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AN INITIAL COMPARISON OF APPLIED BIOSYSTEMS

QUANTIFILER® DUO AND PROMEGA PLEXOR® HY

REAL-TIME PCR DNA QUANTIFICATION SYSTEMS

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AN INITIAL COMPARISON OF APPLIED BIOSYSTEMS QUANTIFILER® DUO AND PROMEGA PLEXOR® HY REAL-TIME PCR DNA QUANTIFICATION SYSTEMS

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By

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CHAPTER I

INTRODUCTION

The analysis of deoxyribonucleic acid (DNA) has become a key element in the forensic sciences for the comparison of known and evidentiary samples. The quality and quantity of DNA recovered from extraction procedures is of utmost importance in obtaining a usable genetic profile. Current analysis techniques examine DNA locations (loci) that contain short tandem repeats (STRs). STR analysis is dependent upon the polymerase chain reaction that amplifies, or copies, these specific regions of the DNA. In order to complete PCR and downstream STR genetic analysis, the quantity of DNA in evidentiary samples must be assessed. The amount of DNA in a sample is important for PCR-based assays because of the narrow ideal concentration range of input template DNA for optimal STR genetic results. The addition of excess DNA results in off-scale data or split peaks. Insufficient amounts of DNA can result in allele dropout, which is the failure to detect an allele in a sample. Another purpose of quantification is to determine the amount of amplifiable DNA in order to minimize excess consumption of the sample that may need to be saved for post-conviction testing or reanalysis by an opposing counsel. Therefore, it is important to make sure accurate quantification and typing results are obtained with the first attempt.

The DNA Advisory Board (DAB) was established by the Federal Bureau of Investigation (FBI) under the DNA Identification Act of 1994 [1]. The primary purpose of the DAB was to recommend to the Director of the FBI a set of national quality assurance standards for implementation in forensic DNA laboratories throughout the country. The DAB addressed DNA quantification in standard 9.3 which states "The laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample where possible" [2].

Several commercially available quantification systems are available to forensic DNA laboratories. Applied Biosystems currently markets the Quantifiler® Human DNA Quantification kit and the Quantifiler® Y Human Male DNA Quantification kit, which quantifies human autosomal and male DNA in two separate reactions. The Promega Corporation promotes the use of the Plexor® qPCR System that quantifies only autosomal human DNA. In the last year, both Applied Biosystems and the Promega Corporation have developed novel multiplex real-time PCR DNA quantification systems. Although their chemistry is different with regard to labeled probes and primers, the purpose of the systems is the same. Applied Biosystems Quantifiler® Duo and the Promega Corporation Plexor® HY systems are both designed to quantify the concentration of human (autosomal) DNA and human male (Y) DNA simultaneously. These novel multiplex systems have the potential for reducing labor, supplies, and the consumption of evidentiary DNA samples by eliminating the need to perform two separate quantification reactions to determine the amount of autosomal DNA and male-specific DNA.

The University of North Texas Center for Human Identification currently uses the Applied Biosystems Quantifiler® Human system. They are looking into choosing one of the new systems for future internal validation and implementation into the laboratory protocol. An initial comparison of Quantifiler® Duo and Plexor® HY must be performed before any final decision can be made, which takes into account the reliability and reproducibility of each kit as well as logistical features such as ease of use, software, and total cost (in time and in money).

CHAPTER II

BACKGROUND

Early Methods of DNA Quantification

Early approaches to DNA quantification were not very sensitive or species-specific and tended to waste valuable forensic samples. These methods mainly included ultraviolet (UV) spectrometry at 260 nm and 280 nm and fluorescence detection with dye staining of a yield gel [1]. UV absorption occurs because of the purine and pyrimidine bases in the DNA molecule. Unfortunately, this assay cannot distinguish between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) [3]. Falsely high absorbance signals were often the result of protein contamination or phenol left from the extraction, both of which contain conjugated ring systems that also will absorb UV light at 260 nm. Sensitivity is limited to 500 ng. However, most forensic samples yield less than 50 ng [3].

Fluorescence analysis requires appropriate dyes that will interact with the DNA. Usually ethidium bromide is applied, which intercalates between the DNA bases and causes an increase in fluorescence by 50 to 100 times. The sensitivity of this assay reaches approximately 10 ng and is dependent on factors such as agarose concentration, running buffers, run conditions, and volume of sample applied to gel [3]. Other dyes, such as PicoGreen®, may also be used with sensitivities reaching 0.05 ng. Post yield gel

electrophoresis visualization can be done with SYBR Green I dye. Again, co-extracted fluorescent molecules may interfere with these assays. Care must by taken as well, as some of the dyes, especially ethidium bromide, are potent mutagens. The specificity of all of these methods is only for autosomal DNA and could detect non-human DNA as well as human DNA [3]. Fungal, bacterial, animal, or plant DNA could also be present in the DNA recovered from a sample. It is critical to determine the amount of human DNA in a forensic sample of unknown origin since all sources of DNA are extracted.

As technology progressed from a RFLP-based (restriction fragment length polymorphism) system to a PCR approach, the need for human-specific DNA quantification increased. The DNA Advisory Board standard 9.3 requires quantification of *human* DNA in forensic samples before amplification [2]. An early method of human-specific quantification is the slot blot technique that utilizes the hybridization of probes specific to human (and higher primate) DNA. The 40 base pair biotinylated probe is complementary to the alpha satellite DNA sequence D17Z1 on human and higher primate chromosome 17 [1]. The DNA is bound to a membrane using a slot blot apparatus. The bound DNA is hybridized with a primate-specific probe and is processed through several chemical steps involving an enzyme conjugate: HRP-SA (horseradish peroxidase-streptavidin) and washes. The bound probe is then visualized by use of either a colorimetric or a chemiluminescence method. The intensity of the slot blot band is proportional to the amount of human DNA in the sample [3]. A standard dilution curve is inserted in one row of the membrane and is used to compare the unknown samples to the standards to determine DNA concentration in the unknown sample.

Applied Biosystems (Foster City, CA) manufactured the Quantiblot® Human DNA Quantitation system, which has since been discontinued. The sensitivity of Quantiblot® was 0.03125 ng/μl and required several initial sample dilutions to obtain results in the range of the standard curve [3]. The main problem with slot blot technology is the subjectivity of the assay. A forensic analyst visually interprets the color intensity of the bands and estimates the DNA concentration. This leads to significant inaccuracies of very high and very low concentrations and variability between analyst interpretations. Despite negative results with the Quantiblot®, in most cases DNA testing will continue forward and often generate a full genetic profile. The dynamic range of Quantiblot® was not sufficient to detect low copy samples routinely handled in forensics.

The slot blot technique was introduced during the Polymarker and DQ alpha era [3]. The decreased sensitivity of these novel PCR technologies worked well with the limited range and inaccuracies of the slot blot. Even if the DNA concentration estimates were off, these techniques would still work. Later STR analysis would eventually render the capabilities of slot blot inadequate due to the new increased sensitivity of STR analysis methods. Since we can use much less DNA than before, we need a quantification system that can detect lower concentrations of DNA.

The Promega Corporation (Madison, WI) developed the AluQuant® Human DNA Quantitation system that probes for *Alu* repeats that occur frequently in the human genome [3]. A series of enzyme coupled reactions leads to the oxidation of luciferin and the production of light [1]. The intensity of the light is proportional to the amount of DNA in the sample. The assay's sensitivity ranges from 0.1 to 50 ng [1]. An advantage to

the AluQuant® is that the DNA is not bound to a membrane. However, AluQuant® requires the use of at least twice the amount of evidentiary sample as slot blot techniques and much more analyst hands-on time. This assay failed to be completely accepted by the forensic science community [4].

Real-Time Polymerase Chain Reaction

Real-time PCR (also called quantitative or qPCR) analyzes the cycle-to-cycle change in fluorescence signal (increase or decrease) that results from accumulation of amplified target product. The reaction consists of three phases as seen in amplification plots: exponential, linear, and plateau (Figure 1). In the exponential phase, there is an excess of reagents, the number amplicons double with each cycle (assuming 100% efficiency), and kinetics drive the reaction forward. The plot of cycle number versus a log scale of DNA concentration should yield a linear relationship during the exponential phase. In the linear phase, the availability of reagents is decreasing and the efficiency drops considerably. In the plateau phase, reagents have been completely consumed and amplification ceases. In end-stage (gel-based) PCR, detection occurs in the plateau phase. In real-time PCR, analysis is conducted during the exponential phase of amplification, producing quantifications that are more accurate.

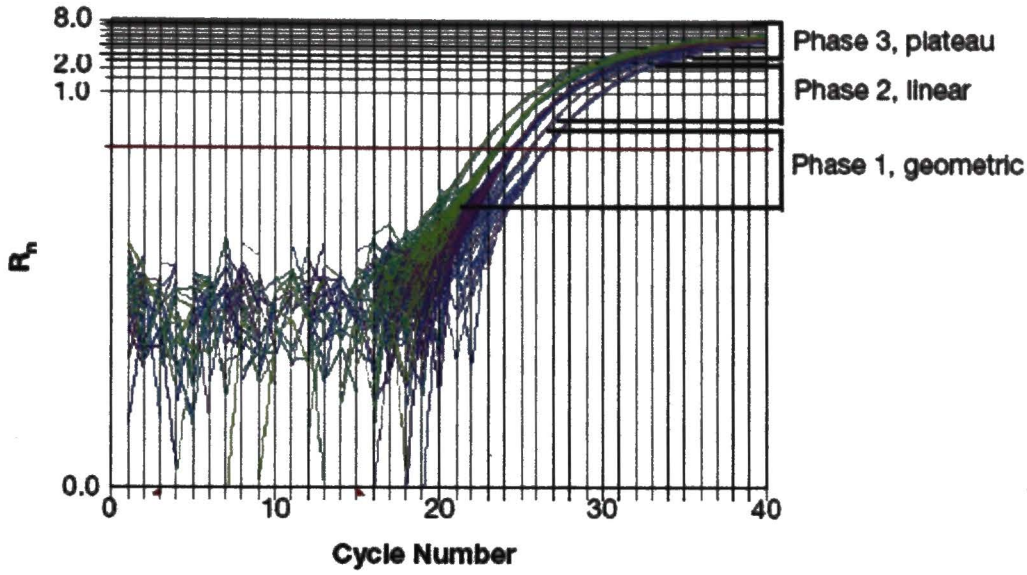

Figure 1. Phases of real-time PCR amplification [5].

The instrument uses the cycle threshold (C_T) for calculations of quantity. The C_T value is the cycle number in which the level of fluorescence crosses the threshold set by the software. The C_T value depends on the starting template copy number and the efficiency of the amplification [6]. The purpose of this threshold value is to detect real data as opposed to background noise in the early PCR stages [1]. The fewer cycles it takes to cross the C_T, the greater the concentration of DNA in that sample. Theoretically, a difference of 1 in C_T values equals a two-fold difference in the amount of starting template [6]. The change in fluorescence is correlated to the standard curve, which is generated from a dilution series of known DNA template amounts in duplicate. Initial DNA concentration is calculated by comparison to the standard curve, providing that there is good consistency and precision among the standard dilutions.

The standard curve results include a graph of the C_T of the standards vs. the starting quantity of the standards. A line of best fit is calculated from these data points. Important values that describe the accuracy and quality of the results are the R^2 value (correlation coefficient), the slope, and the Y-intercept [6]. The R^2 value assesses the closeness of fit of the regression line to the C_T points of the standards. The ideal value is 1.00 (a perfect fit); however, anything greater than 0.99 is acceptable. The PCR amplification efficiency is indicated by the slope. One hundred percent efficiency equates to a -3.3 slope. The Y-intercept is the C_T value at which a sample with a concentration of 1.00 ng/μl would cross the fluorescence threshold.

Samples are assayed on the ABI PRISM® 7500 Sequence Detection System, which is compatible with both Applied Biosystems and Promega technology. The 7500 is a 96-well real-time PCR unit that uses a five-color platform that is calibrated for a range of dyes: NED™, FAM™/SYBR® Green I, VIC®/JOE™, TAMRA™/CY3, ROX™/TEXAS RED, and CY5 dyes [7]. Light is directed to each well by a tungsten-halogen lamp, exciting the fluorescent dyes. The charge-coupled device (CCD) camera detects the emission of the fluorescent dyes. The Sequence Detection Software (SDS) gathers the CCD camera data and applies the algorithms for data analysis. The software is specific for Applied Biosystems technology and is used for instrument control and data analysis. The 7500 has capabilities of calibration of new dyes and is able to run multiplex reactions. Dye chemistries will be discussed under the appropriate system explanations.

Applied Biosystems Quantifiler® Human and Quantifiler® Y Systems

The Quantifiler® Human and Y quantification assays measure the increase in fluorescence using a 5' nuclease assay. Each are duplex assays, containing two independent sets of primers and probes: a target-specific (human DNA or human male DNA) assay and an internal PCR control (IPC) [6]. The target-specific assay consists of the two primers necessary for amplification of either human DNA or human male DNA and one TaqMan® probe labeled with FAM™ dye for detecting the target sequence. The gene target for the human DNA is the human telomerase reverse transcriptase gene (hTERT) located on chromosome 5 [6]. This amplicon is 62 bases in length. The 64 base pair gene target for the male DNA is the sex-determining region Y gene (SRY) located on the Y chromosome [6]. The IPC assay consists of the template DNA, primers specific for the template, and a VIC® labeled TaqMan® probe. The IPC template DNA is a synthetic sequence, not found in nature, which monitors the conditions of the reactions and acts as a predictor of possible failure of genetic analysis and provides a positive control.

The 5' nuclease assay is described in Figure 2. The probes contain a reporter dye linked to the 5' end and a minor groove binder (MGB) linked to the 3' end [6]. The MGB functions to increase the melting temperature without compromising probe length [8]. A nonfluorescent quencher (NFQ) also at the 3' end of the probe aids in measuring the reporter dye fluorescence more accurately [8]. During each cycle of PCR, the probe anneals to a complementary sequence in the template DNA between the forward and reverse primer locations. For this reason, nonspecific amplification will not be detected.

An intact probe will not emit fluorescence because the reporter dye and quencher dye are within a close enough proximity to experience Förster-type energy transfer (also known as fluorescent resonance energy transfer, or FRET)[6]. The 5' nuclease activity of AmpliTaq® Gold DNA polymerase cleaves the reporter dye from the probes hybridized to the target DNA. The separation of the reporter dye from the quencher dye results in the loss of FRET and causes increased fluorescence. The probe is removed from the strand, allowing primer extension to continue. This fluorescence is proportional to the number of amplicons produced.

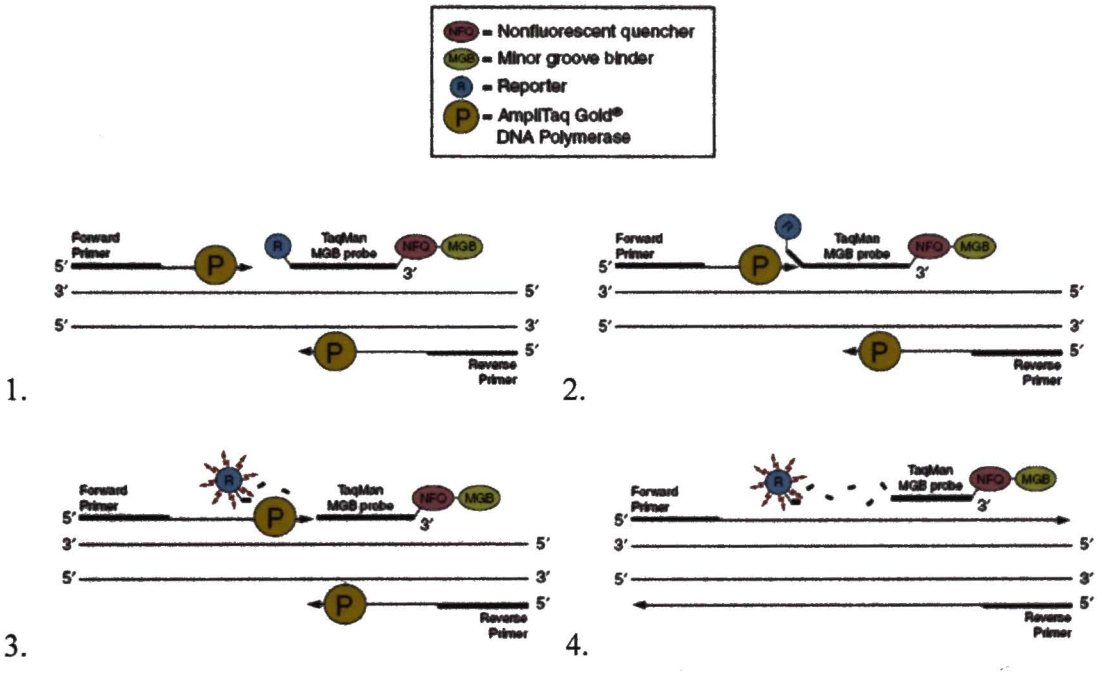

Figure 2. 5' Nuclease Assay [5, 6]. 1. Probe anneals to specific sequence in template DNA between forward and reverse primer. Polymerization of growing strands by AmpliTaq® Gold DNA polymerase. 2. Strand displacement of probe by growing strand. 3. Cleavage of hybridized probes and separation of reporter dye from quencher dye. 4. Completion of polymerization.

The SDS software analyzes the raw spectral data collected from 500 nm to 660 nm [6]. An algorithm is used to remove background fluorescence, and the software uses pure dye standards to express the composite spectrum. Another algorithm is used to calculate the contributions of each dye to the composite spectrum. The reporter dye signals are then normalized (producing a normalized reporter, or R_n, value) based on the passive reference dye that is present in the PCR master mix and thus in all wells of the plate (the reporter signal is divided by the fluorescent signal of the passive reference dye) [6]. Normalization allows the software to account for minor variations in signal strength, which could be due to pipetting differences. The results are displayed in an amplification plot (C_T cycle vs. R_n) and in a spreadsheet format displaying the C_T value, C_T standard deviations for duplicates, sample quantity, mean quantity, and quantity standard deviations for duplicates [4].

The Quantifiler® kits contain either human or male Primer Mix, the human male DNA standard for creating the standard dilutions, and the PCR Reaction Mix. The human male DNA standard is provided at 200 ng/µl, which is diluted to obtain standards of 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µl. The standard curve is composed of this set of eight serial dilutions run in duplicate. The Primer Mix contains the forward and reverse primers and the probe for the appropriate target (human or male DNA) as well as the IPC primers and probe. The PCR Reaction Mix contains AmpliTaq® Gold DNA polymerase, dNTPs (free deoxynucleotides triphosphates), the passive reference dye ROX, and buffer components. Each kit can perform 400 reactions at 25-µl volumes. Quantifiler® Human and Quantifiler® Y are sold separately or as a package.

Applied Biosystems Quantifiler® Duo System

The Quantifiler® Duo System is very similar to the original Quantifiler® Human and Quantifiler® Y Systems, both in protocol and in chemistry. The system uses three 5' nuclease assays simultaneously: a target-specific human DNA assay, a target-specific human male DNA assay, and an internal PCR control assay [5]. The human gene target is the ribonuclease P RNA component H1 (RPPH1) located on chromosome 14. It is important to note that the amplicon length has been increased to 140 bases (as opposed to 62 bases in Quantifiler® Human) [5]. The target-specific assay for human DNA consists of two primers and a TaqMan® MGB probe labeled with VIC® dye. The 130 base pair human male DNA target is the sex-determining region Y (SRY) of the Y chromosome [5]. This amplicon has also been lengthened from 64 base pairs to 130 base pairs. The target-specific assay for male DNA consists of two primers and a TaqMan® MGB probe labeled with FAM™ dye. The IPC assay consists of the 130 base pair template DNA (a synthetic sequence not found in nature), two primers, and a TaqMan® MGB probe labeled with NED™ dye [5]. The mechanism of the 5' nuclease assay and data analysis is the same as explained in detail for the Quantifiler® Human and Quantifiler® Y systems.

The Quantifiler® Duo kit contents include the Primer Mix, the PCR Reaction Mix, the DNA Standard, and the DNA Dilution Buffer. The human male DNA standard is provided at 200 ng/µl, which is diluted with the DNA Dilution Buffer (10mM Tris HCl buffer, pH 8.0, containing 0.1 mM EDTA) to obtain standards of 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µl. The standard curve is generated from this set of eight serial dilutions run in duplicate. The Primer Mix contains the primer pairs and the probes

for RPPH1, SRY, and the IPC (including the IPC template). The PCR reaction mix contains AmpliTaq® Gold DNA polymerase, dNTPs, $MgCl_2$, bovine serum albumin, and 0.02% w/v sodium azide (preservative). Each kit can perform 400 reactions at 25-µl volumes.

Promega Plexor® HY System

The Promega technology measures the decrease in fluorescence by utilizing specific interactions between two modified nucleotides, isoC and isoG [9-11]. The principles are the same as Quantifiler® in that a cycle threshold must be crossed; however, the amplification curve is reversed (Figure 3).

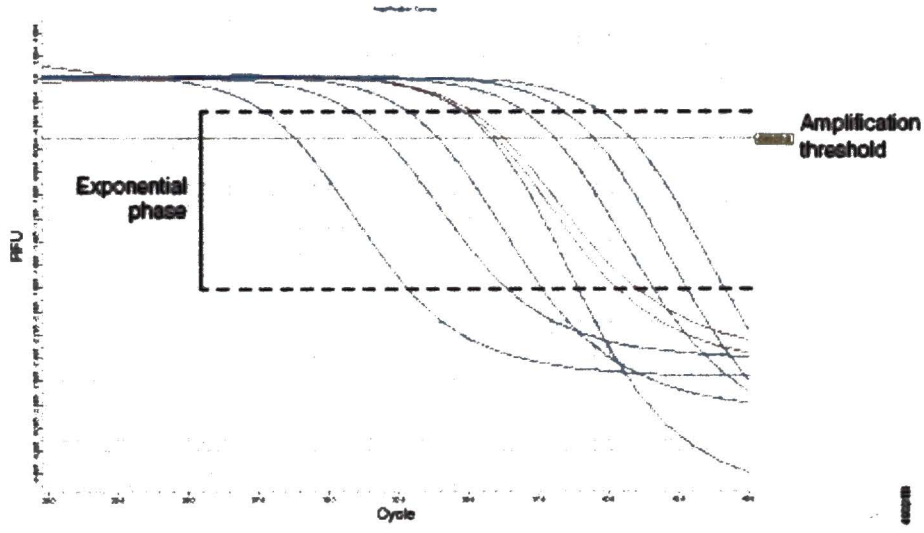

Figure 3. Plexor® amplification curves [10]. Fluorescence is shown at each cycle of the reaction. The amplification threshold is used to determine the C_T for the samples. Note that the exponential phase is occurs below this threshold.

One of the primers instead of a probe is fluorescently labeled, while the second primer remains unlabeled. The primer contains iso-dC, a modified nucleotide, which is linked to the fluorescent reporter at the 5' end. The PCR reaction mix includes deoxynucleotides as well as another modified nucleotide, iso-dGTP linked to the quencher, dabcyl. The dabcyl-iso-dGTP complex is complementary to the iso-dC nucleotide in the primer [9]. The incorporation of dabcyl-iso-dGTP opposite iso-dC results in the quenching of the fluorescent reporter attached to iso-dC (Figure 4). This quenching and consequent decrease in fluorescence is directly proportional to the amount of PCR product accumulated [11].

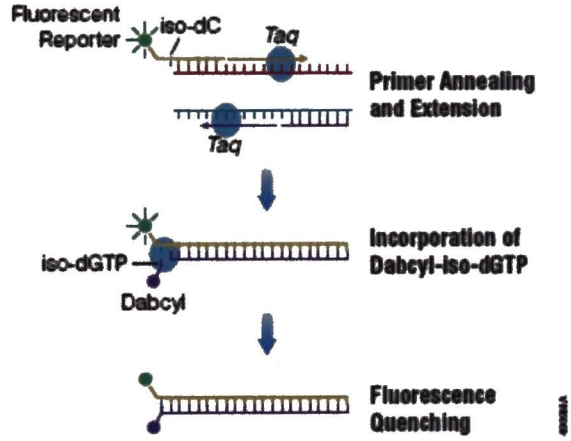

Figure 4. Plexor® real-time PCR amplification chemistry [10].

Because the fluorophore and quencher reside on opposite strands, the melting temperature (T_m) of the product can be determined following amplification [10, 11]. A melting or dissociation curve is generated from slow heating and optical analysis of the amplicons. The expected product has a unique T_m dependent on base composition, which

allows for determination of the specificity of the reaction [10, 11]. The resulting melting curve plot (change in fluorescence with temperature vs. temperature) depicts the temperature at which the greatest rate of change in fluorescence occurs (Figure 5).

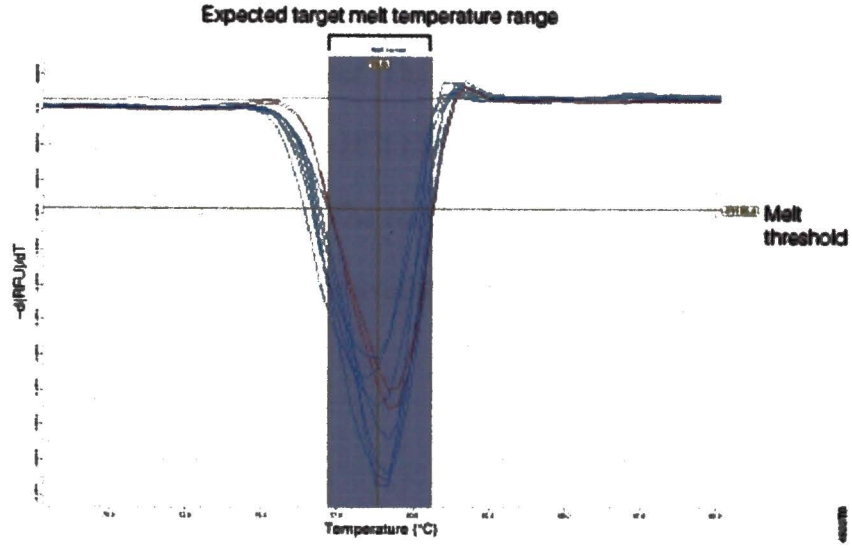

Figure 5. Plexor® melt curves [10]. A plot of change in fluorescence with temperature versus temperature allows for the determination of sample melting temperatures (where the greatest rate of change in fluorescence occurs).

Plexor® HY can run on the Applied Biosystems machinery, however the dye systems differ. For this reason, it is necessary to calibrate the 7500 with the dyes specific to the HY system. Fluorescein dye is used to detect the human autosomal DNA target, a multicopy, 99 base pair segment on chromosome 17 [10]. CAL Fluor® Orange 560 dye detects the 133 base pair Y-chromosome target. CAL Fluor® Red 610 dye detects the internal PCR control in every reaction. The novel sequence is 150 base pairs. The IC5 dye is the passive reference added to each sample, which is used to normalize the data from the other three dyes to this signal. Although the reaction runs successfully on the

Applied Biosystems equipment, the SDS software cannot determine the C_T values or the T_m. Run data must be exported from the SDS software to the Plexor® Analysis Software forensic release version.

Each kit contains the 2X Master Mix, 20X Primer/IPC Mix, Male Genomic DNA Standard, and amplification grade water. The Male Genomic DNA Standard is provided at 50 ng/µl and is diluted in TE^{-4} (Tris-HCl EDTA) buffer to generate standards of 50, 10, 2, 0.4, 0.08, 0.016, and 0.0032 ng/µl. The standard curve is composed of this set of seven standards run in duplicate. The kits are available in 200 and 800 reaction denominations, each using a 20-µl reaction volume.

CHAPTER III

RESEARCH DESIGN

Objective 1 – Sensitivity Study

This study was designed to determine the quantity of template DNA below which amplification is not expected to yield a DNA profile. Dilution series of male and female stock DNA ranging from 0.003 ng/µl to 50 ng/µl will independently be run with both Quantifiler® Duo and Plexor® HY. These samples will be run in duplicate per plate, with duplicate plates being run. We want to determine if the published lowest detection thresholds (0.023 ng/µl for Duo; 0.0032 ng/µl for HY) are concordant with the data obtained.

Objective 2 – Mixture Study

The purpose of this study is to obtain quantification results for mixtures of male and female DNA, which should allow for calculations of autosomal:Y ratios that can be helpful in determining what type of genetic analysis to pursue (autosomal STR, Y-STR, or both). Mixtures of female and male DNA ranging from 1:1 to 1024:1 (female:male) will be run in duplicate per plate, with duplicate plates being run. We want to find out how minor of a contributor the male can be in an excess of female DNA and still be

detected. This is especially important in sexual assault cases where the major contributor is usually female or when the offender is a vasectomized male.

Objective 3 – Concordance Study

The purpose of this study is to compare quantification results from Quantifiler® Duo and Plexor® HY with those from Quantifiler® Human, specifically in cases when samples are degraded. The majority of these samples originate from unidentified human remains. Patterns of overestimation or underestimation of DNA concentration can help determine which system will be most beneficial in these cases. This is where the new amplicon size featured in Quantifiler® Duo is important in comparing the values with previous results for Quantifiler® Human. Sample choice will be at the discretion of the laboratory technical leader and Unidentified Human Remains section analysts. These samples will be ones that are known to be degraded and have previously yielded overestimated results from the Quantifiler® Human quantification system, resulting in poor STR data.

CHAPTER IV

MATERIALS AND METHODS

Quantifiler® Duo Protocol

Manufacturer's protocol was followed directly for the purposes of these studies since internal validation has not otherwise stated acceptable deviations. Refer to the Quantifiler® Duo DNA Quantification Kit User's Manual (P/N 4391294 Rev. A) for specific details on software setup.

1. Prepare the DNA quantification standards dilution series. Label eight 0.5 ml tubes standards 1-8. Add 30 µl DNA Dilution Buffer to the tube labeled standard 1, and add 20 µl DNA Dilution Buffer to tubes 2-8. To prepare standard 1, vortex the 200 ng/µl DNA Standard stock 3-5 seconds and add 10 µl to tube 1. Vortex. This creates the 50 ng/µl standard, a four-fold dilution of the stock. Change pipette tips and add 10 µl of the 50 ng/µl standard to tube 2 containing 20 µl DNA Dilution Buffer. Vortex. This creates the 16.7 ng/µl standard, a three-fold dilution of standard 1. Change tips and continue with three-fold serial dilutions until all standards are made. The concentrations of the standards are 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µl. Standards prepared in DNA Dilution Buffer can be stored up to two weeks at 2 to 8°C.

2. Using the SDS software, create a 96-well plate layout. Layout is flexible with regard to standard and sample placement. In this study, the standards were in duplicate in wells A1-A12 and B1-B4. Non-template control (NTC) duplicates were in wells B5 and B6, and positive control duplicates of 9947A 1.0 ng/µl and 9947A 0.1 ng/µl were in wells B7-B8 and B9-B10, respectively. See chart below. Create and save this document as a new session in the software.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	16.7	16.7	5.56	5.56	1.85	1.85	0.62	0.62	0.21	0.21
B	0.068	0.068	0.023	0.023	NTC	NTC	1.0	1.0	0.1	0.1		
C												
D												
E												
F												
G												
H												

3. Calculate the volumes of Primer Mix and PCR Reaction Mix needed for the master mix. Each reaction calls for 10.5 µl Primer Mix and 12.5 µl PCR Reaction Mix. Include a couple of extra reactions in the calculations to account for loss during reagent transfer and pipetting.
4. Thaw Primer Mix and vortex 3-5 seconds. Swirl, but do not vortex, the PCR Reaction Mix. Pipette calculated volumes of each component into a new tube (appropriately sized). Vortex master mix 3-5 seconds.

5. Dispense 23 µl of the master mix into each well of a MicroAmp Optical 96-well Reaction Plate that will contain standards, controls, or sample. It is recommended that an automatic pipettor be used for consistence and accuracy, as it was in these studies. Do not include master mix in wells that will remain empty.
6. Add 2 µl of standard, sample, or control to the appropriate wells, making sure that each sample is mixed well by pipetting up and down a few times. For standards, controls, and samples that will be run in duplicate, vortex 3-5 seconds before and between duplicates to keep them as consistent as possible.
7. Seal the plate with an Optical Adhesive Cover. Use the sealing tool to ensure that all corners and spaces between wells are adhered properly to the plate. Check that no air bubbles interfere with cover adhesion. Centrifuge plate to remove bubbles from well bottoms.
8. Place plate in the ABI PRISM® 7500 with well A1 oriented in the upper left corner.
9. For the first time running the Duo system in the SDS software, add the appropriate detectors for the reporter dyes of Duo Human (VIC), Duo Male (FAM), and Duo IPC (NED). Select ROX as the Passive Reference dye. For this and subsequent runs, add these detectors to the plate document. Assign sample name, task, and quantity to standards. Assign sample name and task to unknown samples and controls.
10. Set thermal cycler conditions:

a. Stage 1 - 50°C for 2 minutes

b. Stage 2 - 95°C for 10 minutes

c. Stage 3 - 40 cycles of 95°C for 15 seconds/ 60°C for 1 minute

11. Save document and press start. Assay takes approximately 1 hour and 45 minutes to complete.

12. At completion of assay, analyze the run. View the standard curves generated for Duo Human and Duo Male. At optimal, 100% PCR amplification efficiency, the slope will be -3.3. An acceptable slope range is -3.0 to -3.6. The R^2 value should be 0.99 or greater. Note the Y-intercept. Refer to the troubleshooting section of the User's Manual if any of the parameters are out of the expected ranges to determine the course of action. Generally, standard 8 may be omitted to increase fit of the standard curves. At UNTCHI, it is also acceptable to omit up to three standards provided they are not the same standard concentration.

13. Use the report tab to view and save the data in spreadsheet format, which can be exported for later use.

Plexor® HY Protocol

Manufacturer's protocol was followed directly. Refer to the Plexor® HY Systems for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems Technical Manual (P/N TM293) for specific details on calibration and software setup.

1. Before Plexor® HY can be used on the AB PRISM® 7500, the instrument must be calibrated for the specific pure dyes that are not included with the machine. This requires the use of a separate kit, the Plexor® Calibration Kit, Set A (P/N DC1500). Perform the regions of interest calibration, the background calibration, and optical calibration (all as specified in the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide) prior to the pure dye calibration. This requires the use of the Applied Biosystems 7500 or 7500 Fast Real-Time PCR Systems Spectral Calibration Kit I.
2. Dilute Male Genomic DNA Standard in a two-fold fashion. Label seven 1.5 ml tubes standards 1-7. Vortex Male Genomic DNA Standard after initial thawing (store long-term at 4°C). In the tube labeled standard 1, add stock 50 ng/µl Male Genomic DNA Standard (note: protocol does not specify amount, however in order to perform the dilutions and reactions, there must be at least 15 µl). Vortex. This creates the 50 ng/µl standard. In tubes 2-7, add 40 µl TE^{-4} buffer. Change pipette tips and add 10 µl of the 50 ng/µl standard to the second tube. Vortex. This creates the 10 ng/µl standard. Continue performing serial dilutions until all standards are made. The concentrations of the standards are 50, 10, 2, 0.4, 0.08, 0.016, and 0.0032 ng/µl. Note that no statement on the longevity of the standard set is made.
3. Create the plate layout of the 96-well plate. It is recommended that the standards be run in duplicate vertically in wells A1-G1 and A2-G2. Non-

template control duplicates are in wells H1 and H2. Positive controls 9947A 1.0 ng/µl and 9947A 0.1 ng/µl for these studies were in wells A3-D3. See chart below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	1.0									
B	10	10	1.0									
C	2	2	0.1									
D	0.4	0.4	0.1									
E	0.08	0.08										
F	0.016	0.016										
G	0.0032	0.0032										
H	NTC	NTC										

4. Calculate volumes of 2X Master Mix, 20X Primer/IPC Mix, and water for the master mix. Each reaction requires 10 µl 2X Master Mix, 7 µl water, and 1 µl 20 Primer/IPC Mix. Allow for a few extra reactions due to reagent transfer and pipetting. Thaw and vortex reagents. Pipette calculated volumes of each component into a new tube (appropriately sized). Vortex master mix 3-5 seconds.
5. Aliquot 18 µl of the master mix into each well of a MicroAmp Optical 96-well Reaction Plate that will contain standards, controls, or samples. The use of an automatic pipettor is recommended for consistency and accuracy. Do not add master mix to wells that will not contain sample.

6. Add 2 µl of standard, sample, or control to the appropriate wells, making sure that each sample is mixed well by pipetting up and down a few times. For standards, controls, and samples that will be run in duplicate, vortex 3-5 seconds before and between duplicates to keep them as consistent as possible.
7. Seal the plate with an Optical Adhesive Cover. Use the sealing tool to ensure that all corners and spaces between wells are adhered properly to the plate. Check that no air bubbles interfere with cover adhesion. Centrifuge plate to remove bubbles from well bottoms.
8. Place plate in the ABI PRISM® 7500 with well A1 oriented in the upper left corner.
9. For the first time running the HY system in the SDS software, add the appropriate detectors for the reporter dyes for Autosomal (FL), Y (CO650), and IPC (CR610). Select IC5 as the Passive Reference dye. For this and subsequent runs, add these detectors to the plate document. Do not assign task or quantity to any of the samples. The addition of sample names is optional.
10. Set the thermal cycling program:
    a. Stage 1- 95°C for 2 minutes
    b. Stage 2 - 38 cycles of 95°C for 5 seconds/ 60°C for 35 seconds
    c. Stage 3 – default "Dissociation Function"
        i. 95°C for 15 seconds
        ii. 60°C for 1 minute
        iii. 95°C for 15 seconds

11. Save document and press start.
12. Press analyze at the completion of the run. Export amplification data and melt/dissociation data.
13. Download the Plexor® Analysis Software (forensic release) free of charge from the Promega website at www.promega.com/plexorhy/. Import amplification data and melt/dissociation data into the software. Define sample names, standards and quantities, and controls. Adjust the expected target melt temperature. The average melt temperature for the autosomal target is 79-81°C, 81-83°C for the Y-chromosome target, and 79-81°C for the IPC. Generate the standard curves for the autosomal and Y-chromosome targets. Note the R^2 values, Y-intercept, and the PCR efficiency (%).

Sensitivity Study

Sensitivity studies were performed in duplicate for each sex with Quantifiler® Duo and Plexor® HY to establish the sensitivity levels of each quantification assay. Female K562 high molecular weight control DNA (~240 ng/µl, confirmed by quantification) from Promega and male Quantifiler® Human (QH) DNA standard (200 ng/µl stock) from Applied Biosystems were diluted in a two-fold fashion. Due to constraints with the number of reactions left in each kit, every other dilution was chosen instead to achieve concentrations of 50, 12.5, 3.13, 0.78, 0.2, 0.05, 0.012, and 0.003 ng/µl. Samples were run in duplicate per plate, with two plates being run on separate days. Serial dilution was performed at follows:

K562

1. Placed 50 µl of 240 ng/µl K562 DNA into a 0.5 ml tube containing 190 µl of sterile water to create the 50 ng/µl concentration. Tube was vortexed and pipette tip was changed.
2. Placed 120 µl of 50 ng/µl K562 DNA into a 0.5 ml tube containing 120 µl of sterile water. Tube was vortexed and pipette tip was changed.
3. Serial dilutions were continued in a 1:2 manner. Every other dilution was selected for quantification assays.

Quantifiler Human (QH)

1. Placed 50 µl of 200 ng/µl QH DNA into a 1.5 ml tube containing 150 µl of sterile water to create the 50 ng/µl concentration. Tube was vortexed and pipette tip was changed.
2. Placed 100 µl of 50 ng/µl K562 DNA into a 1.5 ml tube containing 100 µl of sterile water. Tube was vortexed and pipette tip was changed.
3. Serial dilutions were continued in a 1:2 manner. Every other dilution was selected for quantification assays.

Mixture Study

Mixture studies were performed in duplicate for with Quantifiler® Duo and Plexor® HY. Male:female DNA mixtures were created from K562 control DNA and Quantifiler® Human (QH) DNA standard. Male:female ratios included 1:2, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. Female K562 DNA (~240

ng/µl) was diluted to 20 ng/µl and was kept constant in each mixture. Male QH DNA (200 ng/µl) was diluted to 20 ng/µl and then concentration was decreased in a two-fold manner for each mixture. Mixtures were run in duplicate per plate, with two plates being run on separate days. Mixtures were created as follows:

1. Diluted QH male DNA (200 ng/µl) down to 20 ng/µl by pipetting 10 µl DNA into a 1.5 ml tube containing 90 µl sterile water.
2. Serially diluted male DNA in a two-fold fashion. First tube contained 50 µl of the 20 ng/µl DNA. Tube was vortexed and pipette tip changed. Transferred 20 µl of 20 ng/µl DNA to a tube containing 20 µl sterile water. This created the 10 ng/µl male DNA. Continued diluting until serial dilutions complete.
3. Diluted K562 female DNA (~240 ng/µl) down to 20 ng/µl by pipetting 25 µl DNA into a 1.5 ml tube containing 275 µl sterile water.
4. Created mixtures by combining 20 µl of K562 female DNA (20 ng/µl) with 20 µl of QH male DNA at the various concentrations.

Concordance Study

Selected casework samples were provided by analysts at UNTCHI based on prior Quantifiler® Human quantification results. Samples with known degraded DNA were run on Quantifiler® Duo and Plexor® HY. Previous quantities from Quantifiler® Human were compared to the new data to look at the effect of the increased amplicon sizes in both kits. Samples were not run in duplicate, and duplicate plates were not run in order to mimic the actual laboratory procedures for casework quantification.

CHAPTER V

RESULTS AND DISCUSSION

Sensitivity Study

Both Quantifiler® Duo and Plexor® HY quantification assays were tested for sensitivity. The Quantifiler® Duo manual states a dynamic range of 0.023 to 50 ng/µl for both autosomal and male DNA. However, quantities as low as 0.0115 ng/µl were also reproducibly detected by studies at Applied Biosystems, while concentrations below 0.00575 ng/µl could not be detected. A human diploid cell contains approximately 0.007 ng of DNA. The kit was evaluated using known DNA in concentrations that ranged from 0.003 to 50 ng/µl. The concentrations determined by real-time PCR quantification assays are not absolute but provide a fairly accurate estimate of the amount of DNA in a sample. For this study, the concentrations determined are the average of two replicates. Using the new Quantifiler® Duo system, the concentration of autosomal DNA obtained from the K562 female DNA ranged from 0.0014 to 53.56 ng/µl (Figure 6). Typically, Y-intercept values greater than 28.5 indicate an overestimation of the sample's concentration. Utilizing Quantifiler® Duo system, Y-intercept values were consistently around 29.2, indicating a slight overestimation as seen by the upper concentration value (53.56 versus 50 ng/µl). In this study, the Quantifiler® Duo system was able to detect lower concentrations than previously stated by Applied Biosystems.

Using the male DNA standard provided in the Quantifiler® Human kit, the concentrations of male DNA detected by the Quantifiler® Duo system ranged from 0.0066 to 101.66 ng/µl (Figure 7). However, using this same male DNA sample, the Quantifiler® Duo kit indicated that the range of autosomal DNA was 0.0053 to 156.98 ng/µl (Figure 8). It is not clear why the concentration range would differ between the reading for autosomal DNA and male DNA since the sample quantified only contained DNA from a male. As previously seen using the female DNA sample, the male DNA sample also provided concentrations that fell below the reported detection limit for Quantifiler® Duo. Using this male control DNA sample, the Y-intercept values were consistently around 30.5, which may account for the overestimation of male DNA. In this study, the male DNA (autosomal and Y) was consistently overestimated by the Quantifiler® Duo assay. The Applied Biosystems manual does not provide any information as to why the male (Y) DNA was consistently overestimated. It is not apparent that the algorithms employed by Applied Biosystems take into consideration that male DNA contains only a single copy of the Y-chromosome. Since the quantification of male DNA appears to be consistently overestimated, a correction factor can be utilized to estimate the final concentration of a male DNA sample.

Since real-time PCR assays are extremely sensitive, the observation of high C_T values (greater than 35) could indicate the presence of very low quantities of recovered DNA. DNA with C_T values greater than 35 usually do not produce reliable STR results and may even represent the presence of random contamination. It is suggested that laboratories establish a maximum C_T value threshold in determining whether a sample

should be amplified for either autosomal or Y-STR markers. Samples with C_T values above that threshold could be considered for mitochondrial DNA analysis.

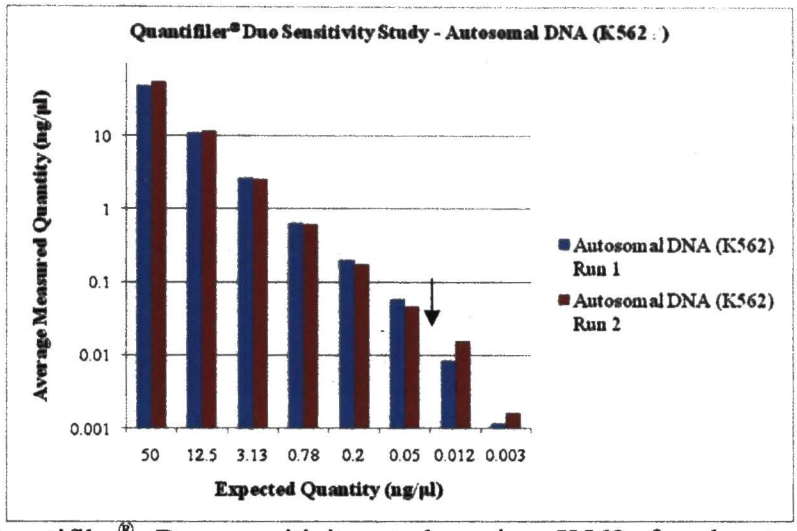

Figure 6. Quantifiler® Duo sensitivity study using K562 female stock DNA for quantification of human autosomal DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.0014 to 53.56 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.023 ng/µl).

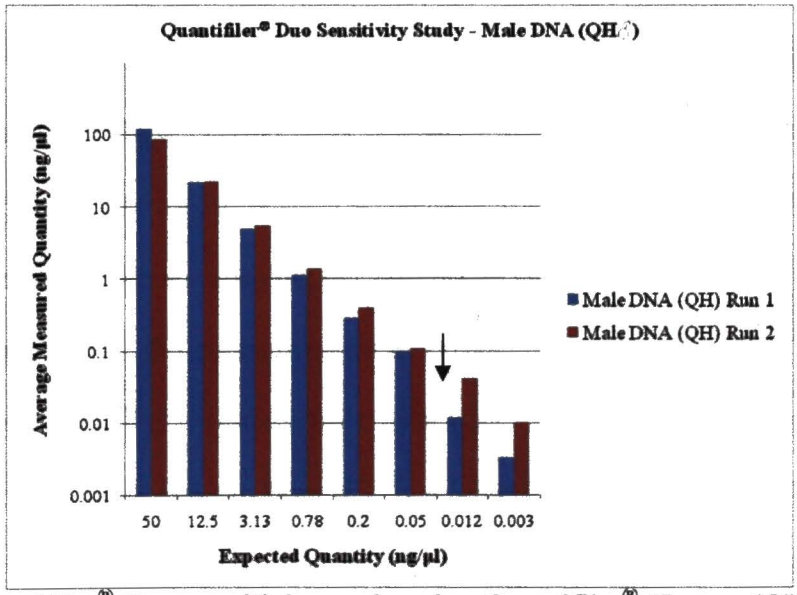

Figure 7. Quantifiler® Duo sensitivity study using Quantifiler® Human (QH) male stock DNA for quantification of male DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.0066 to 101.66 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.023 ng/µl).

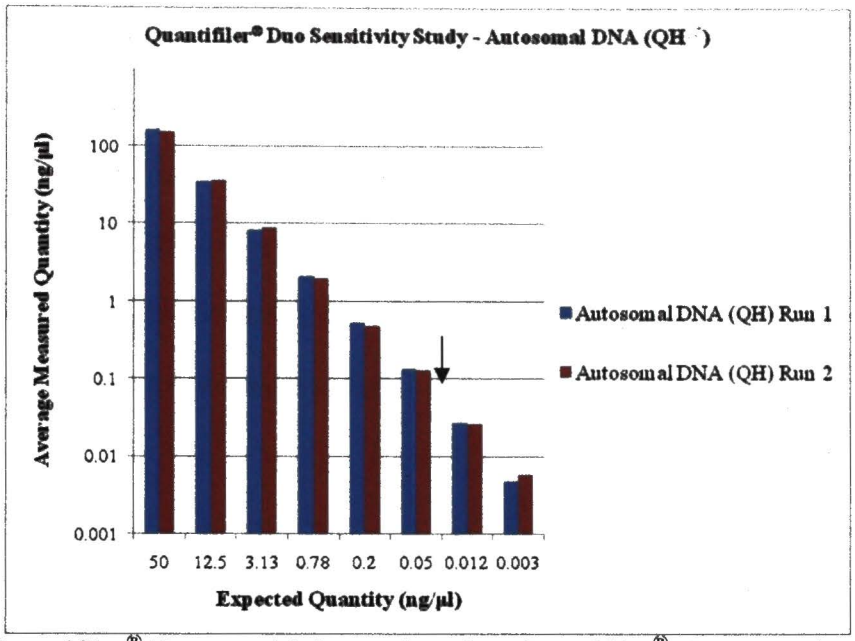

Figure 8. Quantifiler® Duo sensitivity study using Quantifiler® Human male stock DNA for quantification of human autosomal DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.0053 to 156.98 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.023 ng/µl).

The Plexor® HY manual states a dynamic range of 0.0032 to 50 ng/µl for both autosomal and male DNA. The kit was evaluated using known DNA in concentrations that ranged from 0.003 to 50 ng/µl. The concentrations determined by real-time PCR quantification assays are not absolute but provide an estimate of the amount of DNA in a sample. For this study, the concentrations determined are the average of two replicates. Using the new Plexor® HY system, the concentration of autosomal DNA obtained from the K562 female DNA ranged from 0.133 to 222.5 ng/µl (Figure 9). At the highest concentration, the expected DNA concentrations with the female sample were 50 ng/µl and the Plexor HY estimated this concentration at 222.5 ng/µl. This represents an approximate 4.5-fold estimate of the amount of female DNA present.

Using the male DNA standard provided in the Quantifiler® Human kit, the concentrations of male DNA detected by the Plexor® HY system ranged from 0.0635 to 125 ng/µl (Figure 10). Using this same male DNA sample, the Plexor® HY kit indicated that the range of autosomal DNA was 0.0925 to 73.5 ng/µl (Figure 11). It is apparent that autosomal and male DNA is greatly overestimated with Plexor® HY, with female DNA in particular having the most extreme values.

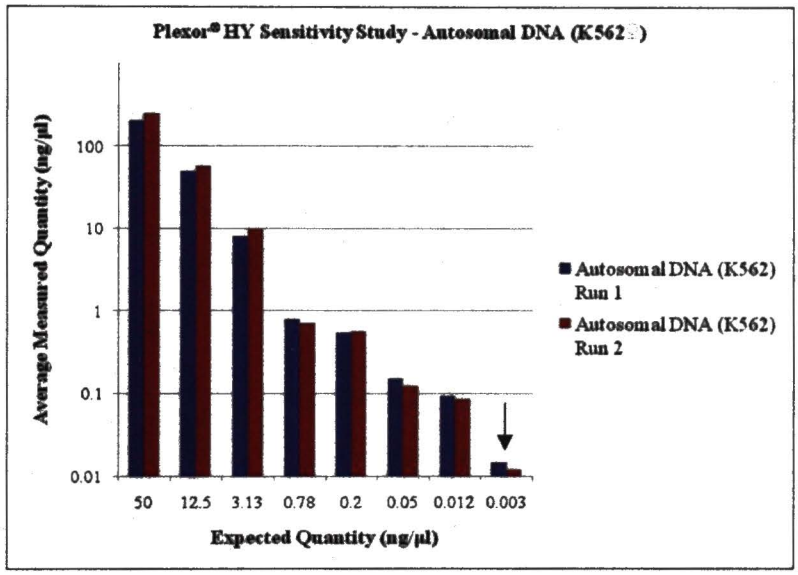

Figure 9. Plexor® HY sensitivity study (runs 1 and 2) using K562 female stock DNA for quantification of human autosomal DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.133 to 222.5 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.0032 ng/µl).

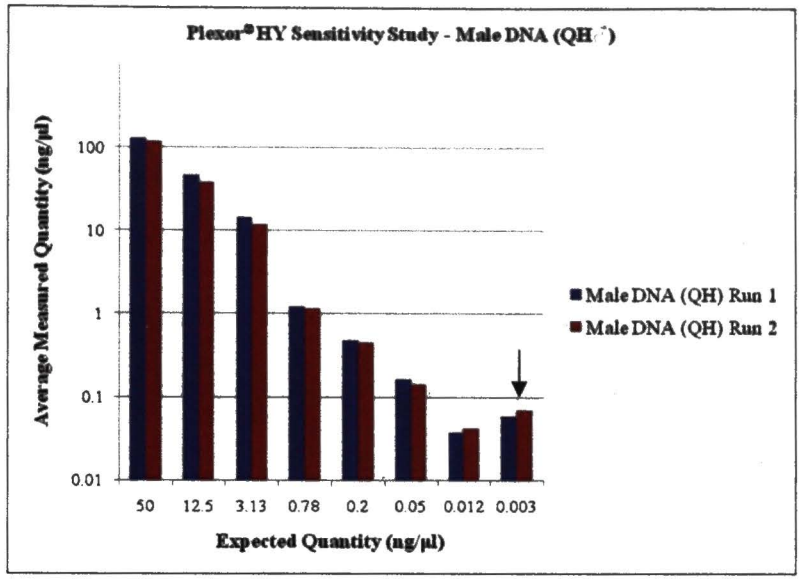

Figure 10. Plexor® HY sensitivity study using Quantifiler® Human (QH) male stock DNA for quantification of male DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.0635 to 125 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.0032 ng/µl).

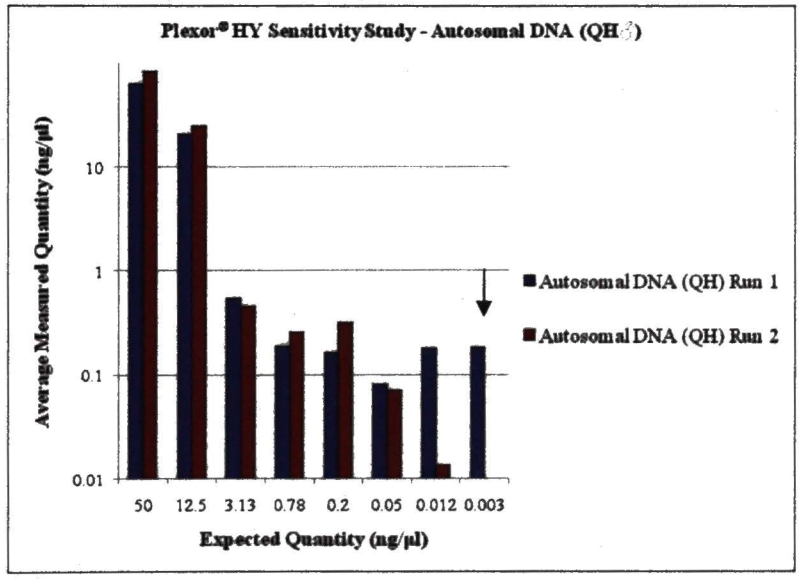

Figure 11. Plexor® HY sensitivity study using Quantifiler® Human male stock DNA for quantification of human autosomal DNA. Expected quantities ranged from 0.003 to 50 ng/µl. Average measured quantities ranged from 0.0925 to 73.5 ng/µl. Arrow indicates lowest concentration threshold of the standard curve (0.0032 ng/µl).

The results of the sensitivity studies were then compared between the two assays to identify any consistent trends in autosomal and male DNA quantification. For these comparisons, the exact same DNA samples were quantified with both systems. In comparison with Quantifiler® Duo, data obtained with the Plexor® HY routinely overestimated the amount of autosomal DNA (Figures 12 and 13) and male DNA (Figures 14 and 15). The overestimation of the DNA concentrations as determined by Plexor® HY may have a significant impact on the quality of the STR typing results. Although initially less sample would be used, this overestimation will likely result in no or partial STR profiles. Ultimately, additional amplification reactions would have to be performed which results in overconsumption of the forensic evidentiary sample. When utilizing a male control DNA sample, the autosomal DNA quantification using Quantifiler® Duo was greater than that of Plexor® HY (Figures 16 and 17).

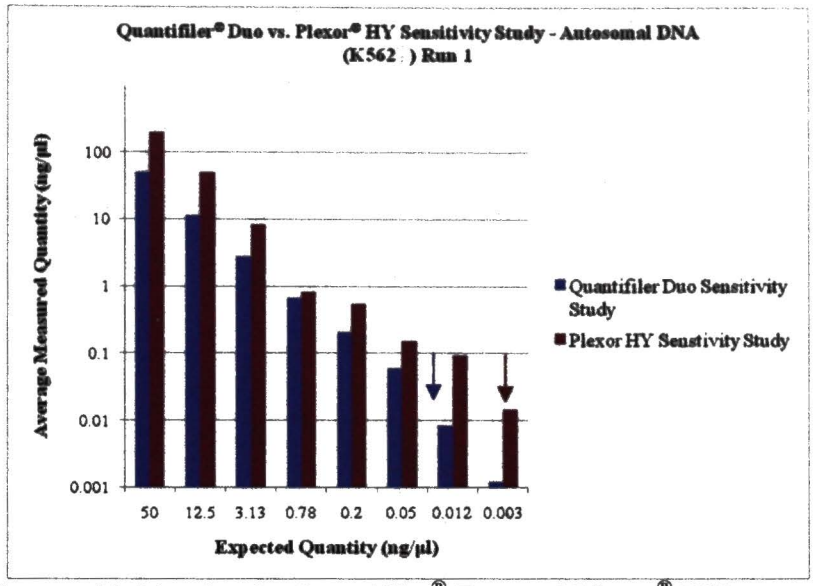

Figure 12. The sensitivities for the Quantifiler® Duo and Plexor® HY systems autosomal DNA estimation using K562 female DNA. This data is obtained from the first plate. Blue arrow indicates lowest concentration threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration detection of Plexor® HY standard curve (0.0032 ng/µl).

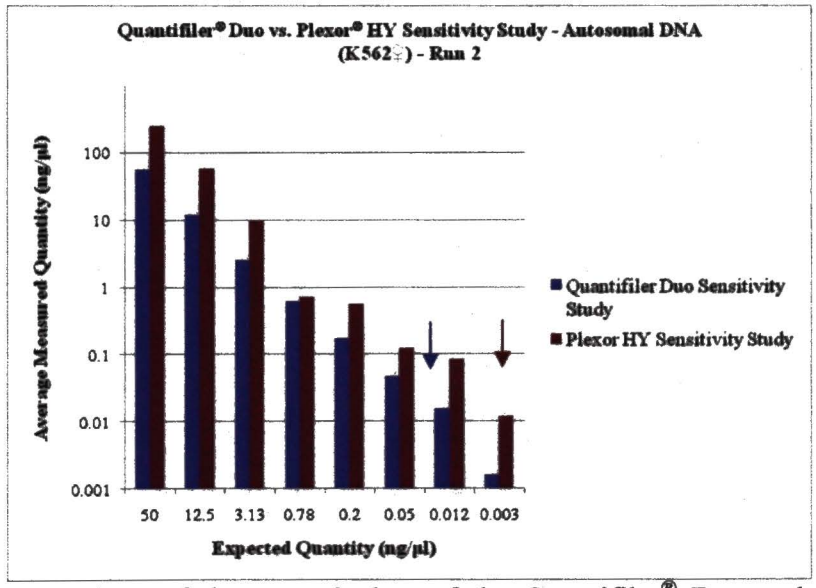

Figure 13. Comparison of the second plate of the Quantifiler® Duo and Plexor® HY sensitivity studies for autosomal DNA estimation using K562 female DNA. Blue arrow indicates lowest concentration threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration threshold of Plexor® HY standard curve (0.0032 ng/µl).

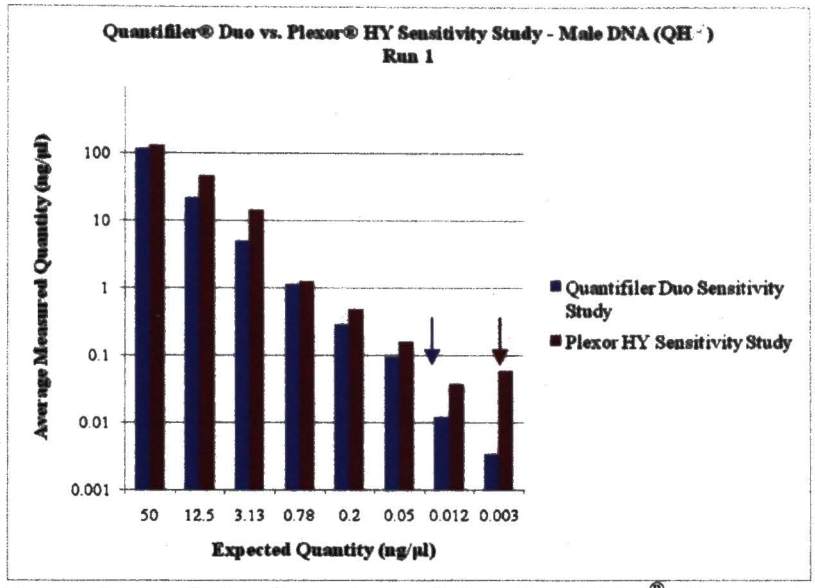

Figure 14. Comparison of the first plate of the Quantifiler® Duo and Plexor® HY sensitivity studies for male DNA estimation using Quantifiler® Human (QH) male DNA. Blue arrow indicates lowest concentration detection threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration detection threshold of Plexor® HY standard curve (0.0032 ng/µl).

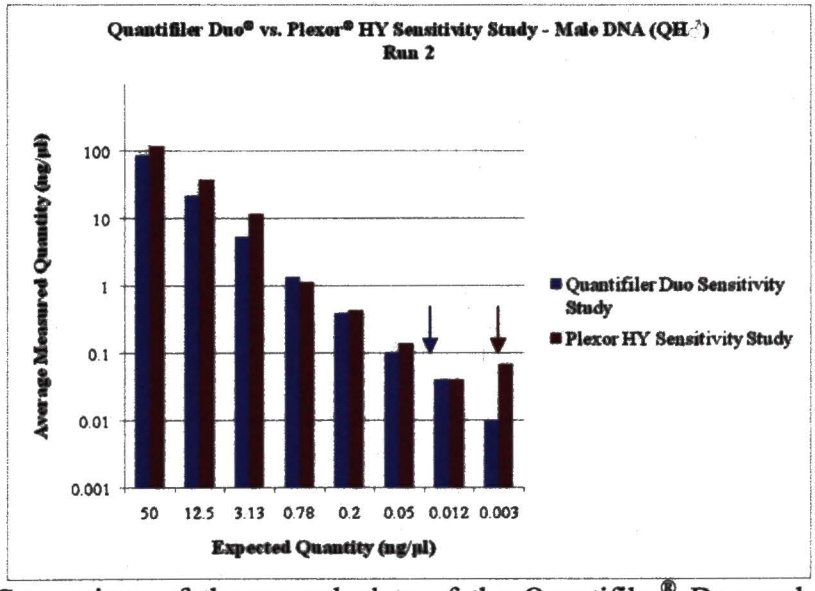

Figure 15. Comparison of the second plate of the Quantifiler® Duo and Plexor® HY sensitivity studies for male DNA estimation using Quantifiler® Human (QH) male DNA. Blue arrow indicates lowest concentration detection threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration detection threshold of Plexor® HY standard curve (0.0032 ng/µl).

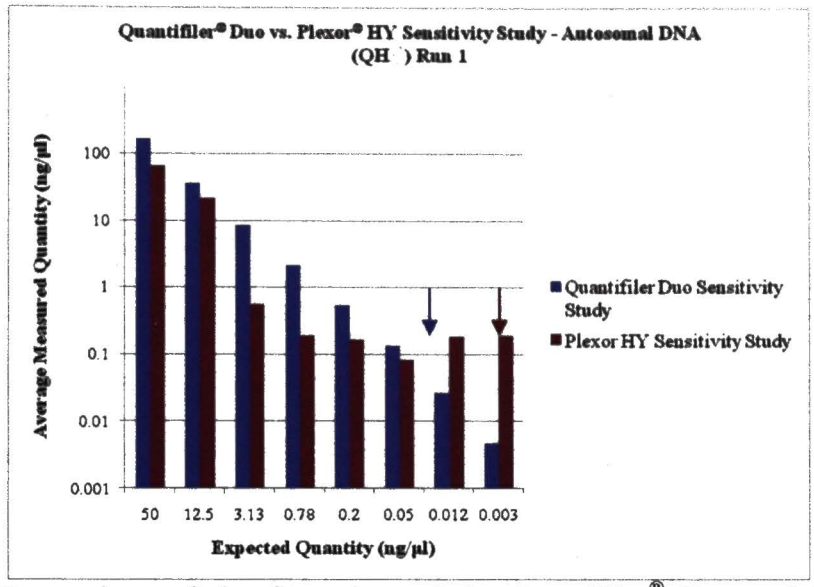

Figure 16. Comparison of the first plate of the Quantifiler® Duo and Plexor® HY sensitivity studies for autosomal DNA estimation using Quantifiler® Human (QH) male DNA. Blue arrow indicates lowest concentration detection threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration detection threshold of Plexor® HY standard curve (0.0032 ng/µl).

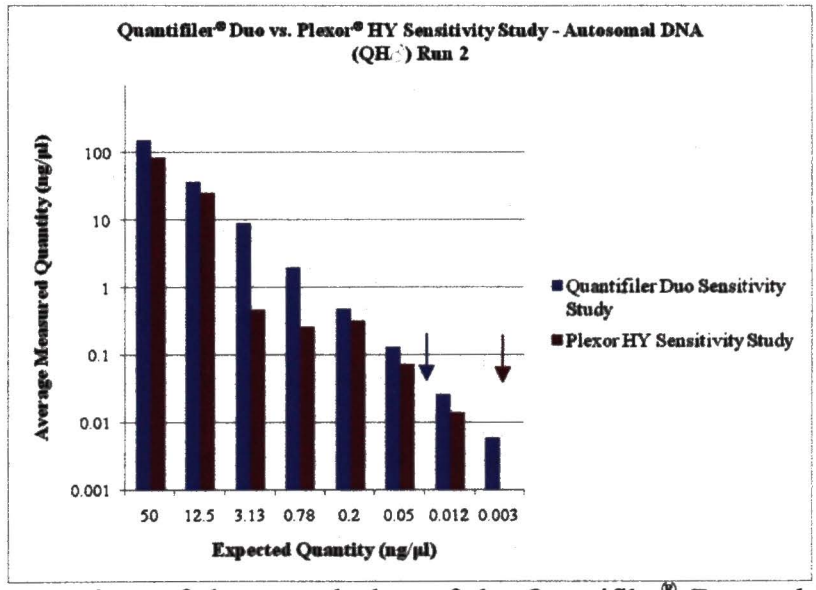

Figure 17. Comparison of the second plate of the Quantifiler® Duo and Plexor® HY sensitivity studies for autosomal DNA estimation using Quantifiler® Human (QH) male DNA. Blue arrow indicates lowest concentration detection threshold of Quantifiler® Duo standard curve (0.023 ng/µl). Red arrow indicates lowest concentration detection threshold of Plexor® HY standard curve (0.0032 ng/µl).

Mixture Study

Male and female DNA from Quantifiler® Human and K562, respectively, were combined in the following ratios of male to female DNA: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. Each mixture was quantified using Quantifiler® Duo and Plexor® HY assays. Figures 18 – 21 show that male DNA was detected in all samples, including the 1:1024 mixture in which the male DNA was only 0.1% of the entire sample. The quantification value of that male DNA was 10 pg/µl, equivalent to approximately three sperm heads/µl. In both assays, the ability to quantify male DNA was not affected by the range of the quantities of female DNA.

Since Quantifiler® Duo allows for the determination of human and male DNA in a sample, the ratio of male and female DNA can be calculated with the following equation:

Male DNA : Female DNA Ratio =

Male DNA/Male DNA : (Human DNA – Male DNA)/Male DNA

Figures 18 and 19 show the mixture ratio ranges. Note that the concentration of male DNA steadily decreases in both runs while the female concentration remains fairly consistent. Duplicate mixture samples were run on duplicate plates. The actual male: female ratios ranged from 1:0.989 to 1:1132.3 in the first plate (Figure 18). These values are fairly consistent with the expected ratios. The actual male: female ratios ranged from 1:1.1 to 1:316.4 in the second plate (Figure 19). The male: female ratio is useful in determining whether to continue with autosomal STR or Y-STR analysis. Mixture experiments as part of an internal validation study would designate the male:female ratio

at which Y-STR analysis would be preferred over autosomal STR analysis to obtain the male DNA profile.

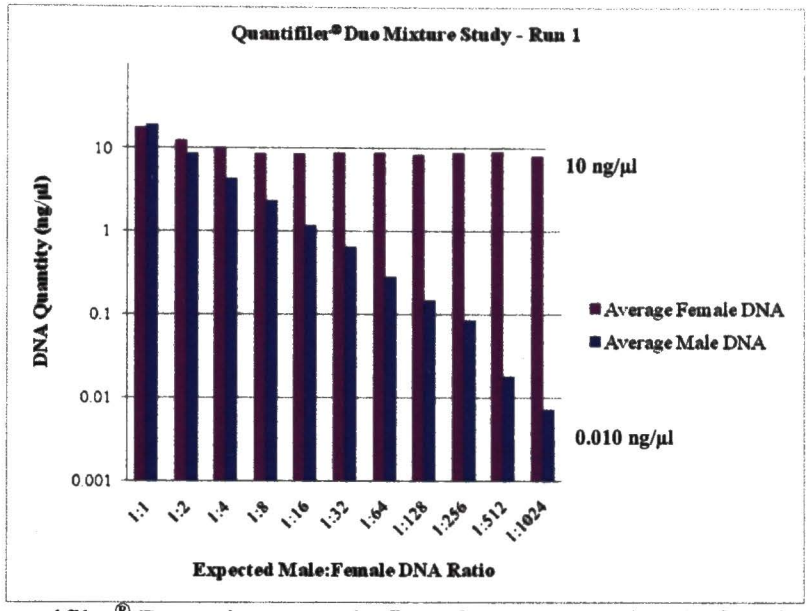

Figure 18. Quantifiler® Duo mixture study first plate. Expected male:female ratios ranged from 1:1 to 1:1024. Actual male:female ratios ranged from 1:0.989 to 1:1132.3.

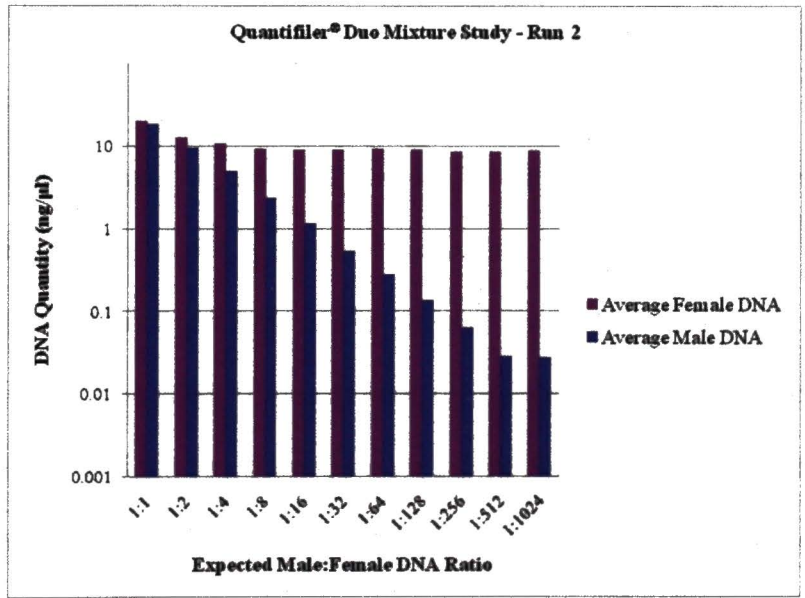

Figure 19. Quantifiler® Duo mixture study second plate. Expected male:female ratios ranged from 1:1 to 1:1024. Actual male:female ratios ranged from 1:1.1 to 1:316.4.

Plexor® HY uses the [AUTO]/[Y] ratio to predict the likelihood of obtaining a successful male autosomal STR profile from a mixed sample. A full male autosomal profile is expected when the [AUTO]/[Y] is less than ten, and no male autosomal profile is expected when [AUTO]/[Y] is 100 or greater [12]. The expected range of [AUTO]/[Y] ratios in this study is 2 to 1000. In Figure 20, the male: female ratios ranged from 1:0.635 to 1:3090 (0.03% of the mixture was detected to be male). This translates into [AUTO]/[Y] ratios ranging from 1.63 to 3091. In Figure 21, the male:female ratios ranged from 1:1.14 to 1:5219 (0.02% of the mixture was detected to be male). The [AUTO]/[Y] ratio ranges from 2.14 to 5220. It would be expected that the ability to obtain a male full male autosomal profile would cease around the 1:8 ratio, where the [AUTO]/[Y] ratio is nine. Loss of all male autosomal alleles would occur around the 1:128 ratio, where the [AUTO]/[Y] ratio is 129.

Again, notice the steady decrease in the concentration of the male portion of the mixtures of both runs. The female DNA concentration does not remain as steady as expected. The unexpected increase in the male fraction of the mixture at the 1:32 ratio is likely the result of a well-dependent fluorescence detection problem that will be discussed further in later sections. It is suggested that laboratories generate guidelines that distinguish the [AUTO]/[Y] ratio at which the path of analysis will include Y-STRs instead of autosomal STRs to obtain the male profile from a mixture. Mixture studies as part of an internal validation would establish the [AUTO]/[Y] ratio at which Y-STR analysis would be preferred over autosomal STR analysis.

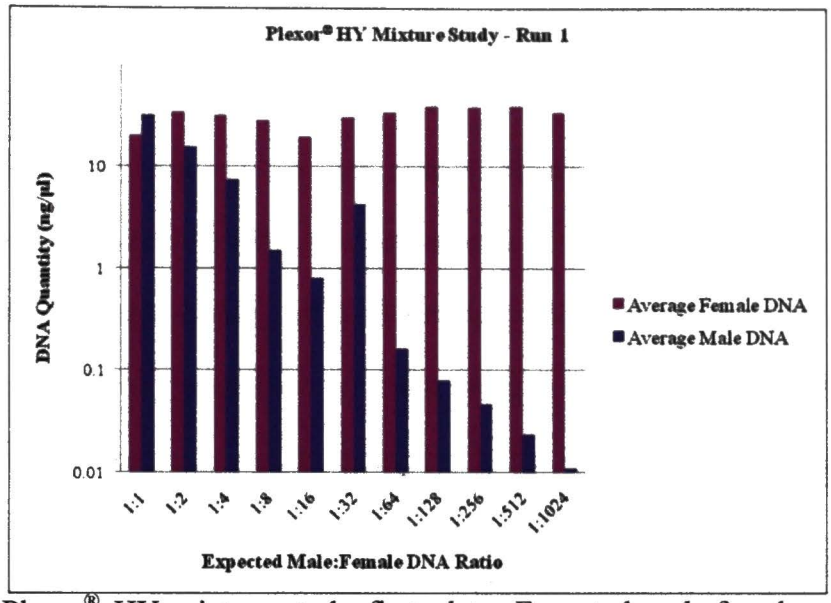

Figure 20. Plexor® HY mixture study first plate. Expected male:female ratios ranged from 1:1 to 1:1024. Actual male:female ratios ranged from 1:0.635 to 1:3090.

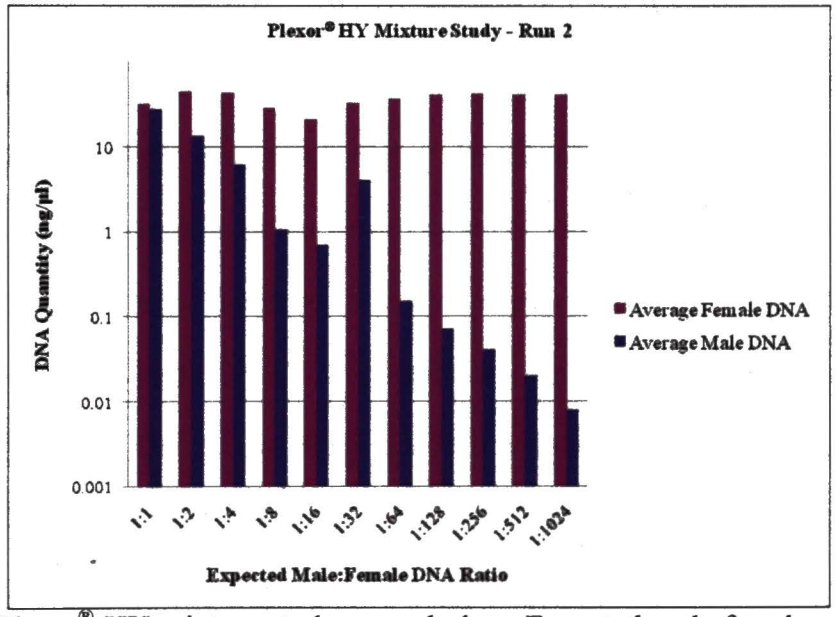

Figure 21. Plexor® HY mixture study second plate. Expected male:female ratios ranged from 1:1 to 1:1024. Actual male:female ratios ranged from 1:1.14 to 1:5219.

Concordance Study

Eight known degraded casework samples from the Unidentified Human Remains section of UNTCHI were run on both assays (Figure 22). Quantifiler® Human had previously overestimated the DNA quantity in some of these samples, which mislead the analysts in their decision-making for further analysis. This lead to very poor to no usable STR results despite the quantification estimate being in an acceptable range (Figures 23 - 27). One goal of this study was to determine if the lengthened amplicon featured in Quantifiler® Duo helped to overcome this issue with degraded samples. Sample 07-4925.1.1A did not yield any results with Quantifiler® Duo, but gave an estimate of 0.223 ng/µl with Quantifiler® Human. All but one sample, F-2454.2A1, gave reduced concentration estimates with Quantifiler® Duo, which is to be expected.

Another goal was to compare the estimates between the assays (Figure 22). Plexor® HY consistently gave much higher concentration estimates than Quantifiler® Duo. According to the results of the sensitivity study, this discrepancy is very significant and could lead to similar problems that were experienced with Quantifiler® Human. Samples 07-4925.1.1A and 07-4925.1.1B did not yield any results on Plexor® HY and showed IPC failure. This is most likely due to a well-dependent fluorescence detection problem with all three dyes (autosomal, Y, and IPC). Sample 07-5216.1.1A also did not yield any autosomal results but did get male results (0.058 ng/µl). This indicates another well-dependent fluorescence detection problem with the autosomal dye. See the *Time and Cost Analysis* section for more details regarding this problem.

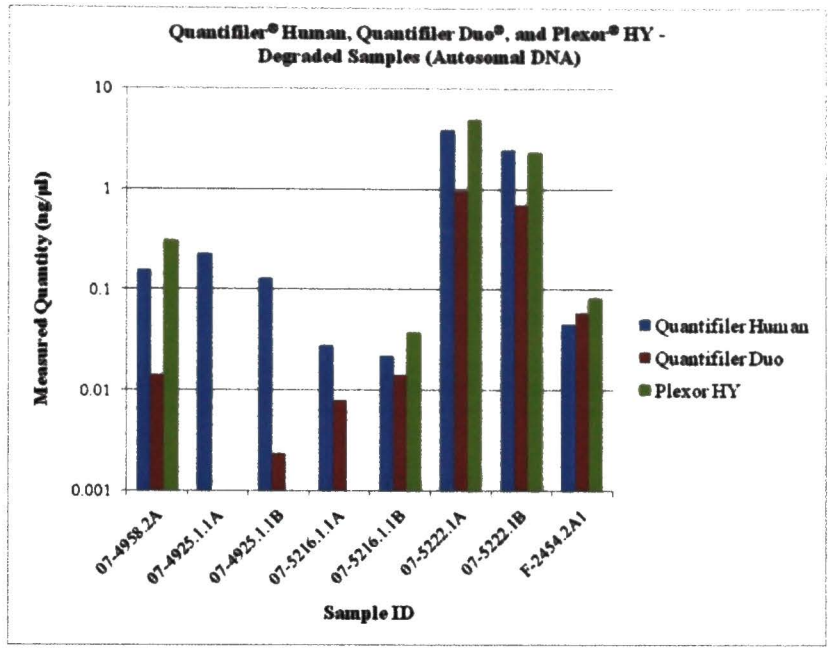

Figure 22. Comparison of the Quantifiler® Human, Quantifiler® Duo, and Plexor® HY concordance study using known degraded samples. The lack of results for samples 07-4925.1.1B and 07-5216.1.1A using Plexor® HY is most likely due to the well-dependent effects of the spectral calibration.

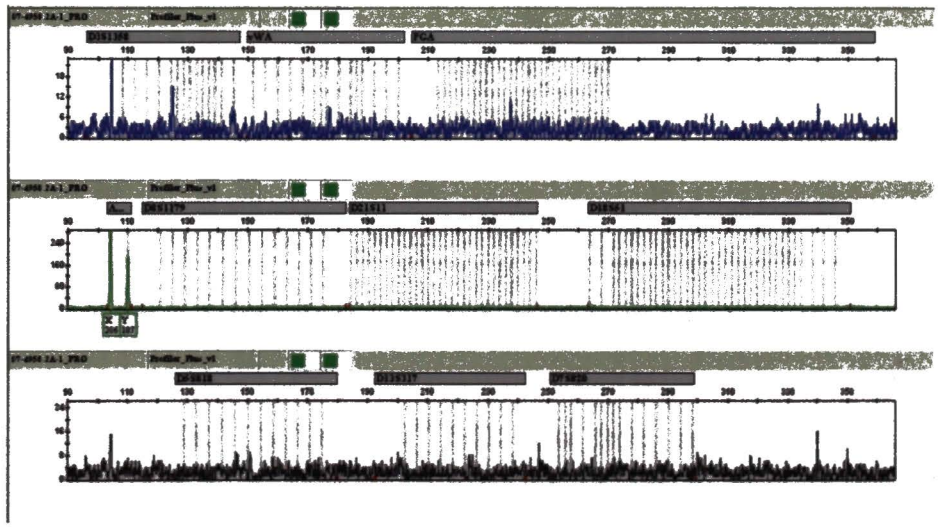

Figure 23. AmpFℓSTR® Profiler Plus® STR data from sample 07-4958.2A. Only amelogenin produced a result. Allele dropout occurred at all other loci.

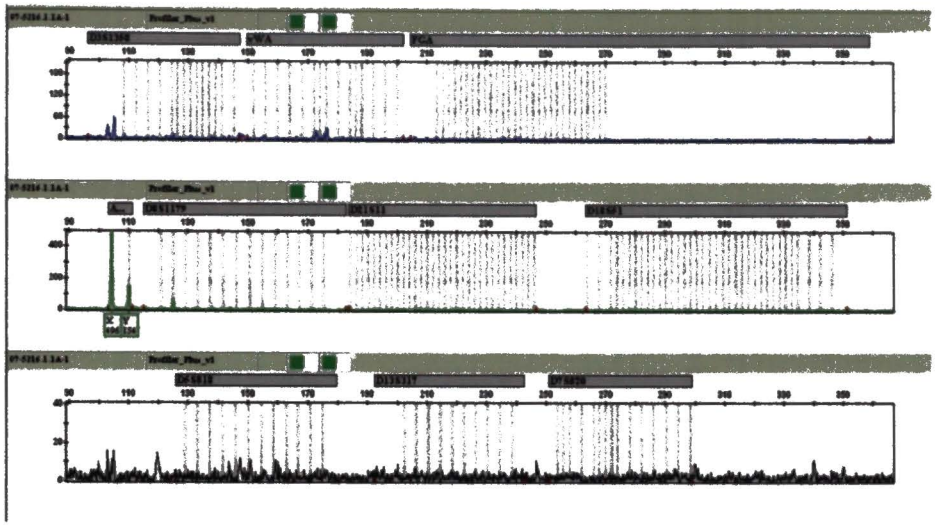

Figure 24. AmpFℓSTR® Profiler Plus® STR data from sample 07-5216.1.1A. Only amelogenin produced a result. Allele dropout occurred at all other loci.

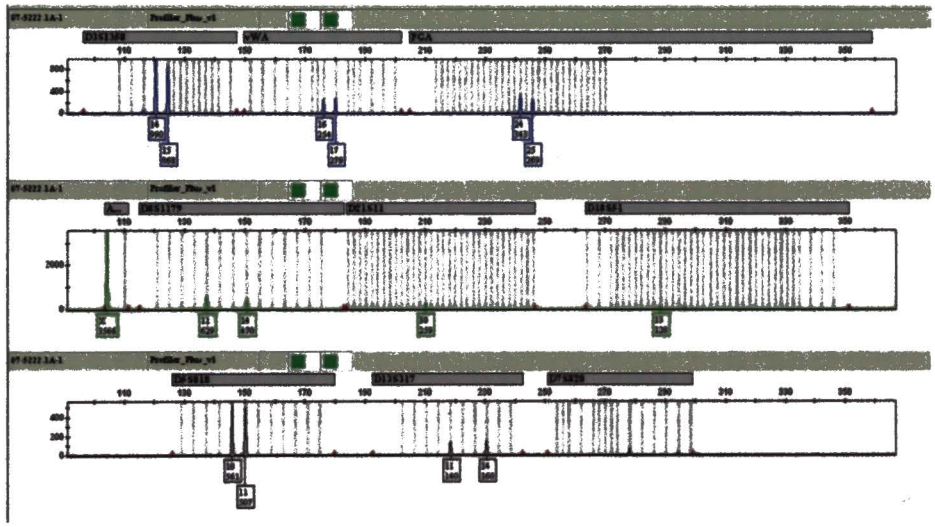

Figure 25. AmpFℓSTR® Profiler Plus® STR data from sample 07-5222.1A. Allele dropout is apparent in the D7S820 locus, and possible dropout has occurred at D21S11 and D18S51.

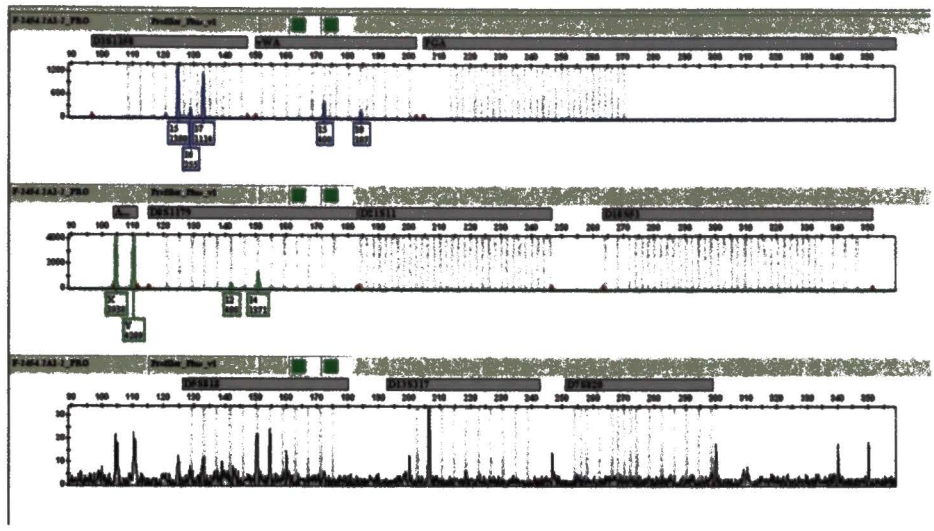

Figure 26. AmpFℓSTR® Profiler Plus® STR data from sample F-2454.2A1. Allele dropout has occurred at six of the ten loci.

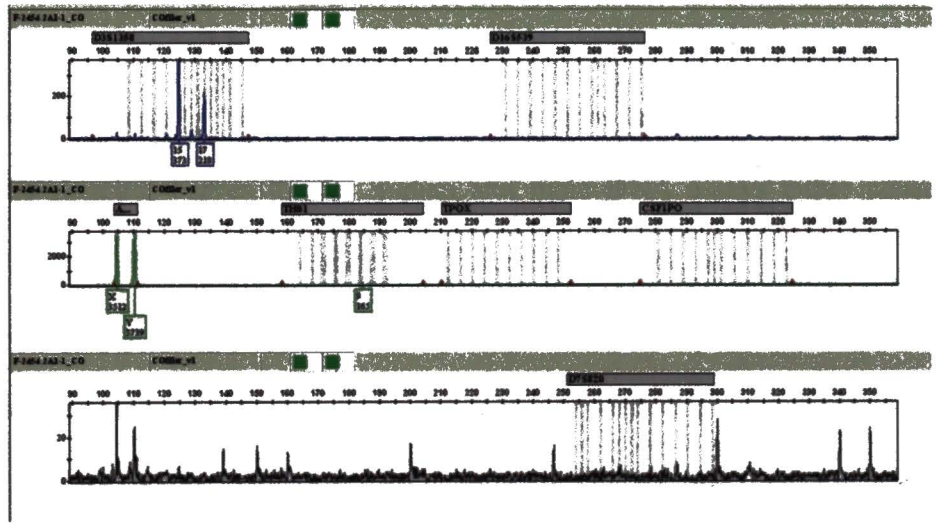

Figure 27. AmpFℓSTR® COfiler® STR data from sample F-2454.2A1. Allele dropout has occurred at five of the seven loci.

Software Analysis

The ease of use of the analysis software was also considered in the comparison of the two assays. Quantifiler® Duo utilizes the same 7500 System SDS Software as Quantifiler® Human, which is already implemented in the UNTCHI laboratory. This made the transition to using Duo seamless. The setup only required the addition of the dye detector for Y-chromosome target, which was as simple as a few mouse clicks. The only addition to the analysis itself was the need to inspect the male DNA standard curve along with the human DNA standard curve. After analysis, the results can be viewed a variety of ways under different tabs. The plate tab is identical to the plate setup tab, except that the quantification values are displayed for the human and male DNA targets (Figure 28). The amplification plot tab displays the raw data for the run (Figure 29). This data provides a way to visualize the C_T values, closeness of fit between duplicates, and which samples actually crossed the threshold to give results. The standard curve tab shows the standard curves for the human and male DNA assays either separately or together (Figure 30). The correlation coefficient (R^2), slope, and y-intercept values are displayed adjacent to the standard curve. The reports tab displays the results in an exportable spreadsheet format (Figure 31). The spreadsheet gives information on well position, sample name, detector (human, male, IPC), task (unknown or standard), C_T value, C_T value standard deviation (if duplicates), quantity, mean quantity (if duplicates), and quantity standard deviation (if duplicates). Filtered and T_m are not used in analysis for Quantifiler® kit purposes.

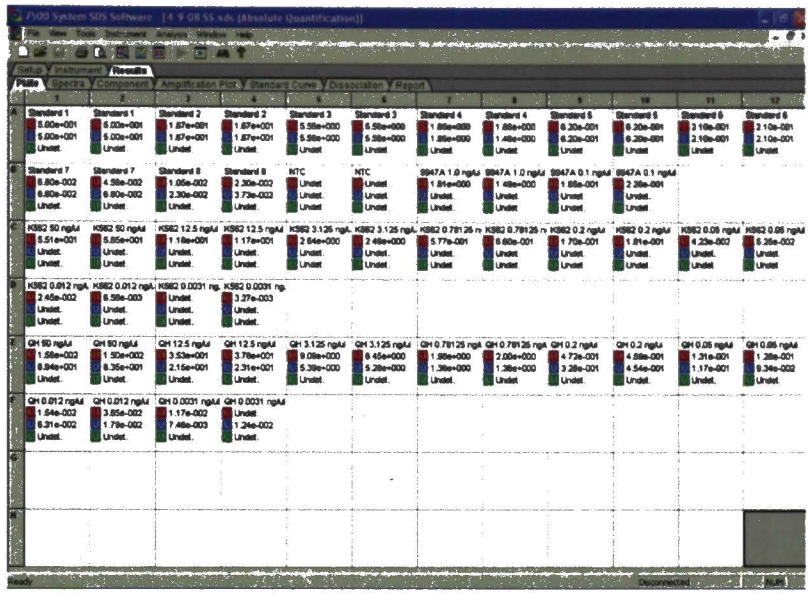

Figure 28. 7500 System SDS Software post-analysis sample sheet page depicting sample names, dye designation (unknown or standard), and measured quantities of autosomal, Y, and IPC detection.

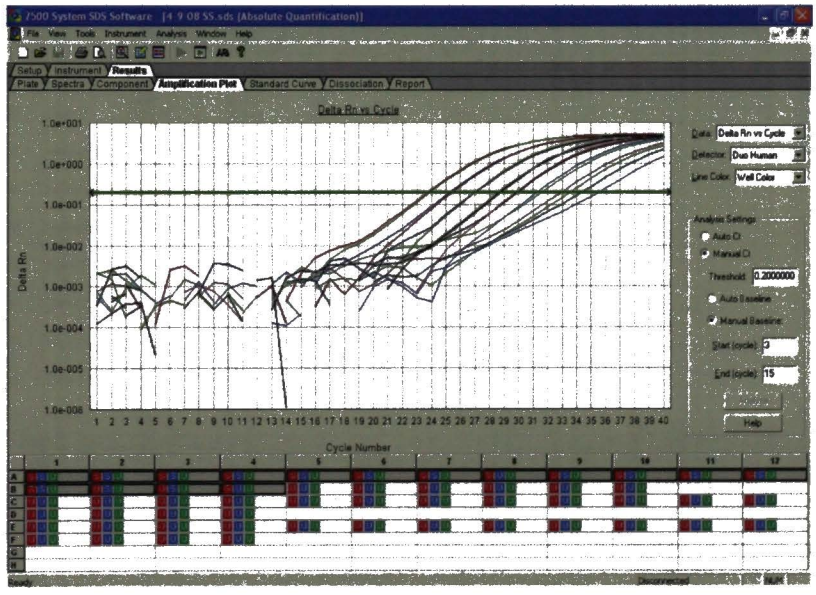

Figure 29. 7500 System SDS Software amplification plot depicting the human standards.

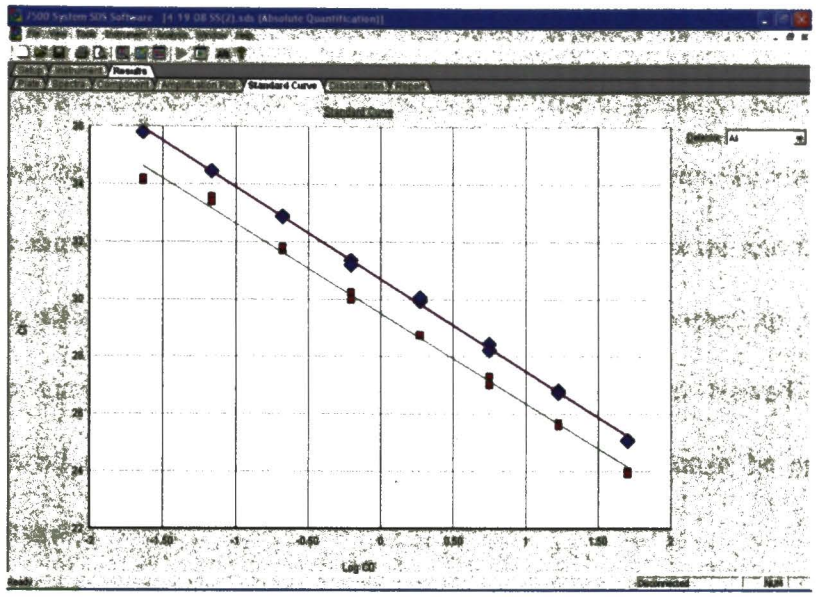

Figure 30. 7500 System SDS Software standard curve tab depicting both the human (red/green) and male (blue/purple) standard curve.

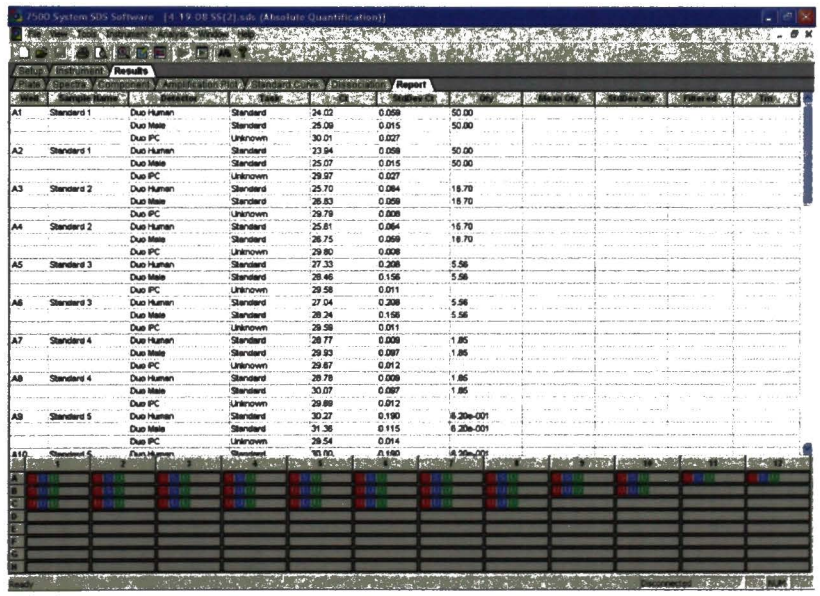

Figure 31. 7500 System SDS Software reports tab displaying well position, sample name, detector, task, C_T, C_T standard deviation, quantity, mean quantity, and quantity standard deviation (filtered and Tm are not used in analysis for Quantifiler® purposes).

The Plexor® Analysis Software (forensic release) is free for download (www.promega.com/plexorhy/); however, the cost in time to actually analyze the results is much greater than that for the Applied Biosystems SDS Software. To analyze Plexor® data, samples must be analyzed (green arrow) first in SDS Software and then two files (amplification data and melt/dissociation data) must be exported to the Analysis Software. No quantification results or standard curves can be viewed on the SDS Software, and all sample names and designation of standards must be entered in the Analysis Software.

Figure 32 displays the sample ID tab, where each sample name is entered independently. The manual states that it is possible to copy and paste a spreadsheet with the names, but this feature did not work during this study. The PCR curves tab displays the amplification plot, melt curve plot, the well selector area, and a legend showing selected wells (Figure 33). Unlike the SDS software, this program does not allow the simultaneous viewing of the autosomal, Y, and IPC data. The standard curve feature has separate tabs for the autosomal and Y standard curves (Figure 34). In order for samples to be analyzed, they are placed on the standard curve itself, making viewing rather cluttered. The samples detail tab is similar to the report tab of the SDS software (Figure 35). Here the data, which includes the C_T values, melting temperatures, and concentrations, is displayed in a spreadsheet format.

One major difference with the Plexor® Analysis Software is the option of generating a forensics report (Figure 36). This report is derived from analyst input values concerning target ranges for sample input volume (normalization parameters). Included

in the report are the [AUTO]/[Y] ratio, the STR dilution status, and STR volume, quantity, and dilution factor. The STR dilution status indicates whether the sample contains enough DNA to use directly or if a dilution is required. The STR volume shows the volume of input DNA that should be used for STR analysis. The STR quantity indicates the amount of sample that will be input if the STR volume is followed. The STR dilution factor is the factor that is necessary to obtain the concentration target inputted by the analyst. This feature has the potential to save time in the laboratory that would be devoted to performing dilution calculations.

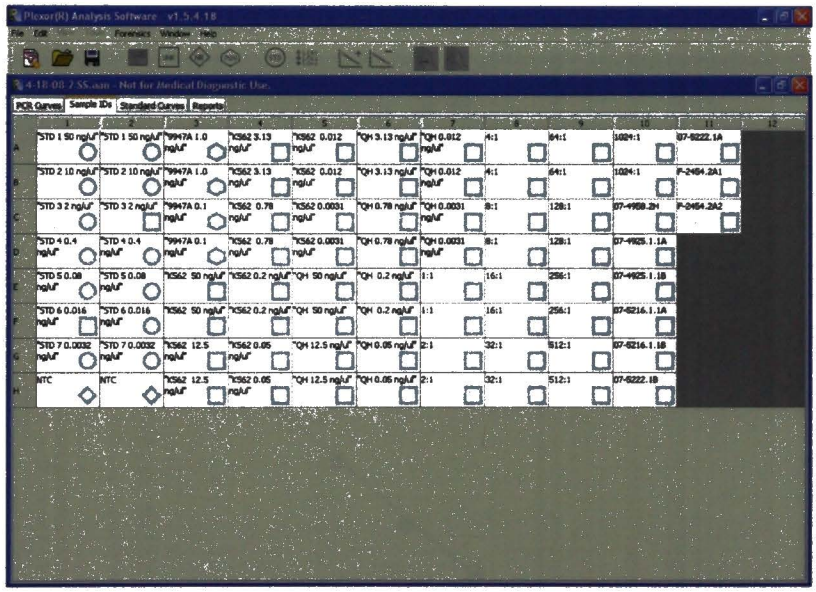

Figure 32. Plexor® Analysis Software sample ID tab.

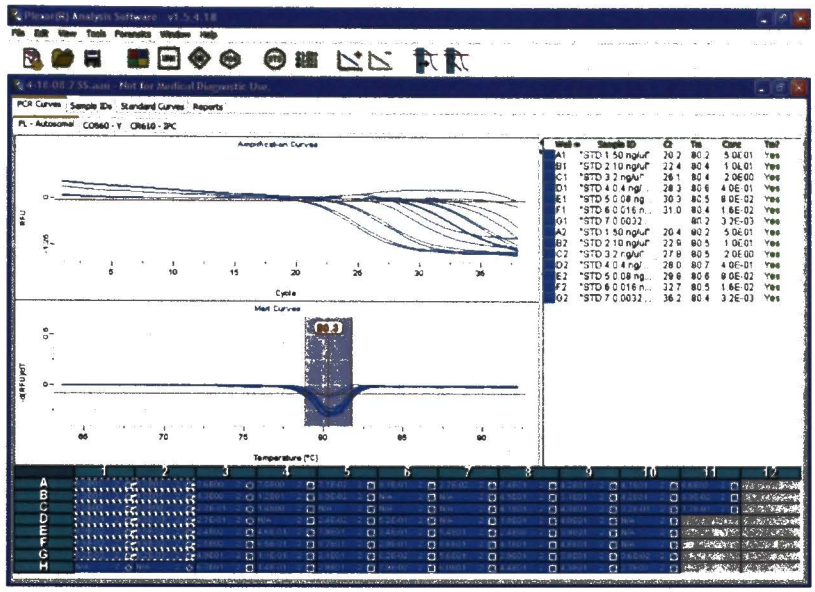

Figure 33. The PCR Curves tab of the Plexor® Analysis Software showing the amplification curves window, the melt curves window, and the well selector area.

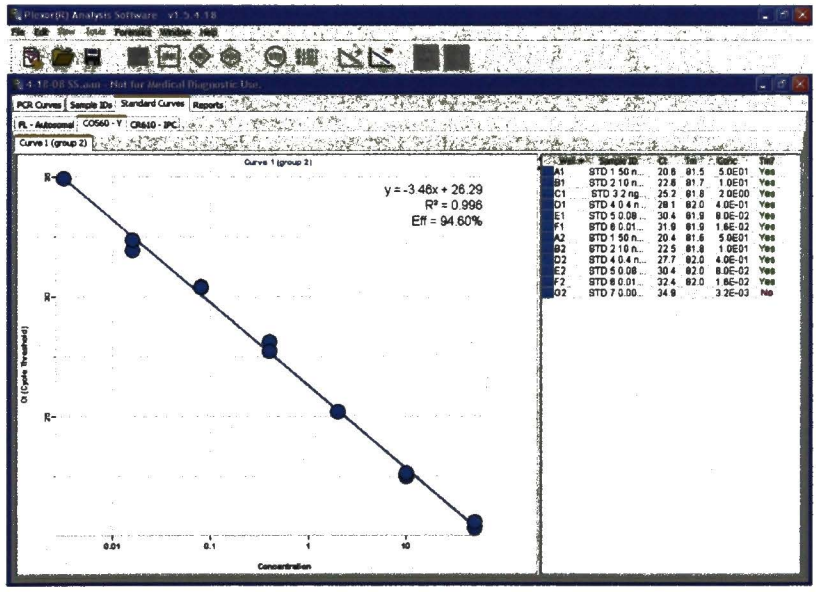

Figure 34. Plexor® Analysis Software standard curves tab showing an autosomal standard curve.

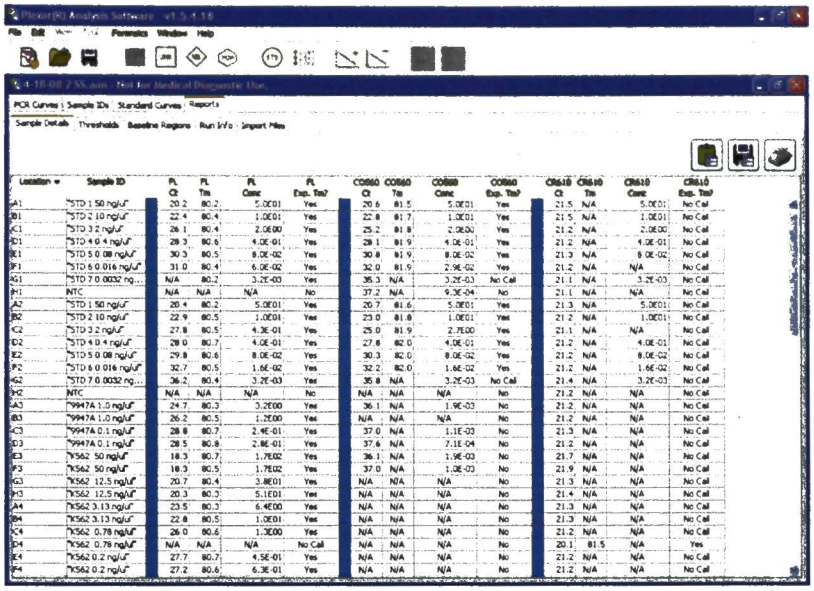

Figure 35. Plexor® Analysis Software sample details tab displaying C_T values, melting temperatures, concentration for each sample at each of the dyes (autosomal, Y, and IPC).

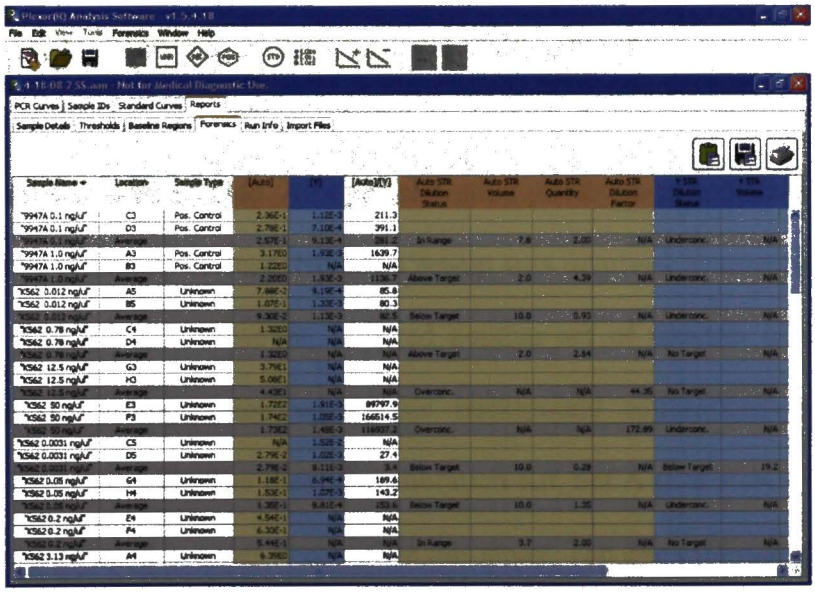

Figure 36. The Forensics Report displaying [AUTO], [Y], [AUTO]/[Y], STR Dilution Status, STR Volume, STR Quantity, STR Dilution Factor, IPC Status, and Curves Status.

Time and Cost Analysis

Applied Biosystems offers Quantifiler® Duo in a $1500, 400-reaction kit ($3.75/reaction). Promega offers Plexor® HY in a $750, 200-reaction kit ($3.75/reaction) or a $2800, 800-reaction kit ($3.50/reaction). In this study, two of the 200 reaction Plexor® HY kits and a 400 reaction Quantifiler® Duo kit were used, making each reaction cost the same. The 200 reaction Plexor® HY kit seems too small, as it would last for only two 96-well plates' worth of quantification. However, the 800 reaction kit, even though it is cheaper to "buy in bulk", seems to be almost too much. Repeated use of the kit could lead to contamination from all the different analysts using it. The Quantifiler Duo® 400 reaction kit seemed to be just the right amount, especially for the twice-weekly quantifications that take place at UNTCHI.

The Applied Biosystems 7500 Spectral Calibration Kit I (P/N 4349180, which was available in the lab) cost $775 and contained the dyes necessary to run Quantifiler® Human, Y, and Quantifiler® Duo. All dye calibration plates are manufactured by Applied Biosystems and are ready for use. No extra set-up was involved in running Quantifiler® Duo assays. Plexor® HY, however, required another set of spectral calibrations with Promega's dyes in conjunction with basic background calibrations using Applied Biosystems' calibration kit. The Plexor® Calibration Kit, Set A (Cat.# DC1500), cost $50 and had to be purchased in order to complete this study. The dye calibration plates do not come pre-made, and hands-on time is required to make them, taking up time and introducing human variation and possibly error. In this study, the calibration took an

entire day and was not entirely precise. Several dye plates had to be remade in order for the instrument to recognize the new dyes.

Human pipetting variation from well to well (despite the use of an automatic pipettor) was apparent in all of the Plexor® HY results as shown earlier. Several wells consistently produced no result in selected dyes, implying that even though all wells "passed" the calibration, something was still not right. If duplicates were not run, it would not have been apparent that the lack of fluorescence detection was due to specific well issues and not the sample being low-copy. This could affect how the analyst decides following analysis. Other problems included drastically different concentrations between duplicate samples on a single plate, which also could be due to the well effects.

CHAPTER VI

CONCLUSIONS

Further Research

This project provided some preliminary data on the comparison of the two competing assays. Upon the final selection of either Quantifiler® Duo or Plexor® HY, additional studies would need to be done as a part of the required internal validation. Replicates of the sensitivity studies, mixture studies, and concordance studies would assist in confirming results obtained in this study. If possible, STR analysis of the degraded samples should be attempted with altered input DNA amounts to see if better data is obtained. Studies on the detection of PCR inhibition were not able to be included in this study but would be beneficial for a more comprehensive analysis of the assays. Analysis of National Institute of Standards and Technology (NIST) standard reference materials would provide a good check of the accuracy of each of the assays. Upon internal validation, an upper limit C_T value would need to be determined. If Plexor® HY is chosen, the data provided in the forensics report would need to be validated for accuracy. It may even be a good idea to contact other DNA laboratories that use either of these assays to see if they have encountered similar trends.

Suggestions and Feedback

The overall purpose of this study was to aid in the decision between implementing Quantifiler® Duo or Plexor® HY to replace Quantifiler® Human. Several of the forensic analysts at UNTCHI provided input to this study. The general consensus was that implementing Quantifiler® Duo would be the better choice for the DNA lab at UNTCHI. For example, a good point was mentioned in that it would be worth it to pay more to get reliable company-manufactured spectral calibration plates. Transitioning into the Quantifiler® Duo system would be fairly seamless, which would limit the amount of time required for analysts who are already overworked. Overall, the results of this initial comparison greatly favor Quantifiler® Duo for implementation at UNTCHI.

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