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1970

EVALUATION OF Y-STR DATA USING A DUPLEX GENDER REAL-TIME PCR ASSAY ON AN ABI PRISM® 7000 SDS FOLLOWED BY AMPLIFICATION WITH APPLIED BIOSYSTEMS AMPFℓSTR® YFILER™ PCR AMPLIFICATION KIT

INTERNSHIP PRACTICUM REPORT

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

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Fort Worth, Texas

August 2007

EVALUATION OF Y-STR DATA USING A DUPLEX GENDER REAL-TIME PCR ASSAY ON AN ABI PRISM® 7000 SDS FOLLOWED BY AMPLIFICATION WITH APPLIED BIOSYSTEMS AMPFℓSTR® YFILER™ PCR AMPLIFICATION KIT

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ACKNOWLEDGEMENTS

I would like to give my appreciation to my supervisor George Duncan, Ph.D. and all the analysts in DNA Unit of the Broward Sheriff's Office in Fort Lauderdale, Florida for their time, guidance, support, and friendship during my internship. I would also like to thank my fellow classmates, friends, and family for their continued support and guidance during my studies at the University of North Texas Health Science Center. Finally, I would like to thank my professors and committee members, John Planz, Ph.D., Arthur Eisenberg, Ph.D., and Joseph Warren, Ph.D.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES:

FIGURES:

CHAPTER I

INTRODUCTION

Quantification is the process of determining the concentration of DNA in a sample and plays an extremely important role in the processes of amplification and STR typing. A method of quantification is mandated for a laboratory conducting forensic DNA analysis by National Standard 9.3 (1). Furthermore, anytime a forensic laboratory chooses to implement a new or novel methodology for any step in DNA analysis, a laboratory must conduct an internal validation to ensure the quality of the method and any results generated on the equipment used within that laboratory are reliable, reproducible, and accurate before the method is utilized for casework analysis (1). Prior to an internal validation, the method or technology must undergo a developmental validation by the developer or manufacturer to determine conditions or limitations of the method or technology on DNA analysis of forensic samples (2).

A study has shown that Y-STR results can be obtained even when the quantification of samples yields a value of 0.00ng/μl (4). The issue of the absolute lowest limit of detection in the quantification process versus input DNA concentrations of the unknown samples to yield any valuable Y-STR typing data has not been addressed. A duplex gender assay developed by Nicklas and Buel (3) has a reported detection limit of 0.5pg for the *Alu* probe of the duplex assay and quantification will be evaluated on a different qPCR platform than originally reported and followed by amplification using Applied Biosystems' AmpFℓSTR® Yfiler™ PCR Amplification Kit to assess quantification limits.

The goal of this internship project was to complete a preliminary evaluation of the sensitivity of a quantification methodology on a different qPCR platform under different detection parameters utilizing Y-chromosome DNA in correlation to Y-STR typing results and evaluate the data qualitatively.

CHAPTER II

BACKGROUND

Gender Duplex Assay (3)

A duplex real-time PCR assay was developed that simultaneously determines the amount of total human and male DNA (3). The assay uses the multicopy *Alu* gene for the detection of the human DNA and a human Y chromosomal repeat (DYZ5) for the detection of male DNA.

This duplex assay is time saving and may also aid in the process of selecting probative samples suited for STR testing. Multiplexing gender typing with DNA quantification allows a crime scene stain or sample to be screened for further analysis while simultaneously performing the necessary DNA quantification of the stain or sample (3). The quantification of male DNA can give information on the probability of obtaining a complete male genetic profile or aid in the decision of conducting Y-STR analysis (3).

The duplex assay utilizes TaqMan® MGB probes that are labeled with the fluorescent dyes VIC and FAM; the VIC dye detects the *Alu* sequence and the FAM dye

detects the DYZ5 sequence. The sensitivity of the assay is reported to be as low as 4.0 pg of male DNA and 0.5 pg of human DNA. No internal control is needed in the assay because the Alu probe in the NTC, or no template control, should amplify by ~38 cycles simply due to what has been attributed to ambient DNA in the air and water (Figure 6). The NTC is an equivalent to an endogenous control and if it does not amplify, then some sort of inhibition is in effect or some component of the reaction is missing (3).

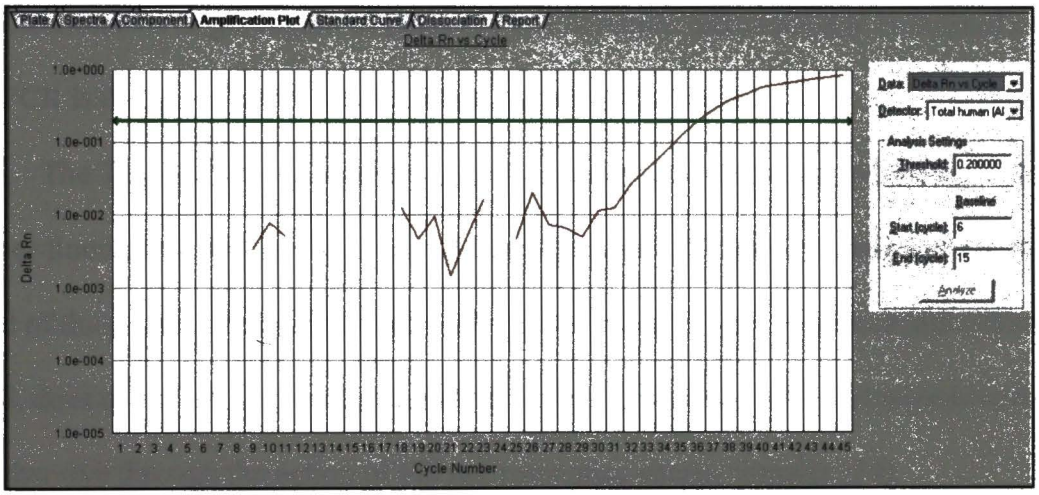

Figure 1: Amplification Plot of NTC

Real-Time Polymerase Chain Reaction

In order to have sufficient amounts of DNA to successfully type STR loci, an enzymatic reaction is necessary to copy certain regions of the DNA. The process is called the polymerase chain reaction (PCR) and simply creates multiple copies of a target region of DNA (13). The copying process occurs in a pattern of heating and cooling the samples in cycles. The PCR product is a copy of the sequence of interest because oligonucleotide

primers are used that are complementary to the 3' ends of the region of interest on the DNA. These primers anneal in the correct locations to direct the copying process (13).

Real-time PCR is a process that actually enables data to be collected as the amplification process is occurring. Real-time PCR is also sometimes referred to as quantitative PCR or qPCR because it determines the amount of DNA in each sample by measuring the changes in fluorescence from cycle to cycle during PCR created by the amplification of a target region of DNA (13). A common technique that is utilized in real-time PCR is the use of the 5' nuclease assay or TaqMan® (13).

The TaqMan® assay consists of an oligonucleotide probe that is labeled with a reporter fluorescent dye on the 5' end of the probe and a fluorescent quencher dye on the 3' end of the probe. The dual-labeled probe anneals to the target sequence and when bound, does not allow fluorescence of the reporter dye because of the closeness to the quencher dye through the action of Förster resonance energy transfer (14).

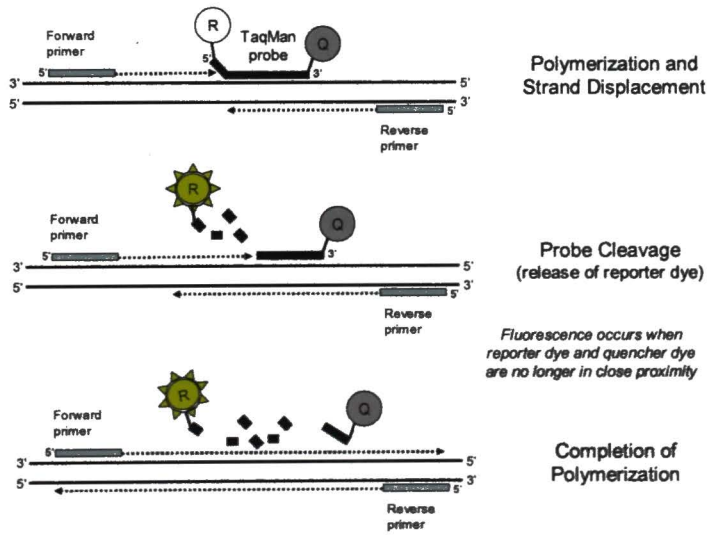

Figure 4.4, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Figure 2: Mechanism of the (5' Nuclease) TaqMan® Assay (13)

Figure 2 depicts the processes involved in a TaqMan® assay. In the first step, the probe will anneal in between the forward and reverse primers. Polymerization occurs and strand synthesis displaces any probe that is bound to the target sequence. In the second step, the probe is cleaved by the 5'-exonuclease activity of the *Taq* DNA polymerase and causes the reporter dye to be released. In the release of the reporter dye, fluorescence occurs because the reporter dye is no longer in close proximity to the quencher dye. Finally, the polymerization is complete and the target sequence is copied accurately (14, 13). Two common dyes used for the reporter dye are FAM and VIC, and a common dye used for a quencher dye is TAMRA.

In addition to the fluorescent dyes that are attached to the TaqMan probes, some instruments such as the ABI PRISM® 7000 Sequence Detection System utilize another dye 'like ROX' that is included in the PCR reaction mix. This dye functions as a passive reference to normalize reporter fluorescence signal (14).

There are three phases that define the polymerase chain reaction: exponential phase, linear phase, and plateau phase. During the exponential phase, the reaction is at the highest efficiency and most accurately depicts the true amount of PCR product being produced. In the linear phase, one or more of the critical reaction components falls below a critical concentration and the amplification reaction begins to lose efficiency. This phase is not used for comparison purposes between the known standard concentrations and the unknown samples. The plateau phase is the final phase in the PCR reaction and is the phase when the product accumulation stops and the assay no longer has any efficiency (13).

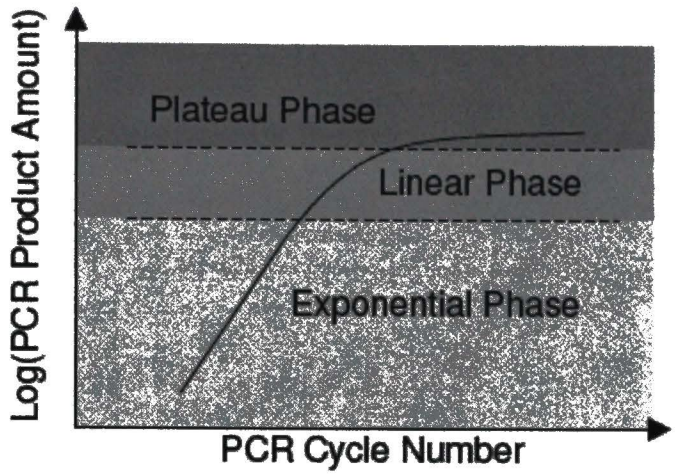

Figure 3: Three Phases of PCR Amplification (15)

The measurement of fluorescence versus cycle number is most accurate during the exponential phase because of the correlation between the amount of PCR product and starting template DNA concentration (13). A value called the cycle threshold (C_T) is the number of amplification cycles that it takes for the fluorescence level to pass a threshold set by the user; this threshold is set above the baseline and the fewer cycles it takes for the fluorescence generated from a particular sample to pass the threshold (the lower the C_T value), the greater the initial concentration of DNA in the sample before the PCR process (13). Ultimately, the concentration of DNA in each unknown sample is determined by the instrument by comparing these values from standards of known concentrations to samples of unknown DNA concentrations.

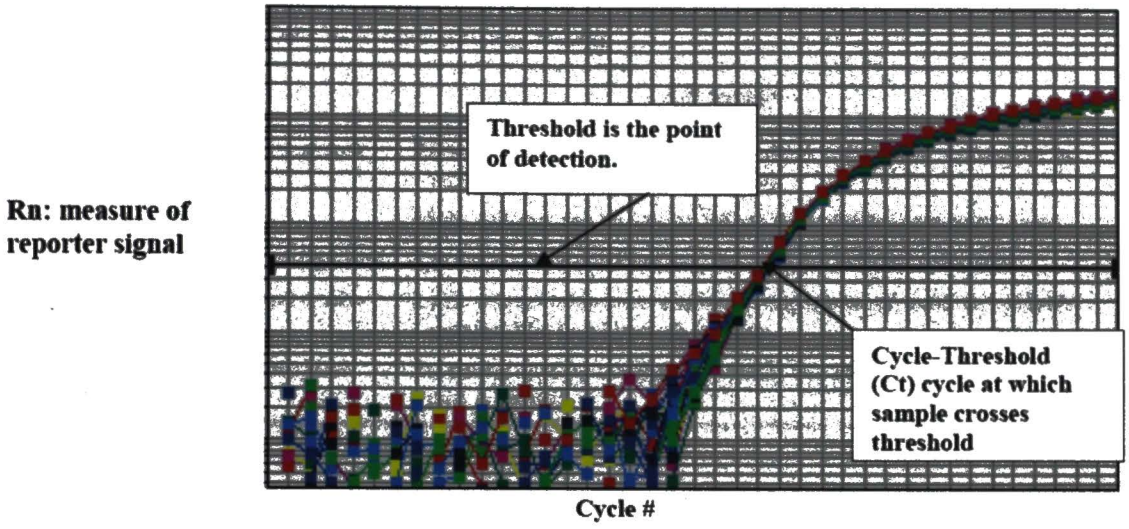

Figure 4: Example of an Amplification Curve (16)

Y-Short Tandem Repeats (Y-STRs)

Y-STRs are short tandem repeats found along the Y-chromosome and can be utilized in forensic DNA testing as an investigative tool with different discriminatory capabilities than autosomal STRs. The Y-chromosome DNA is passed down from father to son and therefore is inherited as a haplotype. Y-STR testing can have many applications including casework involving sexual assault evidence in the instance where a small percentage of male DNA may be masked by a large percentage of female DNA, and scenarios where the perpetrator may be a vasectomized individual and no spermatozoa may be able to be visualized (5, 6, 7).

Table 1. Areas of use in Y-chromosome testing

Use	Advantage
Forensic casework on sexual assault evidence	Male-specific amplification (can avoid differential extraction to separate sperm and epithelial cells)
Paternity testing	Male children can be tied to fathers in motherless paternity cases
Missing persons investigations	Patrilineal male relatives may be used for reference samples
Human migration and evolutionary studies	Lack of recombination enables comparison of male individuals separated by large periods of time
Historical and genealogical research	Surnames usually retained by males; can make links where paper trail is limited

Table 1: Areas of Use in Y-Chromosome Testing (8)

Y-STRs have been used in conjunction with autosomal STR typing in cases of mass disaster victim identification (9), in cases of historical research (10, 11), and examining phylogenetic relationships between human populations (12). Table 1 from Butler (8) on Y-STRs and gives a brief overview of the multiple applications of Y-STR testing.

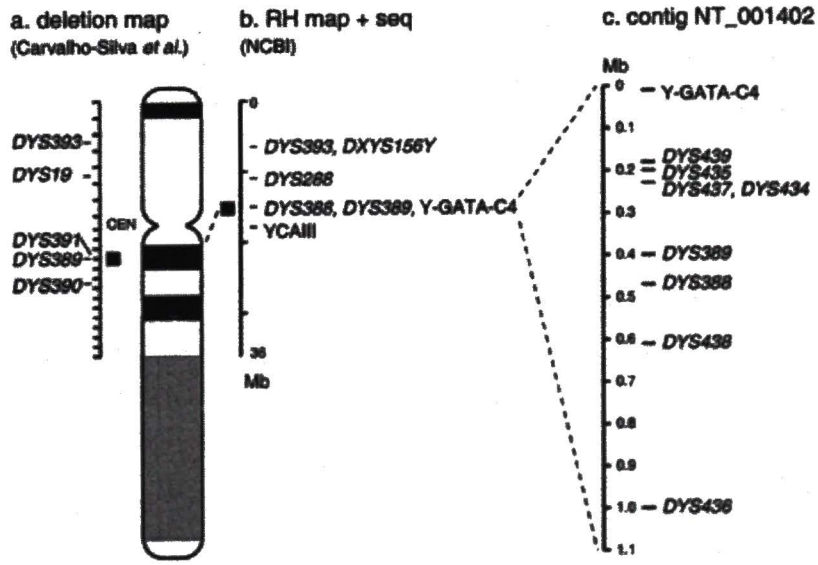

Figure 5: Locations of Common and New Y-STR Markers (21)

A minimal haplotype was decided upon in 1997 by Kayser et al. in order to create a male identification system for forensic applications and to capitalize on the discriminatory potential of the Y-chromosomal DNA (6). The minimal haplotype included the Y-STRs DYS19, DYS389I/II, DYS390, DYS391, DYS392, DYS393 for standard Y-typing with the addition of DYS385a/b, and YCAIIa/b which allows for additional discrimination (6). Additional markers continue to be developed and validated for use in forensic casework (8). Figure 5 depicts chromosomal locations for mapped loci, known loci identified in the sequence information from GenBank, and positions of known and novel loci in more detail (21).

Applied Biosystems' AmpFℓSTR® Yfiler™ PCR Amplification Kit

The kit is a short tandem repeat (STR) multiplex assay that amplifies 17 Y-STR loci within a single PCR reaction (17). The loci amplified in the kit include: the European minimal haplotype (DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393), the SWGDAM recommended Y-STR panel (the European minimal haplotype plus DYS438 and DYS439), and additional loci (DYS437, DYS448, DYS456, DYS458, DYS635 (Y GATA C4), and Y GATA H4).

Table 2 shows the loci that are amplified by the Yfiler™ kit and corresponding dyes that are used in the kit. The Yfiler™ ladder is used to assign the samples genotypes. The table shows the alleles contained within the ladder and also shows the genotype of the Control DNA 007.

Locus Designation	Alleles Included in Yfiler Kit Allelic Ladder[a]	Dye Label	DNA 007 Genotype
DYS456	13–18	6-FAM™	15
DYS389I	10–15		13
DYS390	16–27		24

Locus Designation	Alleles Included in Yfiler Kit Allelic Ladder[a]	Dye Label	DNA 007 Genotype
DYS389II	24–34		29
DYS458	14–20	VIC®	17
DYS19	10–19		15
DYS385 a/b	7–25		11,14
DYS393	8–16	NED™	13
DYS391	7–18		11
DYS439	8–15		12
DYS635	20–26		24
DYS392	7–16		13
Y GATA H4	8–13	PET®	13
DYS437	13–17		15
DYS438	8–13		12
DYS448	17–24		19

Table 2: AmpFℓSTR® Yfiler™ Kit Loci and Alleles (17)

Figure 6 depicts the Yfiler™ ladder that is used to genotype and analyze the unknown samples. The typical workflow for obtaining Y-STR data is fairly straightforward and identical to obtaining autosomal STR data with the exception of the use of different kits and reagents. The first step always includes DNA extraction followed by quantification. However, Figure 7 depicts the use of either the Quantifiler® kit or the Quantiblot® kit. The Quantifiler® kit is commonly used in forensic labs today. The Quantiblot® kit, on the other hand, is no longer available for purchase and has been discontinued because of the advances in the quantification technology.

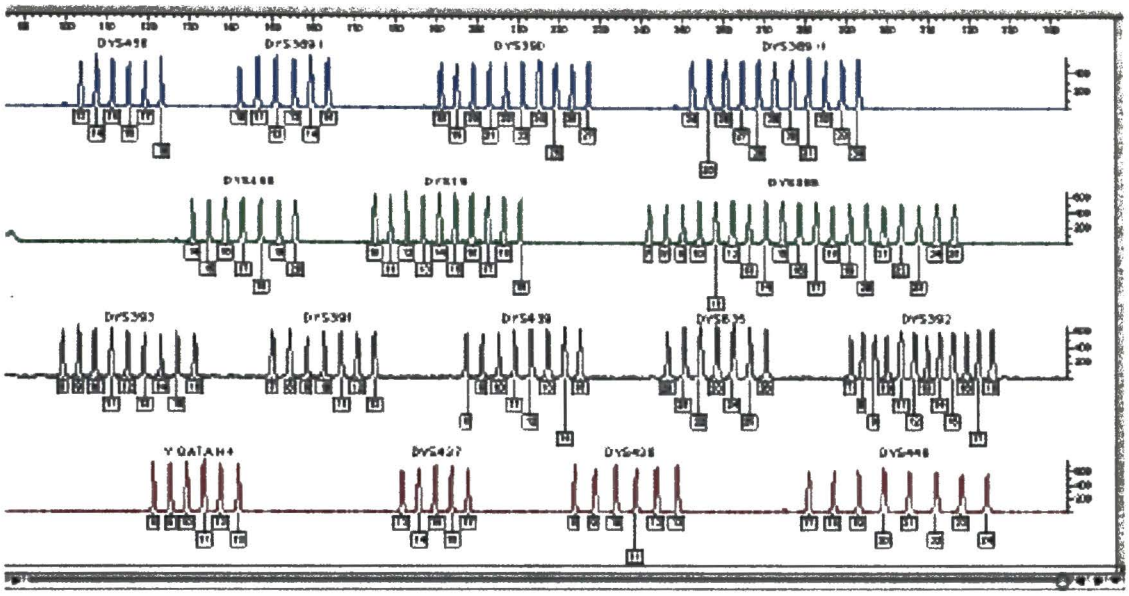

Figure 6: Genotyper® software plot of the AmpFℓSTR® Yfiler™ Allelic Ladder (17)

As for the quantification step, for this project, the duplex gender real-time PCR assay was used to quantify the Y-DNA. The PCR process, for this project, was performed on the GeneAmp® PCR System 9700 Thermal Cycler. Capillary electrophoresis was performed on an ABI PRISM® 310 Genetic Analyzer, and the data was analyzed on the GeneMapperID™ Software v.3.2.

The fluorescent multi-color dye technology allows multiple loci to be analyzed, including loci that have alleles with overlapping size ranges. Any alleles that fall within overlapping loci are differentiated by labeling locus-specific primers with different dye colors (17).

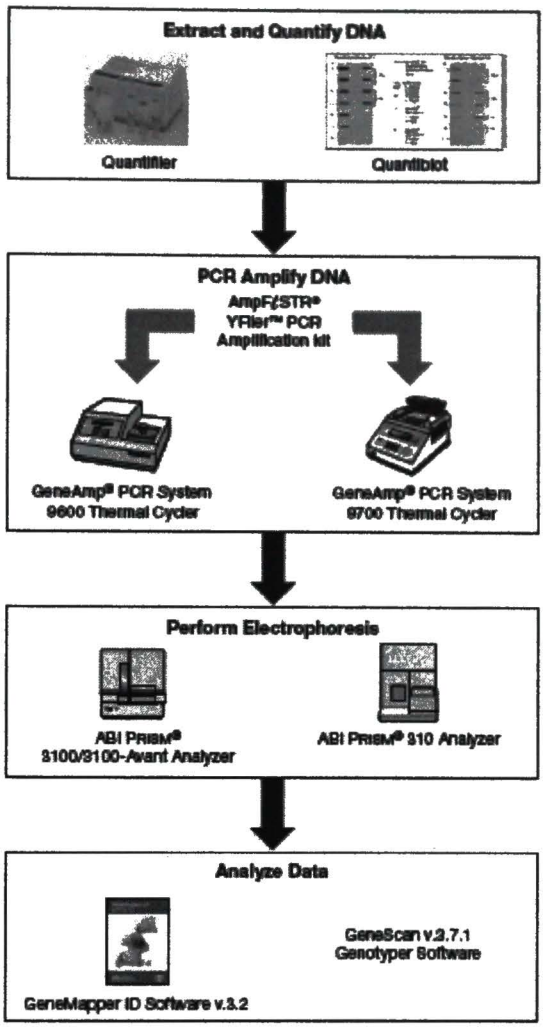

Figure 7: Y-STR Workflow (17)

In order to analyze the data, a process called multicomponent analysis is necessary to separate the five different fluorescent dye colors into specific spectral components. The fluorescent sample labeling dyes used in the Yfiler™ kit are 6-FAM™, VIC®, NED™, and PET® dyes. LIZ®, a fifth dye is used to label the GeneScan™-500 Size Standard (17).

Each of the fluorescent dyes emits a maximum fluorescence at a different wavelength. The data collection process on the ABI PRISM® instruments includes a procedure that separates the fluorescent signals by a diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera in a predictably spaced pattern. There is some overlap in the emission spectra between dyes and the multicomponent analysis aims to effectively correct for the spectral overlap (17).

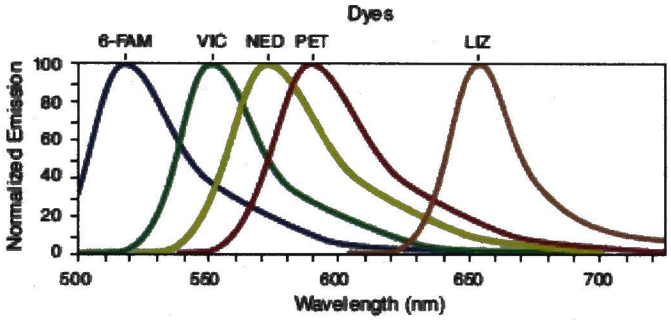

Figure 8: Emission Spectra of Dyes used in the AmpFℓSTR® Yfiler™ PCR Amplification Kit (17)

The standards used in the kit included Control DNA 007 which is a positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR® Yfiler™ Kit Allelic Ladder. Furthermore, another standard used is the GeneScan-500 LIZ Size Standard which is used for obtaining base pair sizing results and this standard has been evaluated as an internal lane size standard yielding precise sizing results for AmpFℓSTR® Yfiler™ kit PCR products (17). And finally the AmpFℓSTR® Yfiler™ Allelic Ladder is used in the kit as previously described.

Capillary Electrophoresis

Capillary electrophoresis is the process of separating and detecting DNA fragments. DNA fragments are drawn into a capillary through the action of electrokinetic injection and pass through a polymer which functions as a sieve that allows the faster migration of smaller fragments of DNA and the slower migration of larger fragments of DNA. During cycle sequencing, samples are labeled with dye that will fluoresce upon laser excitation as it passes through a detection window on the capillary electrophoresis instrument. The fluorescence is measured as a function of the time from the sample injection to the time the sample DNA fragments pass by the detection window (13). Figure 4 represents a schematic illustration of the process of separation and detection of STR alleles using an ABI PRISM® 310 Genetic Analyzer (13).

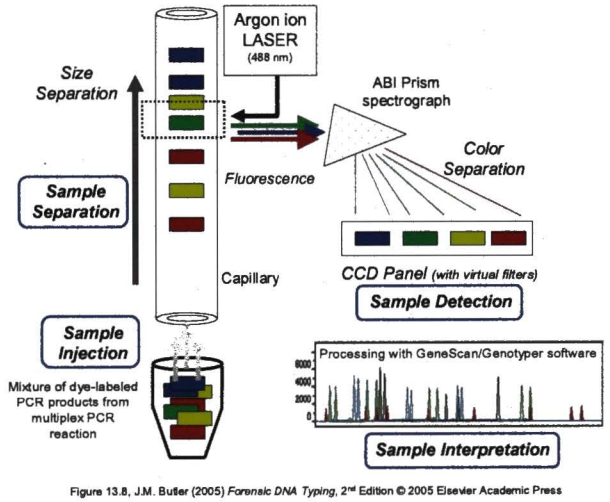

Figure 9: Schematic Illustration of Capillary Electrophoresis (13)

CHAPTER IV

MATERIALS AND METHODS

All reagents necessary for the duplex gender assay were provided by Eric Buel, Ph.D. and Janice Nicklas, Ph.D. at the Vermont Forensic Laboratory in Waterbury, VT. All other reagents necessary for performing DNA analysis including the AmpFℓSTR® Yfiler™ PCR Amplification Kit were provided by the Broward Sheriff's Office (BSO) DNA Unit in Fort Lauderdale, FL. All of the research conducted for this project was performed in the Broward Sheriff's Office DNA Laboratory.

STUDY 1: Assay Sensitivity

The purpose of this study was to evaluate and determine the lowest concentration of DNA that could be added to the duplex gender assay and still obtain any useful genetic information with the Yfiler™ kit. The study consists of serial dilutions of known concentrations of DNA (500pg, 250pg, 125pg, 62.5pg, 31.25pg, 15.63pg, 12.5pg, 6.25pg, 3.13pg, 1.6pg, 0.8pg, 0.4pg). There are optimal amounts or concentrations of input DNA that are suggested and must be validated in a laboratory that will provide complete profiles when analyzed. However, even if a complete profile is not possible or is not obtained, any Y-STR data could be useful in the exclusion of a male individual. In validation studies, the optimal concentration of input DNA into the PCR reaction is determined in order to provide a complete genetic profile. In the sensitivity study, a threshold of quantifiable DNA was attempted to be determined to provide any useful

genetic material or that will yield any degree of a partial genetic profile. The same analyses were done on diluted samples from unrelated male volunteers.

STUDY 2: Standard Curve Consistency Study

The purpose of this study was to evaluate the consistency of the standards used to generate the standard curve and in turn, provide consistent quantification values on samples of unknown DNA concentrations. The study consisted of triplicate samples for each standard as well as four NTC samples that were run on the same 96-well plate. The Ct values were compared for each repeat for each standard and then the means and standard deviation were calculated to give an estimate as to the consistency of the standards used in the duplex gender assay.

STUDY 3: Instrument Adaptation Study

The purpose of this study was to evaluate the performance of the duplex assay on an ABI PRISM® 7000 Sequence Detection System. The real-time PCR research and development for this duplex assay was performed primarily on a Stratagene MX 3000P and occasionally on a Corbett Rotorgene 3000 (San Francisco, CA). For the possibility of future applications in the forensic field, it is extremely important that any kits or assays be adaptable to the instruments and technology being used in most forensic laboratories.

DNA Sample Preparation

Pre-quantified, human genomic DNA extracts were obtained from Janice Nicklas, Ph.D. and Eric Buel, Ph.D. that were originally purchased from Promega Corporation

(male control DNA G1471, Madison, WI). Pre-quantified DNA extracts obtained from Applied Biosystems (male DNA control 007 from AmpFℓSTR® Yfiler™ PCR Amplification Kit and Human DNA Standards from Quantifiler® Kit) were used to generate a dilution series to evaluate the assay's sensitivity. Buccal swabs were taken from three unrelated male volunteers to be used in the sensitivity study.

A dilution series was prepared using two DNA samples: the known male control DNA 007 from Applied Biosystems (Foster City, CA) and the extracted samples from male volunteers. The male volunteer sample that yields the lowest quantification value was taken and further diluted to decrease the reported concentrations even more.

Table 3 depicts the concentrations and preparation instructions of standards that were used in the assay.

	[Concentration]:	Preparation:
Sample 1	64ng/µl Stock	
Sample 2	16ng/µl	10µl [64ng/µl] + 30µl TE-4
Sample 3	4ng/µl	10µl [Sample 2] + 30µl TE-4
Sample 4	1ng/µl	10µl [Sample 3] + 30µl TE-4
Std. 1	0.25ng/µl	10µl [Sample 4] + 30µl TE-4
Std. 2	0.0625ng/µl	10µl [Std. 1] + 30µl TE-4
Std. 3	0.01563ng/µl	10µl [Std. 2] + 30µl TE-4
Std. 4	0.00391ng/µl	10µl [Std. 3] + 30µl TE-4
Std. 5	0.00098ng/µl	10µl [Std. 4] + 30µl TE-4

Table 3: Duplex Gender Assay Quantification Standards

DNA Extraction

DNA extracts from the male volunteer samples were obtained using the BSO's DNA Unit Laboratory Automated Extraction Protocol. The process of DNA extraction is very important because it lyses the cells found in biological material so that the genetic

material can be drawn into solution. The extraction process also separates the DNA from other cellular materials and proteins which can adversely affect the DNA analysis process. For this project, the QIAGEN® BioRobot® 8000 liquid handler robot along with the QIAsoft 4.1 software set-up were used for the automated extraction of buccal swabs. The automated extractions were performed with the Promega DNA IQ™ System DNA extraction kit (Madison, WI). The extraction kit utilizes a magnetic Resin particle with an affinity for binding DNA. The extraction protocols were performed in accordance with the Broward Sheriff's Office Crime Laboratory DNA Unit-Analytical Methods Manual

qPCR Quantification

The gender duplex assay developed by Nicklas and Buel (3) was used to quantify male DNA on an Applied Biosystems' 7000 Prism® Sequence Detection System qPCR instrument. The duplex human/Y DNA quantitation assay utilized the Absolute QPCR Mix (ABgene, Rochester, NY). The master mix included 10µl of ABgene Mix, 1.912µl of the Primer/Probe/BSA Mix, and 6.088µl of H_2O per sample or standard. The total reaction volume was 20 µl: 18 µl of reaction mix and 2 µl of sample, standard, or control. The 20µl reactions contained 1X mix, 100 µM *Alu* forward primer, 200 µM *Alu* reverse primer, 200 µM DYZ5 forward primer, 100 µM DYZ5 reverse primer, 200 µM each probe, and 160 ng/µL BSA (A-9647, Sigma, St. Louis, MO).

The cycling parameters were: 95°C for 15 min hotstart, followed by 45 cycles of 95°C for 30 s, 60°C for 1 min. Data was collected using the ABI 7000 PRISM® SDS Collection Software.

Y-STR Amplification

DNA was amplified with the AmpFℓSTR® Yfiler™ PCR Amplification Kit using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The input volume of DNA was 10µl and 15µl of PCR reaction mix, for a total of 25µl total reaction volume. Negative controls (TE-4 buffer), positive controls (007), and a female control cell line 9947a (Applied Biosystems, Foster City, CA) were used to check for contamination. The cycling parameters for Applied Biosystems' AmpFℓSTR® Yfiler™ PCR Amplification Kit were: initial incubation step- 95°C for 11 min; 30 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 1 min; final extension of 60°C for 80 min; and a final hold of 4°C.

Electrophoresis and Analysis using ABI PRISM® 310 Genetic Analyzer

Conditions for the electrophoretic runs were according to manufacturer's specifications and injection times were adjusted if needed to obtain the best results possible. Data was collected by the ABI PRISM® 310 Genetic Analyzer Data Collection Software v. 1.2.2 and analyzed using the GeneMapperID™ Analysis Software (Applied Biosystems, Foster City, CA). The data was analyzed at a threshold level of 75 RFU and with the Yfiler™ panel.

Statistical Analysis

When comparing values, the standard deviation is commonly used to assess the spread of a set of values. The standard deviation is the square root of the variance where the variance is an average of the squared differences between data points and the mean

(18). The percent standard deviations were calculated by dividing the standard deviations by the mean and multiplying by 100 (20).

Sensitivity Study

The RFU levels, base pair sizes, and number of loci called were analyzed for the sensitivity study samples. The concentration values that were obtained in the quantification step were compared to the number of loci called in each sample and a consistent correlation, if any, between quantification value and loci called were determined.

Standard Curve Consistency Study

The mean values, standard deviations, and percent standard deviations of the Ct values generated by the duplex gender assay were calculated to assess the consistency in Ct values for the standard curve triplicate values on the same 96-well plate and then were compared to the original values calculated in the developmental research of the duplex gender assay. According to Janice Nicklas, the percent standard deviations were calculated by dividing the standard deviations by the mean and multiplying by 100 (Janice Nicklas, electronic mail, July 10, 2007). The range of the percent standard deviations and the range of the standard deviations were compared to the values obtained in the original publication of this duplex assay.

CHAPTER V

RESULTS AND DISCUSSION

Sensitivity Study

The sensitivity study was performed by creating serial dilutions from known DNA concentrations in order to evaluate the performance of the duplex gender real-time PCR assay. The dilution concentrations were calculated from two control DNA extracts (200ng/µl and 0.1ng/µl) to be: 500pg, 250pg, 125pg, 62.5pg, 31.25pg, 15.63pg, 12.5pg, 6.25pg, 3.13pg, 1.6pg, 0.8pg, and 0.4pg. The values determined for each probe for every sample run in triplicate in the dilution series were evaluated. The quantification values are listed in Table 4(A-C) and the determined concentrations are listed in (pg/µl). Table 4-A lists the quantification values obtained for the *Alu* probe over the replicate sampling as well as the mean, standard deviation, and percent standard deviation.

(A)	*Alu* Quantification Values				Standard	% Standard
Sample	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Mean**	**Deviation**	**Deviation**
~500pg	305.00	348.00	273.00	308.67	37.63	12.19
~250pg	141.00	147.00	166.00	151.33	13.05	8.62
~125pg	76.60	66.20	78.10	73.63	6.48	8.80
~62.5pg	29.20	32.30	28.30	29.93	2.10	7.01
~31.25pg	16.70	14.50	14.60	15.27	1.24	8.14
~15.63pg	7.13	7.13	6.63	6.96	0.29	4.15
~12.5pg	7.06	8.91	8.14	8.04	0.93	11.56
~6.25pg	3.82	4.69	4.61	4.37	0.48	11.00
~3.13pg	2.10	2.04	1.93	2.02	0.09	4.26
~1.6pg	1.20	0.87	0.96	1.01	0.17	16.89
~0.8pg	0.56	0.44	0.57	0.52	0.07	13.82
~0.4pg	0.27	0.22	0.27	0.25	0.03	11.40

(B)	DYZ5 Quantification Values				Standard	% Standard
Sample	Repeat 1	Repeat 2	Repeat 3	Mean	Deviation	Deviation
~500pg	233.00	231.00	244.00	236.00	7.00	2.97
~250pg	119.00	99.50	117.00	111.83	10.73	9.59
~125pg	58.40	60.50	64.20	61.03	2.94	4.81
~62.5pg	28.40	30.00	34.60	31.00	3.22	10.38
~31.25pg	16.10	14.50	13.70	14.77	1.22	8.28
~15.63pg	6.22	6.29	5.95	6.15	0.18	2.92
~12.5pg	13.00	6.46	6.88	8.78	3.66	41.69
~6.25pg	3.63	2.13	3.26	3.01	0.78	25.99
~3.13pg	1.57	0.96	1.66	1.40	0.38	27.27
~1.6pg	1.90	0.20	0.96	1.02	0.85	83.49
~0.8pg	0.67	NR	0.20	0.44	0.34	79.07
~0.4pg	0.26	NR	0.43	0.35	0.22	62.77

(C)	DYZ5:*Alu* Ratio				Standard	% Standard
Sample	Repeat 1	Repeat 2	Repeat 3	Mean	Deviation	Deviation
~500pg	0.76	0.66	0.89	0.77	0.12	14.90
~250pg	0.84	0.68	0.70	0.74	0.09	12.06
~125pg	0.76	0.91	0.82	0.83	0.08	9.16
~62.5pg	0.97	0.93	1.22	1.04	0.16	15.22
~31.25pg	0.96	1.00	0.94	0.97	0.03	3.20
~15.63pg	0.87	0.88	0.90	0.88	0.01	1.43
~12.5pg	1.84	0.73	0.85	1.14	0.61	53.88
~6.25pg	0.95	0.45	0.71	0.70	0.25	35.24
~3.13pg	0.75	0.47	0.86	0.69	0.20	28.94
~1.6pg	1.58	0.23	1.00	0.94	0.68	72.39
~0.8pg	1.20	NR	0.35	0.77	0.62	79.50
~0.4pg	0.96	NR	1.59	1.28	0.80	62.77

Table 4: Quantification Values of Sample Replicates

Table 4-B lists the quantification values obtained for the DYZ5 probe over the replicate sampling as well as the mean, standard deviation, and percent standard deviation. Table 4-C lists the DYZ5: *Alu* ratio that was calculated by taking the DYZ5 determined concentration and dividing it by the *Alu* determined concentration, the mean, standard deviation, and percent standard deviation for each of the ratios. Even though some of the ratios are larger that 1.0, it has been reported that normal males can vary between 67% and 150% in the ratio calculation (3).

Overall, the *Alu* probe quantified the samples at a higher concentration than the DYZ5 probe. The difference in quantification values is most likely due to the nature of the multicopy *Alu* gene being reported to have a higher sensitivity when compared to a single-copy gene (3). The results from Table 4-C appear to be consistent with the nature of the samples being all male samples. The known DNA standard used to prepare the serial dilutions was purified from a single male source. The ratio functions to determine the approximate percentage of male DNA to female DNA present within a sample. An ideal ratio value for a single source male sample would be approximately 1.0, while a single source female sample should have a ratio value of 0.0.

Furthermore, the difference in the standard dilution calculations as compared to the actual quantified values is fairly significant. Beyond the normal variation due to pipetting errors or inaccuracies, the difference seen might be able to be attributed to the actual concentration of the known DNA standard. The control DNA from Promega (Madison, WI) was used to prepare the standards in the assay while a control DNA from Applied Biosystems (Foster City, CA) was used to prepare most of the samples with the exception of the volunteer samples. The difference may lie in the reported concentrations of the standards.

The DNA control that was used for the lower threshold samples (15.63pg-0.4pg) was the 007 Control DNA from the Yfiler™ kit (0.1ng/µl). The DNA control that was used for the upper threshold dilution samples (500pg-15.65pg) was the Human DNA Standards from the ABI Quantifiler Y Kit (200ng/µl). The profiles are different; however, the information obtained is still valuable.

Locus	~500pg	~250pg	~125pg	~62.5pg	~31.25pg	~15.63pg	~12.5pg	~6.25pg	~3.13pg	~1.6pg	~0.8pg	~0.4pg
B_DYS456	1652	575	603	331	240	114	NR	NR	NR	NR	NR	NR
B_DYS389I	1378	336	603	231	NR	NR	NR	76	NR	NR	NR	NR
B_DYS390	1640	477	627	224	NR	NR	128	105	NR	NR	NR	NR
B_DYS389II	1346	390	529	207	110	NR	NR	88	NR	NR	NR	NR
G_DYS458	2256	816	1111	318	339	106	188	119	NR	NR	NR	NR
G_DYS19	2483	729	1156	337	130	83	251	NR	NR	81	NR	NR
G_DYS385-Allele 1	2058	561	686	313	220	83	NR	NR	100	NR	NR	NR
Allele 2	1841	577	845	296	121	NR	NR	110	NR	NR	NR	NR
Y_DYS393	1677	555	992	207	209	NR	NR	113	NR	NR	NR	NR
Y_DYS391	3069	885	1193	399	307	211	NR	NR	NR	NR	NR	NR
Y_DYS439	1590	585	537	307	185	NR	NR	NR	NR	NR	NR	NR
Y_DYS635	1065	318	450	196	NR	NR	NR	NR	NR	NR	NR	NR
Y_DYS392	1692	540	588	200	NR	95	95	NR	NR	NR	80	NR
R_Y_GATA_H4	1883	652	649	304	225	NR	147	77	NR	NR	NR	NR
R_DYS437	1657	552	584	165	140	100	116	NR	NR	NR	NR	NR
R_DYS438	1562	544	402	168	NR	NR	87	NR	NR	NR	NR	NR
R_DYS448	1455	371	583	190	140	104	127	NR	NR	NR	NR	NR

Table 5-A: RFU Values of Repeat 1 Dilution Samples

Locus	~500pg	~250pg	~125pg	~62.5pg	~31.25pg	~15.63pg	~12.5pg	~6.25pg	~3.13pg	~1.6pg	~0.8pg	~0.4pg
B_DYS456	1436	697	666	386	189	NR	587	126	188	NR	NR	NR
B_DYS389I	1347	OL-577	436	133	OL-86	NR	90	144	NR	NR	NR	NR
B_DYS390	1587	720	707	93	86	106	315	NR	161	NR	NR	NR
B_DYS389II	1279	659	501	187	88	127	NR	269	NR	NR	NR	NR
G_DYS458	1908	1142	835	169	101	NR	1022	NR	194	NR	NR	NR
G_DYS19	2306	992	1042	245	301	156	434	203	124	NR	NR	NR
G_DYS385-Allele 1	1749	683	413	143	190	NR	158	213	NR	NR	NR	NR
Allele 2	OL-1618	727	484	OL-156	OL-89	NR	580	NR	NR	NR	NR	NR
Y_DYS393	1748	746	615	139	145	88*	224*	NR*	NR*	NR*	NR*	Drop-In*
Y_DYS391	2292	1080	734	216	OL-76	129	114	123	NR	NR	NR	NR
Y_DYS439	1308	615	679	107	146	79	262	131	NR	NR	NR	NR
Y_DYS635	1051	444	298	91	NR	NR	164	100	NR	NR	NR	NR
Y_DYS392	1156	511	420	207	104	NR	129	NR	NR	NR	NR	NR
R_Y_GATA_H4	1597	724	524	112	165	NR	NR	204	307	NR	NR	NR
R_DYS437	1556	691	409	105	131	NR	203	NR	79	NR	NR	NR
R_DYS438	1612	746	423	205	123	78	222	86	NR	NR	NR	NR
R_DYS448	1192	355	417	94	NR	84	337	123	124	155	NR	NR

Table 5-B: RFU Values of Repeat 2 Dilution Samples

Locus	~500pg	~250pg	~125pg	~62.5pg	~31.25pg	~15.63pg	~12.5pg	~6.25pg	~3.13pg	~1.6pg	~0.8pg	~0.4pg
B_DYS456	853	657	555	439	350	183	261	284	NR	NR	NR	NR
B_DYS389I	449	474	217	197	97	93	246	NR	81	NR	NR	109
B_DYS390	697	628	790	527	88	208	237	241	193	208	NR	NR
B_DYS389II	599	549	341	281	169	88	434	NR	154	NR	86	90
G_DYS458	993	774	758	627	339	195	648	152	104	120	NR	91
G_DYS19	1189	1064	818	693	288	NR	454	NR	178	NR	NR	131
G_DYS385-Allele 1	977	780	546	278	308	101	233	NR	NR	222	NR	NR
Allele 2	893	635	627	605	220	124	319	81	NR	NR	NR	NR
Y_DYS393	779	544	736	287	126	93	147*	121*	164*	NR*	Drop-In*	Drop-In*
Y_DYS391	1089	1025	748	339	416	76	233	NR	NR	NR	NR	NR
Y_DYS439	584	569	391	485	132	NR	NR	123	NR	NR	NR	NR
Y_DYS635	423	373	421	239	87	NR	76	101	NR	NR	NR	NR
Y_DYS392	780	566	610	372	243	NR	154	NR	NR	80	NR	NR
R_Y_GATA_H4	904	658	574	586	162	101	176	NR	82	NR	NR	78
R_DYS437	583	571	545	352	214	81	205	161	NR	99	NR	NR
R_DYS438	671	537	326	427	147	156	669	88	NR	NR	NR	NR
R_DYS448	560	398	456	286	138	NR	306	NR	108	NR	NR	NR

Table 5-C: RFU Values of Repeat 3 Dilution Samples

The RFU levels were evaluated for the repeat sampling of the dilution samples (Table 5-A-C). The lowest value that provided a complete Y-STR profile was 62.5pg sample for the three repeats. However, on repeat three, a full profile is seen as low as the 31.25pg sample, with allelic drop-out beginning with the 15.63pg sample. In Table 5, NR designates no result and the * designates an artifact that was seen regularly at the locus DYS393. The artifact was seen in repeat 2 and 3 beginning with the 12.5pg sample for each repeat. The artifact is visible in the first repeat but because it was run separately from the other repeat samplings, the artifact most likely shifted and did not fall within a bin for those samples in repeat 1. When the artifact fell within a bin, it was consistently called as an allele 9, however, the peak has a wider base than a true allele peak. Figure 10 depicts the artifact seen within many of the samples at the DYS393 locus.

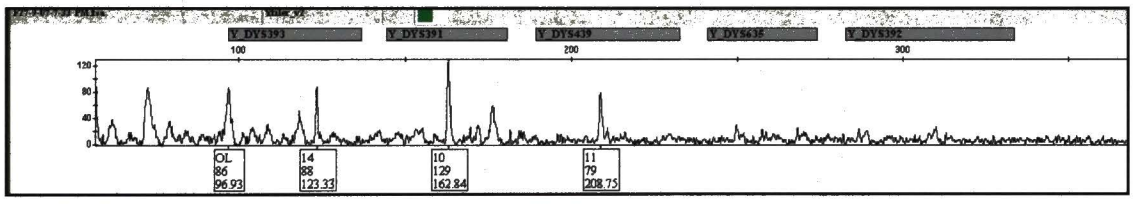

Figure 10: Repeat 2 15.63pg Sample

The samples for repeat 2 are depicted in Figures11-A-L. The figures are depicting the profiles from one sampling batch showing allelic dropout, a stochastic effect, which is caused from too little DNA being present in the sample. As the input concentration of DNA decreases, the baselines for the electropherograms become extremely noisy. The noisy baseline is also due to the low level of DNA present within the samples.

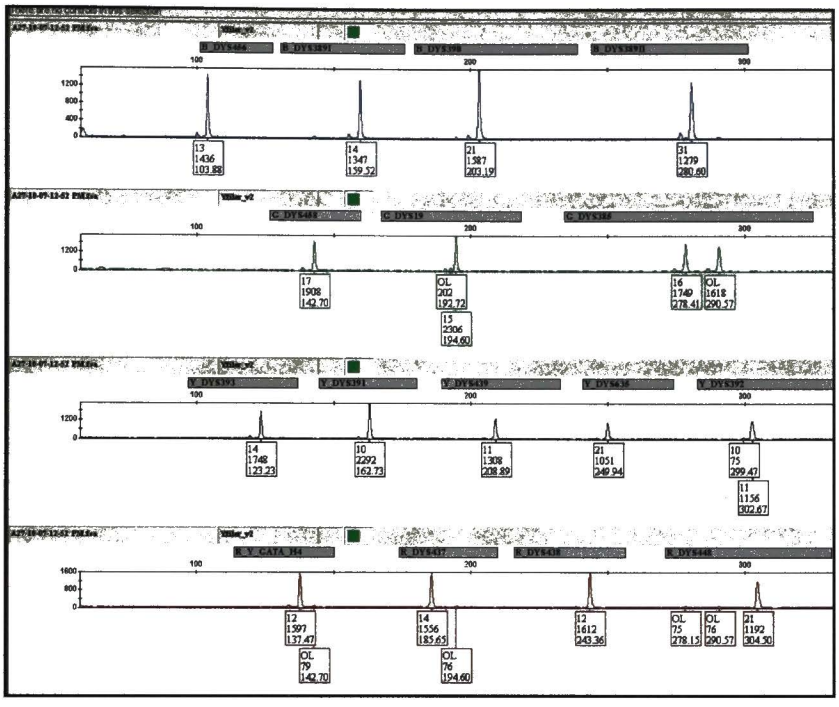

Figure 11-A: ~500pg Sample from Repeat 2

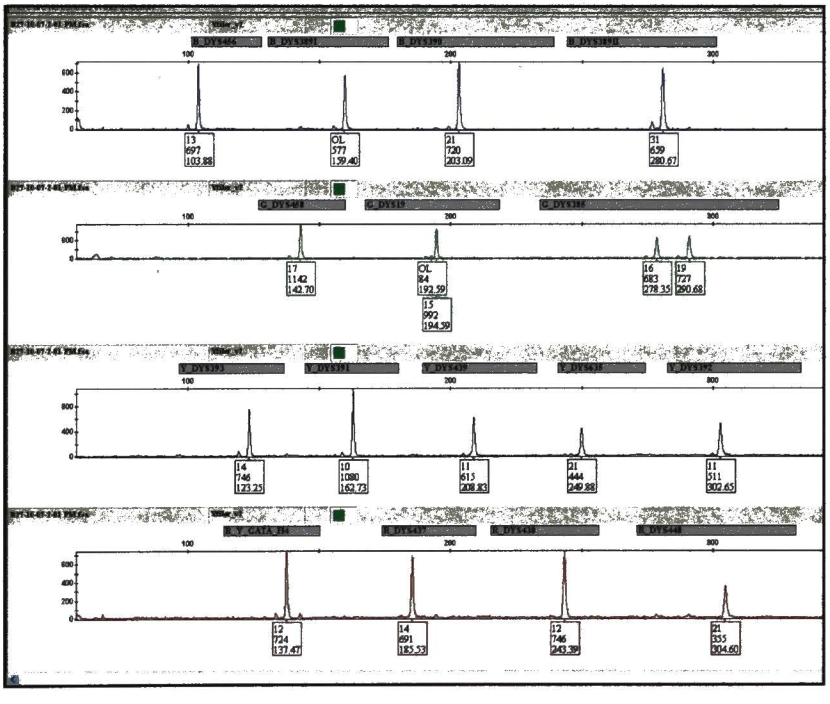

Figure 11-B: ~250pg Sample from Repeat 2

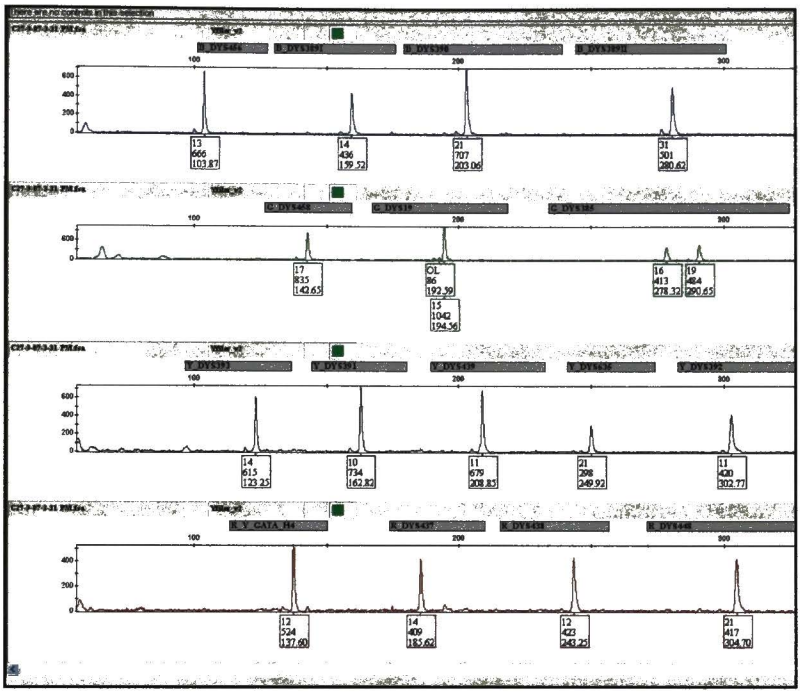

Figure 11-C: ~125pg Sample from Repeat 2

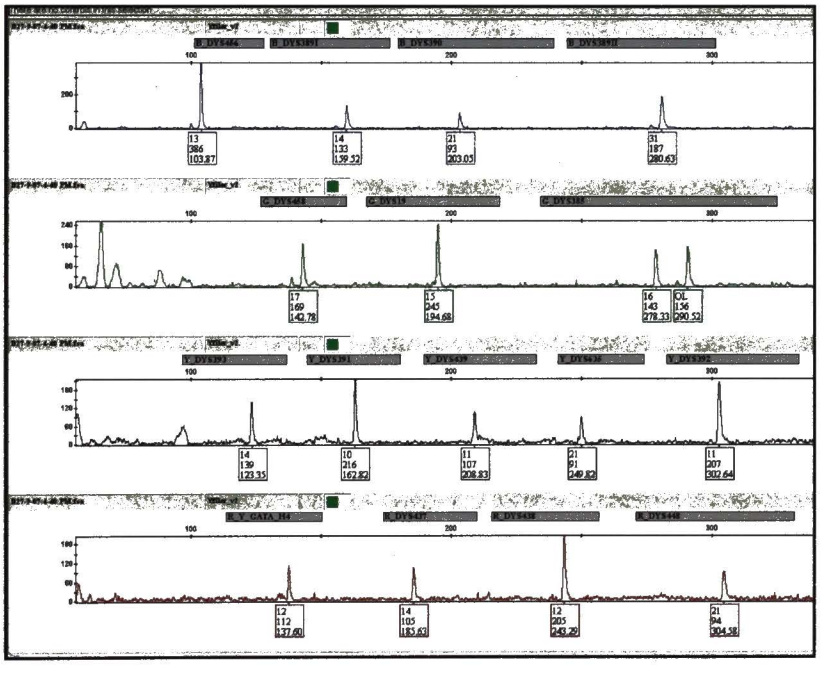

Figure 11-D: ~62.5pg Sample from Repeat 2

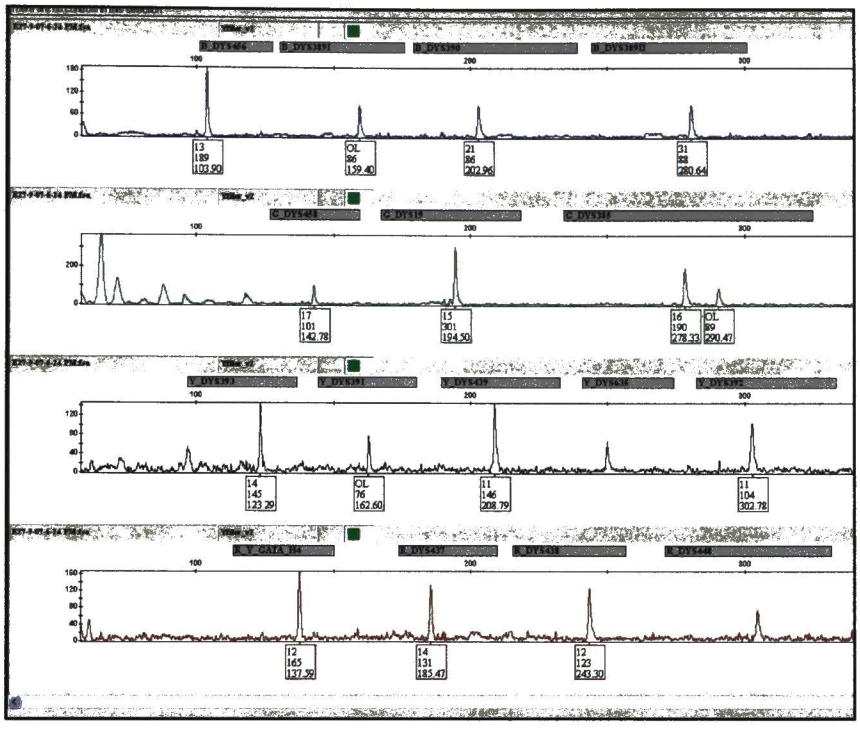

Figure 11-E: ~31.25pg Sample from Repeat 2

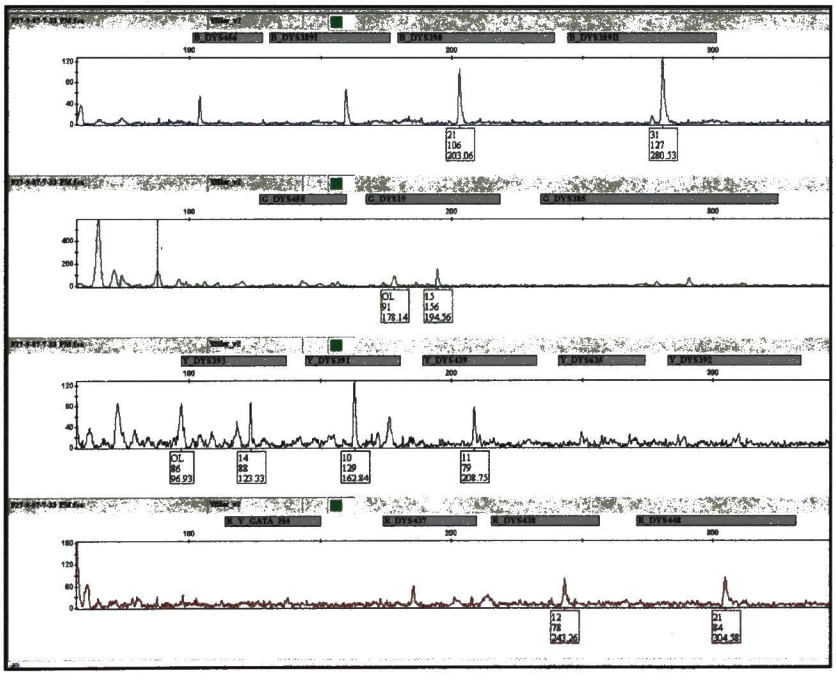

Figure 11-F: ~15.63pg Sample from Repeat 2

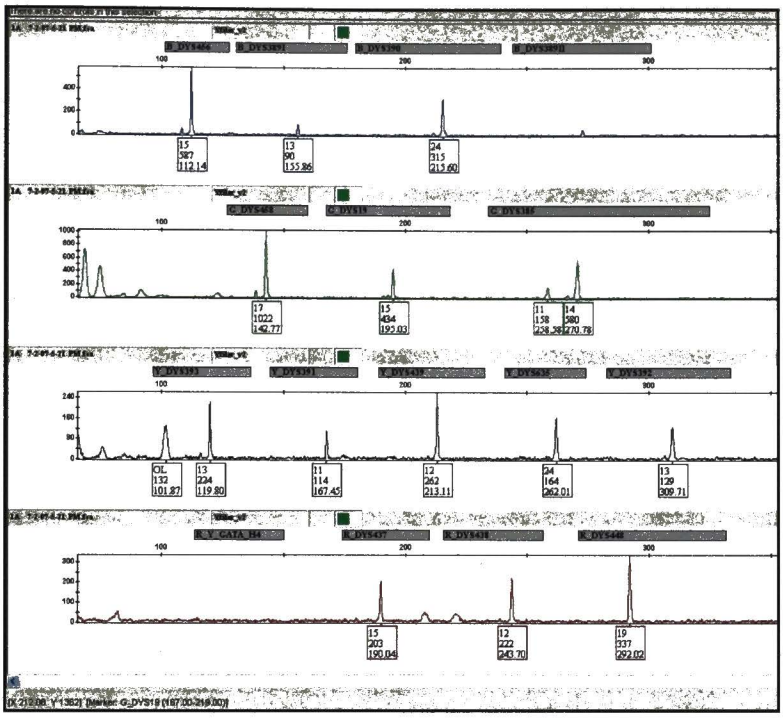

Figure 11-G: ~12.5pg Sample from Repeat 2

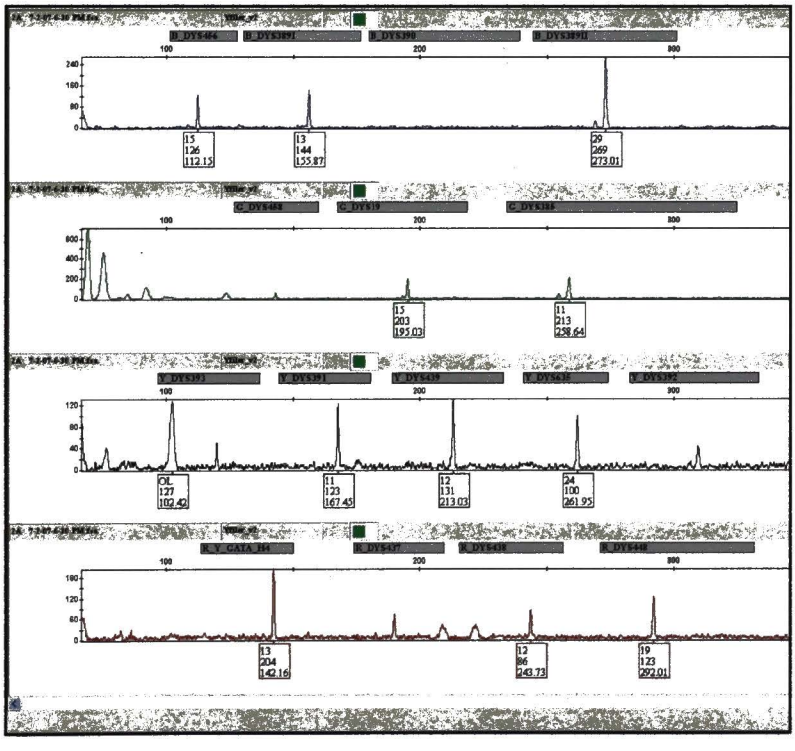

Figure 11-H: ~6.25pg Sample from Repeat 2

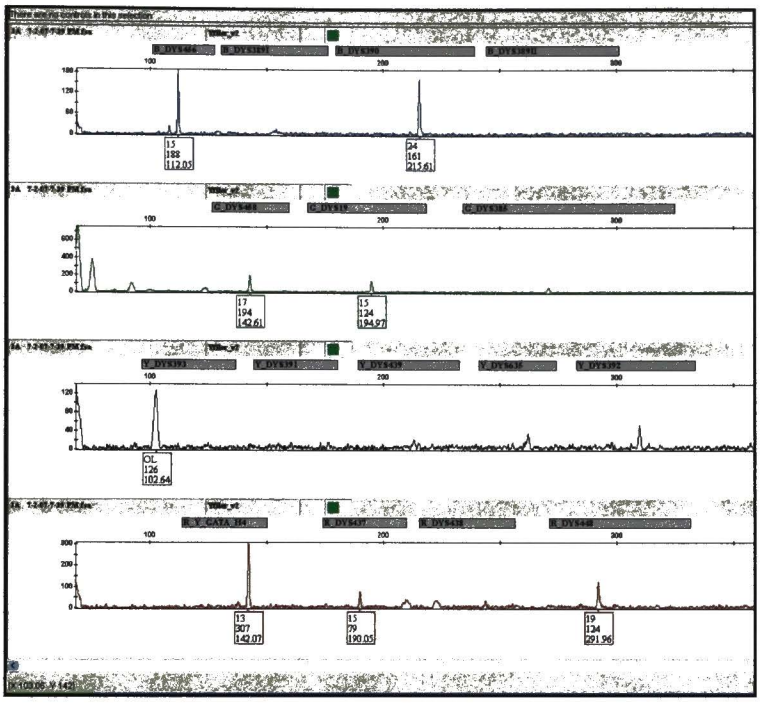

Figure 11-I: ~3.13pg Sample from Repeat 2

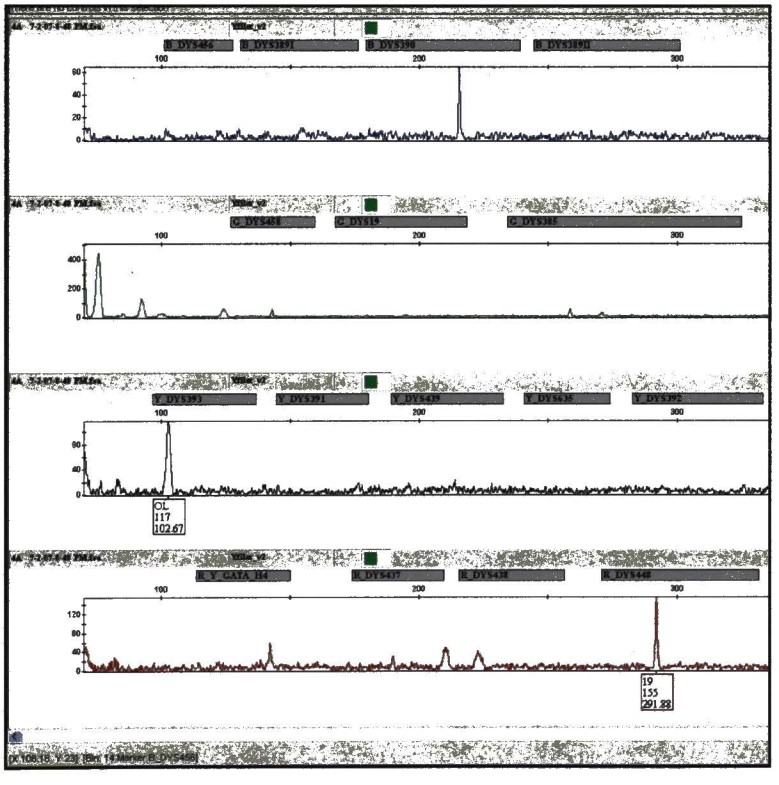

Figure 11-J: ~1.6pg Sample from Repeat 2

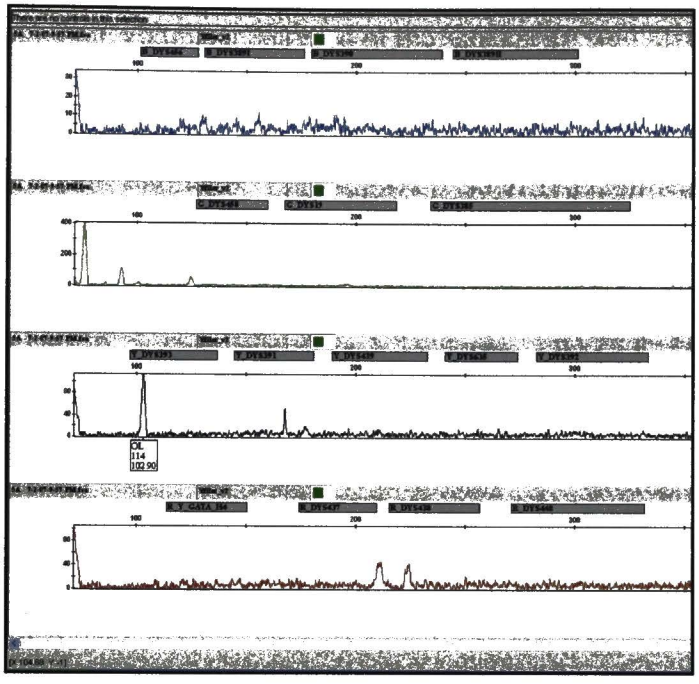

Figure 11-K: ~0.8pg Sample from Repeat 2

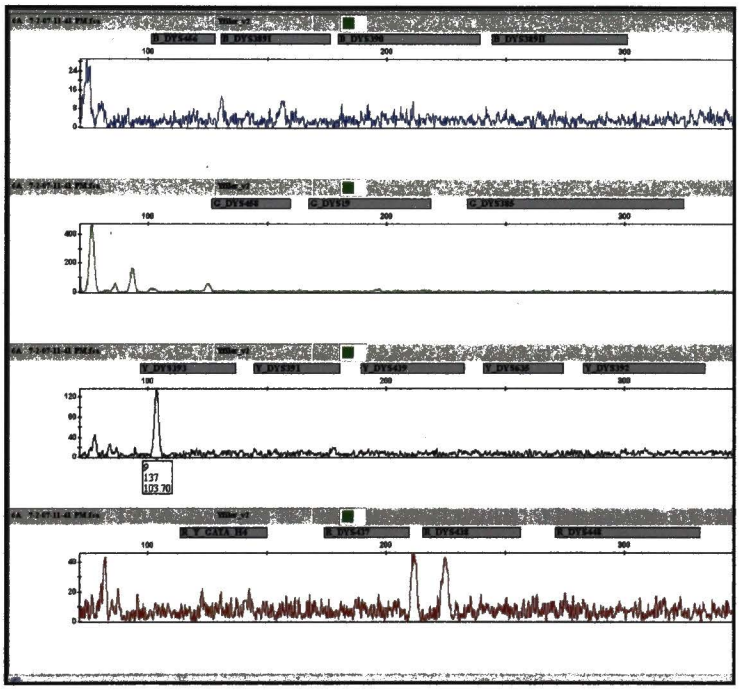

Figure 11-L: ~0.4pg Sample from Repeat 2

The unrelated male volunteer samples in the sensitivity study were extracted by automation and quantified using the Quantifiler™ kit as according to the Broward Sheriff's Office DNA Unit Protocols. The samples were then quantified using the duplex assay and the results were briefly compared in Table 6.

Sample ID	Quantifiler Data	Alu Probe	DYZ5 Probe
KR-E	0.300ng	0.503ng	0.249ng
KR-F	0.140ng	0.277ng	0.100ng
GTD-G	0.069ng	0.070ng	0.034ng
GTD-H	0.100ng	0.056ng	0.022ng
CSC-A	0.352ng	0.566ng	0.369ng
CSC-B	0.322ng	0.547ng	0.370ng

Table 6: Preliminary Quantifiler™ Data and Duplex Gender Assay Data

In four out of the six samples, the quantification value for the DYZ5 probe most closely resembled the quantification values determined by the Quantifiler™ kit. This finding, most likely, is attributed to the fact that the *Alu* probe is reported to be more sensitive and therefore, the value assessed by the DYZ5 probe would be the most similar to the values given by the Quantifiler™ kit which is even demonstrated in just six samples. Even though the quantification data generated from the duplex gender assay most closely resembled the quantification data from the DYZ5 probe, the two quantification systems have not been calibrated to each other so Table 6 may not be an accurate representation of the performance of the two quantification systems. The standard curve in Figure 12-A has an R^2 value of 0.9981. The slope of the standard curve in Figure 12-B has an R^2 value of 0.9969. Figure 12-C depicts the two standard curves shown together in the duplex gender assay.

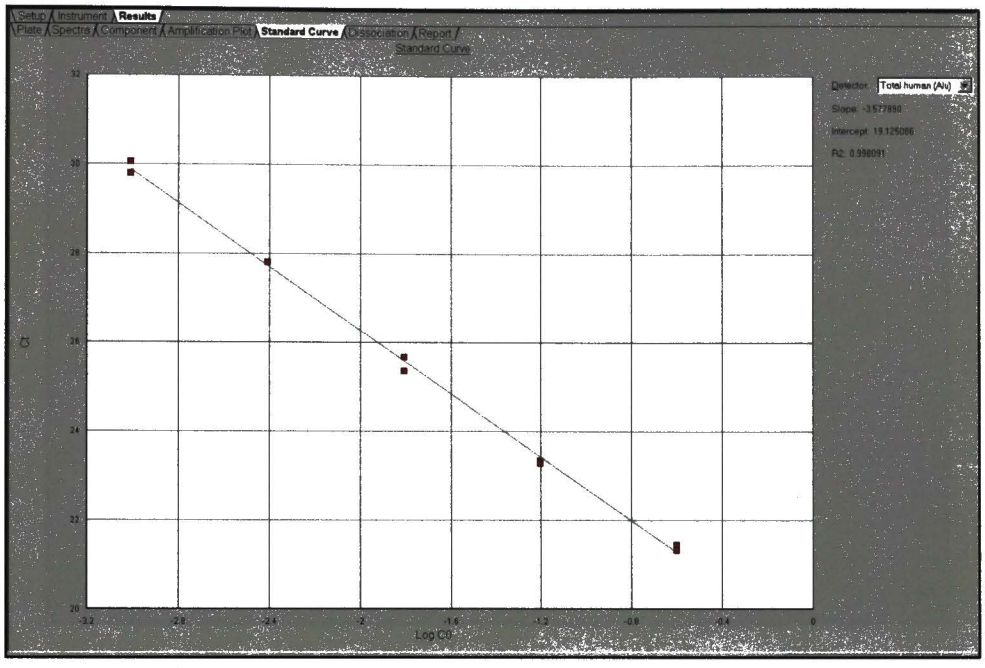

Figure 12-A: Alu Probe Standard Curve of Duplex Assay

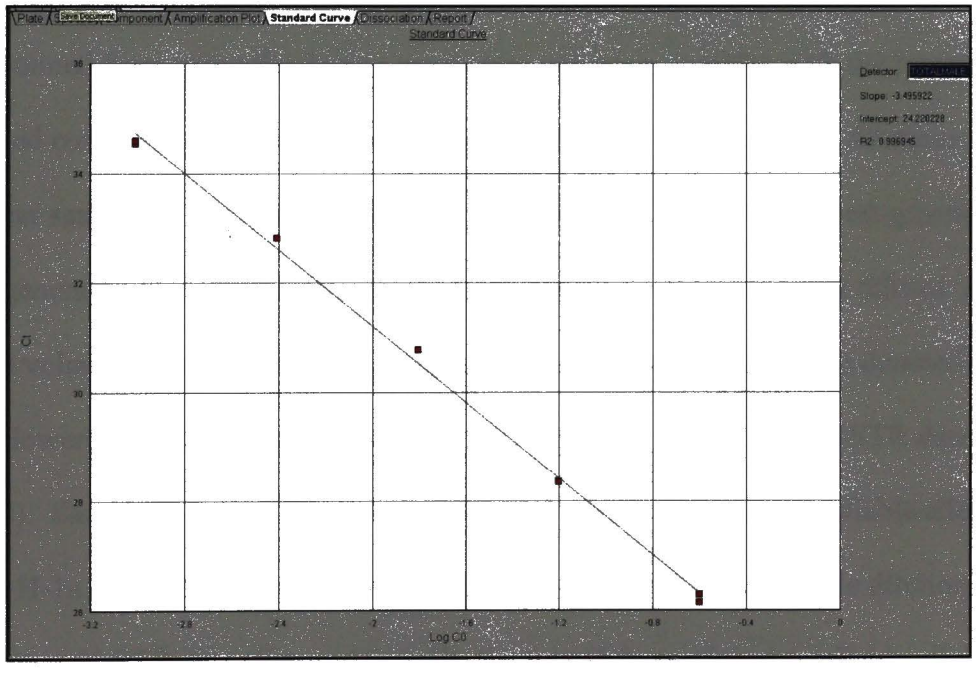

Figure 12-B: DYZ5 Probe Standard Curve of Duplex Assay

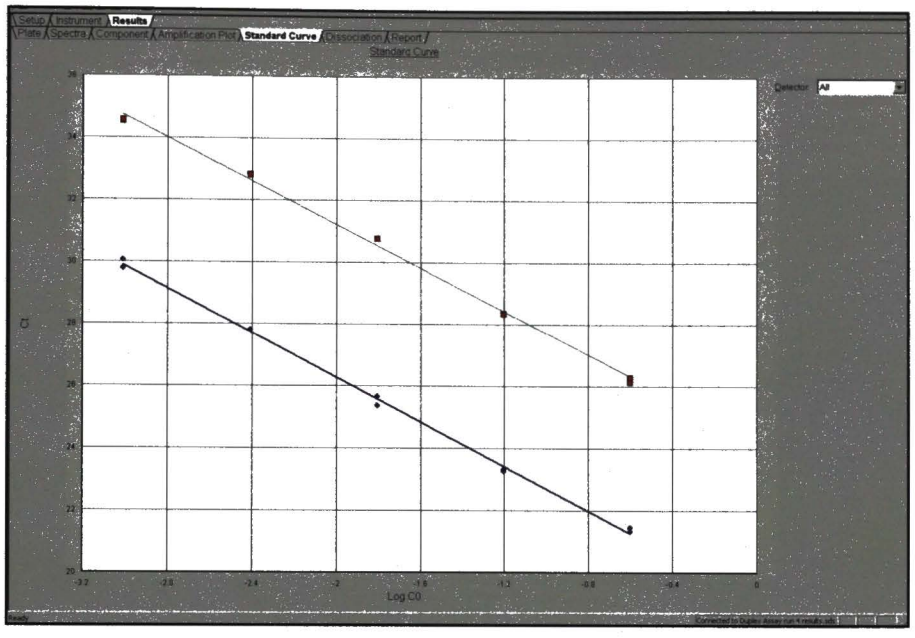

Figure 12-C: Two Standard Curves of Duplex Assay

Figure 13 depicts the standard curve generated from the Quantifiler™ kit. The R^2 value of the standard curve in Figure 16 was 0.995. Once the initial quantification values were obtained from using the duplex assay, two volunteer samples with the lowest quantified concentrations were selected and further diluted in two serial dilutions. Volunteer samples GTD-G and GTD-H were selected as having the lowest quantification values generated from the duplex gender assay (Table 7) and were serial diluted. Each selected volunteer sample was run in duplicate. Each duplicate sample is denoted by using a lowercase 'a' or 'b' after the sample ID. Sample G-1 yielded Y-STR data (Figure 14-A, B). Sample G-1a had 9 callable alleles and sample G-1b had 6 callable alleles. Sample H-1a and H-1b were two samples in the second volunteer sample dilution to produce any Y-STR data (Figure 15-A, B). Sample H-1a produced 4 callable alleles and sample H-1b produced 2 callable alleles.

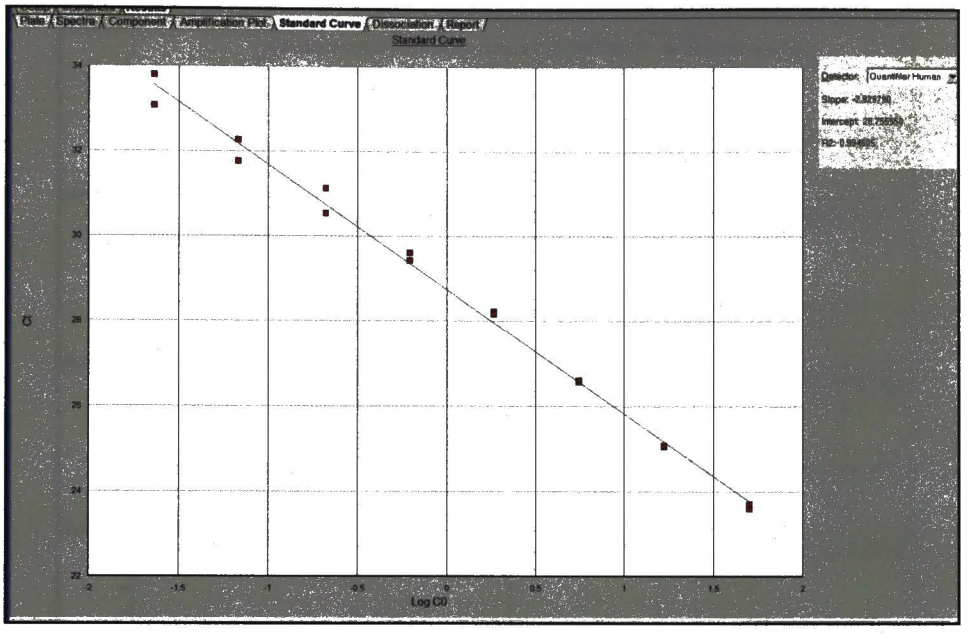

Figure 13: Standard Curve of Quantifiler Kit

Sample:	**GTD-G ~0.0697ng**
G-1	20μl (0.0697ng) + 60μl TE-4 = ~0.0174ng
G-2	20μl (0.0174ng) + 60μl TE-4 = ~0.0044ng
G-3	20μl (0.0044ng) + 60μl TE-4 = ~0.0011ng
G-4	20μl (0.0011ng) + 60μl TE-4 = ~0.00027ng
Sample:	**GTD-H ~0.0556ng**
H-1	20μl (0.0556ng) + 60μl TE-4 = ~0.0139ng
H-2	20μl (0.0139ng) + 60μl TE-4 = ~0.0035ng
H-3	20μl (0.0035ng) + 60μl TE-4 = ~0.00087ng
H-4	20μl (0.00087ng) + 60μl TE-4 = ~0.00022ng

Table 7: Volunteer Sample Dilution Calculations

Sample ID	Calculated [ ]	Alu Probe	DYZ5 Probe
G-1	0.0174ng	0.00762ng	0.00544ng
G-2	0.0044ng	0.00138ng	0.00073ng
G-3	0.0011ng	0.00032ng	0.00013ng
G-4	0.00027ng	0.00012ng	NR
H-1	0.0139ng	0.00727ng	0.00343ng
H-2	0.0035ng	0.00152ng	0.00098ng
H-3	0.00087ng	0.00042ng	0.00025ng
H-4	0.00022ng	0.00073ng	NR

Table 8: Quantification Values of Volunteer Sample Dilutions

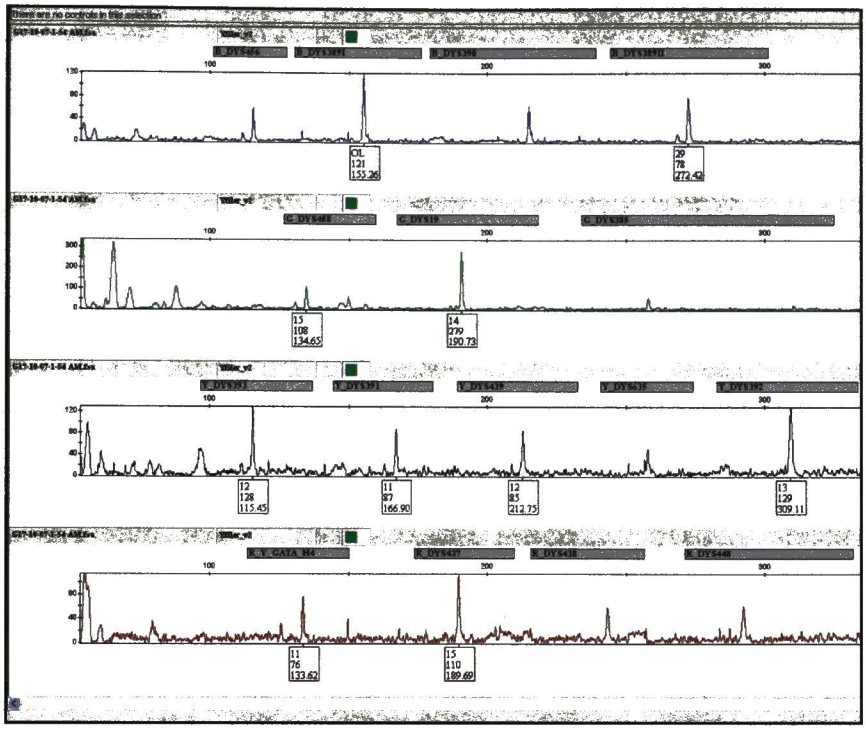

Figure 14-A: Y-STR Data for Sample G-1a

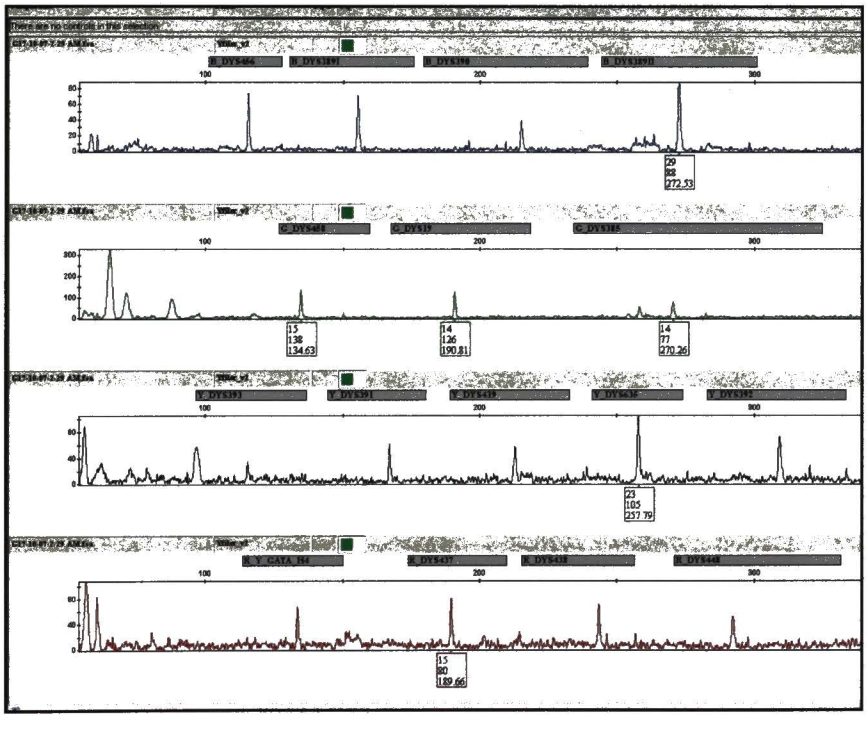

Figure 14-B: Y-STR Data for Sample G-1b

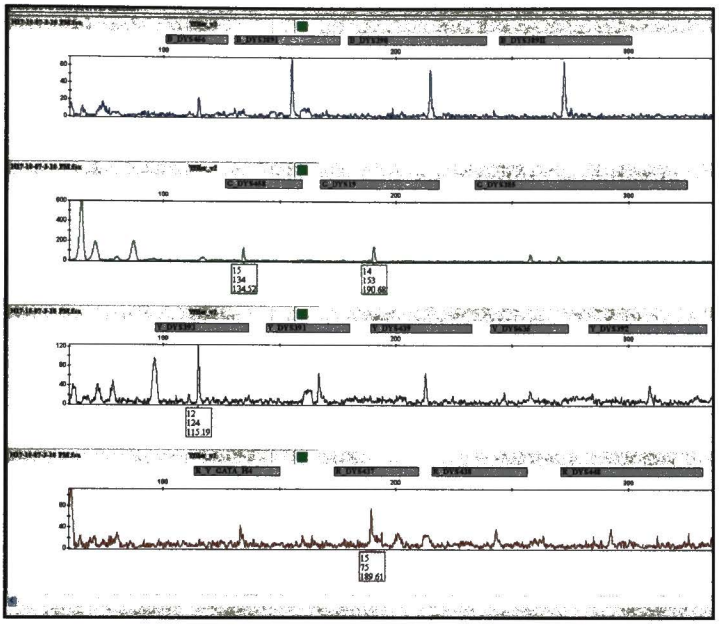

Figure 15-A: Y-STR Data for Sample H-1a

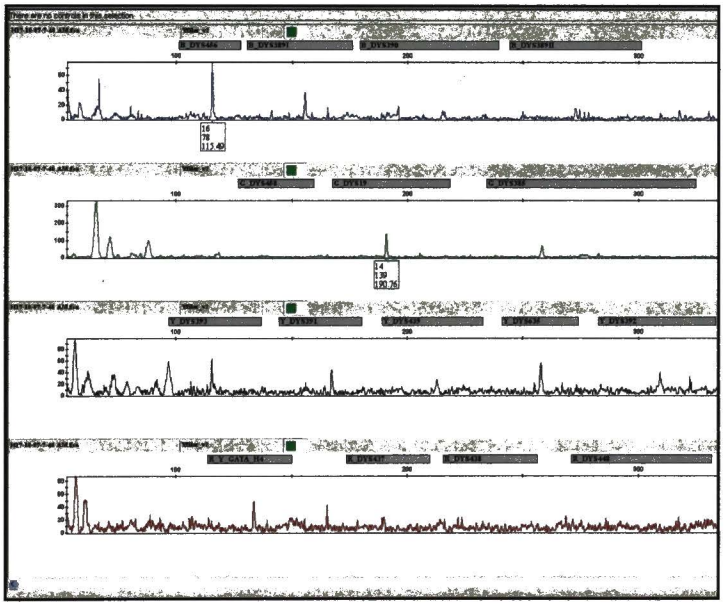

Figure 15-B: Y-STR Data for Sample H-1b

Standard Curve Consistency Study

The purpose of the standard curve consistency study was to assess the consistency of the standards being used in the duplex gender assay. The standards were changed from the original standard dilution series in order to conserve reagents and to target the lower concentrations of DNA. The last four standards of the original standard preparation instructions from the developmental validation were used, however, they were used as the upper threshold and another standard was added to make a fifth standard of the lowest concentration. In addition to conserving reagents, this also created a more specific standard curve based upon lower concentrations of DNA to hopefully aid in more accurate quantification values.

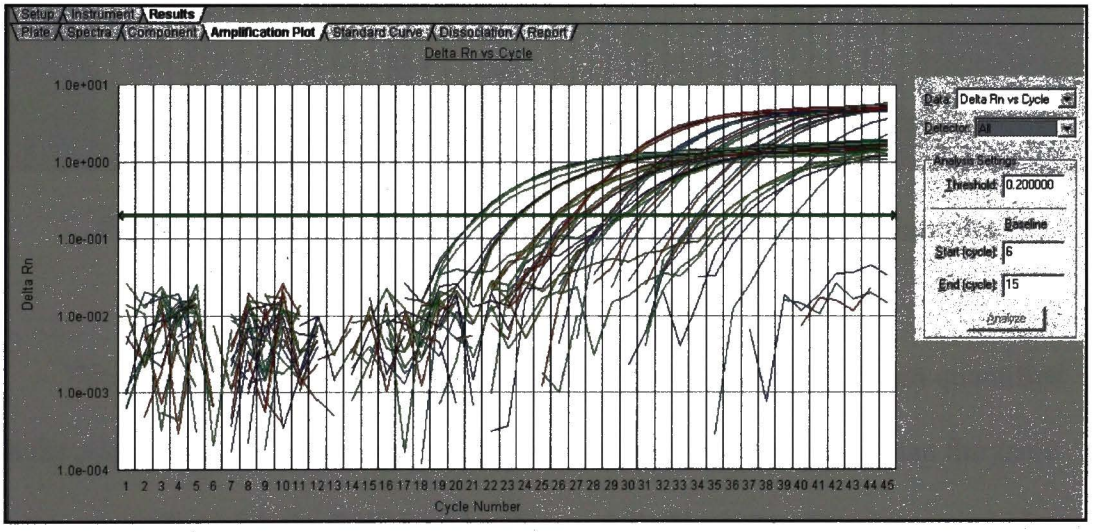

Figure 16: Amplification Plot of Standard Samples

The samples for the standard curve consistency study were originally set up in quadruplicate. However, one set of standards was not included in the calculations because some of the standards were quantified as an undetermined value. This was the only

occasion in all of the duplex gender assay runs where an undetermined value was designated for a standard, so the set of standards was omitted as an outlier from statistical calculations. The undetermined value could have been due to the fact that the last standard was such a small concentration of DNA or it may have been due to pipetting errors or inaccuracies, or for another reason unknown. Figure 16 depicts the amplification plot of all the standard samples run in triplicate.

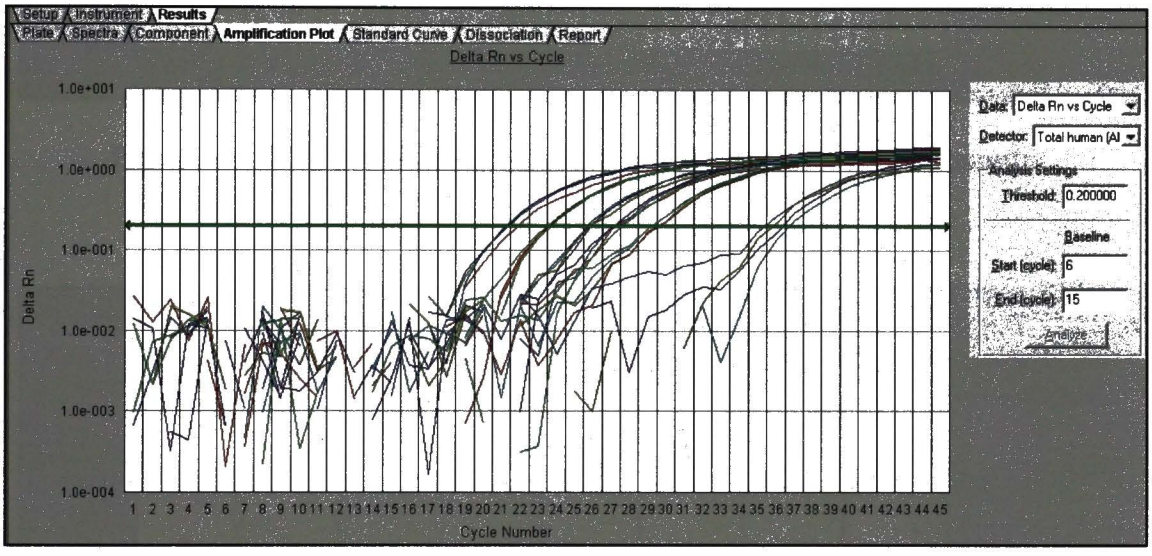

Figure 17: *Alu* Amplification Plot of Triplicate Standard Samples

The amplification plot in Figure 17 depicts how the samples, when quantified with the *Alu* probe, cross the threshold sooner, having lower Ct values than the same samples quantified with the DYZ5 probe (Figure 18). The lower Ct values would be an indication of more DNA present within samples, however in this case, it could be the sensitivity of the *Alu* probe or just due to the nature of the multicopy probe since there should only be one relative amount of DNA within one sample.

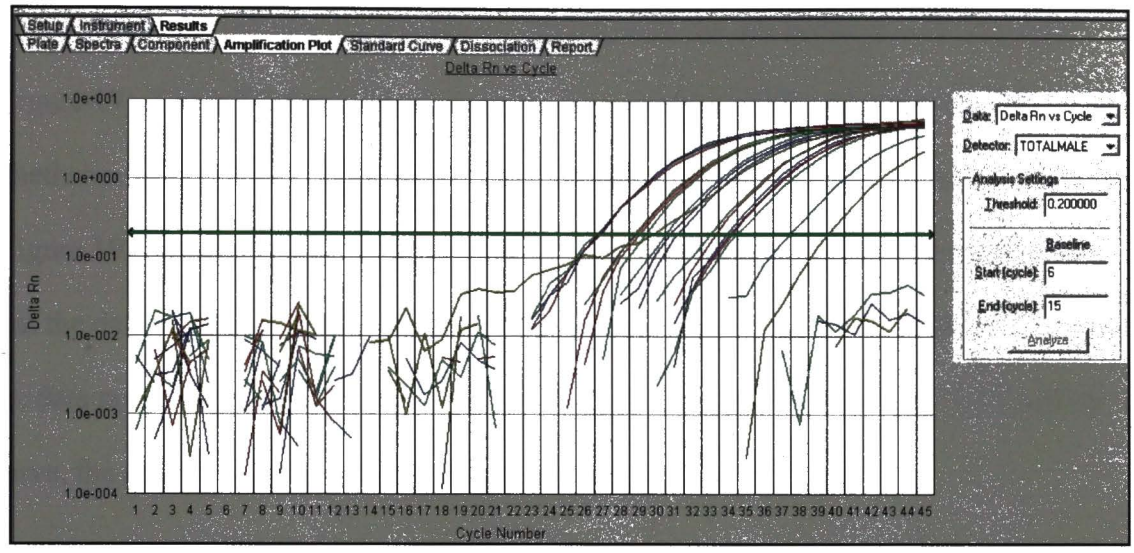

Figure 18: DYZ5 Amplification Plot of Triplicate Standard Samples

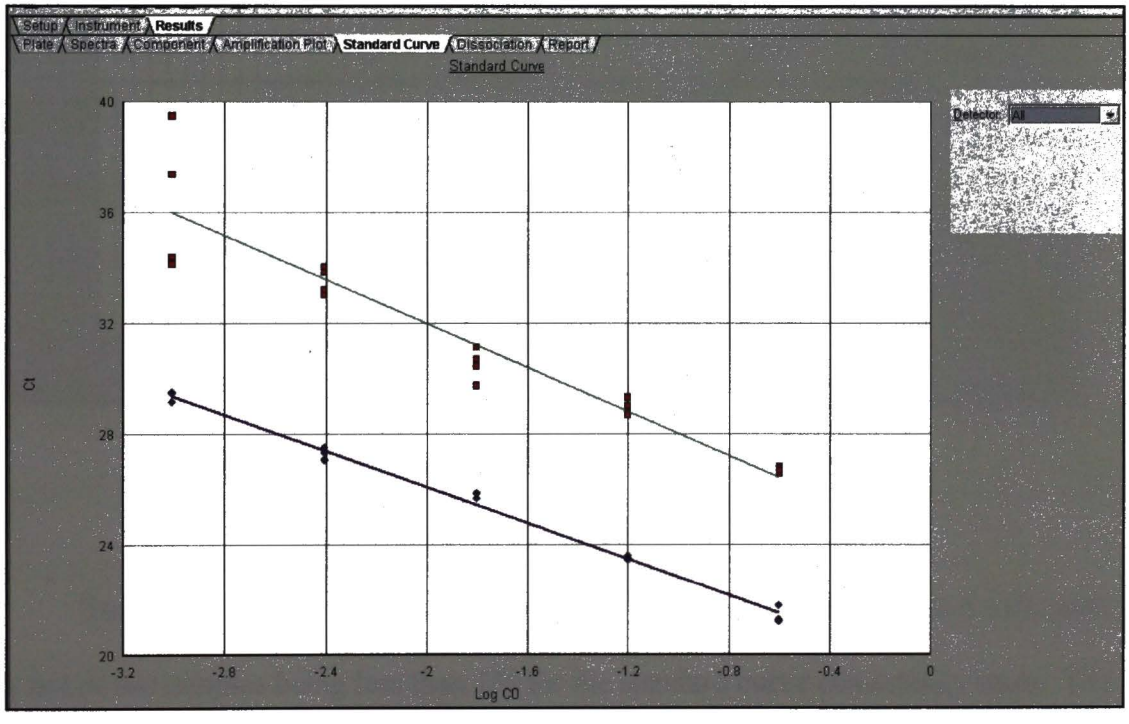

Figure 19: Standard Curves

The NTC samples are the last to amplify with the *Alu* probe at ~35-37 cycles (Figure 19). The NTC functions as the negative control and also provides an indication of whether or not inhibition is in effect. The standard curves that were run on the same plate (Figure 19) indicate that the *Alu* probe (bottom curve) is more sensitive due to the fact that the data points are tightly clustered compared to the DYZ5 curve (top curve), where the data points are very dispersed. Toward the lower concentration end of the standard curve, the data points are more dispersed on the DYZ5 probe curve.

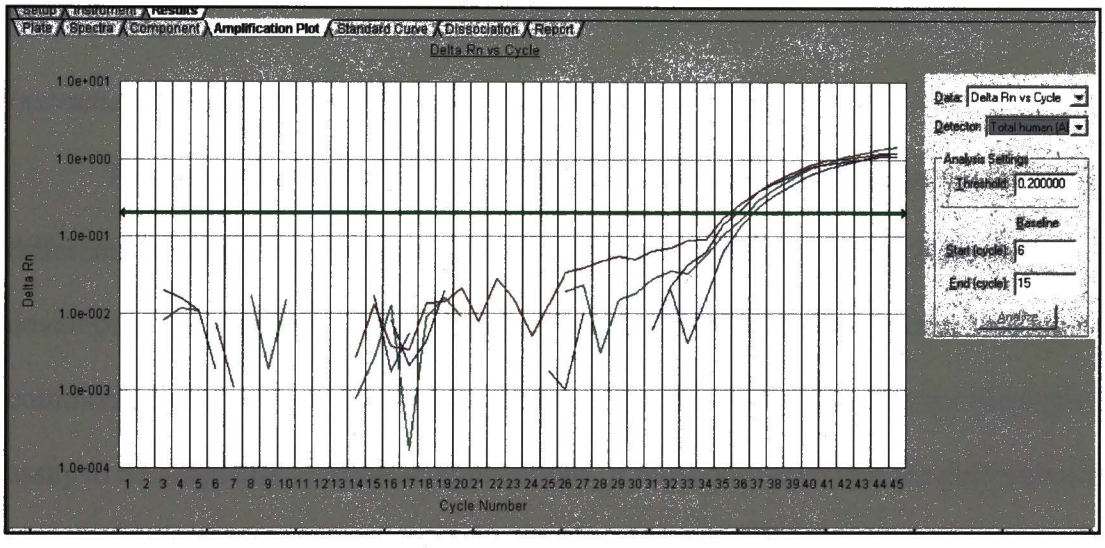

Figure 20: *Alu* Amplification Plot of NTC Samples

The percent standard deviations for the Ct values ranged from 0.22-5.11% with six out of ten samples being less than 1% for the standard curve consistency study. The values indicate that there was consistency within the quantification of the standards and is similar to what has been previously reported by Nicklas and Buel (3).

Standard Value	Repeat 1	Repeat 2	Repeat 3	Mean	Standard Deviation	% Standard Deviation
(a) Alu						
250 pg	21.82	21.3	21.24	21.45	0.32	1.49
62.5 pg	23.53	23.5	23.43	23.49	0.05	0.22
15.63 pg	25.67	25.69	25.85	25.74	0.10	0.38
3.91 pg	27.34	27.07	27.09	27.17	0.15	0.55
0.98 pg	29.16	29.5	29.17	29.28	0.19	0.66
NTC	35.3	36.62	35.7	35.87	0.68	1.89
(b) DYZ5						
250 pg	26.82	26.77	26.68	26.76	0.07	0.27
62.5 pg	29.04	28.8	28.7	28.85	0.17	0.61
15.63 pg	31.16	30.47	29.76	30.46	0.70	2.30
3.91 pg	33.24	33.86	34.05	33.72	0.42	1.26
0.98 pg	37.39	34.15	34.4	35.31	1.80	5.11
NTC	No Ct	No Ct	No Ct			

Table 9: Ct Values for Standard Curve Triplicates

Instrument Adaptation Study

The purpose of this study was to assess the results of the duplex gender assay on a different real-time PCR platform. As previously described, the research for the development of the duplex assay was conducted on two other instruments that are not commonly being utilized by most forensic laboratories today. In order for the duplex assay to be adaptable to forensic applications, it must be shown that the performance of the assay on the equipment in the laboratory meets high standards and produces quality results consistently. All the results indicate that this instrument is very suitable to run the duplex gender assay and achieve reliable and sensitive results.

CHAPTER VI

CONCLUSIONS

The results obtained in this project reflect the sensitivity of the assay in the lower quantification range reported by Nicklas and Buel (3) in the published developmental validation of the duplex gender assay. The quantification assay has a reported sensitivity for the *Alu* probe down to 0.5pg and for the DYZ5 probe, down to 4.0pg (3). The sensitivity study demonstrated the sensitivity of the duplex assay in detecting very small concentrations of DNA in a sample within the range that was previously reported (3) as ~0.5pg for the *Alu* probe and ~4.0pg for the DYZ5 probe. However, even though the samples may have been quantified, does not mean that the samples will produce Y-STR data. Loci were called, with the three repeat sets of samples from the sensitivity study compiled, in the concentrations of ~1.6pg, ~0.8pg, and ~0.4pg. The repeat to repeat variation seen in the Y-STR data from the sensitivity study might be partly attributed to being run on different genetic analyzers. Each instrument functions at a slightly different sensitivity. To obtain a better measure of comparison between quantification values and Y-STR typing data, a full validation study would need to be conducted with access to ample reagents for the assay.

In all three replicates of the dilution samples, a complete Y-STR profile was obtained at a DNA input concentration of ~62.5pg which is more specific than the reported results from the Yfiler™ kit that claims to be able to generate a full Y-profile with less than 125pg(17). As for the volunteer samples, the assay quantified between

5.44pg and 3.43pg of DNA for the DYZ5 probe for sample G-1 and H-1, respectively. The reported sensitivity for the each probe is consistent with the data obtained in this project. However, it still remains difficult to draw a conclusion regarding a quantification threshold for obtaining any detectable Y-STR data. This is simply due to the fact that no quantification standard truly exists to date that is National Institute of Standards and Technology (NIST) certified (20). The development is underway to create a Standard Reference Material (SRM) that will truly represent a known concentration of DNA (20). Once an SRM has been released for DNA quantification, much more research can be conducted to actually determine how accurately quantification assays are generating quantification values for unknown samples. Until that point, the concentrations of the standards must be considered relative along with concentration error due to inaccuracies in pipetting.

The Ct values obtained from the standard curve consistency study were similar to that of the assay development (3). However, even though the values were similar for the standards that were used in this project, almost each Ct value for the *Alu* probe quantification values seemed to be approximately one cycle more than the Ct values obtained for the *Alu* probe in the research conducted for the development of the assay. The Ct values for the DYZ5 probe seemed to be slightly lower than the Ct values that were obtained in the developmental research. This difference may be due to instrument sensitivity variation or other factors unknown. The NTCs all amplified with the *Alu* probe with indicated that inhibition was not an issue in the samples.

In order to implement a new method, technique, kit, or instrument, it must be validated in the forensic laboratory that it is going to be used in to demonstrate that it upholds the high standards set for DNA analysis. The preliminary results indicate that the assay functions on an ABI PRISM® 7000 SDS similarly to the instruments that the developmental research was conducted on. The functionality is important in order for the duplex assay to enter the realm of forensic applications it must be able to function the same on other real-time PCR instruments that would be present in most forensic laboratories in the country today. A full internal validation study would need to be conducted and compared to the results from the developmental validations on the real-time instruments. The only real adjustments that were made to allow the assay to be adapted to the ABI PRISM® 7000 SDS was designating the FAM and VIC dyes, respectively, for the DYZ5 probe and *Alu* probe standards as both standards (in the well inspector option) instead of one standard and one unknown as you would for the IPC in the Quantifiler™ Kit. Once you select both dyes as standards, the quantity must be specified for each standard. Once the run is complete, the NTC must be analyzed to see that the *Alu* probe amplified around ~38 cycles or so.

As a preliminary study, the samples that produced Y-STR data were quantified with extremely small concentrations of DNA in the sample. In the instance that just even one locus has an allele call, the results can be exclusionary in the instance that mutation is not an issue. In addition, the duplex assay can be used as a screening tool to provide male quantification values simultaneously as human DNA quantification values are obtained. The duplex gender assay can be quite an effective measure for screening because the

quantification values are already determined and the DNA extract obtained from the crime scene sample, for example, can always be run for Y-STRs is necessary and the quantification has already been performed and the analyst can directly proceed to the amplification step. Furthermore, due to the increased sensitivity of the assay and the fact that the approximate cost of the reagents per sample is ~$0.70, it could be a major cost saver in many forensic laboratories. The approximate cost of the QuantifilerTM Kit per sample is around three times or more than the cost per sample of the duplex gender real-time PCR assay. The duplex gender assay would just need to be properly validated in order to be used for casework in a forensic laboratory.

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