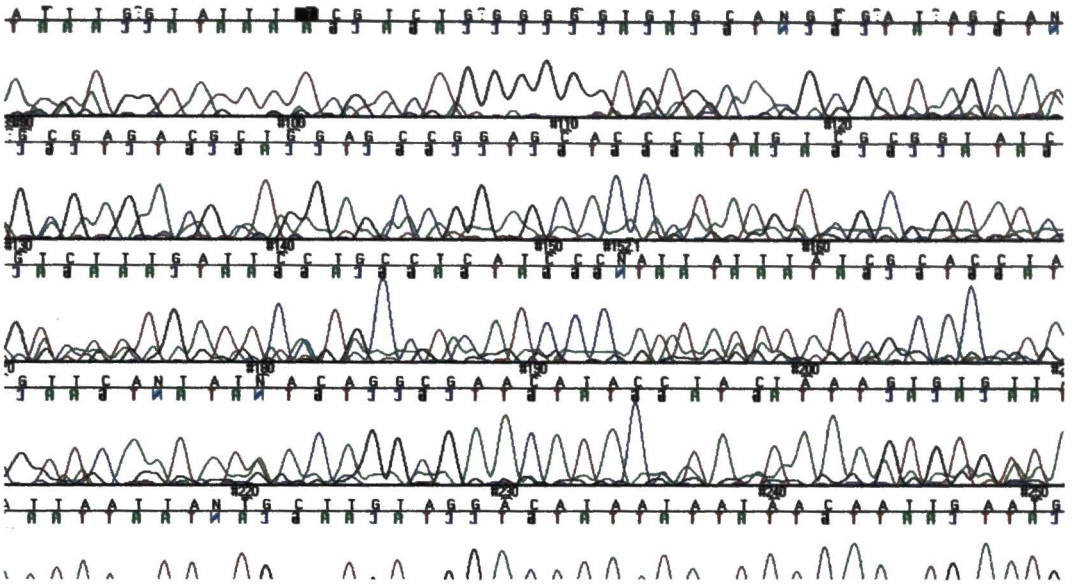
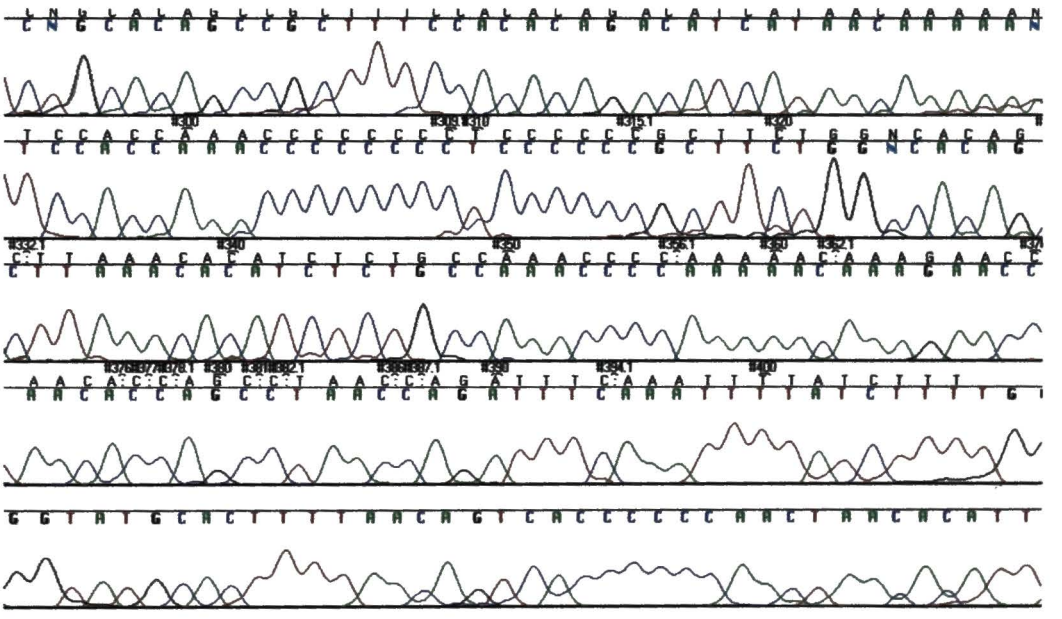


Figure 12: Roby protocol, sample 9948 (0.015ng HV2F)



It was difficult to determine if the poor quality of the sequences was attributed to the primers, or just a bad run. Subsequent runs would have to be completed to effectively assess the performance of the Roby primers.

While these sequences did not appear to offer much in terms of clarity, when paired with their respective complement sequence, analyzed and edited, all the correct polymorphisms were detected, and any ambiguous bases could be resolved by analyzing the complement strand (Figure 13 and 14).

Figure 13: Roby sequence, sample 9948 (0.031ng HV2 F&R)

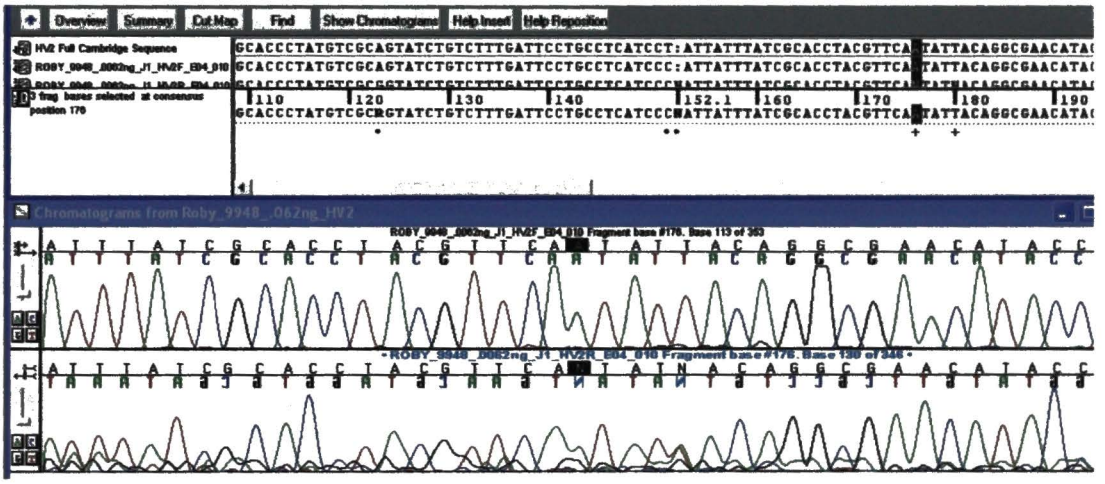
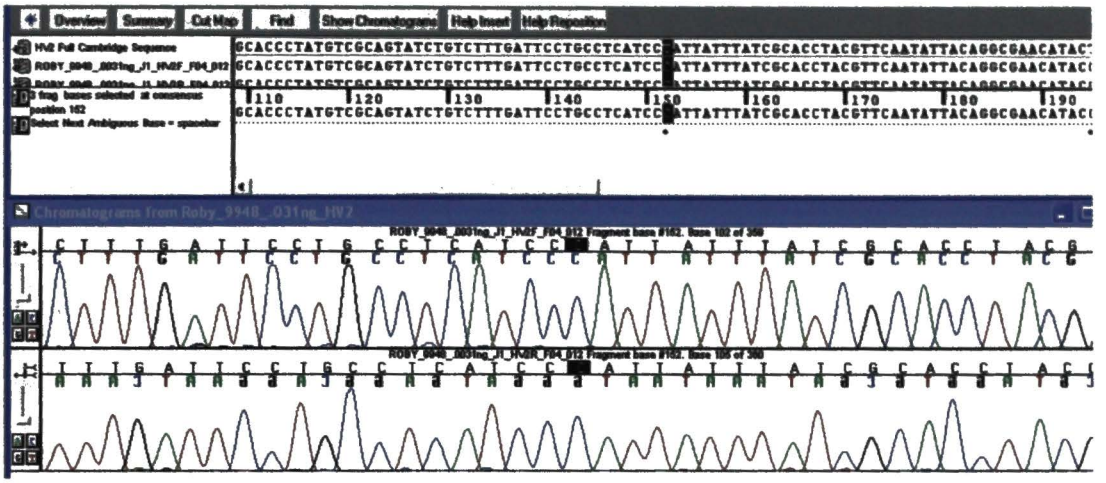


Figure 14: Roby sequence, sample 9948 (0.015ng HV2 F&R)



Base Signal Strength

In determining that the quality of the sequences was acceptable, the sequences were analyzed for trends in signal intensity through out the dilution series. Signal strength in mitochondrial sequencing is analogous to relative fluorescent units (RFUs) in short tandem repeat (STR) analysis; the higher the input DNA the higher the expected frequency. It was hypothesized that the base signal strengths for each sample would decrease as the quantity of input DNA decreased. This hypothesis was tested by collecting the all the base signal strengths for each sample in the forward and reverse direction for hypervariable regions 1 and 2. Since the input quantity of DNA varied amongst the three primer sets, only the values that overlapped (0.25 ng through 0.015 ng) were evaluated. The averages were calculated across the 4 base pairs, G, A, T, and C, and then averaged again down the dilution series and evaluated for trends (See Appendix D).

From the data collected, the signal strengths were found to be extremely variable within the respective primer set (See Appendix C). In many instances the value for the lowest quantity of input DNA was higher than the highest quantity of input DNA. However, there was a clear distinction in the signal intensity between the forward and reverse primers, indicating that generally, the forward primers performed better than the reverse. The only trend observed was between the three primer sets; over all, the Roche amplification had higher signal strength than the UNTHSC or Roby amplification (Figure 15).

Figure 15A: Average Base Signal Strength for Sample 9947A

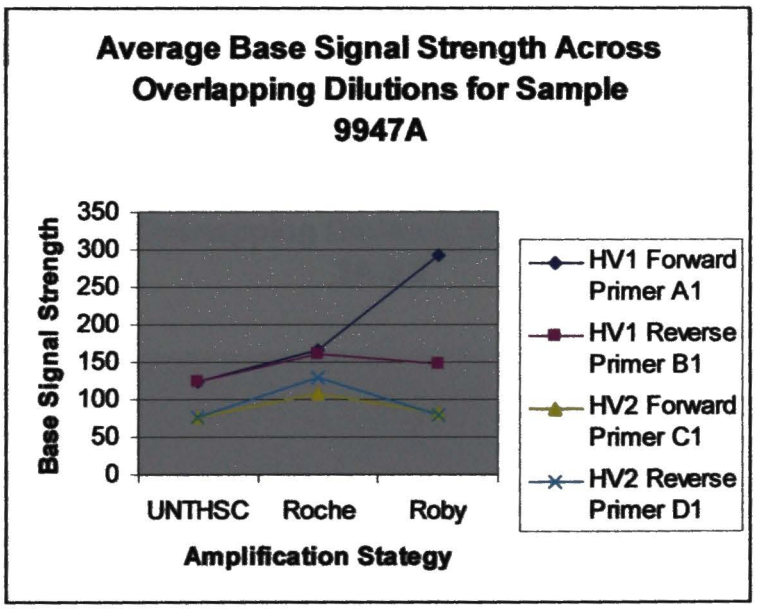


Figure 15B: Average Base Signal Strength for Sample 9948

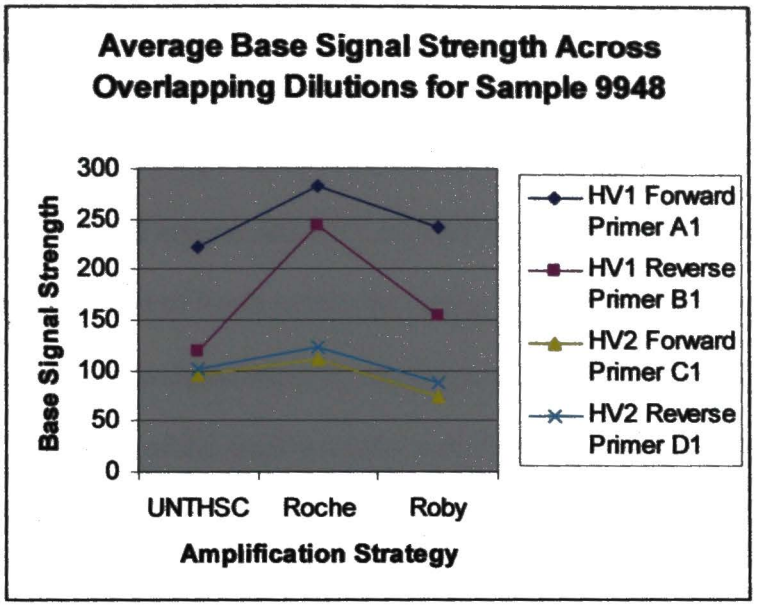
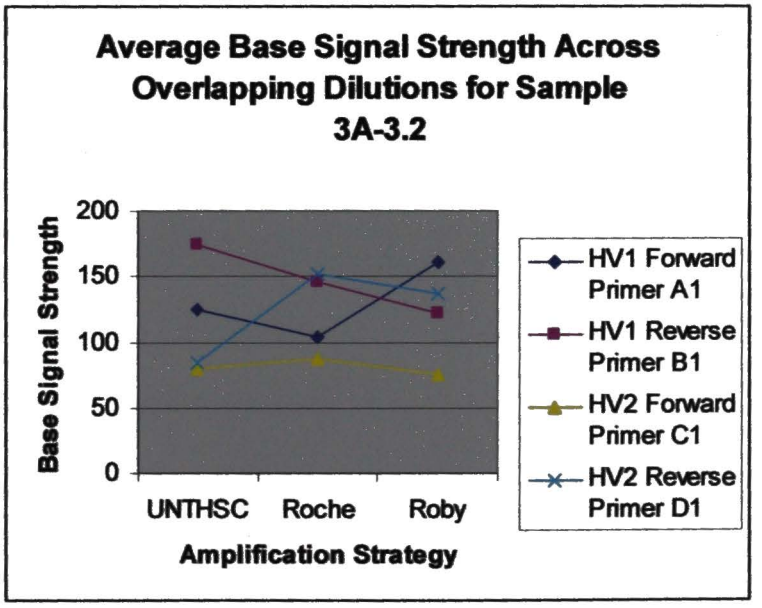


Figure 15C: Average Base Signal Strength for Sample 3A-3.2



Success of Amplifications

The overall success of the three amplification protocols was determined by trimming the un-edited contigs to match that of the reference sequence, counting the number of ambiguous and spacing ambiguous bases for each respective primer (HV1 forward and reverse and HV2 forward and reverse), and subtracting that number from the total amount of bases within the contig. Having already concluded that there was a difference in signal intensity between each amplification technique, it was postulated that the success of the amplifications would differ as well. However, this was rejected when the data of the four respective primers showed very similar results, only differing, at most, by five bases; the amplifications therefore were deemed successful.

CHAPTER V

CONCLUSIONS

The primary purpose of this project was to evaluate the performance of the 3130*xl* Genetic Analyzer and to evaluate the performance of three different approaches to amplifying the mitochondrial DNA control region. Currently, the University of North Texas Health Science Center DNA Identity Laboratory utilizes the ABI Prism® 3100 Genetic Analyzer to perform their entire mitochondrial DNA sequencing analysis. To replace an instrument, it must be proven that the new instrument can perform, at minimum, comparably to the instrument currently used. This includes assessing the quality of the sequences obtained from the new instrument, ensuring that the correct polymorphisms are being detected with the new instrument, and documenting any anomalies that may arise during the validation process.

As previously demonstrated, the quality of the sequences was comparable amongst the three primer sets, and there were no major differences between the results of the amplification protocols. When weighed against the different modes of amplification, the sequences were of excellent quality and the amplifications were all successful. The only incongruity observed were lack of results at the lowest quantity (0.0035ng and 0.007ng, respectively) for the Roby 3A-3.2 sample (HV1 and HV2, forward and reverse) and the UNTHSC 9947A sample (HV1 forward). This occurrence appeared to be an

isolated event, since in both situations the respective other two samples produced data at those values.

The promising results obtained with the validation study and concordant study indicated that other factors had to be evaluated to differentiate between the three amplification techniques. An important question all laboratories should ask is: How do new methodologies and theories affect time and money? The conventional FBI protocol for mitochondrial DNA amplification and sequencing is very labor intensive, time consuming, and expensive [12]. Twice the number of reactions must be set-up for amplification, which requires more reagents (Appendix B), and thus a higher expense for the lab. There is also more room for human error since twice the number of pipetting steps must be performed which can lead to contamination. And, more of the DNA extract is consumed, which can be detrimental in cases of very limited sample. In spite of this, this protocol has been validated, undergone quality control (QC) measures, and is in current use in the laboratory.

The co-amplification of HV1 and HV2 regions in a duplex reaction is beneficial because it reduces the amount human labor and is useful when DNA sample is limited [8]. While this project focused solely on evaluating the duplex amplification protocol, Chong et al also evaluated the LINEAR ARRAY™ mtDNA HVI/HVII Region Sequence Typing Kit for use as a template for sequencing reactions [2005]. The authors used the same primers as those used for amplification in the cycle sequencing step. Their results indicate that the use of this kit for amplification and sequencing is sensitive and robust enough to be used in forensic evidence cases [11]. This may be a path the DNA Identity

Laboratory will want to pursue if the Roche Kit is utilized for mitochondrial amplification. The Roche kit arrives having undergone quality control measures so the lab would not have to worry about this step, but this does increase the cost of the kit.

While the Roche Applied Sciences kit is very appealing, one must be careful with contamination. Unlike conventional methods of mitochondrial DNA amplification, where if contamination occurs in the HV1 region the HV2 region may be clean, if the duplex reaction is contaminated this affects both regions of interest.

The third amplification protocol evaluated was a large mitochondrial DNA amplicon for amplification and sequencing designed by Rhonda Roby, Director of Forensic Program at Celera. This single amplification reaction amplifies an 1100 base pair fragment that spans the mitochondrial control region. Much like the Roche amplification, the Roby amplification is appealing due to the fewer steps required for successful amplification of mitochondrial regions HV1 and HV2. Also, although not demonstrated in this project, the Roby protocol utilizes the same two primers for amplification and sequencing, as opposed to the use of four primers with the UNTHSC protocol for mitochondrial sequencing.

As discussed in chapter 4, it was noted that the full potential of the RF1 and RR2 primers may not have been reached by failing to use the same primers in the cycle sequencing step (Rhonda Roby). While this may be true, and should be further evaluated, the lower PCR reaction volume size, 10 µL, when compared to a 25 µL or 50 µL reaction volume, prohibits extensive cycle sequencing analysis subsequent to PCR amplification. After running samples on the Agilent 2100 which requires 1 µL of PCR

product, and setting up a cycle sequencing reaction, which requires 2 uL of PCR product per region of interest, or 9 μL of PCR product if it is a negative control, there is no sample left. This is not practical when working in an environment where instruments may malfunction, as did the case with the Agilent 2100 BioAnalyzer, or when human error is incorporated into the equation.

However, with family reference samples where there is an abundance of DNA, the Roby amplification protocol is ideal. It has been documented that the Roby amplification protocol demonstrates optimal performance when used with high quality and quantity DNA [14]. This indicates that incorporation into forensic casework may not be the best route, but as suggested, use in family reference samples may prove to be helpful. This is a decision that would need to be made by the technical leader and further evaluated in the lab. The Roby amplification protocol is the least expensive, in terms of number of reactions obtained for the amount of money spent, of all three protocols but would have to go through quality control measures before it could be used in the lab. The DNA Identity Laboratory currently has in place a quality control protocol that would expedite this process.

Overall, the Roche Applied Sciences LINEAR ARRAY™ mtDNA HVI/HVII Region Sequence Typing Kit fits best for what the DNA Identity Laboratory is looking for in an amplification protocol of forensic evidentiary samples. It cuts back on analyst operation and performs comparably to current means of amplification. In conjunction, the Roby protocol fits best with amplifying and sequencing family reference samples.

This compromise allows for the elimination of the labor intensive FBI amplification protocol altogether and cuts back on costs.

One of the main goals projected to accomplish was to assess the sensitivity of the 3130*xl* for human mtDNA. This was not achieved because the serial dilutions were not quantified to establish the amount of mitochondrial copies in the sample, but rather only the total nuclear DNA. Mitochondrial DNA can be present in levels 10 000 times that of nuclear DNA in a sample extract, so quantifying total nuclear DNA does not provide sufficient information. The absence of the accurate mitochondrial DNA quantities in the samples made the sensitivity study of mtDNA ineffective and may be the cause of the inconsistent results with the signal intensities. In spite of this, the sensitivity of the 3130*xl* was completed for total human nuclear DNA. This project illustrates that quality sequences and results are obtainable at input nuclear DNA values ranging from 1ng to .00035ng. The 3130*xl* produced consistent, accurate, reproducible results for the three amplification sets, and it is now at the labs discretion to choose an amplification strategy that best fits with their projected goal.

APPENDIX A

AMPLIFICATION STATEGY

Appendix A

Table 4: Amplification Strategy- Volume per reaction

Reagents	UNTHSC	Roche	Roby
LINEAR ARRAY mtDNA Reaction mix	X	20 µL of pre-made master mix	X
LINEAR ARRAY HVI/HVII mtDNA Primer Mix	X	10 µL of pre-made primer mix	X
Sterile Water	5.5 µL	X	0.8 µL
Forward Primers (10 µmM)	0.5 µL	X	X
Reverse Primers (10 Mm)	0.5 µL	X	X
Forward Primers (30 µmM)	X	X	0.2 µL
Reverse Primers (30 Mm)	X	X	0.2 µL
10X PCR Buffer	2.5 µL	X	1 µL
25mM MgCl	2.0 µL	X	0.6 µL
dNTP mix (10 mM)	1.0 µL	X	0.8 µL
BSA (1.6 µg/ µL)	2.5 µL	X	1 µL
AmpliTaq Gold (5U/ul)	0.5 µL	X	0.4 µL

APPENDIX B

AGILENT RESULTS

Appendix B

Figure 16A - Chip 1: Agilent 2100 BioAnalyzer results for UNTHSC samples. Note: sample label values are [DNA]/10 µl.

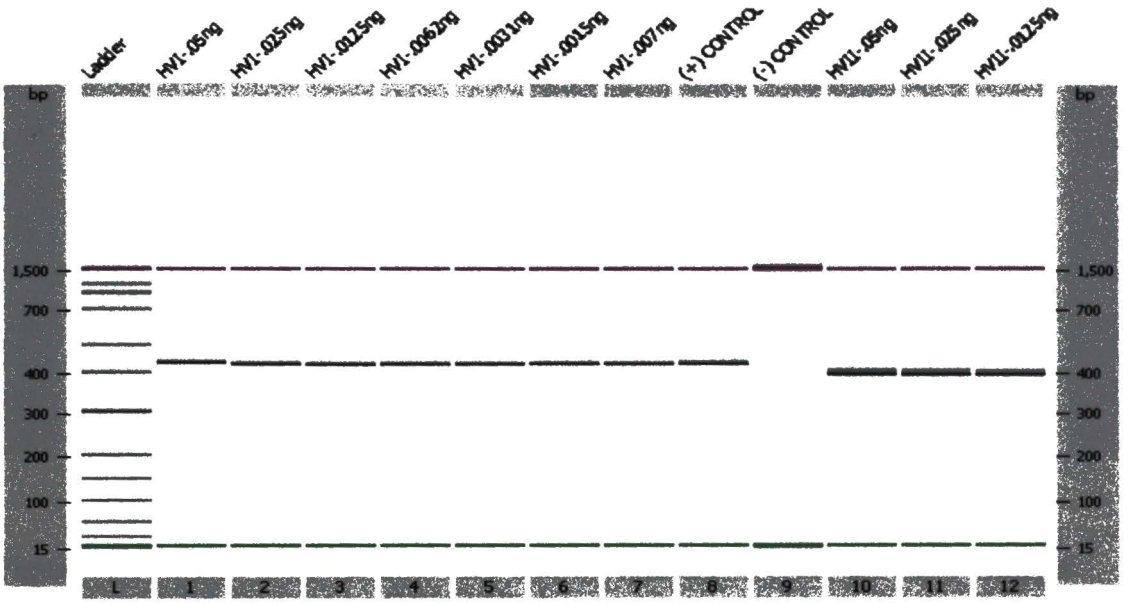


Figure 16B - Chip 2: Agilent 2100 BioAnalyzer results for UNTHSC HV2 and Roche samples. Note: sample label values are [DNA]/10 µl.

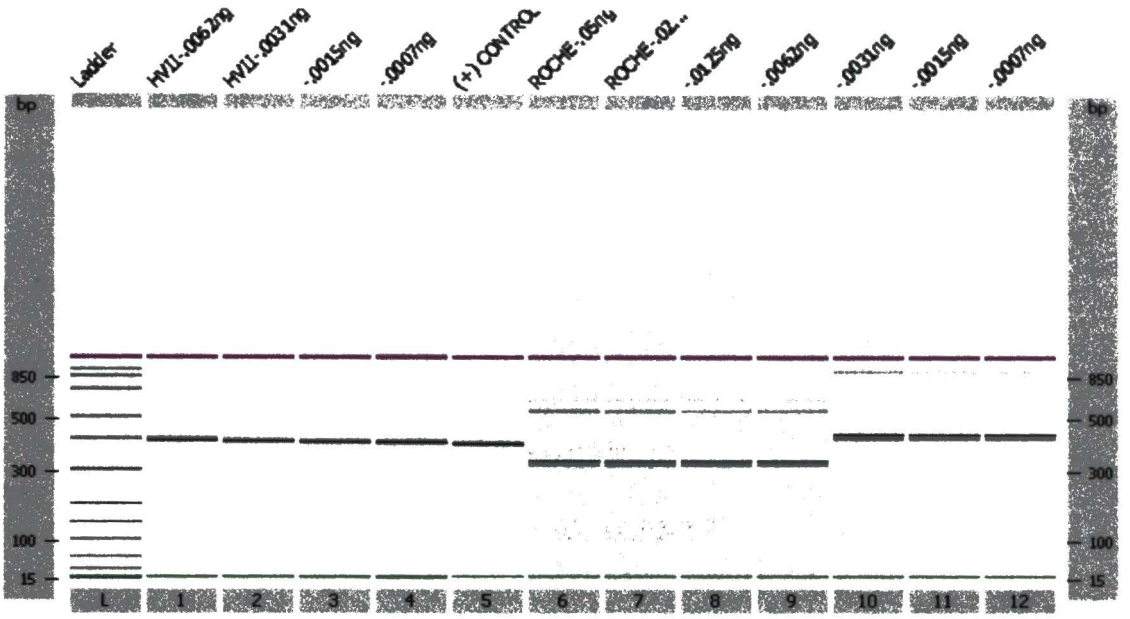
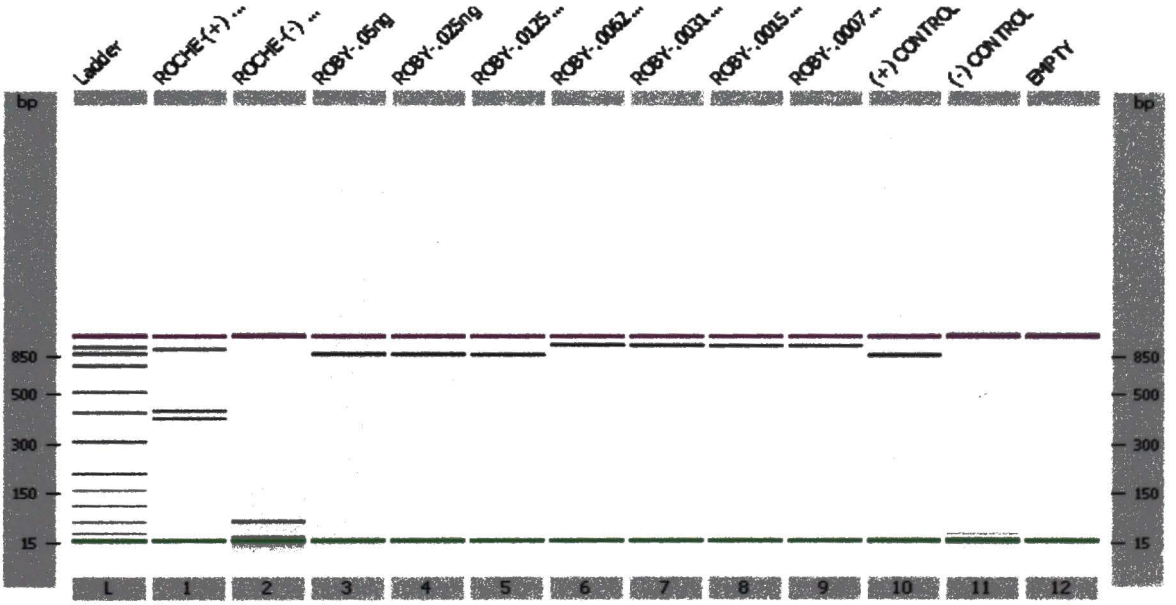


Figure 16C - Chip 3: Agilent 2100 BioAnalyzer results for Roche and Roby samples. Note: sample label values are [DNA]/10 µl.



APPENDIX C

COST OF REAGENTS

Appendix C

Table 5: Cost of reagents in bulk

Reagents	UNTHSC	Roche	Roby
LINEAR ARRAY KIT	X	$1591.00/ 50 Reactions	X
Primers A1, B1, C1, D1	$20.00 EACH	X	X
Primers RF1, RR2	X	X	$20.00 EACH
10X PCR Buffer	$20.00/ 1.5 ml	X	$20.00/ 1.5 ml
25mM MgCl	$20.00/ 1.5 ml	X	$20.00/ 1.5 ml
dNTP mix (10 mM)	$50.00/ 1 ml	X	$50.00/ 1 ml
BSA	$34.00/ 25g	X	$34.00/ 25g
AmpliTaq Gold (5U/ul)	$694.00/ 6 pack	X	$694.00/ 6 pack

Table 6: Cost of reagents utilized per reaction not including the primers

Reagents	UNTHSC	Roche	Roby
LINEAR ARRAY KIT	X	~$31.82/ 1 Reaction	X
10X PCR Buffer	~$1.00/ 1 Reaction	X	~$1.00/ 1 Reaction
25mM MgCl	~$.80/ 1 Reaction	X	~$.80/ 1 Reaction
dNTP mix (10 mM)	~$1.50/ 1 Reaction	X	~$1.50/ 1 Reaction
BSA	~$1.00/ 1 Reaction	X	~$1.00/ 1 Reaction
AmpliTaq Gold (5U/ul)	~$35.00/ 1 Reaction	X	~$35.00/ 1 Reaction
TOTAL/ Reaction	~$78.60 For HVI and HVII amplifications	~$31.82 For HVI and HVII amplifications	~$39.30 For HVI and HVII amplifications

APPENDIX D

AVERAGE BASE SIGNAL STRENGTH

Appendix D

Table 7: Average base signal strength for UNTHSC protocol sample 9947A

	UNTHSC	Base Signal Strength								Average	
	9947A	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.5ng	74	146	196	138	161	52	71	74	**125.5**	**102.5**
	.25ng	91	153	246	132	200	51	87	72	**156**	**102**
	.125ng	77	237	194	199	154	74	70	108	**123.75**	**154.5**
	.062ng	77	168	190	144	154	53	71	74	**123**	**109.75**
	.031ng	72	174	170	130	141	46	68	65	**112.75**	**103.75**
	.015ng	68	248	152	179	132	65	64	90	**104**	**145.5**
	.007ng		211		154		60		84		**127.25**
								Average		**124.167**	**120.75**
HV2											
	.5ng	90	99	170	90	116	47	59	40	**108.75**	**69**
	.25ng	95	145	175	127	120	66	62	56	**113**	**98.5**
	.125ng	78	122	141	104	93	53	47	43	**89.75**	**80.5**
	.062ng	48	150	89	133	56	65	27	35	**55**	**95.75**
	.031ng	55	87	88	69	57	34	28	28	**57**	**54.5**
	.015ng	58	87	100	67	65	32	32	27	**63.75**	**53.25**
	.007ng	25	63	44	45	30	23	14	20	**28.25**	**37.75**
								Average		**84.25**	**69.89**

Table 8: Average base signal strength for UNTHSC protocol sample 9948

	UNTHSC	Base Signal Strength								Average	
	9948	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.5ng	181	271	519	265	376	103	122	141	**299.5**	**195**
	.25ng	172	328	480	303	334	114	108	153	**273.5**	**224.5**
	.125ng	167	257	454	214	320	81	101	105	**260.5**	**164.25**
	.062ng	138	161	375	134	262	51	84	66	**214.75**	**103**
	.031ng	131	134	366	109	255	42	81	55	**184.75**	**85**
	.015ng	113	170	311	124	225	48	72	66	**180.25**	**102**
	.007ng	103	131	260	90	194	38	63	49	**155**	**77**
								Average		**224.04**	**135.82**
HV2											
	.5ng	81	161	155	171	106	87	52	72	**98.5**	**122.75**
	.25ng	115	219	211	234	139	116	70	95	**133.75**	**166**
	.125ng	94	139	178	134	115	66	56	53	**110.75**	**98**
	.062ng	99	138	178	132	121	64	57	53	**113.75**	**96.75**
	.031ng	41	89	75	84	50	42	24	33	**47.5**	**62**
	.015ng	63	122	112	108	80	54	39	45	**73.5**	**82.25**
	.007ng	35	73	65	63	47	32	23	27	**42.5**	**48.75**
								Average		**88.61**	**169.125**

Table 9: Average base signal strength for UNTHSC protocol sample 3A-3.2

	UNTHSC	Base Signal Strength								Average	
	3A-3.2	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.5ng	99	173	285	155	201	56	67	80	**163**	**116**
	.25ng	177	58	156	164	58	112	80	38	**117.75**	**372**
	.125ng	234	33	194	83	70	54	99	17	**149.25**	**46.75**
	.062ng	81	264	230	214	157	79	52	110	**130**	**166.75**
	.031ng	86	274	249	222	177	86	59	121	**142.75**	**175.75**
	.015ng	51	177	144	132	102	50	33	71	**82.5**	**107.5**
	.007ng	78	107	230	74	160	26	54	36	**130.5**	**60.75**
								Average		**130.82**	**149.36**
HV2											
	.5ng	99	201	205	211	133	104	66	86	**125.75**	**150.5**
	.25ng	125	170	240	167	159	79	78	71	**150.5**	**121.75**
	.125ng	50	116	92	119	59	58	28	48	**57.25**	**85.25**
	.062ng	87	170	170	168	112	81	55	68	**106**	**121.75**
	.031ng	11	21	19	16	14	8	6	8	**12.5**	**13.25**
	.015ng	62	111	116	108	79	54	39	43	**74**	**79**
	.007ng	49	53	102	48	63	22	30	18	**61**	**35.25**
								Average		**83.71**	**116.25**

Table 10: Average base signal strength for Roche protocol sample 9947A

	ROCHE	Base Signal Strength								Average	
	9947A	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	1ng	158	269	433	349	319	142	118	193	**257**	**238.25**
	.5ng	125	205	342	265	249	110	92	149	**202**	**182.25**
	.25ng	103	241	276	272	194	105	69	144	**160.5**	**190.5**
	.125ng	97	214	264	235	182	91	65	123	**152**	**165.75**
	.062ng	131	174	359	164	250	61	86	81	**206.5**	**120**
	.031ng	92	250	251	240	177	92	60	124	**145**	**176.5**
	.015ng	103	227	292	199	210	79	73	111	**169.5**	**154**
								Average		**184.64**	**175.32**
HV2											
	1ng	164	206	343	240	235	130	127	116	**217.25**	**173**
	.5ng	91	164	187	192	126	101	66	90	**117.5**	**136.75**
	.25ng	122	220	233	241	154	122	81	107	**147.5**	**172.5**
	.125ng	121	208	232	218	154	111	75	96	**145.5**	**158.25**
	.062ng	87	172	161	173	106	88	55	75	**102.25**	**127**
	.031ng	48	120	82	110	54	56	27	47	**52.75**	**83.25**
	.015ng	75	150	138	133	93	69	48	57	**88.5**	**102.25**
								Average		**105.75**	**136.14**

Table 11: Average base signal strength for Roche protocol sample 9948

	ROCHE	Base Signal Strength								Average	
	9948	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	1ng	224	337	582	466	450	193	160	262	**354**	**314.5**
	.5ng	284	279	751	332	545	132	188	183	**442**	**231.5**
	.25ng	299	282	759	366	559	145	194	192	**452.75**	**246.25**
	.125ng	225	275	589	341	434	132	147	177	**228.75**	**231.25**
	.062ng	141	271	386	376	288	146	100	192	**228.75**	**246.25**
	.031ng	192	335	546	423	416	173	142	231	**324**	**290.5**
	.015ng	107	266	292	272	227	114	76	149	**175.5**	**200.25**
								Average		**315.11**	**251.46**
HV2											
	1ng	147	231	320	311	221	159	114	141	**401**	**210.5**
	.5ng	190	193	394	233	268	113	135	98	**246.75**	**159.25**
	.25ng	141	191	284	228	194	112	97	98	**179**	**157.25**
	.125ng	48	156	93	188	64	93	31	79	**59**	**129**
	.062ng	88	136	205	190	140	97	74	79	**126.75**	**125.5**
	.031ng	96	153	221	197	152	104	78	85	**136.75**	**134.75**
	.015ng	43	89	87	93	64	49	32	41	**56.5**	**68**
								Average		**172.25**	**140.61**

Table 12: Average base signal strength for Roche protocol sample 3A-3.2

	ROCHE	Base Signal Strength								Average	
	3A-3.2	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	1ng	139	267	361	335	259	128	100	174	**214.5**	**226**
	.5ng	165	241	433	297	307	113	109	153	**253.5**	**201**
	.25ng	102	161	265	189	188	74	65	98	**155**	**130.5**
	.125ng	81	162	216	181	156	72	55	96	**127**	**127.75**
	.062ng	30	283	78	288	55	114	17	157	**45**	**210.5**
	.031ng	81	131	225	130	172	54	61	74	**134.75**	**97.25**
	.015ng	36	219	97	225	69	88	26	129	**57**	**165.25**
								Average		**140.96**	**165.46**
HV2											
	1ng	123	257	270	367	173	175	89	159	**163.75**	**239.5**
	.5ng	82	175	178	235	110	112	56	96	**106.5**	**154.5**
	.25ng	55	199	119	246	77	106	39	99	**72.5**	**162.5**
	.125ng	45	212	93	252	60	119	31	101	**57.25**	**171**
	.062ng	91	242	196	288	129	139	65	123	**120.25**	**198**
	.031ng	80	104	173	122	118	60	59	52	**107.5**	**84.5**
	.015ng	56	175	122	201	81	96	45	105	**76**	**144.25**
								Average		**100.54**	**164.89**

Table 13: Average base signal strength for Roby protocol sample 9947A

	ROBY	Base Signal Strength								Average	
	9947A	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.25ng	274	244	594	249	471	108	162	141	**375.25**	**185.5**
	.125ng	290	226	638	213	511	88	180	119	**404.75**	**161.5**
	.062ng	185	240	398	211	313	85	107	111	**250.75**	**161.75**
	.031ng	178	230	384	185	288	74	96	96	**236.5**	**146.25**
	.015ng	151	126	322	97	242	39	80	50	**198.75**	**78**
	.007ng	36	77	86	56	56	23	20	29	**49.5**	**46.25**
	.0035ng	79	98	175	70	131	30	44	38	**107.25**	**59**
								Average		**217.54**	**119.75**
HV2											
	.25ng	70	140	164	161	115	82	48	63	**99.25**	**111.5**
	.125ng	81	132	184	147	134	75	58	57	**114.25**	**102.75**
	.062ng	58	101	126	106	85	51	36	40	**76.25**	**74.5**
	.031ng	48	125	106	121	74	57	31	44	**64.75**	**86.75**
	.015ng	38	21	83	17	57	8	24	6	**50.5**	**13**
	.007ng	50	32	107	27	73	13	30	10	**65**	**20.5**
	.0035ng	35	79	80	72	58	37	24	29	**49.25**	**54.25**
								Average		**74.18**	**66.18**

Table 14: Average base signal strength for Roby protocol sample 9948

	ROBY	Base Signal Strength								Average	
	9948	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.25ng	212	259	465	267	357	115	119	147	**288.25**	**197**
	.125ng	208	229	436	233	338	96	118	125	**275**	**170.75**
	.062ng	236	182	503	168	384	68	132	87	**313.75**	**126.25**
	.031ng	134	221	284	190	218	76	74	98	**177.5**	**146.25**
	.015ng	115	207	246	163	187	66	64	83	**153**	**129.75**
	.007ng	97	96	198	73	154	32	51	38	**125**	**59.75**
	.0035ng	55	70	127	51	99	22	33	26	**78.5**	**42.25**
								Average		**201.57**	**124.57**
HV2											
	.25ng	44	173	110	189	79	89	33	69	**66.5**	**130**
	.125ng	94	167	221	179	157	83	67	64	**134.75**	**123.25**
	.062ng	63	113	129	115	94	52	38	41	**81**	**80.25**
	.031ng	14	8	31	7	21	4	8	3	**18.5**	**5.5**
	.015ng	52	141	111	137	79	64	32	49	**68.5**	**97.75**
	.007ng	26	12	54	10	39	6	16	4	**33.75**	**8**
	.0035ng	13	28	32	26	24	15	10	11	**19.75**	**20**
								Average		**60.39**	**66.39**

Table 15: Average base signal strength for Roby protocol sample 3A-3.2

	ROBY	Base Signal Strength								Average	
	3A-3.2	G		A		T		C			
		F	R	F	R	F	R	F	R	F	R
HV1											
	.25ng	177	261	393	255	288	100	106	133	**241**	**187.25**
	.125ng	148	198	325	183	235	73	83	95	**197.75**	**137.25**
	.062ng	134	199	288	180	209	72	75	95	**176.5**	**136.5**
	.031ng	74	114	158	96	120	39	43	50	**98.75**	**74.75**
	.015ng	67	113	143	95	112	40	40	53	**90.5**	**75.25**
	.007ng	42	70	89	51	70	23	23	27	**56**	**42.75**
	.0035ng										
								Average		**143.42**	**108.96**
HV2											
	.25ng	70	244	174	312	125	151	51	121	**105**	**207**
	.125ng	50	149	121	180	85	86	33	69	**72.25**	**121**
	.062ng	30	161	72	169	50	78	20	62	**43**	**117.5**
	.031ng	77	110	161	122	109	58	51	45	**99.5**	**83.75**
	.015ng	36	197	88	223	64	109	26	84	**53.5**	**153.25**
	.007ng	24	115	51	122	37	62	17	46	**32.25**	**86.25**
	.0035ng										
								Average		**67.58**	**128.13**

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Figure 1: http://www.mitomap.org/mitomapgenome.pdf *Copyright 2002 @ Mitomap.org*

Figure 2: Applied Biosystems. System Profile: Applied Biosystems 3130 and 3130*xl* Genetic Analyzers.