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Quantitation of DNA is an essential step in DNA testing and methods used to quantitate DNA must be human specific. Quantitation of DNA is also important because the PCR reaction performs optimally in a narrow concentration range. Applied Biosystems (Foster City, CA) has recently introduced two new DNA quantitation kits, Quantifiler™ Human and Quantifiler™ Y Human Male DNA Quantification kits, which quantify the total amount of amplifiable DNA present in a sample. Both kits are based on the 5' nuclease assay and Taqman® probe-based technology which allow for a more accurate result which will minimize further problems downstream. To validate the use of the Quantifiler™ kits, a series of studies were performed to determine the reliability and performance of the kits. Results demonstrate this quantitation method produces similar results to those obtained by the Quantiblot® method. In addition, results demonstrate that this quantitation method is highly sensitive and yields consistent results.

Internal Validation of Quantifiler™ Human and Quantifiler™ Y Human male DNA Quantification Kits Using Applied Biosystems 7500 Real-Time PCR

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# INTERNAL VALIDATION OF QUANTIFILER™ HUMAN AND QUANTIFILER™ Y HUMAN MALE DNA QUANTIFICATION KITS USING APPLIED BIOSYSTEMS 7500 REAL-TIME PCR SYSTEM

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# Chapter I

## INTRODUCTION

While several steps are involved in forensic DNA testing procedures, quantitation of deoxyribonucleic acid (DNA) is one of the most critical steps in the process of determining a person's DNA profile. Based on the initial amount of DNA present in a sample, the input DNA concentration must be adjusted for the polymerase chain reaction (PCR) to perform optimally. There are several methods which may be used to determine the quantity of DNA present in a sample. However, due to standard 9.3 issued by the Director of the FBI, forensic laboratories are limited to quantitation methods that are human specific (1). Due to harsh environments or poor evidence collection and storage, the DNA may be present in limited quantities or contaminants may be present. The human specificity requirement ensures that the quantitation results will not be exaggerated due to bacterial growth or other contaminants which may be present in evidentiary samples.

The polymerase chain reaction (PCR), an enzymatic process first described in 1986, allows for the synthesis of specific DNA sequences (2). PCR is used to replicate specific regions of DNA during repetitive cycles which consist of denaturing the DNA template followed by annealing the primers to complimentary sequences and extension of the bound primers by a DNA polymerase (3). In forensics, PCR is typically used to

amplify thirteen short tandem repeat (STR) loci. A person's unique DNA profile is determined by combining the results for each of the thirteen loci. The PCR reaction used for amplification performs optimally in a narrow concentration range since problems may occur with STR analysis when DNA is added at a level outside the range (3). Too much DNA can result in incomplete 3' adenylation and therefore split peaks. Low levels of DNA can result in stochastic fluctuations and therefore allele dropout. Because of these problems, it is important to have a reliable method of quantitating the amount of DNA present in a casework sample.

Probably the most popular method in forensic laboratories today is the 'slot-blot' procedure for DNA quantitation performed via the Quantiblot® Human DNA Quantitation kit (Applied Biosystems, Foster City, CA). Quantitation is based upon the hybridization of a 40 base pair probe that is complimentary to a primate-specific alpha satellite DNA sequence D17Z1 located on chromosome 17. Known DNA standards, as well as unknown DNA samples, are blotted onto a nylon membrane, denatured, and hybridized with D17Z1 probe (3, 4). This method is at best semi-quantitative as the results are based on visual interpretation of the samples to the known standards.

Real-time PCR (RT-PCR) via the Quantifiler™ kits allows for a more accurate quantitation result because it has the ability to monitor the progress of PCR as it occurs. RT-PCR uses a fluorogenic probe containing a reporter fluorescent dye on the 5' end and quencher dye on the 3' end. While the probe is intact the proximity of the quencher dye greatly reduces the fluorescence signal emitted by the reporter dye by Fluorescence Resonance Energy Transfer (FRET) through space. If the target sequence is present, the

probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of the DNA polymerase as the primer is extended. Additional reporter dyes are cleaved from their respective probes with each cycle of the PCR reaction, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced (5). A standard curve is then generated through the incorporation of a set of DNA concentrating standards which are used to determine the amount of total amplifiable DNA present in a sample (6).

The Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit were recently introduced by Applied Biosystems (ABI) to provide the forensic community a more accurate quantitation result based on RT-PCR (6, 7). Both kits use the same, simple protocol based on the 5' nuclease activity of Taq DNA polymerase which involves minimal hands-on time with results obtained in less than two hours. Each kit contains an internal positive control which allows for monitoring of PCR inhibition which may affect the kits ability in detecting presence of human DNA. Prior to the development of these kits, inhibition could only be detected after samples were unsuccessfully amplified. Inhibitors of the PCR reaction are usually co-precipitated out with the DNA during extraction. Heme, found in blood, is a major inhibitor of the PCR reaction. Unlike other inhibitors, heme may be detected through visual observation of the extracted sample (9). These kits provide the analyst with information about which sample will fail to provide a DNA profile, reducing STR analysis time and cost of re-processing. The Quantifiler™ Y Human Male DNA Quantification kit is the first commercially available kit for male DNA quantitation. The

presence of male DNA in a sexual assault case is usually not detected due to the high background of female DNA. By amplifying a Y chromosome specific gene, SRY region, the amount of male DNA only in a sample may be determined. Each kit is designed to be used independently; however, when both kits are used for the same set of samples, it will be possible to resolve mixtures of male and female DNA (7, 8).

Although this method appears to be more beneficial than current methods, the method must be validated for use in casework before it can be applied. Validation studies are a requirement in the forensic community because of standard 8.1 issued by the Director of the FBI (1). This standard separates validation studies into two categories, developmental and internal. The developmental validation is performed by the company that develops the new technique or method whereas an internal validation is performed before a new method or technique is implemented into use in a forensic laboratory (1). Validation studies provide documentation which shows methods and techniques used in the DNA testing process are reliable. Interpretation guidelines and protocols for the analyst to follow are determined after validation studies are performed.

Validation studies also play a role in legal proceeding. Through the use of discovery orders, defense attorneys are allowed access to validation studies. Defense attorneys rarely attack the science behind DNA testing anymore; instead emphasis is placed on the manner in which the testing is conducted. Because of this, validation studies may pose challenges to admitting DNA evidence under Daubert (Expert Witness lecture notes).

As such, an internal validation of RT-PCR via the Quantifiler™ kits using the Applied Biosystems 7500 Real-Time PCR System must be performed before this method may replace the existing quantitation method.

# CHAPTER II

# BACKGROUND

## *Current Quantitation Methods*

Early methods of DNA quantitation typically involved either absorbance at 260 nm wavelength or fluorescence after staining a yield gel with ethidium bromide. Unfortunately, because those approaches are not very sensitive, they consume valuable forensic specimens that are irreplaceable. Absorbance measurements are not specific for DNA and contaminants may give false results. In addition, UV absorbance and fluorescent dyes are not human specific. Other quantitation methods include fluorescence-based microplates, enzymatic hybridizations, and 'slot-blot' procedures (3).

PicoGreen® double-stranded DNA (dsDNA) quantitation reagent (Molecular Probes, Eugene, OR) is an ultra sensitive fluorescent nucleic acid stain for quantifying dsDNA in solution. The PicoGreen reagent used in conjunction with fluorescence-based microplate reader can detect as little as 250 pg/mL dsDNA. The major disadvantage of this system is failure to meet the human specificity requirement of the national standards (10).

The AluQuant™ Human DNA Quantitation System (Promega Corporation, Madison, WI) provides a simple and sensitive method for quantitating the concentration of human DNA in forensic samples. The system uses a series of enzymatic reactions to

produce a luminescent signal proportional to the quantity of human DNA present. The sensitivity level of the system, 0.1-50 ng, is at a level that the forensic community requires (11); however, it is not widely used because labs had tremendous problems performing the test manually and robotics is required to perform analysis accurately.

The Quantiblot® Human DNA Quantification kit is based on hybridization of a biotinylated oligonucleotide probe to extracted DNA samples immobilized on a nylon membrane. The probe is complimentary to a primate-specific alpha satellite DNA sequence at the D17Z1 locus. The sensitivity level of this system ranges from 10 ng to 0.15 ng of human or primate DNA (12). Although this method is used by most forensic laboratories, it has several disadvantages. The major disadvantage of this method is the subjective manner in which the results are obtained. This method is at best semi-quantitative as the results are based on visual interpretation of the samples to the known standards. When a sample falls outside the systems range, the analyst must determine the quantity based on how the sample corresponds to the bands on the membrane. This can lead to inaccurate results which may affect STR analysis results.

*Background Information on Real-Time PCR*

The polymerase chain reaction (PCR) has revolutionized the scientific community because of its broad applications in several disciplines of science including molecular biology, medical diagnostics, and forensics. Since Mullis described this technique for the synthesis of specific DNA sequences (2), PCR has become the most widely used technique in science. The method consists of repetitive cycles of denaturation, hybridization, and polymerase extension. There are three basic PCR phases: geometric,

linear, and plateau. The geometric phase is the initial phase of the reaction where exact doubling of PCR product is accumulating at every cycle. During the linear phase the reaction components are being consumed and the amplification efficiency begins to decrease. The plateau phase occurs when the PCR reaction has stopped and no more product is made. It is during the geometric phase that the fluorescent signal is detected during RT-PCR because all the reagents are fresh and available making the reaction more precise (6, 13).

The concept behind real-time PCR (RT-PCR) has been around for little over a decade. In 1993, Higuchi et al. (14) was the first to describe a quantitative assay that monitored multiple PCRs simultaneously. His assay used ethidium bromide to measure real-time product and also showed that inhibition of PCR could be detected in real-time assays. Prior to the discovery of RT-PCR, Holland et al (15) introduced the idea of utilizing the inherent 5'→ 3'activity of *Taq* DNA polymerase and also envisioned the use of this technique to create a method in which amplification and detection occur in one tube.

Improvements have been made that allow for RT-PCR to be performed more efficiently. When Higuchi developed the idea of real-time monitoring of PCR products he used an intercalating dye, ethidium bromide, which binds to all double-stranded DNA (dsDNA). This allows for nonspecific amplification of DNA. Also, the radio-labeled probes used by Holland have now been replaced. Applied Biosystems (ABI) has developed a RT-PCR system which incorporates fluorogenic-labeled probes that are dissociated by the 5' nuclease activity of AmpliTaq Gold® DNA polymerase. The

availability of these probes enabled the development of a real-time method for detecting only specific amplification product (16).

AmpliTaq Gold® DNA polymerase has 5' exo-nuclease activity. The 5' exo-nuclease activity and FRET (Fluorescent Resonant Energy Transfer) makes it possible to detect PCR amplification in real-time. The principle behind FRET is that when a high-energy dye is in close proximity to a low-energy dye, there will be a transfer of energy from high to low. In the 5' nuclease activity, an oligonucleotide called a Taqman® minor-groove binder (MGB) probe is designed with a high-energy dye termed a reporter at the 5' end, and a low-energy non-fluorescent quencher dye at the 3' end (Figure 1).

Figures 1- 4. The 5' Nuclease Process which takes place during the cycles of the PCR reaction (6-8).

Figure 1. Polymerization

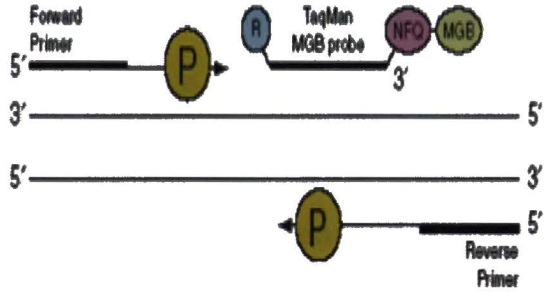



Figure 2. Stand Displacement

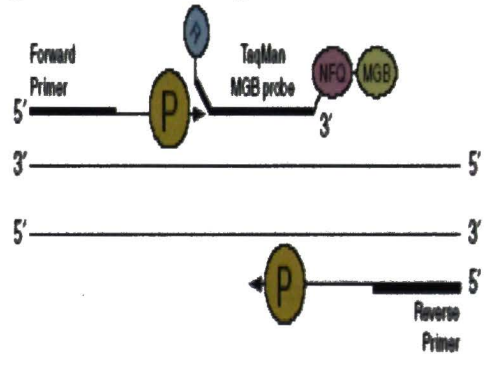



Figure 3. Cleavage

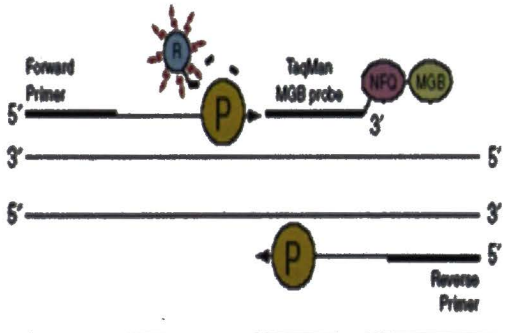
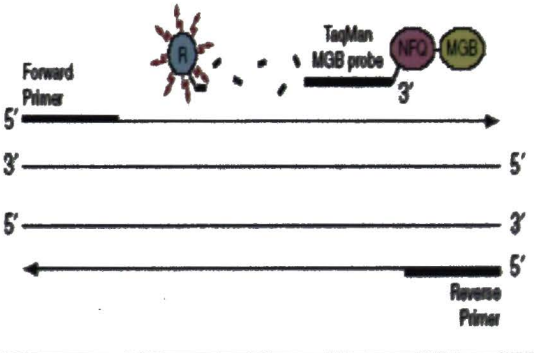



Figure 4. Polymerization Completed



When the probe is intact (Figures 1 and 2) and excited by a light source, the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5' nuclease activity of the AmpliTaq Gold® DNA polymerase (Figure 3), the distance between the dyes increases causing the transfer of energy to stop. Cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal and also removes the probe from the target strand, allowing primer extension to continue to the end of the template strand (Figure 4). The fluorescent emissions of the reporter increase and the quencher decrease. Additional reporter molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescent intensity proportional to the amount of amplicon produced. The increase in reporter signal is captured by the RT-PCR system and displayed by the sequence detection software (SDS). The higher starting copy number of the DNA target, the sooner a significant increase in fluorescence is detected (13, 16).

*Quantifiler™ kits*

The Quantifiler™ Human DNA Quantification kit (Applied Biosystems, Foster City, CA, catalog # 4343895) and Quantifiler™ Y Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA, catalog # 4343906) are designed to quantify the total amount of amplifiable human (and higher primate) DNA or human male DNA in a sample. When the kits are used together for the same set of samples, the gender of the sample can be determined. The Quantifiler™ Y Human Male DNA Quantification kit is the first commercially available kit that allows for a quantitation result to be obtained for male DNA. The results from the Quantifiler™ kits can aid in determining if sufficient

human DNA or human male DNA is present to proceed with STR analysis and how much sample to use in STR analysis applications (6).

Each Quantifiler™ kit includes all the reagents necessary for the amplification, detection, and quantitation of 400 reactions using the ABI 7500 Real-Time PCR System. The kit contains three reagent components: Quantifiler Human DNA Standard, Quantifiler PCR Reaction mix, and Quantifiler human primer mix or Quantifiler Y male primer mix. Both kits utilize the same reagents except for the primer mix, which is pre-formulated with a primer and probe system that amplifies either the Human Telomerase gene (Quantifiler Human kit) or the SRY gene (Quantifiler Y kit). Each kit also contains an Internal Positive Control (IPC), a synthetic DNA template that has been formulated into the primer mix. A second primer and probe system along with the IPC template is added creating a duplex reaction in each tube. By amplifying both systems in a single tube, the kits provide an indication of potential sample inhibition and allow for quick identification of samples that do not contain human DNA (7, 8) .

Both Quantifiler kits are optimized for use with the same simple protocol, based on the 5' nuclease activity and TaqMan® probe-based technology for maximum reliability and ease of use. Unlike other quantitation methods, the kits protocol does not include incubation steps, tubes to open and close, sample-transfer steps, or mid-process washing. The entire quantitation procedure can be completed in less than two hours (7, 8).

*Applied Biosystems 7500 Real-Time PCR System*

The Applied Biosystems 7500 Real-Time PCR System is an integrated and versatile platform for the detection and quantitation of nucleic acid sequences. RT-PCR

combines thermal cycling, fluorescence detection, and application specific software to measure cycle-by-cycle accumulation of PCR products in a single-tube homogenous reaction (17).

Thermal cycling and fluorescent detection can be performed on the ABI 7500 instrument which contains a Peltier-based 96-well block which supports 96-well reaction plates and 0.2 ml strip tubes in volumes ranging from 25 – 100 µl. A tungsten-halogen lamp passes light through the ABI optical adhesive cover and excites the fluorescent dyes in each sample well. Fluorescence emission is then detected through five emission filters to a charge-coupled device (CCD) camera. During the run, the CCD camera detects the fluorescence emission from each well. The sequence detection software (SDS) software obtains the fluorescence emission data from the CCD camera and applies data analysis algorithms.

SDS software for the ABI 7500 system runs on the Windows XP® operating system and is used for instrument control, data collection, and data analysis. After the RT-PCR accumulation of samples using the Quantifiler kits, with just one mouse click, the ABI 7500 Real-Time PCR System SDS software automatically calculates the amount of total amplifiable DNA present in each sample. The calculation is based on the cycle number at which the amount of amplified product crosses the threshold in each reaction. For each sample, the cycle number is then correlated with a standard curve generated from a series of DNA standards, allowing the software to translate the cycle-number into a DNA concentration. Using the amount of amplifiable DNA calculated by the SDS

software, the appropriate amount of sample DNA can be added to the subsequent STR amplification the first time, making the test both cost- and time effective (6-7, 13).

# CHAPTER III

## RESEARCH DESIGN AND METHODOLOGY

Seven studies were performed to validate the use of the Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit using the ABI 7500 Real-Time PCR System for use in casework at Reliagene Technologies Inc. (New Orleans, LA).

*Cross-Contamination Study*

The ABI 7500 Real-Time PCR System has the ability to process 96 samples at once. To demonstrate if cross-contamination of adjacent wells is possible, 24 negative amplification samples (no template DNA) and 24 male DNA samples were placed alternately in a checkerboard fashion for the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit on the same reaction plate to examine cross contamination during the operation.

*Sensitivity Study*

The sensitivity of the Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit was tested by preparing a series of dilutions from a known DNA sample. All samples were male so both kits could be used. The concentration of the seven samples for the Quantifiler™ Human DNA Quantification kit ranged from 100 pg/µl to 10 pg/µl while the concentration of the four

samples for Quantifiler™ Y Human Male DNA Quantification kit ranged from 100 pg/μL to 25 pg/μL.

*Concordance Study*

Seven different male samples were quantified using the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit. These seven samples had been previously quantified using the Quantiblot® Human DNA Quantitation kit and the quantitation results were compared.

*Reproducibility Study*

Seven different male samples were tested for reproducibility of the quantitation results. All samples were tested using the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit. Three different runs were performed to ensure reproducible results could be obtained.

*Mixture Study*

To determine the ability of the Quantifiler™ Y Human Male DNA Quantification kit in obtaining results from mixed source samples, male-female mixture samples were prepared in ratios of 1:20, 1:50, 1:100, 1:150, 1:500, 1:1000, 1:1250, and 1:2500. The mixture samples were tested with the Quantifiler™ Y Human Male DNA Quantification kit to determine the quantity of male DNA only present in a sample.

*Non-probative Study*

Five non-probative cases were tested using the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit. A

total of 22 samples, both males and female were tested for each kit to ensure results could be obtained with actual forensic casework samples.

*Slope Values for Standard Curve*

Because the Quantifiler™ kits were designed to work on the ABI Prism 7000 Sequence Detection System, the slope range provided could not be used on the ABI 7500 RT-PCR system. The ABI 7500 RT-PCR System is one of the latest real-time platforms developed by Applied Biosystems. This system offers advantages over previous real-time platforms since it is smaller and incorporates five excitation and five emission filters which improve the ability to excite dyes at longer wavelengths. For each Quantifiler™ kit, a standard curve was used to evaluate the performance of the eight concentrating DNA standards. A slope value was generated for each standard curve and indicated the PCR amplification efficiency (6). To determine the slope range for the Quantifiler™ kits using the ABI 7500 RT-PCR System, the slope values from each of the previous studies was recorded. The slope range was then determined and included all slope values that were within the highest and lowest recorded values.

# CHAPTER IV

# MATERIALS AND METHODS

*Sample Source and Extraction*

Unless noted otherwise all samples are male. For the cross contamination study, a 96-well Chelex-100 (Bio-Rad, Hercules, CA, catalog #142-1253) DNA extraction method was used to isolate DNA from 92 samples from adjudicated paternity cases. Twenty-four samples were used in the cross-contamination for each Quantifiler™ kit. For the sensitivity study a known DNA sample, SGM AmpF*l*STR positive control DNA (Applied Biosystems, Foster City, CA) containing 100 pg/µl was serially diluted to obtain varying DNA concentrations. For the concordance and reproducibility studies, a total of seven samples were used. Six samples which had been previously extracted using and quantified via the Quantiblot® Human DNA Quantification kit and one known male DNA sample purchased from American Type Culture Collection (ATCC, Manassas, VA, catalog # CCL-256) were quantified using the Quantifiler™ kits. The mixture study samples were a known genomic male and female sample purchased from ATCC (Manassas, VA, catalog # CCL-2). The same known male sample used in the previous studies was also used in the mixture study. Twenty-two non-probative samples from five non-probative cases were used in the non-probative study. Of the twenty-two samples, ten were female.

*Sample Preparation*

The mixture samples were prepared from male and female genomic DNA obtained from ATCC (Manassas,VA). Male-Female mixture samples were prepared in ratios of 1:20, 1:50, 1:100, 1:150, 1:500, 1:1000, 1:1250, and 1:2500. The human female DNA was added at a constant level of 50 ng in all samples, and the human male DNA varied from 2.5 ng in the 1:20 sample to 0.02 ng in the 1:2500 sample. The sensitivity of the Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit was tested by preparing a series of dilutions from a known DNA sample. The positive control DNA, 007, provided with the SGM AmpF*l*STR kit containing 100 pg/µl of human male DNA served as the source of male DNA for the sensitivity study. Samples were prepared by performing dilutions with $T_{10}E_{.1}$ buffer [10 mM Tris-HCl (pH 8), 0.1 mM $Na_2EDTA$, 20 µg/mL glycogen]. The concentration of the seven samples for the Quantifiler™ Human DNA Quantification kit ranged from 100 pg/µL to 10 pg/µL while the concentration of the four samples for Quantifiler™ Y Human Male DNA Quantification kit ranged from 100 pg/L to 25 pg/µL.

*Standard Preparation*

Quantifiler™ Human DNA Standard is supplied in each kit. A set of standards is prepared with a three-fold dilution series with eight concentration points in the standard series for each kit. The eight concentration points for each kit ranged from 50 ng/µL (Standard 1) to 0.023 ng/µL (Standard 8). Samples were prepared by performing dilutions with $T_{10}E_{.1}$ buffer [10 mM Tris-HCl (pH 8), 0.1 mM $Na_2EDTA$, 20

μg/ml glycogen]. Glycogen was added to the buffer to allow for the standard to be stored for up to two weeks. One set of DNA standards and one negative amplification control (no DNA template) was run with each kit. The standard curve generated by the sequence detection (SDS) software for each kit is used to determine the quantity of DNA present in an unknown sample. The SDS software automatically calculates the total amplifiable DNA present in each sample.

*Real-Time PCR Protocol*

DNA samples were quantified using both Quantifiler™ kits except for the mixture study which only used the Quantifiler™ Y Human Male DNA Quantification kit. In all instances, 2 μL of template DNA or standard was amplified using 23 μL of either human or male master mix for a total reaction volume of 25 μL. The negative amplification control (NAC) consisted entirely of 25 μL of master mix for each kit. Amplification was performed using the following PCR conditions: 1 cycle of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds followed by 1 minute at 60°C. Amplification and detection was performed on an ABI 7500 RT-PCR System (Applied Biosystems, Forster City, CA) using either a 96-well tray or 8-strip tubes with optical covers.

# CHAPTER V

## RESULTS AND DISCUSSION

### *Cross-Contamination Study*

Contamination, the accidental transfer of DNA, is a major issue when performing PCR. If contamination is observed or suspected, the results are void unless sufficient sample is available for the tests to be repeated. The ABI 7500 Real-Time PCR System has the ability to process 96 samples at one time. Since 96 samples can be processed at once, there is a possibility for cross-contamination of adjacent wells on the reaction plate to occur. Negative amplification controls (NAC) were used to monitor for contamination of the PCR process and contained no DNA template. Since there is no DNA template in the sample, these samples are not expected to be detected by the software. Cross-contamination of adjacent wells was evaluated because each sample that contained DNA was surrounded by a NAC which did not contain DNA. If DNA is detected, then the NAC has failed and the source of contamination should be determined. Twenty-four negative amplification controls (no DNA template) and 24 human male DNA samples were placed alternately on the same 96 well reaction plate to examine cross-contamination from adjacent wells on the reaction plate during the operation. The samples were quantitated using the Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit. No standard set was run with this

study since the purpose was only to detect for presence of DNA. The cycle-threshold ($C_T$) value was used to determine if DNA was present in a sample. The SDS software uses a threshold setting to define the level of detectable fluorescence. Based on the number of cycles required to reach the threshold, the SDS software can compare test samples quantitatively: A sample with a higher starting template copy number reaches the threshold earlier than a sample with a lower starting template copy number. The threshold-cycle ($C_T$) for a specified amplification plot occurs when the fluorescent signal increases beyond the value of the threshold setting (6). A $C_T$ would only be detected if the sample contained DNA, those samples without DNA present would fail to give $C_T$ and thus be undetermined by the software. Results are shown in Table 1. All 24 negative amplification controls tested for each kit produced no detectable DNA, ensuring that the procedures do not introduce cross-contamination of adjacent wells on the reaction plate. As expected, all 24 male DNA samples produced a detectable amount of DNA. The $C_T$ values for the Quantifiler™ Human kit ranged from 26.5 to 31.15 and the Quantifiler™ Y kit ranged from 26.76 to 31.46. It was concluded that the ABI 7500 Real-Time PCR System does not yield cross-contamination of adjacent wells because no DNA was present in the 24 NAC samples.

Table 1. Results for Cross-Contamination Study.
The reaction plate was divided in half, with one-half of the plate running the Quantifiler™ Human DNA Quantification kit (Qt-H) represented by the color purple and the other half using the Quantifiler™ Y Human Male DNA Quantification kit (Qt-Y) represented by the grey color. The NAC samples are designated as blank on the reaction plate.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Qt-H Blank | Qt-H 02-80187 Ct=26.5 | Qt-H Blank | Qt-H 02-84994 Ct=31.07 | Qt-H Blank | Qt-H 02-87229 Ct=27.22 | Qt-Y Blank | Qt-Y 02-80187 Ct=26.76 | Qt-Y Blank | Qt-Y 02-84994 Ct=31.45 | Qt-Y Blank | Qt-Y 02-87229 Ct=27.92 |
| B | Qt-H 02-88713 Ct=29.81 | Qt-H Blank | Qt-H 02-90695 Ct=26.84 | Qt-H Blank | Qt-H 02-91232 Ct=28.19 | Qt-H Blank | Qt-Y 02-88713 Ct=30.52 | Qt-Y Blank | Qt-Y 02-90695 Ct=27.74 | Qt-Y Blank | Qt-Y 02-91232 Ct=27.75 | Qt-Y Blank |
| C | Qt-H Blank | Qt-H 03-13050 Ct=27.21 | Qt-H Blank | Qt-H 03-14546 Ct=29.26 | Qt-H Blank | Qt-H 03-14818 Ct=29.03 | Qt-Y Blank | Qt-Y 03-13050 Ct=27.76 | Qt-Y Blank | Qt-Y 03-14546 Ct=29.88 | Qt-Y Blank | Qt-Y 03-14818 Ct=29.64 |
| D | Qt-H 03-14981 Ct=28.69 | Qt-H Blank | Qt-H 03-17173 Ct=29.15 | Qt-H Blank | Qt-H 03-21687 Ct=29.50 | Qt-H Blank | Qt-Y 03-14981 Ct=29.05 | Qt-Y Blank | Qt-Y 03-17173 Ct=30.02 | Qt-Y Blank | Qt-Y 03-21687 Ct=29.28 | Qt-Y Blank |
| E | Qt-H Blank | Qt-H 03-18265 Ct=27.64 | Qt-H Blank | Qt-H 03-19798 Ct=28.12 | Qt-H Blank | Qt-H 03-21180 Ct=31.15 | Qt-Y Blank | Qt-Y 03-18265 Ct=28.38 | Qt-Y Blank | Qt-Y 03-19798 Ct=28.95 | Qt-Y Blank | Qt-Y 03-21181 Ct=33.00 |
| F | Qt-H 03-22560 Ct=29.07 | Qt-H Blank | Qt-H 03-22562 Ct=28.06 | Qt-H Blank | Qt-H 03-22566 Ct=30.48 | Qt-H Blank | Qt-Y 03-22560 Ct=29.67 | Qt-Y Blank | Qt-Y 03-22562 Ct=28.49 | Qt-Y Blank | Qt-Y 03-22566 | Qt-Y Blank |
| G | Qt-H Blank | Qt-H 03-22569 Ct=31.13 | Qt-H Blank | Qt-H 03-22268 Ct=27.24 | Qt-H Blank | Qt-H 03-22274 Ct=28.13 | Qt-Y Blank | Qt-Y 03-22569 Ct=31.46 | Qt-Y Blank | Qt-Y 03-22268 Ct=27.95 | Qt-Y Blank | Qt-Y 03-22274 Ct=28.89 |
| H | Qt-H 03-22277 Ct=28.00 | Qt-H Blank | Qt-H 03-22280 Ct=26.94 | Qt-H Blank | Qt-H 03-22283 Ct=27.78 | Qt-H Blank | Qt-Y 03-22277 Ct=27.68 | Qt-Y Blank | Qt-Y 03-22280 Ct=27.48 | Qt-Y Blank | Qt-Y 03-22283 Ct=28.02 | Qt-Y Blank |

*Sensitivity Study*

A sensitivity study was performed to evaluate the range of detection for the Quantifiler™ kits. The sensitivity of the Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit was evaluated by preparing a series of dilutions from the positive control DNA, 007, provided with the SGM

AmpF*l*STR kit which contained 100 pg/µl of human male DNA. The concentration of the seven samples for the Quantifiler™ Human DNA Quantification kit ranged from 100 pg/µl to 10 pg/µl while the concentration of the four samples for Quantifiler™ Y Human Male DNA Quantification kit ranged from 100 pg/µL to 25 pg/µL. The variation in the amount of DNA input and the amount of DNA detected by the Quantifiler™ kits is probably due to the different methods used for the quantitation. Results are shown in Table 2. The Quantifiler™ Human DNA Quantification kit was able to detect and quantify all seven concentration ranges. The lowest concentration level able to be detected and quantified was 0.0084 ng/µL DNA. The Quantifiler™ Y Human Male DNA Quantification kit detected and quantified the human male DNA down to 0.0445 ng/µl. The sensitivity of both kits described by the manufacture is 0.023 ng/µL (7, 8).

Table 2. Results for the Sensitivity Study

| **Quantifiler Human Kit** | |
|---|---|
| **Sample** | **Quantity (ng/µL)** |
| 100 pg/µL | 0.121 |
| 75 pg/µL | 0.0784 |
| 50 pg/µL | 0.0645 |
| 30 pg/µL | 0.0391 |
| 25 pg/µL | 0.0384 |
| 20 pg/µL | 0.0137 |
| 10 pg/µL | 0.0084 |
| **Quantifiler Y Kit** | |
| **Sample** | **Quantity (ng/µL)** |
| 100 pg/µL | 0.121 |
| 50 pg/µL | 0.0768 |
| 37.5 pg/µL | 0.0445 |
| 25 pg/µL | undetermined |

One limitation for this study is that the samples were not evaluated for STRs to determine a DNA profile. Since no profiles were obtained, it is unclear if this quantitation method will pose any interpretation problems when analyzing a DNA profile. Also, it would have been advantageous to determine a profile of samples that quanted below the manufactures sensitivity level since interpretational issues arise when low levels of DNA are amplified via the PCR reaction.

*Concordance Study*

A concordance study was performed to compare the quantitation results obtained using the Quantiblot® method and the Quantifiler™ kits. Before the kits can be accepted into use for casework, the results obtained with the new method should be similar to or better than the existing method currently in use. Seven different human male samples were quantified using the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit. These seven samples had been previously quantified using the Quantiblot® Human DNA Quantitation kit and the results were compared. Quantiblot® is an hybridization based assay while Quantifiler™ assays, on the other hand, are real-time PCR-based assays. Since these two methods are based on very different principles, the values for the quantity of DNA do not match exactly. The difference between the quantities of DNA obtained from Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit are attributed to variations in PCR conditions. Results are shown in Tables 3 and 4. The different methods produced similar quantification results for all seven samples.

Table 3. Results from Concordance Study.
The values in the table below represent the percent differences between the Quantiblot results and each Quantifiler kit.

| Sample | Quantiblot | Quantifiler Human Kit | | Quantifiler Y kit | |
|---|---|---|---|---|---|
| | Result | Result | % Difference | Result | % Difference |
| | ng/μL | ng/μL | from Quantiblot | ng/μL | from Quantiblot |
| MC-107 | 2.500 | 3.000 | 20.000 | 2.960 | 18.400 |
| JS | 1.250 | 1.000 | 20.000 | 0.979 | 21.680 |
| SS | 1.250 | 1.480 | 18.400 | 1.480 | 18.400 |
| DS | 1.250 | 1.650 | 32.000 | 1.530 | 22.400 |
| 8864 | >5 | 15.460 | | 13.010 | |
| 29453-F | 6.000 | 7.210 | 20.167 | 6.750 | 12.500 |
| 219140-F | 1.000 | 0.936 | 6.400 | 0.818 | 18.200 |

Table 4. Average Differences from Quantiblot and Quantifiler

| Method | Average Differences (%) | |
|---|---|---|
| | Quantifiler Human Kit | Quantifiler Y kit |
| Quantiblot | 19.495 | 18.597 |

The major limitation to this study was that no DNA profiles were obtained which would have been demonstrated whether DNA profiles obtained were concordant since profile were obtained previously with quantitation results from Quantiblot®. For example, the sample number, 8864, was quanted with Quantiblot® to be >5, while the Quantifiler™ Human and Quantifiler™ Y Human kits quanted the sample to be 15.46 and 13.01, respectively. Comparisons of DNA profiles would have shown which quantitation method will reduce interpretational issues, such as stutter and split peaks. Also, this study should have included more samples for comparison between the two quantitation methods. Again, since Quantiblot® results are based on visual

interpretation, the more results compared to Quantifiler™ results may have shown a more significant difference.

*Reproducibility Study*

A study was performed to evaluate the reproducibility of the system. A reproducible system should give the same or similar results when the same set of samples is examined. Seven different human male samples were tested for reproducibility of the quantitation results in three separate reactions for each kit. Since the same set of samples was also used in the concordance study, the results from the concordance study were also the results for the first reproducibility study. All seven different male samples tested gave comparable quantitation results for each run. The results were also comparable between both kits. Results are shown in Tables 5.

Table 5. Statistical Results of Reproducibility Study for each Quantifiler™ kit

| | **Quantifiler Human Kit** | |
|---|---|---|
| **Sample** | **Average** | **Standard Deviation** |
| MC-107 | 2.717 | 0.319739477 |
| JS | 1.073 | 0.094516313 |
| SS | 1.540 | 0.471487009 |
| DS | 1.747 | 0.231156513 |
| 8864 | 16.737 | 1.710389819 |
| 29453-F | 7.740 | 0.556686626 |
| 219140-F | 0.999 | 0.080727938 |
| | **Quantifiler Y Kit** | |
| **Sample** | **Average** | **Standard Deviation** |
| MC-107 | 2.507 | 0.407717222 |
| JS | 1.021 | 0.162555632 |
| SS | 1.540 | 0.265141472 |
| DS | 1.837 | 0.351330803 |
| 8864 | 14.793 | 1.701891105 |
| 29453-F | 7.307 | 0.491358661 |
| 219140-F | 1.006 | 0.165553617 |

Since no DNA profiles were obtained for this study, it is uncertain whether the profiles for this study would have been reproducible.

*Mixture Study*

A study was performed to evaluate the ability of the Quantifiler™ Y Human DNA Quantification kit in detecting male DNA in the presence of excess female DNA. Male-Female mixture samples were prepared in ratios of 1:20, 1:50, 1:100, 1:150, 1:500, 1:1000, 1:1250, and 1:2500. The human female DNA was added at a constant level of 50 ng in all samples, and the human male DNA varied from 2.5 ng in the 1:20 sample to 0.02 ng in the 1:2500 sample. Results are shown in Table 6. The mixture samples were tested with the Quantifiler™ Y Human Male DNA Quantification kit to determine the quantity of male DNA which. could be detected in the ratios up to 1:500. Thus, the Quantifiler™ Y Human Male DNA Quantification kit is specific for male DNA and it is possible to obtain quantitation results in presence of excess amounts of female DNA (0.1 ng of male DNA and 50 ng of female DNA).

Table 6. Results of the Mixture Study

| **Female DNA Quantity(ng)** | **Male DNA Quantity (ng)** | **Ratio** | **Concentration of male DNA taken (ng/ul)** | **Results for Quantifiler Y (ng/ul)** |
|---|---|---|---|---|
| 50 | 2.5 | 1:20 | 1.25 | 1.0200 |
| 50 | 1 | 1:50 | 0.5 | 0.3830 |
| 50 | 0.5 | 1:100 | 0.25 | 0.1800 |
| 50 | 0.3 | 1:150 | 0.15 | 0.0482 |
| 50 | 0.1 | 1:500 | 0.05 | 0.0296 |
| 50 | 0.05 | 1:1000 | 0.025 | undetermined |
| 50 | 0.04 | 1:1250 | 0.02 | 0.0063 |
| 50 | 0.02 | 1:2500 | 0.01 | undetermined |

Limitations to this study are similar to ones previously mentioned. No DNA profiles were obtained for any of the samples. It would have been interesting to observe whether major and minor components could be detected in the ratios quanted by the Quantifiler™ kits and if full profiles could be obtained. Also, if both kits had been run, it would have been possible to compare the input quantities with those detected by the kits.

*Non-Probative Study*

A study was performed to evaluate the Quantifiler™ kits ability to produce quantitation results for actual adjudicated forensic casework samples. All twenty-two samples from five non-probative cases that were tested gave a quantitation result for both Quantifiler™ kits. Ten of the twenty-two samples were female and none of the female victim reference samples provided results with Quantifiler™ Y Human Male DNA Quantification kit. This study also demonstrates the male DNA specificity of the Quantifiler™ Y Human Male DNA Quantification kit. One sample, 3005, gave an extremely high result for both kits suggesting that too much DNA was present in the sample. Therefore, the sample was repeated with a 1:40 dilution for both kits. After the sample was repeated, the value obtained by the software was within the range of standards, 8.48 and 7.2 for the Quantifiler™ Human and Y Human Male DNA Quantification kits respectively. The value obtained by the software was then multiplied by 40 to reflect the quantity of DNA present in the sample. Another sample, 3024, was repeated because the Quantifiler™ Y Human Male DNA Quantification kit gave a quantitation result that was half of the value given by the Quantifiler™ Human DNA

Quantification kit. The sample was repeated with a 1:10 dilution for both kits. and the Quantifiler™ Y Human Male DNA Quantification kit can be used for casework samples. After the sample was repeated, the values obtained by the software were 4.95 and 3.5 for the Quantifiler™ Human and Y Human Male DNA Quantification kits respectively. The values were then multiplied by 10 to obtain results shown in Table 7.

Table 7. Results for the Non-Probative Study

| | | Quantifiler Human | Quantifiler Y |
|---|---|---|---|
| **Sample Info** | **Description** | **Quantity (ng/ul)** | **Quantity (ng/ul)** |
| **Case #1** | | | |
| 10081V1 | Victim Ref | 5.5700 | |
| BC12875 | Suspect Ref | 3.9800 | 3.7800 |
| 10081E | Evd "E" fraction | 0.3210 | 0.1030 |
| 10081S | Evd "S" fraction | 0.3270 | 0.3880 |
| **Case #2** | | | |
| 3004 | Victim Ref | 15.2700 | |
| 3005 * | Suspect Ref | 339.2000 | 283.0000 |
| 3006E | Evd "E" fraction | 10.1300 | 3.4100 |
| 3006S | Evd "S" fraction | 21.5400 | 20.8800 |
| **Case #3** | | | |
| 3023 | Victim Ref | 6.7100 | |
| 3024 ** | Suspect Ref | 49.5000 | 35.0000 |
| 3025E | Evd "E" fraction | 0.7590 | 0.3170 |
| 3025S | Evd "S" fraction | 9.5200 | 4.2900 |
| **Case #4** | | | |
| 3032 | Victim Ref | 31.0500 | |
| 3033 | Suspect Ref #1 | 46.0400 | 37.1000 |
| 3034 | Suspect Ref #2 | 0.9510 | 0.8040 |
| 3035 | Suspect Ref #3 | 7.3300 | 6.2500 |
| 3036E | Evd "E" fraction | 0.4400 | 0.1530 |
| 3036S | Evd "S" fraction | 0.9200 | 1.3200 |
| **Case #5** | | | |
| 71609V1 | Victim Ref | 1.9800 | |
| S2 | Suspect Ref | 13.2900 | 10.1000 |
| 71609E | Evd "E" fraction | 0.0491 | 0.0226 |
| 71609S | Evd "S" fraction | 0.3840 | 0.3490 |
| *=this sample was diluted 1:40 | | | |
| **= this sample was diluted 1:10 | | | |

Unfortunately, Quantiblot® results from these samples were not available for comparison. It would have been interesting to compare the quantification results for sample 3005, for which a dilution had to be performed to give a reliable Quantifiler™ result. This sample contained a high quantity of DNA and might have caused interpretational issues when determining the Quantiblot® result since it was based on visual observation. Also, since DNA profiles had already been obtained for these samples, profiles should have been obtained for comparison between the two quantitation methods.

*Slope Values Study*

The Quantifiler™ kits have not been optimized for use on the 7500 RT-PCR System; therefore, the slope ranges for the Quantifiler™ kits were determined. The slope values from the standard curve generated for the Quantifiler™ Human DNA Quantification kit and the Quantifiler™ Y Human Male DNA Quantification kit were evaluated to determine the slope range for each kit. For each run performed, a slope value was determined from the standard curves generated for each kit. Results are shown in Table 8. The slope values for the Quantifiler™ Human DNA Quantification kit ranged between -2.920803 to -3.364452 and the Quantifiler™ Y Human Male DNA Quantification kit between -3.759541 to -4.232018. Therefore, values within these ranges are acceptable. If the slope value generated does not fall within the range, the samples must be repeated. The slope ranges using the ABI Prism 7000 Sequence Detection System are -2.9 to -3.3 and -3.0 to -3.6 for the Quantifiler™ Human DNA

Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit respectively.

Table 8. Results for the Slope Values Study

| Run Number | Quantifiler Human | Quantifiler Y |
|---|---|---|
| 1 | -3.335279 | -4.047353 |
| 2 | -3.200183 | -3.810937 |
| 3 | -2.980675 | -4.06108 |
| 4 | -3.205130 | -4.232018 |
| 5 | -3.277318 | -3.759541 |
| 6 | -3.203513 | -4.232018 |
| 7 | -3.224062 | -4.075242 |
| 8 | -3.364452 | -3.811838 |
| 9 | -2.920803 | -3.918922 |
| 10 | -3.218311 | -3.851291 |
| 11 | -3.262270 | -4.047353 |
| | | |
| | **Range of slope values** | **Range of slope values** |
| | -2.920803 to -3.364452 | -3.759541 to -4.232018 |

The major limitation for this study was that standards were not run in duplicate as recommended by the manufacturer. The results from the Quantifiler™ Human kit seem to fall within the slope range of the ABI Prism 7000 Sequence Detection System despite not being run in duplicate. However, the Quantifiler™ Y kit does not fall within the range of the ABI Prism 7000 Sequence Detection System. If the standards had been run in duplicate for the Quantifiler™ Y kit, the slope values may have been closer to those reported by the ABI Prism 7000 Sequence Detection System.

# CHAPTER VI

## CONCLUSIONS

The use of RT-PCR for quantitation purposes is an improvement over current methods used by the forensic community. Seven studies were evaluated in the validation process and the results show the system offers several advantages over current methods. One major advantage of these kits is the simple and easy protocol. Results can be obtained in less than two hours with limited hands on time. The analyst is then available to carry out other duties in the laboratory. Another advantage of these kits is the increased level of sensitivity. Perhaps the best feature is the system's ability to detect for the presence of inhibitors before the STR analysis is performed. This feature will no doubt save time and money as the analyst will be warned that a sample will be unsuccessful.

There are also limitations to this quantitation method. The major limitation is both kits require the purchase of costly instrumentation. A thermal cycler with a laser detector is needed to perform (RT-PCR). Instrument price can range from around $40,000 to about $90,000 depending on the model chosen by the user. The price alone is enough to hinder some forensic laboratories from taking advantage of the benefits RT-PCR offers. Another limitation of the Quantifiler kits is they require excellent pipetting techniques because of this calibration of pipetters should be verified before using the kits

as to avoid wasting the reagents. Although the master mix was prepared for additional reaction to account for pipetting errors, there were a few occasions when there would be insufficient master mix for the reactions and additional reagents would be prepared. This results in consumption of the reagents at a faster rate and provides an added cost to the laboratory. This limitation may be overcome by the use of electronic pipetters or liquid handling robots. By using electronic pipetters, the desired volume is set and the does not change unlike with manual pipetters, the adjustable dial may change while pipetting the reagents into the appropriate tubes. Liquid handling robots are machines which automatically dispense a selected quantity of liquid reagent, solvent, or sample (18). It is important to minimize pipetting issues especially when preparing the standards as they will be used to determine the quantity of DNA present in the sample.

To implement this method into use at Reliagene Technologies Inc. (New Orleans LA), an internal validation of the Quantifiler™ kits using the ABI 7500 Real-Time PCR System was performed. Twenty-four samples and negative amplification controls were analyzed in the cross-contamination study with no evidence of contamination detected. The sensitivity study demonstrated that the level of sensitivity of the Quantifiler™ kits is superior to that of the Quantiblot® Human DNA Quantification kit (Applied Biosystems, Forster City, CA). The concordance study showed similar trends in quantitation results. Because the two methods being compared are based on different principles, the exact values were not expected. The reproducibility study demonstrated that reliable, reproducible results could be obtained using the Quantifiler™ kits on the 7500 RT-PCR System. The mixture study demonstrated that Quantifiler™ Y Human

Male DNA Quantification kit is specific for male DNA and it is possible to obtain quantitation results in presence of excess amounts of female DNA. The non-probative study showed that quantitation results could be obtained from casework samples using the Quantifiler™ kits. Unfortunately, the results from Quantiblot® This study also reinforced the male specificity of the Quantifiler™ Y Human Male DNA Quantification kit because none of the female samples were detected with this kit. Finally, the slope values study determined acceptable slope ranges to be used when analyzing the standard curve generated by the SDS software.

A major limitation to this validation study is that no DNA profiles were obtained for any of the studies. Therefore, it is unclear whether this new quantitation method will pose any interpretational issues when analyzing a DNA profile. Nonetheless, the Quantifiler™ kits have been validated for use in casework at Reliagene Technologies, Inc. (New Orleans, LA).

## CHAPTER VII

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