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To produce the most accurate and reliable results, forensic laboratories, such as AFDIL, must examine new technologies in the field of DNA analysis. The present study is the beginning of an internal validation of the most recent development in DNA quantitation, RT-PCR. The Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Identification Kit are RT-PCR assays that quantitate human and male DNA, and detect PCR inhibitors which may hinder the ability to produce a reliable STR profile. Sensitivity and non-probative case sample studies were performed according to the DAB guidelines. The Quantifiler™ kits were not as sensitive as had been previously reported by Applied Biosystems. The non-probative case sample study demonstrated results two fold greater than results from the Taqman® *Alu*-PCR Quantitation System, a RT-PCR assay developed and validated at AFDIL. At this time, it appears it may be in the best interest of AFDIL to continue its use of the Taqman® *Alu*-PCR Quantitation System.

THE VALIDATION OF APPLIED BIOSYSTEMS QUANTIFILER™ HUMAN DNA QUANTIFICATION KIT AND QUANTIFILER™ Y HUMAN MALE DNA QUANTIFICATION KIT FOR THE ARMED FORCES DNA IDENTIFICATION LABORATORY

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LIST OF ABBREVIATIONS

ABI 3100, ABI PRISM® 3100 Genetic Analyzer

ABI 7000, ABI PRISM® 7000

ABI 7700, ABI PRISM® 7700

AFDIL, Armed Forces DNA Identification Laboratory

bp, base pair

Ct, cycle threshold

DAB, DNA Advisory Board

DNA, deoxyribonucleic acid

dsDNA, double stranded DNA

FRET, fluorescence resonance energy transfer

HRP, horse radish peroxidase

hTERT, human telomerase reverse transcriptase gene

IPC, internal positive control

MGB, minor groove binder

NFQ, non fluorescent quencher

NIST, National Institute for Standards and Technology

OD, optical density

PCR, polymerase chain reaction

RFU, relative fluorescence unit

RNA, ribonucleic acid

rpm, rotation per minute

RT-PCR, real-time PCR

SRY, sex determining region Y

ssDNA, single stranded DNA

STR, short tandem repeat

TE, Tris-EDTA

TMB, 3',3',5',5'-tetramethylbenzidine

UV, ultraviolet

CHAPTER 1

INTRODUCTION

Quantitation of deoxyribonucleic acid (DNA) is essential in the analysis of forensic samples. The analysis of DNA recovered from evidentiary samples requires the use of the polymerase chain reaction (PCR), which yields optimal results within a limited range of input DNA. Therefore, in order to ensure an optimum DNA profile forensic scientists must accurately determine the amount of DNA recovered from evidentiary samples. Recognizing the importance of DNA quantitation, the DNA Advisory Board (DAB), a congressionally established organization, set forth guidelines and standards for forensic laboratories to follow. Among them is 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"(1). A requirement detailed in standard 9.3 is the specification of human DNA. Often forensic samples may be mixed, containing genetic material from other sources such as bacteria. Although the primers used for PCR are higher primate specific, a highly sensitive, human specific quantitation system is highly desirable in forensic laboratories.

Conventional spectrophotometric quantitation methods used in research labs do not provide the sensitivity and specificity required for the analysis of

DNA recovered from forensic samples. Among these are ultraviolet (UV) and fluorescent spectroscopy. Ultraviolet spectroscopy is a simple method that has been widely used for many years. However, UV spectroscopy is not human specific and requires at least 10 ng/μl of DNA for detection. The purine and pyrimidine bases that comprise DNA absorb the UV light. An optical density (OD) reading of 1.0, in a quartz cuvette with a 1 cm light path, is equivalent to: 50 μg/ml of double stranded DNA (dsDNA); 40 μg/ml of single stranded DNA (ssDNA); or 20 μg/ml of synthetic oligonucleotides. UV light at 260nm and 280 nm are used to measure the amount of DNA present, and the 260/280 ratio is used to determine purity. A ratio of 1.8 to 2.0 indicates a pure DNA sample, free of contaminants (2). The presence of contaminants such as ribonucleic acid (RNA), proteins, phenol, or high concentrations of salt will alter the UV absorbance measurement (3). Although UV spectroscopy is a simple and rapid technique the lack of sensitivity is a disadvantage for its use in forensics.

Fluorescence spectrophotometric analysis has been utilized in forensics due to its ability to quantitate smaller amounts of DNA. The analysis of DNA with fluorescence spectroscopy requires the addition of an appropriate fluorescent dye. The emission spectrum of the dye changes upon binding with, or intercalating into the DNA molecule. Unlike UV spectroscopy, fluorescent spectroscopy requires the use of DNA samples of known concentration to develop a standard curve. The fluorescent measurement of

an unknown sample is then compared to the standard curve, and the DNA concentration determined.

Several fluorescent dyes have been used to quantitate DNA. The most common fluorescent dye is Ethidium Bromide, which has been used extensively in solution based analysis and in gel electrophoresis. The intercalation of Ethidium Bromide into dsDNA increases its fluorescent emission by a factor of 50 to 100. Binding of the dye to RNA also contributes to the fluorescence emission. The addition of an RNAse to a sample can increase the selectivity of the assay. Ethidium bromide can quantitate DNA in the range of 1 to 5 ng/µl (2).

Other fluorescent dyes such as bisbenzimidazole, commonly known as Hoechst 33258, and PicoGreen have been utilized to quantitate DNA. The Hoechst dyes have been shown to bind to the minor groove of the DNA helix, binding preferentially to A-T rich regions. The dye binds twice as well to double-stranded DNA as to single-stranded DNA, but does not appear to intercalate. When the appropriate wavelength of light is applied to the bound Hoechst dye, there is a fluorescent enhancement. The fluorescence enhancement has been shown to be highly specific for DNA. RNA enhances the fluorescence to a much smaller extent; usually well below 1% of that produced by the same concentration by weight of DNA. Hoechst dyes can be used to quantitate samples containing as little as 2.5 ng/ml of DNA. An alternative to Hoechst is the dye Picogreen. A fluorometer used in

conjunction with Molecular Probes' PicoGreen dsDNA Quantitation Reagent enables scientists to quantitate as little as 250 pg/ml of dsDNA. The sensitivity of this assay exceeds that achieved with the Hoechst 33258. The standard PicoGreen assay protocol is also simpler than the Hoechst 33258 method, because a single concentration of the PicoGreen Reagent allows detection over the full dynamic range of the assay. The linear detection range of the PicoGreen assay in the appropriate fluorometer extends four orders of magnitude in DNA concentration, from 100 pg/ml to 1000 ng/ml with a single dye concentration. The linearity of the assay is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations, including salts, urea, ethanol, chloroform, detergents, proteins and agarose. The assay protocol has been developed to minimize the fluorescence contribution of RNA and single-stranded DNA (ssDNA) (3). The fluorescent spectrophotometric quantitation methods previously described are not useful for routine forensic casework. They are not sensitive enough for the amounts of DNA typically recovered from forensic samples, and they do not meet standard 9.3, since the methods are not human specific. Methods with greater sensitivity and specificity were required.

Walsh et al. (4) described the first method using a slot blot hybridization assay for the quantitation of DNA extracted from forensic evidentiary samples. The hybridization probe was a 40-base oligonucleotide which was complementary to the alpha satellite region on human chromosome 17

(D17Z1). The oligonucleotide probe was labeled at the 5' end with a molecule of Biotin. The detection system utilized the chemical Streptavidin, which has an extremely high binding affinity for biotin. In order to visualize the presence of the bound probe, the enzyme Horse Radish Peroxidase (HRP) was conjugated to Streptavidin. Additional components of the detection system were a Luminol reagent and autoradiography film. Luminol is a chemiluminscent reagent that can be activated and will emit light. In the presence of HRP the Luminol reagent is cleaved and emits photons. The emission of photons or light is used to expose the autoradiography film. The intensity of the light is proportional to the amount of HRP, which is directly related to the amount of bound probe, or ultimately the quantity of DNA in a sample. The slot blot hybridization assay contained a standard dilution ranging from 10 ng to 150 pg of DNA. The first step of the slot blot hybridization assay was to denature both the unknown DNA samples and the DNA standards and bind them to a solid support (nylon membrane). The nylon membrane was then hybridized with D17Z1 probe. Excess probe was removed following a series of wash steps, and the Streptavidin-HRP complex was added to the membrane. Excess Streptavidin-HRP was removed and the Luminol reagent was added. The nylon membrane was then placed in contact with the autoradiography film and allowed to expose for a specified period of time. The intensity of the exposed film from the unknown samples was then compared with the standards to determine the DNA concentrations (4).

Several variations of the D17Z1 slot blot hybridization assay have been developed, each using a different method to visualize the bound probe. The most commonly used assay in forensic labs is a commercially available product, Quantiblot™ Human DNA Quantitation Kit, manufactured by Applied Biosystems, Foster, City, CA. This kit can be used with either a Luminol film based detection system or with a 3',3',5',5'-tetramethylbenzidine (TMB) colorimetric detection system. In the colorimetric detection system, the enzyme HRP cleaves the TMB chromogen substrate and a blue color is deposited on the nylon membrane. With the colorimetric readout the concentration range of detection is limited, ranging from 10 ng to 150 pg of total DNA. Samples must often be diluted to ensure that the concentration will fall within the detectable range. The limit of detection with HRP and a fluorescent substrate is 40 pg of bound DNA. With the Quantiblot™ system, the choice of an appropriate exposure time is often difficult or may require an expensive detection instrument, such as a CCD camera (5). The majority of laboratories that utilize the Quantiblot™ Human DNA Quantitation Kit estimate the concentration of their unknown samples by a visual comparison with the intensity of the known standards. This visual comparison of band or slot intensities is inherently inaccurate. The National Institute for Standards and Technology (NIST) conducted a study to examine the ability of forensic examiners/analysts to quantitate DNA. The study showed variations in the precision and accuracy of the quantitation methods used. Those laboratories,

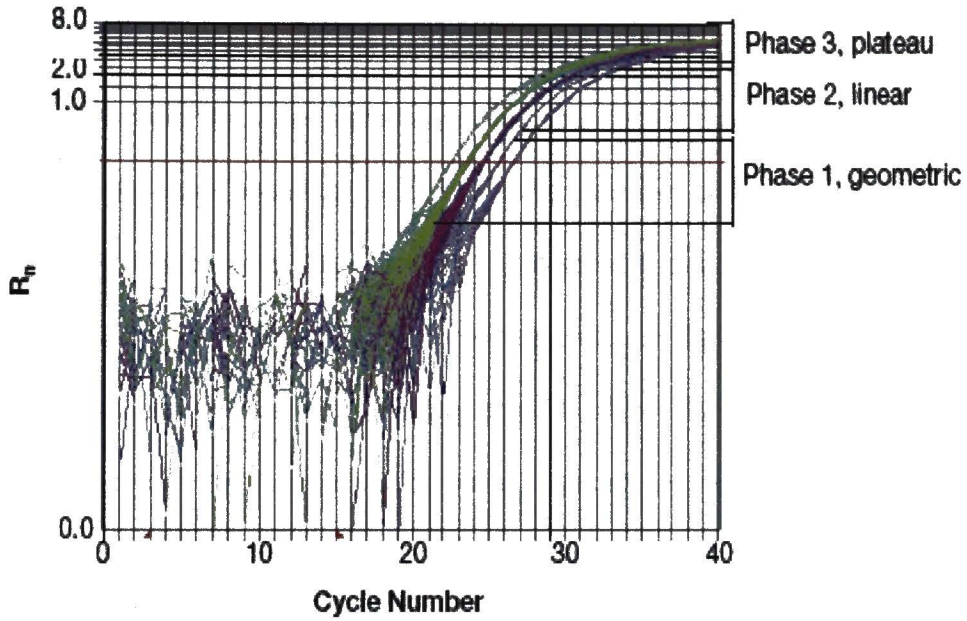
which relied on the Quantiblot™ technique to estimate the quantity of DNA, demonstrated a greater variation at lower concentrations (6).

Although the Quantiblot™ Human DNA Quantitation Kit provided increased sensitivity and human specificity, it still did not provide the optimum system for routine forensic use. In addition to the question of precision and accuracy, the Quantiblot™ assay is both time consuming and laborious. The entire process from sample application through signal detection and interpretation requires more than two hours of hands on time to complete (2). The limitations associated with the Quantiblot™ Human DNA Quantitation Kit have led to the further development of newer quantitation methods. Most recently, a new commercially available quantitation system has been developed which utilizes the sensitivity and specificity of a real time-PCR (RT-PCR) assay.

The amplification of DNA using the PCR process consists of three phases: 1.exponential (geometric); 2. linear; and 3. plateau (Figure 1). The exponential (geometric) phase occurs first, in which the amplification reagents are plentiful and readily available, resulting in the doubling of the amplicons with each cycle. The linear phase is next, the amplification reaction are less efficient due to the consumption of reagents. During the linear phase, the amplicons are not doubling with each cycle. Finally, during the plateau phase all of the reagents have been consumed, and the amplification process has ceased. Research has demonstrated that variations in the rate of amplification

occur during the linear and plateau phases (2, 4). Therefore, data collection and quantitation for a RT-PCR assay occurs during the exponential phase.

Figure 1. Amplification plot demonstrating phases of PCR.

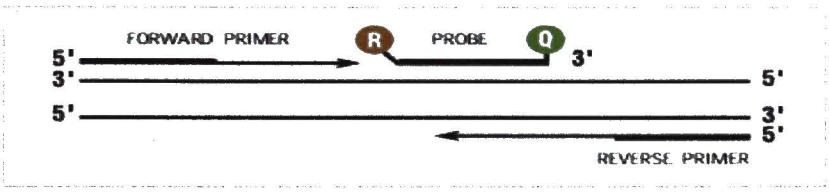


RT-PCR assays have a large dynamic range of detection, are extremely sensitive, highly reproducible, and require limited hands on time (2, 3). Another advantage to this technique is there is no post-PCR clean up steps required. The detection is done in sealed amplification tubes decreasing the possibility of cross contamination from PCR products. RT-PCR also offers the ability to detect the amplification of multiple target sequences simultaneously. The combination of the availability of a high throughput format and the ability to multiplex the detection of different target sequences, is a significant advantageous for forensic laboratories. However, RT-PCR does require careful optimization of the amplification and detection assay (7).

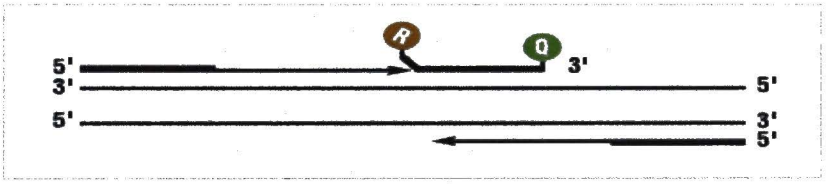
A variety of RT-PCR assays have been developed, including several which utilize a Taqman probe. In a Taqman assay, a uniquely labeled probe, complementary to a region within the amplification target sequence is included in addition to the normal PCR primers. The Taqman probe will bind to the template DNA during the annealing phase of PCR. The probe has a reporter molecule bound on the 5' end and a quencher molecule attached at the 3' end. The reporter is typically a fluorescent dye such as FAM, and the quencher dye is often TAMRA. The emission of the fluorescent reporter dye is suppressed by the proximity of the quencher dye. The suppression of the reporter dye by the quencher is known as fluorescence resonance energy transfer (FRET). In a Taqman PCR assay, the Taq polymerase elongates an extension primer, moving closer to the probe. Once the Taq polymerase reaches the 5' end of the probe, the reporter dye is cleaved off the probe by the 5' nuclease activity of the enzyme. The fluorescence emitted by the reporter dye (fluorophore), once separated from the quencher can be detected (Figure 2). During each PCR cycle the amount of free fluorophore increases yielding fluorescence directly proportional to the cycle number and the amount of input DNA (2). In the early cycles of PCR, the change in fluorescence is negligible and is too low for detection. The baseline of the amplification plot is defined during those first cycles of amplification. During the geometric/exponential phase of PCR, the detection system registers a significant change in fluorescence. The detection point used for the assay is

set at ten times the standard deviation of the baseline. This detection point is known as the cycle threshold (Ct). Within the exponential/geometric phase, at any given cycle, the Ct is proportional to the log of the initial amount of DNA (8).

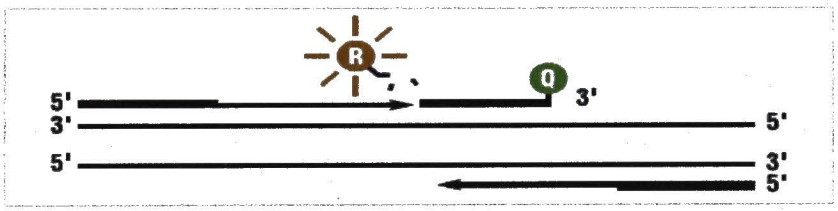
Figure 2. Real-time PCR



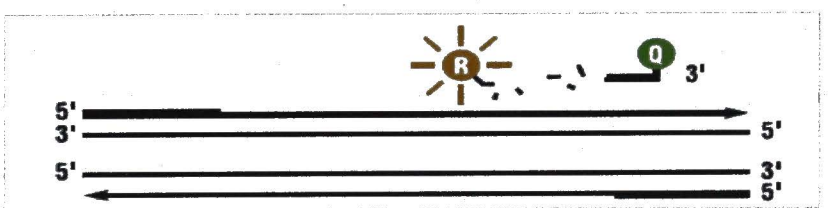
1. Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe, respectively.



2. Strand displacement: When the probe is intact, the reporter dye emission is quenched.



3. Cleavage: During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

The detection of *Alu* sequences within the human genome has been used as the basis for a PCR based quantitation system. *Alu* is the name given to a family of highly repeated sequences found in 500,000 to 1,000,000 copies throughout the human genome. They make up 6-13% of the haploid genome. The consensus Alu sequence is approximately 280 bp consisting of two similar monomers connected by an A-rich region. It has been postulated that *Alu* sequences were derivatives of 7SL RNA. 7SL RNA is found in all species and has been significantly amplified over millions of years of primate evolution. Rodents have a repetitive element known as B1 which was also derived from 7SL RNA. However, the rodent B1sequence is completely distinct from the Primate *Alu* sequences (9).

The Armed Forces DNA Identification Laboratory (AFDIL) has developed and validated a Taqman® *Al*u-PCR Quantitation system. The assay is based on the hybridization of a fluorogenic probe (6-FAM TAGTGGCGGGCGCCTGTAATCCCAGCTA-TAMRA). The probe was constructed with a 6-FAM fluorescent dye at the 5'end and a quencher dye, TAMRA, on the 3' end. The probe is complimentary to a consensus human specific *Alu* sequence. Forward and reverse primers were developed to provide the specificity for the human *Alu* sequence amplification. This assay is performed on the ABI 7700 Detection System and based upon AFDILS validation studies is able to detect as little as 2 pg/µl of DNA (10). The assay requires only minimal only hands on time for both PCR and the instrument

software setup. The entire assay takes approximately 2 hours and12 minutes to quantitate. the amount of DNA in 80 unknown samples.

The Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit (Applied Biosystems, Foster City, CA) are the newest commercially available quantitation assays. They provide the ability to quantify both total human DNA and human male DNA. In addition, these two assays provide the ability to detect the presence of PCR inhibitors, which could ultimately affect the quality of the STR profiles generated for forensic identity testing. Both of these kits utilize the Taqman assay technique, and detect two target sequences simultaneously. One is a target specific assay for either total human DNA or for human male DNA and the second assay is for an internal PCR template control (IPC) sequence which monitors for the presence of PCR inhibition. The probe used for the target specific assay is a Taqman MGB probe that contains a FAM reporter dye at the 5' end, and a non-fluorescent quencher (NFQ), and a minor groove binder (MGB) at the 3' end (Figure 3). The MGB has been attached to the synthetic oligonucleotide probe in order to stabilize the binding of the probe to its target region. As in the Taqman® *Alu*-PCR Quantitation System the probe anneals to the DNA and fluorescence is inhibited by the quencher dye. The probe is cleaved off the DNA when the Taq polymerase moves closer and the separation of the reporter and quencher results in an increase of fluorescence (11).

Figure 3. Quantifiler™ Taqman MGB probe.

The Quantifiler™ Human DNA Quantification Kit targets the human telomerase reverse transcriptase gene (hTERT)(11). The hTERT gene has been mapped to chromosome 5p15.33. It is a single copy gene of approximately 40 kilo bases and its location is very close to the telomere. There are no known markers more distal than hTERT (12). The Quantifiler™ Human DNA Quantification Kit targets an intronic, non-translated region within the hTERT gene, and PCR amplification generates a 62 bp product. The Quantifiler™ Y Human Male DNA Quantification Kit also targets a single copy gene. The target is a non-translated region of the sex determining region Y gene (SRY). It is located on chromosome Yp11.3 and produces an amplicon of 64 bases in length (11).

The Armed Forces DNA Identification Laboratory currently utilizes the Taqman® *Alu*-PCR Quantitation System. The components for their quantitation system are generated and quality control tested in house. Most operational forensic laboratories have found it more cost effective and easier to purchase commercially available kits. The conversion to an alternate quantitation system, the Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit, would require both an internal validation study (1), and a demonstration that the new system is either more sensitive or more cost effective for the laboratory. A direct comparison

of AFDIL's Taqman® *Alu*-PCR Quantitation System and Applied Biosystems Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit was conducted in order to determine which quantitation system is most advantageous for use in their lab.

CHAPTER 2

MATERIAL AND METHODS

I. Quantifiler™ Human DNA Quantification Kit

a. Sensitivity

The sensitivity of the Quantifiler™ Human DNA Quantification Kit was evaluated by running serial dilutions of the genomic DNA control 9947a. The DNA control was provided at 10 ng/µl and diluted with TE to 5 ng/µl, 2.5 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.25 ng/µl, 0.125 ng/µl, 0.0625 ng/µl, 0.03125 ng/µl, 0.025 ng/µl, and 0.015625 ng/µl. Each DNA concentration was run in duplicate within a 96-well plate at various positions to determine spatial reproducibility and sensitivity. For each 96-well plate, serial dilutions were performed with the DNA standard provided by Applied Biosystems. The standard was supplied at 200 ng/µl and diluted with TE to the concentrations recommended by Applied Biosystems; 50 ng/µl, 16.7 ng/µl, 5.56 ng/µl, 1.85 ng/µl, 0.62 ng/µl, 0.21 ng/µl, 0.068 ng/µl, and 0.023 ng/µl. The DNA standards were run in duplicate to create a standard curve. The standards were also examined to determine the affect of time on the DNA concentrations. For each 96-well plate, the Primer Mix and PCR Reaction Mix, provided in the kit, were combined to create a master mix. A total of 23 µl of the master mix was placed into the appropriate wells of the 96-well plate. A 2 µl aliquot of the appropriate standard or sample was added to each well

containing master mix. TE buffer was used as a negative control to determine if samples and/or reagents have been contaminated.

According to the manufacturer's manual, each 96-well plate should be centrifuged at 3000 rpm for 30 seconds prior to quantitation on the ABI 7000. To improve the results observed from first run of the Quantifiler™ Human DNA Quantification Kit, the protocol was modified. The 96-well plate was briefly vortexed and then placed in a centrifuge and spun at 1000 rpm for 30 seconds prior to quantitation. For each experiment the real-time PCR cycling conditions were as follows:

1 cycle	10 minute hold	95°C
40 cycles	15 seconds	95°C
	1 minute	60°C

The analysis settings for the experiments included: a threshold detection at 0.2 and a baseline generation starting at cycle 6 and ending at cycle 15. To ensure proper quantitation of the samples, the slope and R^2 of the standard curve were analyzed and adjusted, if necessary. The slope of the standard curve with a range from –2.9 to –3.3 indicates that the PCR reaction proceeded efficiently. An R^2 of 0.98 or higher demonstrates how well the regression line created from the standard curve includes the actual Ct points of the standards. If the standard curve did not fall within the recommended slope and R^2 values, the Ct points that appeared to be outliers were removed from the standard curve. If the elimination/s of those data points did not

improve the standard curve, the results are considered invalid and the experiment should be repeated.

b. Non-probative case samples

To determine the reproducibility of the Quantifiler™ Human DNA Quantification Kit, a set of samples commonly processed at the Armed Forces DNA Identification Laboratory were examined. The samples were previously extracted organically and quantitated with the Taqman® *Alu*-PCR Quantitation System. The undiluted (neat) extraction and a 1:100 dilution of each sample was quantitated with the Quantifiler™ Human DNA Quantification Kit and the results were with the results from the Taqman® Alu-PCR Quantitation System. For further examination of the non-probative case samples, a 1:200 dilution was performed using the neat samples and then quantitated using the Quantifiler™ Human DNA Quantification Kit. The quantitation results from the 1:200 dilution samples were compared with the results from the neat and 1:100 samples, as well the results from the Taqman® *Alu*-PCR Quantitation System. The real-time PCR cycling parameters and the analysis settings were the same as in the sensitivity experiments. Select samples were amplified using the Promega PowerPlex™ 16 STR System, following the manufactures recommended conditions, and analyzed with the ABI 3100 Genetic Analyzer.

II. Quantifiler™ Y Human Male DNA Quantification Kit

a. Sensitivity

The sensitivity of the Quantifiler™ Y Human Male DNA Quantification Kit was evaluated by running serial dilutions of the genomic DNA control 9948, a male cell line. The DNA control was provided at 10 ng/µl and diluted with TE to 5 ng/µl, 2.5 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.125 ng/µl, 0.0625 ng/µl, and 0.025 ng/µl. Each DNA concentration was run in duplicate within a 96-well plate and at various positions to determine spatial reproducibility as well as sensitivity. Applied Biosystems provided a DNA standard at 200 ng/µl, which was diluted with TE to the recommended concentrations; 50 ng/µl, 16.7 ng/µl, 5.56 ng/µl, 1.85 ng/µl, 0.62 ng/µl, 0.21 ng/µl, 0.068 ng/µl, and 0.023 ng/µl. The DNA standards were run in duplicate to create a standard curve. In addition, the standards were also quantitated to determine the affect of time on the DNA concentrations. For each plate, Primer Mix and PCR Reaction Mix, provided by Applied Biosystems, were combined to create a master mix. A total of 23 µl of the master mix was placed into the appropriate wells and a 2 µl aliquot of the appropriate standard or sample was added to each well containing the master mix. As a result of the experiments performed with the Quantifiler™ Human DNA Quantification Kit, each plate was briefly vortexed and centrifuged at 1000 rpm for 30 seconds. For each experiment, the real-time PCR cycling conditions were as follows:

1 cycle	10 minute hold	95°C
40 cycles	15 seconds	95°C
	1 minute	60°C

The analysis settings for the experiments included: a threshold detection at 0.2 and a baseline generation starting at cycle 6 and ending at cycle 15. To ensure proper quantitation of the samples, the slope and R^2 of the standard curve were analyzed and adjusted, if necessary. The slope of the standard curve with a range from –3.0 to –3.6 indicates that the PCR reaction proceeded efficiently. An R^2 of 0.98 or higher demonstrates how well the regression line created from the standard curve includes the actual Ct points of the standards. If the standard curve did not fall within the recommended slope and R^2 values, the Ct points that appeared to be outliers were removed from the standard curve. If the elimination/s of those data points did not improve the standard curve, the results are considered invalid and the experiment was repeated.

b. Non-probative case samples

To determine the reproducibility of the Quantifiler™ Y Human Male DNA Quantification Kit, a set of samples commonly processed at the Armed Forces DNA Identification Laboratory were examined. The samples used were the same samples examined in the non-probative case sample study for the Quantifiler™ Human DNA Quantification Kit. Due to the lack of extracted neat sample, only the 1:100 and 1:200 diluted samples were examined and compared to the previous quantitation with the Quantifiler™ Human DNA Quantification Kit. The real-time PCR cycling parameters and the analysis settings were the same as in the sensitivity experiments.

CHAPTER 3

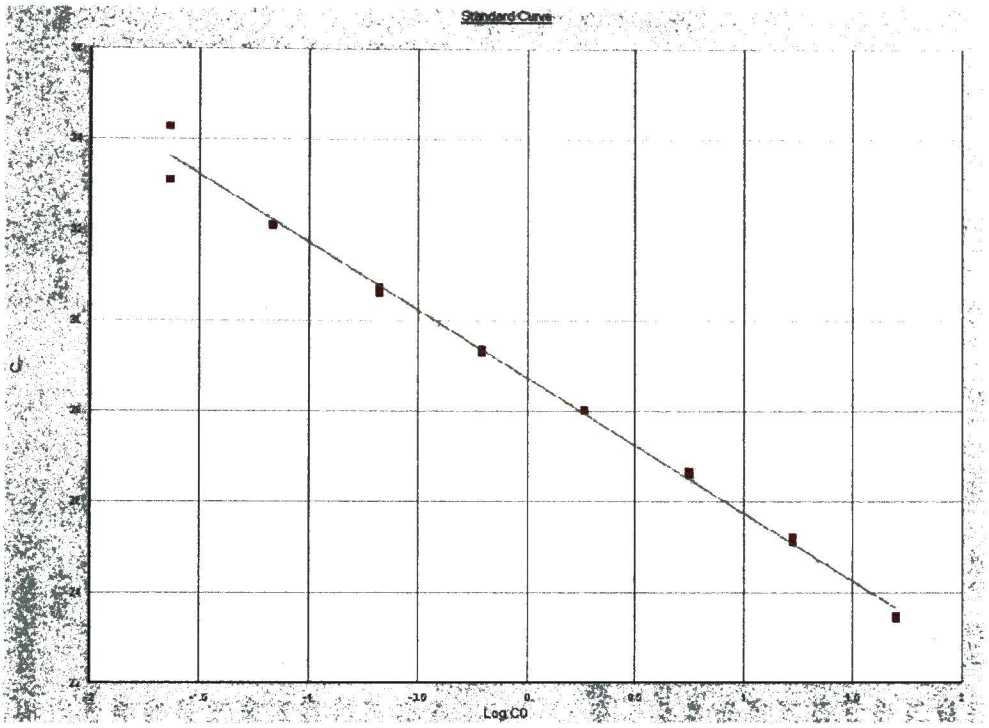
RESULTS

I. Quantifiler™ Human DNA Quantification Kit

a. Sensitivity

Prior to the interpretation of any unknown samples, the standard curve and IPC for each sample on the 96-well plate must be analyzed. Applied Biosystems recommends the slope of the standard curve to fall between –2.9 to –3.3 and the standard curve should possess an R^2 value no less than 0.98. The slopes for all the standard curves generated for the sensitivity study were well within the recommended range. The R^2 was never less then 0.99. The first experiment (Run 1) was the only experiment in which some of the Ct data points needed to be eliminated to improve the standard curve. Both of the standard points for 0.068 ng/µl were removed. Figure 4 is an example of just one of the standard curves. The slope is -3.127 and R^2 is 0.999. All of the standard curves did exhibit the precision between Ct points seen in Figure 4.

Figure 4. The standard curve from Run 3 of the sensitivity study



The duplicate samples analyzed from Run1 showed variations in the DNA quantitation results. Following the first run of the kit, the protocol was adjusted to include a brief vortex of the plate and centrifugation at 1000 rpm for 30 seconds rather than simply centrifuging at 3000 rpm for 30 seconds. The change in the protocol improved the standard deviations within a plate. Tables 1 and 2 demonstrate the improvement noted above. The results highlighted in red indicate the Run 1 in which the plate was not vortexed and was centrifuged for 30 seconds at 3000 rpm.

Table 1. Quantitation results for sensitivity results with the Quantifiler Human DNA Quantification Kit

	Run 1				Run 2				Run 3				Run 4				Run 1-4	
Sample	**Quantity(ng/ul)**		**AVG**	**STDEV**	**Quantity(ng/ul)**		**AVG**	**STDEV**	**Quantity(ng/ul)**		**AVG**	**STDEV**	**Quantity(ng/ul)**		**AVG**	**STDEV**	**AVG**	**STDEV**
10 ng/ul	8.670	14.420	11.545	4.066	16.460	17.180	16.820	0.509	14.460	14.080	14.270	0.269	18.540	13.890	16.215	3.288	14.713	2.965
5 ng/ul	3.910	7.450	5.680	2.503	7.860	7.510	7.685	0.247	6.560	6.330	6.445	0.163	6.810	6.930	6.870	0.085	6.670	1.228
2.5 ng/ul	1.670	2.910	2.290	0.877	2.390	1.100	1.745	0.912	1.930	2.150	2.040	0.156	1.890	2.570	2.230	0.481	2.076	0.563
1 ng/ul	0.647	1.330	0.989	0.483	0.808	0.757	0.783	0.036	0.847	0.666	0.757	0.128	0.947	0.821	0.884	0.089	0.853	0.216
0.5 ng/ul	0.322	0.563	0.443	0.170	0.334	0.348	0.341	0.010	0.345	0.366	0.356	0.015	0.424	0.398	0.411	0.018	0.388	0.079
0.25 ng/ul	0.188	0.417	0.303	0.162	0.180	0.166	0.173	0.010	0.152	0.193	0.173	0.029	0.115	0.208	0.162	0.066	0.202	0.091
0.125 ng/ul	0.089	0.000	0.044	0.063	0.064	0.097	0.080	0.024	0.105	0.065	0.085	0.028	0.072	0.094	0.083	0.016	0.073	0.033
0.0625 ng/ul	0.036	0.044	0.040	0.006	0.026	0.067	0.046	0.029	0.039	0.035	0.037	0.003	0.074	0.053	0.063	0.015	0.047	0.017
0.03125 ng/ul	0.017	0.046	0.031	0.020	0.016	0.021	0.018	0.004	0.027	0.048	0.038	0.015	0.030	0.031	0.030	0.001	0.029	0.012
0.025 ng/ul	0.855	0.944	0.900	0.204	0.421	0.545	0.483	0.088	0.447	0.442	0.445	0.004	0.722	0.773	0.748	0.036	0.619	0.188
0.0156 ng/ul	0.017	0.011	0.014	0.004	0.010	0.013	0.011	0.002	0.016	0.000	0.008	0.011	0.024	0.012	0.018	0.008	0.013	0.007

Table 2. The affect of vortexing and reduced rpm centrifugation on quantitation results

	Run 2				Run 3				Run 4				Runs 2-4	
Sample	**Quantity(ng/ul)**		**AVG**	**STDEV**	**Quantity(ng/ul)**		**AVG**	**STDEV**	**Quantity(ng/ul)**		**AVG**	**STDEV**	**AVG**	**STDEV**
10 ng/ul	16.460	17.180	16.820	0.509	14.460	14.080	14.270	0.269	18.540	13.890	16.215	3.288	15.768	1.910
5 ng/ul	7.860	7.510	7.685	0.247	6.560	6.330	6.445	0.163	6.810	6.930	6.870	0.085	7.000	0.580
2.5 ng/ul	2.390	1.100	1.745	0.912	1.930	2.150	2.040	0.156	1.890	2.570	2.230	0.481	2.005	0.515
1 ng/ul	0.808	0.757	0.783	0.036	0.847	0.666	0.757	0.128	0.947	0.821	0.884	0.089	0.808	0.094
0.5 ng/ul	0.334	0.348	0.341	0.010	0.345	0.366	0.356	0.015	0.424	0.398	0.411	0.018	0.369	0.035
0.25 ng/ul	0.180	0.166	0.173	0.010	0.152	0.193	0.173	0.029	0.115	0.208	0.162	0.066	0.169	0.033
0.125 ng/ul	0.064	0.097	0.080	0.024	0.105	0.065	0.085	0.028	0.072	0.094	0.083	0.016	0.083	0.018
0.0625 ng/ul	0.026	0.067	0.046	0.029	0.039	0.035	0.037	0.003	0.074	0.053	0.063	0.015	0.049	0.019
0.03125 ng/ul	0.016	0.021	0.018	0.004	0.027	0.048	0.038	0.015	0.030	0.031	0.030	0.001	0.029	0.011
0.025 ng/ul	0.421	0.545	0.483	0.088	0.447	0.442	0.445	0.004	0.722	0.773	0.748	0.036	0.558	0.154
0.0156 ng/ul	0.010	0.013	0.011	0.002	0.016	0.000	0.008	0.011	0.024	0.012	0.018	0.008	0.012	0.008

The brief vortex and reduced centrifugation speed, improved the precision of the quantitation results, however, the actual amount of DNA determined in a sample was not as accurate as expected. Although the actual quantity of DNA calculated was not as accurate as what would have been predicted, the Ct values, as shown in Tables 3 and 4, differ by one cycle for those samples in which the DNA concentrations exhibit a two fold difference.

Results of the analysis of the standards showed a decrease in their DNA concentration over time, Table 5.

Table 3. Ct results of sensitivity experiments with the Quantifiler Human DNA Quantification Kit

	Run 1				Run 2				Run 3				Run 4				Run 1-4	
Sample		**Ct**	**AVG Ct**	**Ct STDEV**	**Ct**		**AVG Ct**	**Ct STDEV**	**Ct**		**AVG Ct**	**Ct STDEV**	**Ct**		**AVG Ct**	**Ct STDEV**	**AVG Ct**	**Ct STDEV**
10 ng/ul	25.930	25.210	25.570	0.509	25.090	25.040	25.065	0.035	24.960	25.000	24.980	0.028	24.700	25.110	24.905	0.290	25.130	0.356
5 ng/ul	27.050	26.140	26.595	0.643	26.050	26.110	26.080	0.042	26.030	26.080	26.055	0.035	26.110	26.090	26.100	0.014	26.208	0.342
2.5 ng/ul	28.260	27.470	27.865	0.559	27.600	28.600	28.100	0.707	27.690	27.550	27.620	0.099	27.920	27.490	27.705	0.304	27.823	0.411
1 ng/ul	29.600	28.570	29.085	0.728	29.000	29.090	29.045	0.064	28.810	29.140	28.975	0.233	28.890	29.090	28.990	0.141	29.024	0.299
0.5 ng/ul	30.580	29.790	30.185	0.559	30.150	30.100	30.125	0.035	30.030	29.950	29.990	0.057	30.020	30.110	30.065	0.064	30.091	0.227
0.25 ng/ul	31.350	30.220	30.785	0.799	30.950	31.060	31.005	0.078	31.150	30.820	30.985	0.233	31.860	31.030	31.445	0.587	31.055	0.464
0.125 ng/ul	32.410				32.310	31.750	32.030	0.396	31.650	32.300	31.975	0.460	32.520	32.140	32.330	0.269	32.154	0.332
0.0625 ng/ul	33.680	33.400	33.540	0.198	33.470	32.240	32.855	0.870	32.990	33.140	33.065	0.106	32.490	32.960	32.725	0.332	33.046	0.491
0.03125 ng/ul	34.740	33.350	34.045	0.983	34.130	33.740	33.935	0.276	33.500	32.700	33.100	0.566	33.760	33.710	33.735	0.035	33.704	0.590
0.025 ng/ul	29.580	29.070	29.325	0.361	29.850	29.510	29.680	0.240	29.680	29.690	29.685	0.007	29.270	29.180	29.225	0.064	29.479	0.276
0.0156 ng/ul	35.410	34.780	35.095	0.445	34.700	34.370	34.535	0.233	34.230				34.080	35.060	34.570	0.693	34.661	0.473

Table 4. Ct results of sensitivity experiments demonstrating effect of vortexing prior to amplification.

	Run 2				Run 3				Run 4				Runs 2-4	
Sample	**Ct**		**AVG Ct**	**Ct STDEV**	**Ct**		**AVG Ct**	**Ct STDEV**	**Ct**		**AVG Ct**	**Ct STDEV**	**AVG Ct**	**Ct STDEV**
10 ng/ul	25.090	25.040	25.065	0.035	24.960	25.000	24.980	0.028	24.700	25.110	24.905	0.290	24.983	0.149
5 ng/ul	26.050	26.110	26.080	0.042	26.030	26.080	26.055	0.035	26.110	26.090	26.100	0.014	26.078	0.033
2.5 ng/ul	27.600	28.600	28.100	0.707	27.690	27.550	27.620	0.099	27.920	27.490	27.705	0.304	27.808	0.416
1 ng/ul	29.000	29.090	29.045	0.064	28.810	29.140	28.975	0.233	28.890	29.090	28.990	0.141	29.003	0.130
0.5 ng/ul	30.150	30.100	30.125	0.035	30.030	29.950	29.990	0.057	30.020	30.110	30.065	0.064	30.060	0.073
0.25 ng/ul	30.950	31.060	31.005	0.078	31.150	30.820	30.985	0.233	31.860	31.030	31.445	0.587	31.145	0.368
0.125 ng/ul	32.310	31.750	32.030	0.396	31.650	32.300	31.975	0.460	32.520	32.140	32.330	0.269	32.112	0.367
0.0625 ng/ul	33.470	32.240	32.855	0.870	32.990	33.140	33.065	0.106	32.490	32.960	32.725	0.332	32.882	0.446
0.03125 ng/ul	34.130	33.740	33.935	0.276	33.500	32.700	33.100	0.566	33.760	33.710	33.735	0.035	33.590	0.481
0.025 ng/ul	29.850	29.510	29.680	0.240	29.680	29.690	29.685	0.007	29.270	29.180	29.225	0.064	29.530	0.261
0.0156 ng/ul	34.700	34.370	34.535	0.233	34.230				34.080	35.060	34.570	0.693	34.488	0.393

Table 5. The affect of time on DNA concentrations of the standards

Standard(ng/ul)	Day 2 (ng/ul)		AVG	Day 3 (ng/ul)		AVG
50	54.04	58.01	56.03	53.47	53.26	53.37
16.7	16.61	17.03	16.82	14.74	15.48	15.11
5.56	5.25	5.32	5.29	4.57	4.64	4.61
1.85	1.62	1.66	1.64	1.45	1.63	1.54
0.62	0.40	0.48	0.44	0.37	0.38	0.37
0.21	0.14	0.16	0.15	0.15	0.14	0.14
0.068	0.03	0.05	0.04	0.04	0.07	0.06
0.023	0.01	0.03	0.02	0.01	0.02	0.02

Table 6, shows a comparison of the sensitivity of the Quantifiler™ Human DNA Quantification Kit quantitation system, as indicated by the Applied Biosystems, with the results I obtained at AFDIL. The Taqman® *Alu*-PCR Quantitation System was developed and manufactured at AFDIL for their own internal use.

Table 6. A comparison of the sensitivity levels of the quantitation systems.

Quantitation System	Sensitivity Level	
	Manufacturer's Specifications	Observed
Quantifiler™ Human DNA Quantification Kit	23 pg/µl	125 pg/µl
Taqman® *Alu*-PCR Quantitation System	2 pg/µl	2 pg/µl

b. Non-probative case samples

The slopes from all the standard curves generated, fell within the range of –2.9 to –3.3, and the R^2 values were never less than 0.99. In Runs 1 and 2, the data points for the 0.023 ng/µl were eliminated from the standard curve and in Run 4, the data points for 0.21 ng/µl were removed from the standard curve.

The results obtained between the undiluted neat samples and their corresponding 1:100 dilutions, did not demonstrate the expected proportional decrease (Table 7). These results may indicate the presence of a inhibitor in the undiluted neat samples.

Table 7. Comparison of quantitation results between the Quantifiler™ Human DNA Quantification Kit and the Taqman® *Alu*-PCR Quantitation System

Sample Name	Quantity (ng/ul)						
	Run 1	Run 2	Run 3	Run 4	AVG	7700 AVG	STDEV
04M0407 44A1	72.120	81.710	152.870	360.100	166.700	-1.000	133.870
04M0407 44A1 1:100	63.900	57.540	44.130	50.120	53.923	7.892	8.621
04M0407 44A1 1:200		14.340	16.200	17.970	16.170		1.815
04M0407 43A1	0.007	0.002	0.216	0.012	0.059	-0.074	0.091
04M0407 43A1 1:100	183.340	175.680	120.590	145.230	156.210	5.702	25.022
04M0407 43A1 1:200		52.210	45.560	51.200	49.657		3.584
04M0407 40A1	0.000	0.000	0.002	0.000	0.000	-1.000	0.001
04M0407 40A1 1:100	21.960	23.590	21.630	25.140	23.080	6.850	1.619
04M0407 40A1 1:200		9.940	8.710	10.730	9.793		
04M0407 39A1	2505.360	2887.240	1602.790	2394.030	2347.355	-1.000	539.443
04M0407 39A1 1:100	78.070	64.930	54.670	66.060	65.933	7.200	9.577
04M0407 39A1 1:200		5.210	6.100	8.970	6.760		1.604
04M0407 33A1	213.860	254.320	134.160	354.120	239.115	-1.000	91.489
04M0407 33A1 1:100	5.180	3.360	3.980	4.260	4.195	2.427	0.757
04M0407 33A1 1:200		1.810	1.730	2.080	1.873		0.183
04M0407 32A1	521.130	92.110	100.340	518.870	308.113	-1.000	244.691
04M0407 32A1 1:100	7.110	5.770	4.950	6.980	6.203	3.649	1.030
04M0407 32A1 1:200		2.810	2.930	3.120	2.953		0.156
04M0407 30A1	0.046	0.735	0.045	1.460	0.572	-0.750	0.676
04M0407 30A1 1:100	0.989	0.876	1.020	1.260	1.036	0.668	0.161
04M0407 30A1 1:200		0.539	0.557	0.920	0.672		0.215
04M0407 24A1	668.390	1157.210	486.180	648.360	740.035	-1.000	289.836
04M0407 24A1 1:100	15.250	13.430	11.960	13.280	13.480	5.536	1.352
04M0407 24A1 1:200		5.990	5.450	5.960	5.800		0.303
04M0407 23A1	41.560	119.050	60.970	139.800	90.345	-1.000	46.593
04M0407 23A1 1:100	10.060	7.970	7.690	9.110	8.708	4.939	1.091
04M0407 23A1 1:200		3.910	3.280	3.790	3.660		0.335
04M0407 22A1	84.360	19.800	26.690	27.250	39.525	-1.000	30.081
04M0407 22A1 1:100	5.660	5.650	3.690	6.640	5.410	2.941	1.237
04M0407 22A1 1:200		2.560	2.590	3.010	2.720		0.252
04M0407 19A1	887.830	927.170	530.000	810.570	788.893	-0.494	179.262
04M0407 19A1 1:100	9.260	6.080	5.810	7.280	7.108	3.907	1.571
04M0407 19A1 1:200		2.660	2.280	2.830	2.590		0.282
04M0407 13A1	485.230	486.330	287.860	495.600	438.755	-0.750	100.704
04M0407 13A1 1:100	5.700	5.680	5.470	5.740	5.648	2.958	0.121
04M0407 13A1 1:200		2.460	3.080	2.760	2.767		0.310

Table 7 cont'd.

Sample Name	Quantity (ng/ul)						
	Run 1	Run 2	Run 3	Run 4	AVG	7700 AVG	STDEV
04M0407 76A1	8.910	10.630	10.640	12.410	10.648	-1.000	1.429
04M0407 76A1 1:100	7.330	7.280	6.900	8.160	7.418	4.767	0.531
04M0407 76A1 1:200		2.970	3.690	4.050	3.570		0.550
04M0407 56A1	0.000	0.350	882.320		294.223	-1.000	509.307
04M0407 56A1 1:100	145.440	110.640	92.390	113.380	115.463	5.992	22.050
04M0407 56A1 1:200		45.880	38.630	52.920	45.810		7.145
04M0407 57A1	11.700	6.260	4.830	28.170	12.740	-1.000	3.625
04M0407 57A1 1:100	46.040	42.810	30.170	38.340	39.340	7.443	6.880
04M0407 57A1 1:200		10.610	9.760	8.970	9.780		0.820
04M0407 74A1	2784.960	3217.820	1653.790	2338.230	2498.700	-1.000	668.007
04M0407 74A1 1:100	43.830	31.370	33.040	36.970	36.303	7.130	5.540
04M0407 74A1 1:200		9.750	7.530	11.020	9.433		1.766
04M0407 75A1	2.060	3.710	4.010	16.670	6.613	-1.000	6.760
04M0407 75A1 1:100	70.960	75.340	51.210	69.330	66.710	6.793	10.640
04M0407 75A1 1:200		27.080	22.260	29.150	26.163		3.535
04M0407 52A1	792.270	824.930	439.410	687.080	685.923	-0.738	174.550
04M0407 52A1 1:100	5.360	4.630	5.030	5.740	5.190	3.431	0.473
04M0407 52A1 1:200		1.980	1.730	2.040	1.917		0.164
04M0407 51A1	230.380	244.630	336.290		270.433	-0.750	57.477
04M0407 51A1 1:100	9.730	8.510	8.350	9.450	9.010	4.490	0.683
04M0407 51A1 1:200		3.770	3.920	2.990	3.560		0.499
04M0407 50A1	0.454	4.190	3.570	1322.030	332.561	-0.750	659.648
04M0407 50A1 1:100	50.350	48.940	34.410	27.190	40.223	6.046	11.287
04M0407 50A1 1:200		21.790	18.200	22.430	20.807		2.280
04M0407 59A1	108.930	330.000	200.490	355.820	248.810	-1.000	115.389
04M0407 59A1 1:100	79.400	92.030	73.200	79.790	81.105	6.222	7.884
04M0407 59A1 1:200		27.770	22.620	28.530	26.307		3.215
04M0407 RB1	0.000	0.008	0.011	0.015	0.008	-0.500	0.006
04M0407 RB1 1:100	0.004	0.003	0.000	0.034	0.010	-0.500	0.016
04M0407 RB1 1:200		0.000	0.001	0.010	0.004		0.006
04M0407 RB2	0.004	0.011	0.004	0.051	0.018	-0.250	0.022
04M0407 RB2 1:100	0.001	0.000	0.008	0.013	0.005	-0.500	0.006
04M0407 RB2 1:200		0.000	0.000	0.000	0.000		0.000

The IPC from a number of the neat samples and the 1:100 dilutions provide by AFDIL displayed the presence of an inhibitor. Therefore, 1:200 dilutions were prepared using the neat samples and compared to the 1:100 dilutions. To determine the accuracy

and reproducibility of the Quantifiler™ Human DNA Quantification Kit, the results were also compared to the quantitations previously obtained with the Taqman® Alu-PCR Quantitation System. The quantitation results obtained from the 1:200 dilutions using the Quantifiler™ Human DNA Quantification Kit were similar to the 1:100 dilutions obtained from the Taqman® Alu-PCR Quantitation System (Table 7).

The amount of DNA determined using the Quantifiler™ Human DNA Quantification Kit appeared to be two times the amount obtained from the Taqman® Alu-PCR Quantitation System. As a result of the observed difference between the two systems, select samples were amplified using Promega's PowerPlex™ 16 STR System and profiled on the ABI 3100 (Table 8). Promega's PowerPlex™ 16 STR System has been shown to produce optimal results with the input of 1ng of DNA. The 1:100 dilution samples were previously prepared at AFDIL. Those that were chosen for amplification with the PowerPlex™ 16 STR System were diluted to 1 ng/μl based on the original Taqman® Alu-PCR Quantitation System results. The 1:200dilution samples, which had been prepared for this study, were diluted based on the results from Quantifiler™ Human DNA Quantification Kit. Table 8 shows a list of the samples chosen for amplification. The average reading for the 1:200 dilutions are similar to the 1:100 dilutions on the ABI 7700 using the Taqman® Alu-PCR Quantitation System. The amount of DNA added based upon these readings should have been very similar.

Table 8. Samples chosen for amplification with Promega's PowerPlex™ 16 STR System.

Sample Name	Quantity (ng/ul)							Fold
	Run 1	Run 2	Run 3	Run 4	STDEV	AVG	7700 AVG	Difference
04M0407 33A1 1:100	5.18	3.36	3.98	4.26	0.76	4.20	2.43	1.73
04M0407 33A1 1:200		1.81	1.73	2.08	0.18	1.87		
04M0407 32A1 1:100	7.11	5.77	4.95	6.98	1.03	6.20	3.65	1.70
04M0407 32A1 1:200		2.81	2.93	3.12	0.16	2.95		
04M0407 30A1 1:100	0.99	0.88	1.02	1.26	0.16	1.04	0.67	1.55
04M0407 30A1 1:200		0.54	0.56	0.92	0.21	0.67		
04M0407 24A1 1:100	15.25	13.43	11.96	13.28	1.35	13.48	5.54	2.43
04M0407 24A1 1:200		5.99	5.45	5.96	0.30	5.80		
04M0407 23A1 1:100	10.06	7.97	7.69	9.11	1.09	8.71	4.94	1.76
04M0407 23A1 1:200		3.91	3.28	3.79	0.33	3.66		
04M0407 22A1 1:100	5.66	5.65	3.69	6.64	1.24	5.41	2.94	1.84
04M0407 22A1 1:200		2.56	2.59	3.01	0.25	2.72		

The peak heights from the genetic profiles obtained from the 1:100 and 1:200 dilutions were not the same. The RFU levels for the 1:200 samples were approximately two fold less than the 1:100 samples (Table 9). This would indicate that the amount of input DNA from the 1:200 samples was approximately half of what the intended amount was. The RFU peak heights obtained from the 1 ng positive control sample, was closer to the RFU values obtained from the 1:100 samples which were previously quantitated using he ABI 7700 and the Taqman® Alu-PCR Quantitation System (Figure 5). These results would confirm the observation that the amount of DNA determined using the Quantifiler™ Human DNA Quantification Kit is two times the amount obtained from the Taqman® Alu-PCR Quantitation System.

Figure 5. Electropherogram comparing the peak heights generated from the amplification of the 1:100, 1:200, and positive control samples using the Promega's PowerPlex™ 16 STR System.

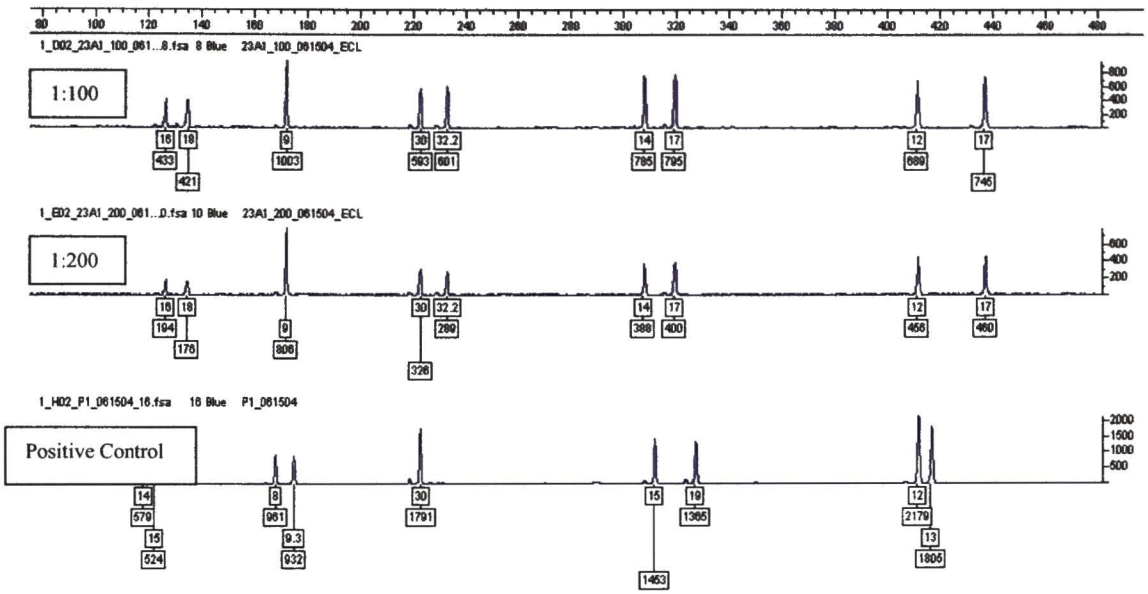


Table 9. Peak heights of the samples amplified with the PowerPlex™ 16 STR System

LOCUS		04M0407 24A1			04M0407 30A1		
		1:100	1:200	Fold Difference	1:100	1:200	Fold Difference
D3S1358	PEAK 1	369.00	249.00	1.48	395.00	149.00	2.65
	PEAK 2	346.00	239.00	1.45	246.00	135.00	1.82
THO1	PEAK 1	1167.00	489.00	2.39	618.00	375.00	1.65
	PEAK 2						
D21S11	PEAK 1	665.00	231.00	2.88	344.00	140.00	2.46
	PEAK 2	519.00	268.00	1.94	266.00	148.00	1.80
D18S51	PEAK 1	761.00	485.00	1.57	301.00	217.00	1.39
	PEAK 2	801.00	352.00	2.28	319.00	168.00	1.90
PENTA E	PEAK 1	591.00	545.00	1.08	210.00	124.00	1.69
	PEAK 2	624.00	277.00	2.25	132.00	101.00	1.31
D5S818	PEAK 1	714.00	388.00	1.84	582.00	280.00	2.08
	PEAK 2						
D13S317	PEAK 1	226.00	134.00	1.69	147.00	89.00	1.65
	PEAK 2	223.00	156.00	1.43	140.00	83.00	1.69
D7S820	PEAK 1	401.00	233.00	1.72	158.00	92.00	1.72
	PEAK 2	340.00	254.00	1.34	173.00	101.00	1.71
D16S539	PEAK 1	345.00	187.00	1.84	127.00	80.00	1.59
	PEAK 2	387.00	180.00	2.15	196.00	66.00	2.97
CSF1PO	PEAK 1	601.00	332.00	1.81	272.00	104.00	2.62
	PEAK 2						
PENTA D	PEAK 1	458.00	281.00	1.63	158.00	104.00	1.25
	PEAK 2	419.00	237.00	1.77	130.00	76.00	1.71
AMEL	PEAK 1	543.00	351.00	1.55	693.00	283.00	2.45
	PEAK 2	731.00	338.00	2.16	637.00	262.00	2.43
VWA	PEAK 1	781.00	372.00	2.10	434.00	268.00	1.62
	PEAK 2	731.00	277.00	2.64	473.00	263.00	1.80
D8S1179	PEAK 1	483.00	346.00	1.40	190.00	140.00	1.36
	PEAK 2	386.00	226.00	1.71	277.00	82.00	3.38
TPOX	PEAK 1	627.00	274.00	2.29	400.00	180.00	2.22
	PEAK 2	633.00	329.00	1.92	263.00	129.00	2.04
FGA	PEAK 1	713.00	365.00	1.95	246.00	111.00	2.22
	PEAK 2	693.00	259.00	2.68	229.00	104.00	2.20
Average				1.89			1.95

Table 9 cont'd

LOCUS		04M0407 32A1			04M0407 33A1		
		1:100	1:200	Fold Difference	1:100	1:200	Fold Difference
D3S1358	PEAK 1	342.00	110.00	3.11	334.00	183.00	1.83
	PEAK 2	417.00	116.00	3.59	358.00	176.00	2.03
THO1	PEAK 1	1383.00	797.00	1.74	1506.00	662.00	2.27
	PEAK 2						
D21S11	PEAK 1	612.00	290.00	2.11	815.00	284.00	2.87
	PEAK 2	551.00	350.00	1.57	613.00	247.00	2.48
D18S51	PEAK 1	841.00	287.00	2.93	823.00	523.00	1.57
	PEAK 2	635.00	233.00	2.73	940.00	393.00	2.39
PENTA E	PEAK 1	1054.00	458.00	2.30	899.00	398.00	2.26
	PEAK 2	831.00	418.00	1.99	1093.00	434.00	2.52
D5S818	PEAK 1	801.00	233.00	3.44	781.00	382.00	2.04
	PEAK 2						
D13S317	PEAK 1	284.00	82.00	3.46	233.00	162.00	1.44
	PEAK 2	278.00	108.00	2.57	318.00	116.00	2.74
D7S820	PEAK 1	432.00	200.00	2.16	480.00	170.00	2.82
	PEAK 2	467.00	166.00	2.81	265.00	177.00	1.50
D16S539	PEAK 1	548.00	164.00	3.34	615.00	239.00	2.57
	PEAK 2	472.00	166.00	2.84	502.00	247.00	2.03
CSF1PO	PEAK 1	874.00	258.00	3.39	883.00	369.00	2.39
	PEAK 2						
PENTA D	PEAK 1	528.00	242.00	2.18	516.00	254.00	2.03
	PEAK 2	616.00	202.00	3.05	622.00	235.00	2.65
AMEL	PEAK 1	523.00	218.00	2.40	558.00	223.00	2.50
	PEAK 2	667.00	270.00	2.47	740.00	301.00	2.46
VWA	PEAK 1	765.00	378.00	2.02	755.00	389.00	1.94
	PEAK 2	753.00	349.00	2.16	641.00	328.00	1.95
D8S1179	PEAK 1	439.00	173.00	2.54	563.00	267.00	2.11
	PEAK 2	581.00	176.00	3.30	617.00	297.00	2.08
TPOX	PEAK 1	577.00	371.00	1.56	733.00	319.00	2.30
	PEAK 2	580.00	322.00	1.80	590.00	320.00	1.84
FGA	PEAK 1	968.00	420.00	2.30	894.00	346.00	2.58
	PEAK 2	761.00	349.00	2.18	1130.00	293.00	3.86
Average				2.53			2.29

Table 9 cont'd

		04M0407 22A1			04M0407 23A1		
LOCUS		1:100	1:200	Fold Difference	1:100	1:200	Fold Difference
D3S1358	PEAK 1	414.00	144.00	2.88	433.00	194.00	2.23
	PEAK 2	271.00	122.00	2.22	421.00	176.00	2.39
THO1	PEAK 1	556.00	318.00	1.75	1003.00	806.00	1.24
	PEAK 2						
D21S11	PEAK 1	412.00	214.00	1.93	593.00	326.00	1.82
	PEAK 2	406.00	224.00	1.81	601.00	289.00	2.08
D18S51	PEAK 1	722.00	237.00	3.05	785.00	388.00	2.02
	PEAK 2	556.00	240.00	2.32	795.00	400.00	1.99
PENTA E	PEAK 1	240.00	193.00	1.24	689.00	456.00	1.51
	PEAK 2	406.00	73.00	5.56	745.00	460.00	1.62
D5S818	PEAK 1	868.00	316.00	2.75	933.00	248.00	3.76
	PEAK 2						
D13S317	PEAK 1	201.00	99.00	2.03	288.00	121.00	2.38
	PEAK 2	174.00	87.00	2.00	242.00	96.00	2.52
D7S820	PEAK 1	339.00	217.00	1.56	483.00	270.00	1.79
	PEAK 2	323.00	174.00	1.86	416.00	144.00	2.89
D16S539	PEAK 1	306.00	87.00	3.52	491.00	144.00	3.41
	PEAK 2	132.00	135.00	1.02	465.00	106.00	4.39
CSF1PO	PEAK 1	188.00	39.00	4.18	681.00	128.00	5.32
	PEAK 2	163.00	57.00	2.86			
PENTA D	PEAK 1	287.00	227.00	1.26	396.00	122.00	3.25
	PEAK 2	342.00	104.00	3.29	364.00	217.00	1.68
AMEL	PEAK 1	568.00	200.00	2.84	562.00	196.00	2.87
	PEAK 2	656.00	264.00	2.48	650.00	275.00	2.36
VWA	PEAK 1	1326.00	563.00	2.36	701.00	366.00	1.92
	PEAK 2				583.00	319.00	1.83
D8S1179	PEAK 1	654.00	193.00	3.39	558.00	171.00	3.26
	PEAK 2	623.00	210.00	2.97	613.00	174.00	3.52
TPOX	PEAK 1	415.00	171.00	2.43	570.00	349.00	1.63
	PEAK 2	409.00	142.00	2.88	794.00	350.00	2.27
FGA	PEAK 1	1041.00	513.00	2.03	684.00	386.00	1.77
	PEAK 2				628.00	357.00	1.76
Average				2.52			2.47

II. Quantifiler™ Y Human Male DNA Quantification Kit

a. Sensitivity

The quantitation of the DNA samples using the Quantifiler™ Y Human Male DNA Quantification Kit provided reliable results down to only 62.5 pg/μl (Table 10). The quantitation results obtained with samples below 62.5 pg/μl showed significant differences from their intended concentration. Applied Biosystems provides a known concentration standard in the Quantifiler™ Y Human Male DNA Quantification Kit used to make the appropriate dilutions to generate the standard curve. The results from Run 2 (Table 10, in red) of the Quantifiler™ Y Human Male DNA Quantification Kit were obtained using a standard curve generated from dilutions of the male cell line 9948 as opposed to the standard provided by Applied Biosystems. The use of this alternative sample to generate a standard curve in order to quantitate samples of known concentrations yielded results with a significant variation. The removal of the results from Run 2 produced a decrease in the standard deviation of the Quantifiler™ Y Human Male DNA Quantification Kit (Table 11). A comparison of the sensitivity levels of all of three of the RT-PCR quantitation systems is shown in Table 12.

Once diluted, the standards generated from both the Quantifiler™ Human DNA Quantification Kit, and the Quantifiler™ Y Human Male DNA Quantification Kit exhibited significant degradation over time. The slope of the standard curves generated for each run fell slightly outside the recommended range of –3.0 to –3.6, therefore, requiring the removal of Ct data points. Once the outlying Ct points were eliminated, the standard curves did fall within the recommended range and R^2 did not fall below 0.98.

Table 10. QuantifilerTM Y Human Male DNA Quantification Kit sensitivity results

	Run 1				Run 2					Run 3				Run 1-3	
Sample	Quantity(ng/ul)		AVG	STDEV	Quantity(ng/ul)			AVG	STDEV	Quantity(ng/ul)		AVG	STDEV	AVG	STDEV
10 ng/ul	16.620	16.430	16.525	0.134	5.380	8.950	8.320	7.550	1.905	13.750	13.130	13.440	0.438	11.797	4.315
5 ng/ul	7.480	8.750	8.115	0.898	5.080	4.500	4.540	4.707	0.324	8.370	7.310	7.840	0.750	6.576	1.825
2.5 ng/ul	3.820	4.820	4.320	0.707	2.970	2.280	2.510	2.587	0.351	4.910	3.380	4.145	1.082	3.527	1.048
1 ng/ul	1.640	1.540	1.590	0.071	1.270	1.030	0.934	1.078	0.173	1.900	1.520	1.710	0.269	1.405	0.345
0.5 ng/ul	0.760	0.826	0.793	0.047	0.657	0.532	0.550	0.580	0.068	0.946	0.633	0.790	0.221	0.701	0.151
0.125 ng/ul	0.182	0.152	0.167	0.021	0.163	0.155	0.167	0.162	0.006	0.088	0.214	0.151	0.089	0.160	0.038
0.0625 ng/ul	0.063	0.041	0.052	0.016	0.050	0.039	0.053	0.047	0.007	0.055	0.068	0.061	0.009	0.053	0.011
0.025 ng/ul	0.013	0.015	0.014	0.001	0.011	0.016	0.017	0.015	0.003	0.002	0.002	0.002	0.000	0.011	0.006

Table 11. Sensitivity results from experiments performed with the standard provided in the kit

	Run 1				Run 3				Run 1&3	
Sample	Quantity(ng/ul)		AVG	STDEV	Quantity(ng/ul)		AVG	STDEV	AVG	STDEV
10 ng/ul	16.620	16.430	16.525	0.134	13.750	13.130	13.440	0.438	14.983	1.801
5 ng/ul	7.480	8.750	8.115	0.898	8.370	7.310	7.840	0.750	7.978	0.694
2.5 ng/ul	3.820	4.820	4.320	0.707	4.910	3.380	4.145	1.082	4.233	0.753
1 ng/ul	1.640	1.540	1.590	0.071	1.900	1.520	1.710	0.269	1.650	0.175
0.5 ng/ul	0.760	0.826	0.793	0.047	0.946	0.633	0.790	0.221	0.791	0.131
0.125 ng/ul	0.182	0.152	0.167	0.021	0.088	0.214	0.151	0.089	0.159	0.054
0.0625 ng/ul	0.063	0.041	0.052	0.016	0.055	0.068	0.061	0.009	0.057	0.012
0.025 ng/ul	0.013	0.015	0.014	0.001	0.002	0.002	0.002	0.000	0.008	0.007

Table 12. A comparison of sensitivity levels of the three RT-PCR quantitation systems.

Quantitation System	Sensitivity Level	
	Manufacturer's Specification	Observed
Quantifiler Human DNA Quantification Kit	23 pg/µl	125 pg/µl
Quantifiler Y Human Male DNA Quantification Kit	23 pg/µl	62.5 pg/µl
Taqman Alu-PCR Quantitation System	2 pg/µl	

The known concentration sample provided to generate the dilution control standards in the Quantifiler™ Y Human Male DNA Quantification Kit showed a greater decrease in DNA concentration over time as compared with the sample provided in the Quantifiler™ Human DNA Quantification Kit (Table 13).

Table 13. Change in the DNA concentrations of the Quantifiler™ Y Human Male DNA Quantification Kit standards over time.

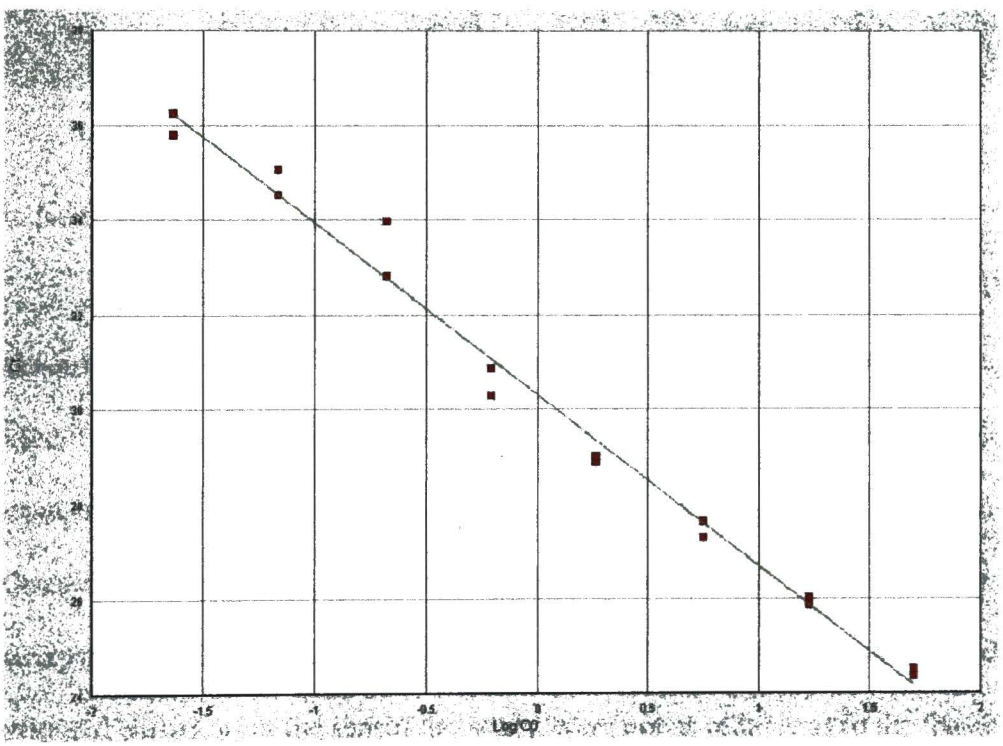
	Day 2			Day 3		
Standard	Quantity(ng/ul)		AVG	Quantity(ng/ul)		AVG
50ng/ul	36.810	39.290	38.050	36.120	36.140	36.130
16.7ng/ul	13.200	12.400	12.800	7.470	12.050	9.760
5.56ng/ul	5.080	4.930	5.005	5.000	4.500	4.750
1.85ng/ul	1.690	1.910	1.800	1.630	1.610	1.620
0.62ng/ul	0.633	0.473	0.553	0.536	0.541	0.539
0.21ng/ul	0.169	0.166	0.168	0.118	0.155	0.137
0.068ng/ul	0.041	0.030	0.036	0.030	0.029	0.030
0.023ng/ul	0.020	0.005	0.013	0.005	0.004	0.005

In addition, the standards in the Quantifiler™ Y Human Male DNA Quantification Kit, demonstrated less precision than those in the Quantifiler™ Human DNA Quantification Kit. Several Ct points were removed in order to create a standard

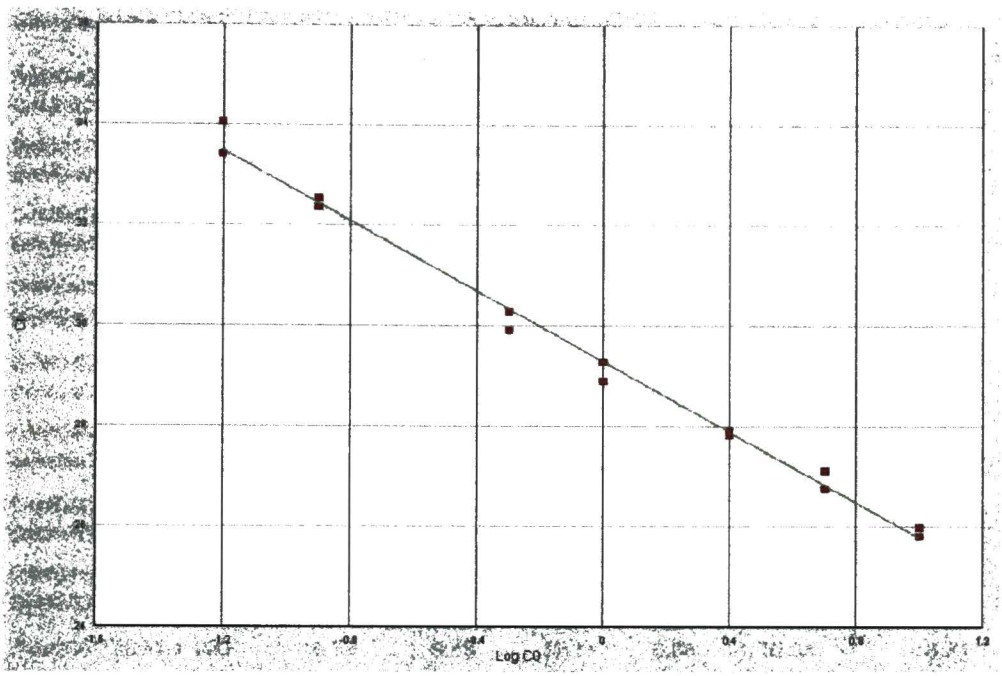
curve that fell within the recommended range for the slope and R^2. Figure 6a shows the standard curve generated prior to removing outlying Ct points, and Figure 6b shows the improvement after the removal of the outlier Ct points. The slope of the standard curve before the Ct points were removed was -3.7, which is outside of the recommended range, and R^2 was 0.978. After the Ct points were removed the slope improved to -3. 4 and R^2 improved to 0.987.

Figure 6. Quantifiler™ Y Human Male DNA Quantification Kit standard curves.

a) The standard curve prior to removing the outlier Ct points.



b) The standard curve after the removal of the outlier Ct points



b. Non-probative case samples

To determine the reproducibility of the Quantifiler™ Y Human Male DNA Quantification Kit, a set of samples commonly processed at the Armed Forces DNA Identification Laboratory were examined. The 1:100 and 1:200 diluted samples were examined and compared to a previous quantitation from Quantifiler™ Human DNA Quantification Kit. Table 12 demonstrates the results of the experiment and a comparison with the result from the Quantifiler™ Human DNA Quantification Kit. The standard curves, as in the sensitivity study, were not as reliable as in the Quantifiler™ Human DNA Quantification Kit. To obtain a standard curve that met the required slope and R^2 values, three data points were removed from the standard curve for each run.

Table 14. Quantifiler™ Y Human Male DNA Quantification Kit non-probative case sample study.

Sample Name	Quantity (ng/ul)					
	Run 1	Run 2	Run 3	STDEV	AVG	Human DNA AVG
04M0407 44A1 1:100	51.36	56.73	61.21	4.93	56.43	53.92
04M0407 44A1 1:200	21.55	25.91	26.11	2.58	24.52	16.17
04M0407 43A1 1:100	125.20	147.85	155.27	12.79	142.77	156.21
04M0407 43A1 1:200	51.51	66.89	61.97	7.85	60.12	49.66
04M0407 40A1 1:100	19.83	24.56	27.62	3.92	24.00	23.08
04M0407 40A1 1:200	8.72	11.99	13.90	2.62	11.54	9.79
04M0407 39A1 1:100	61.34	82.33	64.17	11.39	69.28	65.93
04M0407 39A1 1:200	10.85	13.46	14.04	1.70	12.78	6.76
04M0407 33A1 1:100	4.10	5.31	5.65	0.81	5.02	4.20
04M0407 33A1 1:200	2.52	2.01	3.05	0.52	2.53	1.87
04M0407 32A1 1:100	6.20	7.30	8.51	1.16	7.34	6.20
04M0407 32A1 1:200	3.33	4.56	4.35	0.66	4.08	2.95
04M0407 30A1 1:100	1.26	1.67	1.97	0.36	1.63	1.04
04M0407 30A1 1:200	0.96	0.98	1.01	0.03	0.98	0.67
04M0407 24A1 1:100	13.37	13.45	14.10	0.40	13.64	13.48
04M0407 24A1 1:200	7.14	7.27	8.13	0.54	7.51	5.80
04M0407 23A1 1:100	10.73	9.58	10.71	0.66	10.34	8.71
04M0407 23A1 1:200	5.44	5.41	5.19	0.14	5.35	3.66
04M0407 22A1 1:100	9.67	5.70	9.97	2.38	8.45	5.41
04M0407 22A1 1:200	3.20	5.27	5.56	0.21	4.68	2.72
04M0407 19A1 1:100	6.15	6.90	6.83	0.41	6.63	7.11
04M0407 19A1 1:200	3.42	3.16	3.13	0.16	3.24	2.59
04M0407 13A1 1:100	6.49	6.26	6.29	0.13	6.35	5.65
04M0407 13A1 1:200	4.69	3.16	3.64	0.78	3.83	2.77
04M0407 76A1 1:100	9.81	10.32	8.75	0.80	9.63	7.42
04M0407 76A1 1:200	5.52	5.11	4.27	0.64	4.69	3.57
04M0407 56A1 1:100	89.58	102.64	98.58	6.68	96.93	115.46
04M0407 56A1 1:200	47.94	50.65	49.64	1.37	49.41	45.81
04M0407 57A1 1:100	39.54	41.97	40.62	1.22	40.71	39.34
04M0407 57A1 1:200	13.07	14.59	13.22	0.84	13.63	9.78
04M0407 74A1 1:100	37.10	44.27	40.45	3.59	40.61	36.30
04M0407 74A1 1:200	11.07	13.49	8.86	2.32	11.14	9.43
04M0407 75A1 1:100	65.28	78.67	55.61	11.58	66.52	66.71
04M0407 75A1 1:200	27.25	28.11	33.97	3.66	29.78	26.16
04M0407 52A1 1:100	7.04	7.02	7.65	0.36	7.24	5.19
04M0407 52A1 1:200	2.98	3.69	3.87	0.13	3.51	1.92
04M0407 51A1 1:100	10.75	9.81	10.96	0.61	10.51	9.01
04M0407 51A1 1:200	5.83	5.43	4.90	0.47	5.39	3.56
04M0407 50A1 1:100	45.95	45.12	47.18	1.04	46.08	40.22
04M0407 50A1 1:200	20.60	21.14	23.25	1.40	22.20	20.81
04M0407 59A1 1:100	89.10	0.05	89.23	51.45	59.46	81.11
04M0407 59A1 1:200	28.58	23.18	30.72	3.89	27.49	26.31

CHAPTER 4

DISCUSSION

I. Quantifiler™ Human DNA Quantification Kit

a. Sensitivity

The sensitivity of the Quantifiler™ Human DNA Quantification Kit was evaluated by running serial dilutions of the genomic DNA control 9947a. The precision of the data obtained improved between samples within a 96-well plate after the inclusion of vortexing and reduced rpm centrifugation (Tables 1 and 2). The standard deviations for samples within a 96-well plate and between plates decreased after this modification to the Applied Biosystems protocol was made. Without vortexing, the DNA sample within a well was not thoroughly mixing with the amplification master mix. For optimum amplification and fluorescence signal detection, it is important that the samples are mixed very well. The initial step of centrifuging at 3000 rpm, recommended by Applied Biosystems (11), although removes any residual sample from the walls of the wells, could cause the DNA template to concentrate in the bottom of the well. The requirement for mixing was even more prominent in the results obtained in these experiments because the DNA control 9947a is stored with glycerol. The glycerol increases the density of the sample, making it easier for the DNA to settle at the bottom of the well. With the inclusion of a mixing step, the standard curve generated also improved. With the mixing

step, it was not necessary to remove any Ct points from the standard curves and R^2 was never below 0.99.

The quantitation results obtained (Table 2) were not as accurate as expected. Slight deviations from the expected quantity could occur due to excess sample on the pipette tip, insufficient mixing of the sample with the master mix, or the concentration of the 9947a control DNA was not accurate. The sensitivity of the Quantifiler™ Human DNA Quantification Kit was not as good as the specifications of Applied Biosystems. The results from this study indicated a lower quantitation threshold of 125 pg/µl, as opposed to Applied Biosystems specification of 23 pg/µl. Quantitation of samples at or below 23 pg/µl does not appear realistic since Applied Biosystems indicates that it is often necessary to remove the Ct points of the 23 pg/µl standard from the standard curve. This would strongly indicate that quantitation of 23 pg/µl may not be accurate.

The Ct values for the samples were as expected. Real-time PCR assays use the exponential phase of PCR to quantitate the amount of DNA in a sample. During the exponential phase the amount PCR product doubles with each cycle, therefore, it is expected to see a difference of one cycle between samples that differ by a two fold DNA amount. This can be seen in Tables 3 and 4, as the sample concentration decreases by two fold the Ct increases by one. This is true for all of the samples except the 0.025 ng/µl. During the dilutions, a pipetting error occurred in the preparation of the 0.025 ng/µl sample. However, the mistake was noted and the cycle number was appropriate for the corresponding dilution.

The stability of the standards is shown in Table 5. By the third day, the DNA sample concentrations had decreased significantly. Applied Biosystems recommends storing the standards in glycogen for long-term storage and reuse. The addition of glycogen prevents DNA from sticking, due to the porosity of plastic, to the sides of the tube. It is not advantageous to add glycogen in this particular occurrence. For a reagent to be used at AFDIL, it must go through quality control measures before implementation, requiring more time and excess use of reagents. There is a sufficient amount of DNA standard provided in the Kit to dilute a set of fresh standards each time and experiment is run, therefore, the addition of glycogen is an unnecessary step.

b. Non-probative case samples

To determine the reproducibility of the Quantifiler™ Human DNA Quantification Kit, a set of samples commonly processed at the Armed Forces DNA Identification Laboratory was examined. Due to observed inhibition in the amplification of several of the neat samples and their respective 1:100 dilutions (Table 6), a series of 1:200 dilutions were performed and compared to the 1:100 dilutions (Table 7). The data shown in Tables 6 and 7 demonstrates that it was necessary to dilute the neat samples in order to yield reproducible and reliable results. Examination the IPC within the neat samples indicated that high concentrations of DNA inhibited the PCR process. The guidelines provided in the Quantifiler™ Human DNA Quantification Kit manual for interpreting the IPC results should be followed with caution. In many of the neat samples the IPC did not amplify. According to the manual if no amplification occurred with the IPC and the DNA sample

resulted in a low Ct, the IPC result should be ignored. However, in several cases, inhibition was observed when compared to the 1:100 dilutions (Table 6). The neat DNA samples that failed to amplify with a corresponding IPC that also did not amplify are deemed invalid by Applied Biosystems guidelines. The manufacturer recommends that amplification of that sample should be repeated. However, repeating the quantitation without any further sample manipulation requires the use of more sample, more kit reagents, and an expenditure of additional time. In these experiments, the results of the IPC indicated PCR inhibition, and that dilution of the samples was necessary for accurate results. The results depicted in Figure 7 demonstrate that by diluting the neat samples the PCR inhibitor was removed.

Table 8 also revealed a two fold difference in the results obtained between the Quantifiler™ Human DNA Quantification Kit and the Taqman® Alu-PCR Quantitation System. The quantitation results obtained for 1:200 dilution samples quantitated with Quantifiler™ Human DNA Quantification Kit were similar in quantity to the 1:100 dilution samples quantitated with the Taqman® Alu-PCR Quantitation System. A few select samples, shown in Table 8, were amplified with the PowerPlex™ 16 STR System and analyzed on the ABI 3100 Genetic Analyzer. RFU levels of the positive control, the 1:100, and 1:200 dilution samples indicated that the results from the Quantifiler™ Human DNA Quantification Kit over estimate the true DNA concentration. The PowerPlex™ 16 STR System works optimally with an input of 1ng of DNA. Therefore, the 1:100 samples were diluted based on the results from Taqman® Alu-PCR Quantitation System and the 1:200 samples were diluted based on the Quantifiler™

Human DNA Quantification Kit's results. If the results obtained from the Taqman® Alu-PCR Quantitation System for the 1:100 samples were similar to the results of the 1:200 samples from the Quantifiler™ Human DNA Quantification Kit, the peak heights for the corresponding samples were expected to be similar as well. However, the genetic analysis revealed that the peak heights for the 1:200 were approximately half of the expected peak heights. The peak heights of the 1:100 samples were closer to those of the 1 ng positive control sample. This demonstrated that the Quantifiler™ Human DNA Quantification Kit produced results which were approximately two fold greater than their true concentrations. It also indicted that the Taqman® Alu-PCR Quantitation System was more accurate. It is difficult to determine the specific cause for the increased concentration seen with the Quantifiler™ Human DNA Quantification Kit. A significant difference between the two quantitation systems is that the Taqman® Alu-PCR Quantitation System only requires 1 µl of sample while the Quantifiler™ Human DNA Quantification Kit requires 2 µl. The amount of input sample potentially is the basis for the difference between the two methods. However, the quantitation software provide with the Quantifiler™ Human DNA Quantification Kit, if working properly, should accounts for the 2 µl volume. Based upon the results from the sensitivity study it did not appear as though the software or ABI 7000 instrument was working improperly.

Based upon the results obtained in the sensitivity study in which a low standard deviation for most samples was obtained, the non-probative case samples were not run in duplicate within the same 96-well plate. Case samples that quantitated greater than

13 ng/µl did exhibit higher standard deviations than those samples with lower DNA concentrations. These results could be due to either excess DNA sample on a pipette tip or the inhibition with greater amounts of DNA.

II. Quantifiler™ Y Human Male DNA Quantification Kit

a. Sensitivity

The sensitivity of the Quantifiler™ Y Human Male DNA Quantification Kit was evaluated by running serial dilutions of the genomic DNA control 9948. As seen in the sensitivity experiment (Tables 1 and 2) of the Quantifiler™ Human DNA Quantification Kit, the addition of a brief vortex and centrifugation at 1000 rpm for 30 seconds improved the precision within a 96-well plate and increases the precision of the standard curve. Therefore, all runs using the Quantifiler™ Y Human Male DNA Quantification Kit were vortexed and centrifuged at 1000 rpm for 30 seconds.

Tables 10 and 11 demonstrated that the Quantifiler™ Y Human Male DNA Quantification Kit was able to accurately determine DNA concentrations accurately down to 62.5 pg/µl. The manual, provided by Applied Biosystems, indicated that the kit has the capability of determining DNA concentrations as low as 23 pg/µl. Tables 10 and 11 clearly show that the 25 pg/µl sample is consistently quantitated below the indicated concentration. The other samples quantitate slightly greater than the amount of sample would indicate. However, the samples were serially diluted from a sample of 9948 control DNA at 10 ng/µl. The results shown in Table 10 could indicate that the

concentration of 9948 was greater than 10 ng/µl and/or excess sample was used during the initial dilution.

The Quantifiler™ Y Human Male DNA Quantification Kit standards do not exhibit the same amount of precision as they did with Quantifiler™ Human DNA Quantification Kit (Table 12). Certain runs required up to 4 points to be removed from the standard curve to achieve the recommended values for the slope and R^2.

b. Non-probative case samples

To determine the reproducibility of the Quantifiler™ Y Human Male DNA Quantification Kit, a set of samples commonly processed at the Armed Forces DNA Identification Laboratory were examined. The same samples used in the non-probative case sample study for the Quantifiler™ Human DNA Quantification Kit were used with the Quantifiler™ Y Human Male DNA Quantification Kit. There was not a sufficient amount of the original neat samples, therefore, only the 1:100 and 1:200 diluted samples were examined and compared to the results from the quantitation from the Quantifiler™ Human DNA Quantification Kit.

Table 14 indicated that the results from the Quantifiler™ Y Human Male DNA Quantification Kit were similar to those of the Quantifiler™ Human DNA Quantification Kit. The results were not expected to be exactly the same, since the target genes for amplification are not only on two different chromosomes, but hTERT is diploid and SRY is haploid. These studies also indicate that the results between both Quantifiler™ systems were consistent. Further studies are necessary in order to determine the

sensitivity of the Quantifiler™ Y Human Male DNA Quantification Kit and determine the ability to quantitate the male portion of a male: female mixture.

CHAPTER 5

CONCLUSIONS

The Quantifiler™ Human DNA Quantification Kit have proven to be precise within and between 96-well plates for samples ranging from approximately 5 ng/µl to 125 pg/µl. However, the quantitation results appear to be approximately two fold higher than the actual concentration. More experiments are necessary to determine the cause of a two fold increase in the quantitation of case samples.

To obtain the most reliable results, all samples should be run in duplicate, and the addition of a brief vortex and 1000 rpm centrifugation for 30 seconds prior to quantitation on the ABI 7000. New dilution concentration standards should be made for each analysis in order to generate an accurate standard curve.

The Quantifiler™ Y Human Male DNA Quantification Kit was able to detect DNA down to 62.5 pg/µl. The results obtained with this study did not obtain the same level of sensitivity indicated by Applied Biosystems. However, the results obtained with samples above 62.5 pg/µl were reproducible, as demonstrated by the low standard deviation between runs.

The results obtained from the Quantifiler™ Y Human Male DNA Quantification Kit were consistent with those obtained with the Quantifiler™ Human DNA Quantification Kit.

As with the Quantifiler™ Human DNA Quantification Kit new standards should be made for each analysis. In addition, if four or more data points need to be removed from the standard curve, caution must be taken in the reliability of the quantitation of the samples.

It appears, at this time, to be in the best interest of AFDIL to continue the use of the Taqman® Alu-PCR Quantitation System that they developed. The Taqman® Alu-PCR Quantitation System has proven to be more sensitive and reliable. This system is also more cost efficient than the Quantifiler™ Kits (3).

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