

A COMPARATIVE STUDY OF THE USE OF A  
RAPID THERMAL CYCLER PHILISA® VERSUS THE  
GENEAMP® PCR SYSTEM 9700 ON FORENSICALLY  
RELEVANT SAMPLES

By

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Abstract: Traditional PCR methods for amplification of multiplex STR systems typically take 3-4 hours, due to the limitations of the *Taq* DNA polymerase and the thermal cyclers used for amplification. Heat-stable, 'fast' polymerases, specially engineered for higher processing times, faster activation rates, and faster extension rates can be used in conjunction with rapid thermal cyclers that are designed to have faster ramp rates than traditional thermal cyclers. Together, these have been demonstrated to dramatically decrease the PCR amplification time of forensically relevant samples, to as little as 17 minutes. However, it is important to determine whether rapid thermal cycling can be reliably implemented into a forensic DNA laboratory, for use on forensic casework.

This study compared one such rapid thermal cycling platform, Philisa® (Streck Laboratories Inc., Omaha NE) to the traditional GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA) to determine whether the fast PCR method was as robust, sensitive, and reliable as the traditional Taq-based system. Three multiplex PCR amplification kits, namely AmpFℓSTR® Identifiler™, Yfiler™, and MiniFiler™ were evaluated for use on Philisa® and the quality of the genotyping results obtained from the samples amplified with this instrument were reviewed in the context of an internal validation study performed in tandem with studies carried out with the GeneAmp® PCR System 9700. A novel end-point PCR DNA quantification method, Q-TAT™, was also evaluated using the rapid thermal cycler, comparing its performance to that of the traditional GeneAmp® 9700 thermal cycler. Further, a cost analysis of each system demonstrated the financial investment involved with each thermal cycler.

The results indicate that while there was a dramatic decrease in PCR amplification time with the rapid thermal cycler, there was a significant increase in PCR artifacts such as stutter. Differences in inter-loci peak imbalance and non-adenylated peaks were also noted. Moreover, the amount of input template DNA required for the fast PCR system was considerably higher than that of the traditional thermal cycler. The two systems performed comparably in terms of heterozygote peak height imbalances and drop-in artifacts.

Ultimately, each thermal cycler performed equally well in each study conducted. The Philisa® thermal cycler was faster, but was out-performed by the GeneAmp® PCR System 9700 with the Yfiler™ amplification kit and the Q-TAT™ method.

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## CHAPTER I

### INTRODUCTION

The Polymerase Chain Reaction (PCR) technique, invented in 1983 by Sir Kary B. Mullis, revolutionized the field of forensic science.<sup>1</sup> Because of this discovery, scientists were now able to multiply small amounts of deoxyribonucleic acids (DNA), readily detectable by a variety of methods, into exponential quantities. The discovery also earned Mullis the Nobel Prize in Chemistry for 1993.<sup>2</sup> However, Mullis' first procedure was manually performed, making it a highly labor-intensive and slow process.<sup>3</sup> A simple thermal cycler was created from the partnership between the Cetus Corporation and the Perkin Elmer Corporation, a company that developed instruments and reagents for PCR.<sup>3</sup> This machine used AmpliTaq™ DNA Polymerase to replicate the DNA strands.<sup>4</sup>

In 1993, Perkin Elmer acquired Applied Biosystems. This pioneer in biotechnology had experience in developing and manufacturing biochemical, automated genetic engineering and diagnostic research instruments.<sup>5</sup> With this new arm of the business dedicated specifically to instrument development, the GeneAmp® PCR System 9700 was created. Today, GeneAmp® 9700s are marketed with 96-well capacity and inter-changeable silver-, or gold-plated blocks. These blocks achieve fast temperature changes and distribute the heat more uniformly throughout

the block than aluminium. The 9700 system is self-contained, with user-friendly programming capabilities.

In many forensic laboratories, the GeneAmp® PCR System 9700 is used for reference and evidentiary sample DNA profiling. It has long been the preferred choice of thermal cyclers for DNA laboratories. Moreover, a variety of specialized Short Tandem Repeat (STR) amplification kits equipped with master mixes, primers, and all DNA controls needed to produce a STR profile from a forensic DNA sample have been developed for use with this instrument. Over the years, it has been shown to be a reliable, robust instrument for PCR. In recent times, however, several companies have attempted to develop faster thermal cycling technology, and this study evaluates one such rapid thermal cycler, Philisa® (Streck Laboratories, Omaha NE), released in October 2010.

Philisa® is an 8-well, silver-block, end-point detection rapid PCR thermal cycler that is marketed to cut traditional amplification times from hours to minutes. The instrument has a small footprint and intuitive Windows® based software.<sup>6</sup> With the ever-pressing case backlogs and rush-case scenarios that exist in forensic DNA laboratories, the implementation of such a system could significantly reduce case turn-around times, although with only 8 wells, this could be extremely challenging. But before any new instrument can be used in a DNA laboratory, it must first be validated for use with the respective STR kits that the lab has validated for its human identification (HID) profiling. The Scientific Working Group on DNA Analysis Methods (SWGDM) has provided guidelines for validation for DNA analysis methods since July 2003; the most recent version from December 2012.<sup>7</sup> Forensic laboratories accredited under the Federal

Bureau of Investigations (FBI) Quality Assurance Standards are required to perform these validation studies.

Validation is one aspect of the laboratory's quality assurance program. Validation studies allow the laboratory to set operational parameters; they determine whether the expected results can be obtained, and how those results should be interpreted. According to the Merriam-Webster Dictionary, quality assurance can be defined as a "program for the systematic monitoring and evaluation of the various aspects of a project, service, or facility to ensure that standards of quality are being met" with a stated level of confidence. Quality control encompasses the day-to-day laboratory operational practices that ensure quality. Validation can be broken down into process validation and method validation. Process validation is important to quality control because it is the way in which a laboratory documents the evidence and establishment of quality, to show that the various procedures will consistently produce results that meet the pre-established specifications of quality. Method validation, on the other hand, shows that analytical procedures are suitable for their intended use. Developmental and internal validations are examples of process and method validations, that are required when implementing or modifying technologies for forensic DNA analysis.<sup>7</sup> While developmental validation documents test data in order to determine the conditions and limitations of novel DNA methodology,<sup>7</sup> internal validation demonstrates that existing or established procedures perform as expected in the laboratory.<sup>7</sup> Internal validation studies are therefore conducted prior to using a new procedure, or in this case a new instrument, for forensic DNA analysis.

Thermal cyclers are generally considered as critical instruments in a laboratory since the performance of the thermal cycler can affect the quality of the DNA profiling results and, by



extension, how those results are interpreted. Within the forensic DNA realm, result interpretation can have life or death consequences for a suspect on trial, or affect closure to the victims of crime. It is therefore imperative to know that any thermal cycler intended for use with forensic DNA casework performs within the expected limits for each STR amplification kit used with it.

This study was a comparison of the performance of the GeneAmp® PCR System 9700 with that of the Philisa® rapid thermal cycler using three AmpFℓSTR® STR kits: Identifiler™, Yfiler™, and MiniFiler™ (Applied Biosystems Inc., Foster City, CA). The three kits were evaluated, using various validation criteria, for performance within the context of a forensic DNA laboratory. In addition, a unique end-point PCR multiplex quantification method, developed at Oklahoma State University, was also assessed on Philisa® and compared against the GeneAmp® PCR System 9700. Lastly, a cost analysis was performed for each thermal cycler, based on its capacity

## CHAPTER II

### REVIEW OF LITERATURE

Short Tandem Repeat (STR) genotyping of deoxyribonucleic acids (DNA) found in the nucleus of human cells produces more genetic information than traditional mitochondrial methods. Moreover, the advent of multiplex systems targeting several loci on the human genome, have increased the ability of forensic DNA laboratories to characterize and individualize samples obtained within a forensic context. Not limited to recently-deposited stains or fresh samples, in recent times, STR genotyping of human remains has been used to elucidate the sex, identity or paternity, disease etiology, and possibly post-mortem intervals of bones that have been buried for weeks or hundreds of years.<sup>8</sup> This form of genotyping is facilitated by the polymerase chain reaction (PCR). During PCR, initial template DNA quantities are multiplied exponentially, while each new piece of DNA is tagged with a fluorescent dye, which allows it to be visualized and quantified by a capillary electrophoretic process.

Current STR PCR amplification running times range between 3-4 hours, using traditional thermal cycling parameters for multiplex systems. However, recently there has been a thrust to reduce amplification times, in an effort to maximize analysis times and increase throughput, especially with time-sensitive cases.<sup>9</sup> Consequently, a number of rapid thermal cyclers have been developed for use with special PCR reagents designed specifically for the fast PCR process.

The purpose of this review is three-fold: to discuss rapid PCR and its application to DNA analysis, to discuss the implications of rapid PCR being able to generate reproducible STR profiles from forensically relevant samples and to discuss internal validation studies used in implementing this new technology within the context of a forensic DNA laboratory.

### **Importance of Rapid Thermal Cycling**

Rapid PCR has been touted to reduce traditional PCR run times by half<sup>10</sup> with the AmpFℓSTR® Yfiler™ amplification kit, and even to as little as 17 mins<sup>11</sup> with the AmpFℓSTR® Identifiler™ amplification kit. These considerably low PCR run times, coupled with the mandatory extraction and quantification steps for forensic samples, can potentially allow a DNA laboratory to generate genetic profiles that are ready for analysis in one workday.<sup>12</sup> Rapid PCR would encourage a higher throughput, and reduce the severe backlogs that loom over many forensic DNA laboratories.

Rapid thermal cycling has been applied in the medical field for clinical diagnosis, pathogen detection and specimen identification.<sup>13,14</sup> These applications used multiplex systems showing that fast PCR has been reliably employed with multiplex assays. Three STR PCR multiplex amplification kits widely used in the forensic DNA arena, manufactured by Applied Biosystems Incorporated (Foster City, CA), are the Identifiler™, Yfiler™, and MiniFiler™ kits. These are the three amplification kits used in this comparative study of a new rapid thermal cycler versus a traditionally used thermal cycler. From the scientific literature, STR kits have been validated with different rapid thermal cyclers, and produced complete genetic profiles of forensic samples.<sup>10,11,15,16,17</sup> As a result, the capabilities of DNA analysis have been expanded to include a new technology that can be implemented after internal validations have been sufficiently conducted to document the expected performance of the instrument with each STR kit, as

recommended by the Scientific Working Group of DNA Analysis Methods (SWGDM) in its Validation Guidelines document.<sup>18</sup>

When accessing the available literature discussing rapid thermal cycling, several key words and phrases were used. Some of these were STR genotyping, fast PCR, rapid PCR, mixture interpretation, validation studies of STR kits, DNA stutter, and heterozygous peak ratios. Science Direct and PubMed online search engines provided the most relevant literature since they both are extensive databases with a wide range of scientific articles in forensics and clinical research. Scientific articles dating as far back as twenty years ago to the present were obtained, and the relevance of each article was determined from the abstract available on the Science Direct or PubMed Websites.

The literature stated that rapid PCR has been in use since 1990, where DNA fragments of about 500 base pairs were being copied in 15 mins.<sup>19</sup> Additionally, there was extensive literature showing how special reagents and cycling protocols were optimized to suit rapid thermal cycling.<sup>9,10,17</sup> Several articles included validation studies for the implementation of fast PCR protocols with forensic DNA casework, as well as comparisons of the quality of the genetic profiles produced from fast thermal cycling versus the traditional thermal cycling procedures. The areas focused on were stutter percentages, sensitivity, heterozygous peak ratios, and total PCR run time.

This review illustrates how rapid thermal cycling has been performed in a forensic DNA context. It explores the best methods to maximize the quality of fast PCR mechanisms, thereby increasing the quality of the resulting genetic profiles produced. Discussion of the limitations of rapid thermal cycling is incorporated, with further discussion of the performance of rapid thermal cycling in the areas of stutter, sensitivity, heterozygous peak ratios, and total PCR run time.

## Results and Discussion

### *Rapid Thermal Cycling*

In the context of this study, the rapid thermal cycler used was an end-point detection PCR machine, with ramp rates of up to 15°C/s heating and 12°C/s cooling.<sup>6</sup> The traditional end-point thermal cycler has ramp rates of about 5°C/s heating or cooling.<sup>20</sup> ‘Fast’ DNA polymerases, specially engineered for higher processing rates, faster extension rates, and faster activation times were used with the rapid thermal cyclers.<sup>9</sup> Most of these enzymes were combined with optimized buffers and deoxyribonucleoside triphosphates (dNTPs) that are required to complete the reagents necessary for PCR.

The enzyme SpeedSTAR™ HS (Takara Bio USA, Madison WI) was frequently cited as the polymerase of choice for rapid thermal cycling. One study by Giese et al stated that SpeedSTAR™ HS exhibited a four-fold higher fidelity than *Taq* polymerase for fast PCR.<sup>21</sup> This fidelity was measured by the extent to which the enzyme amplified a DNA template without introducing sequence errors.<sup>21</sup> Additionally, a study by Vallone et al used the SpeedSTAR™ HS polymerase in conjunction with another fast polymerase, PyroStart™ (Fermentas, Glen Burnie, MD) to increase the efficiency of PCR in the system.<sup>17</sup> Interestingly, this study successfully performed fast PCR using the traditional thermal cycler with the combination of these two ‘fast’ enzymes. Consequently, SpeedSTAR™ HS was the DNA polymerase chosen for use with the rapid thermal cycler evaluated in this study.

### *Multiplex Amplification of STR Kits with Rapid Thermal Cyclers*

The use of rapid thermal cycling with multiplex STR kits required optimization of PCR methods, and the use of special enzymes, buffers, and dNTPs designed for a faster cycling process. In some instances, special rapid thermal cyclers, designed to produce faster ramp rates

than traditional thermal cyclers, were also utilized. With all of these essential criteria met, the literature was cited specifically relating to the three STR kits used with this comparative study.

The AmpFℓSTR® Identifiler™ amplification kit was validated for use with rapid thermal cycling platforms by several researchers. Tsukada and associates performed 60-minute (fast) PCR with this kit; a sensitivity range of 500 picograms (pg) to 1 nanogram (ng) of DNA template was obtained.<sup>22</sup> Choung et al. achieved multiplex PCR of the same assay in 36 minutes using a rapid system and the SpeedSTAR™ HS polymerase.<sup>16</sup> Another study with Identifiler™ by Vallone et al. demonstrated amplification of the 15 loci plus the Amelogenin locus in less than 36 minutes.<sup>17</sup> Here, the optimal target DNA amount was 750pg. A more recent validation of the Identifiler™ STR amplification kit, by Halsell, Choquette and Kelly, with the rapid thermal cycler used in this comparative study reported total PCR run times of less than 17 minutes.<sup>11</sup>

Documented validation studies were not as extensive for the AmpFℓSTR® Yfiler™ and AmpFℓSTR® MiniFiler™ STR amplification kits. Tsukada et al. performed fast PCR amplification with Yfiler™, using a TaqGold™ Fast PCR Mix on the traditional GeneAmp® PCR System 9700 thermal cycler.<sup>10</sup> PCR run time was cut in half<sup>10</sup>, from a conventional time of approximately 3 hours. The range of template DNA amount that produced complete genetic profile results was 250pg to 2ng.<sup>10</sup> No validation publications were found for the MiniFiler™ kit from the search engines used for this review.

#### *Analytical Issues Associated with Rapid Thermal Cyclers*

The rapid PCR thermal cycling capability is not without its flaws. Several issues associated with the multiplex amplification of STR amplification kits were noted. These included split peaks<sup>16</sup>, inter-loci peak height imbalances,<sup>9</sup> increased stutter percentages<sup>21</sup>, and more PCR artifacts, especially drop-in.<sup>9</sup>

The most common issue of these was split peaks (minus A), caused by the incomplete adenylation of the new copies of DNA during the extension step. To address the split peak issue a few approaches were employed. Choung et al. extended the final extension step beyond 10 minutes.<sup>16</sup> In a study by Vallone and colleagues, a final 1 minute incubation step was added after the 28-cycle, 3-step rapid PCR method, to promote adenylation.<sup>17</sup> Giese et al. increased the amount of polymerase as the input DNA template amount increased.<sup>21</sup> They also adjusted annealing temperatures from 57°C to 62°C, with hold times increased from 5 seconds to 30 seconds.<sup>21</sup> Each of these adjustments to the protocol resulted in a dramatic decrease, if not complete elimination, of the minus A peaks.

Most of the studies reported an increase in stutter percentages with the rapid thermal cyclers over those observed with the traditional thermal cycling platforms. Stutter is an artifact of PCR where the DNA polymerase slips from the DNA template during extension. The result is a DNA fragment that is one repeat less or one repeat more than that of the true fragment size. These are reverse and forward stutter, respectively. Stutter peaks usually have peak areas about 15% or less that of the true allele peak area.<sup>23</sup> Although forward stutter is less frequent, it still occurs and must be accounted for when interpreting DNA profiles.

From the literature, inter-loci peak height imbalances were similar to those observed with the traditional thermal cycling methods.<sup>9,17</sup> Heterozygous ratios reported were greater than 0.84.<sup>17</sup> Similarly, no marked increase in drop-in alleles was noted from the studies.

## **Conclusions and Implications**

Successful validation of rapid thermal cyclers for use within a forensic context to amplify DNA samples using multiplex STR amplification kits has been demonstrated in the scientific literature. Special polymerases, buffers, and associated dNTPs are required to carry out these fast PCR reactions. This review has shown that the implementation of rapid thermal cycling can

potentially reduce the backlog of forensic DNA labs, increase throughput, and facilitate faster case turn-around times, especially with time-sensitive cases. Rapid PCR has been shown to be as robust, reliable and specific for human profiling as traditional thermal cycling methods.

Interestingly, a study by Verheij et al. utilized rapid multiplex PCR amplification to directly profile DNA samples from extraction to STR typing in 2-3 hours, instead of a conventional 10 - 12 hour time.<sup>12</sup> This assay used an inhibitor-tolerant DNA polymerase, with a non-adenylated allelic ladder, and a rapid thermal cycler using ultra-thin walled PCR tubes.<sup>12</sup> With this system, the researchers increased the allele-calling threshold for profiling interpretation, so as to reduce the incidence of PCR artifacts appearing in the profiles. Their method was found to be effective for high level DNA, such as that obtained from reference samples.<sup>12</sup> This type of assay could be very useful with database DNA casework, where mass numbers of buccal swabs are analyzed for entry into the CODIS database.

Finally, rapid PCR amplification experiments by Laurin and Frégeau demonstrated the ability of the method to reliably profile the minor alleles in mixture samples.<sup>9</sup> This work could have implications for mixture interpretation protocol. Therefore, a comparison of the robustness of fast thermal cyclers to resolve mixtures against the performance of traditional thermal cyclers would be prudent and very significant to the forensic DNA community.



## CHAPTER III

### METHODOLOGY

This comparative study between Philisa® (Streck Inc., Omaha NE) and the GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA) was modeled after the internal validation guidelines articulated in the Scientific Working Group on DNA Analysis Methods (SWGDM) Validation Guidelines for DNA Analysis Methods approved in December 2012.<sup>7</sup> Streck Laboratories Incorporated in Omaha Nebraska provided the Philisa® thermal cycler used for this comparative study. Because this internal study involved new instrumentation in an already-established DNA laboratory, the studies were limited to sensitivity studies, dynamic range studies, stochastic studies, known and non-probative sample studies, and mixture studies. Additionally, contamination was monitored through the use of controls. Material modification studies specifically relating to a reduced reaction volume during PCR, and an increased injection time for Philisa® were also conducted. Each thermal cycler was assessed for total PCR run time per reaction. The quality of the results obtained from each study was an indication of the performance of each instrument.

#### **SENSITIVITY STUDIES**

Sensitivity studies assess the performance of the PCR machine using a standard control DNA sample with a known profile. These controls are provided in the short tandem repeat (STR) genotyping kits. Sensitivity studies define the lowest amount of template DNA that can

reproducibly produce a complete DNA profile, with peak heights above the analysis threshold of 150 relative fluorescence units (RFU) for the ABI 3130 Genetic Analyzer used for this study.

In this study, the Applied Biosystems Incorporated (ABI) AmpF $\ell$ STR® Identifiler™, Yfiler™, and MiniFiler™ STR amplification kits were used. The 10ng/μL 9947A control DNA supplied in the Yfiler™ kit was used with the Identifiler™ studies on the Philisa® rapid thermal cycler because of the large amounts of template DNA needed for this machine. This same control was used with the studies performed with the GeneAmp® 9700, for consistency. The 2800M™ 10ng/μL male control DNA sample provided by the Promega Corporation was used for studies on both thermal cyclers with the Yfiler™ and MiniFiler™ kits. Three replicates of serially diluted template control DNA from 5ng-313pg for Philisa®, and 1ng-63pg for the GeneAmp® 9700, were used. The lowest template mass was where the average peak height for the locus with the smallest RFU minus three standard deviations was greater than 150RFU.<sup>24</sup>

## **DYNAMIC RANGE STUDIES**

The dynamic range of the thermal cycler was determined as the range between the lowest amount of input DNA that could be reproducibly amplified on the system to produce a full profile with peak heights above 150RFU, and the highest amount of input DNA that produced fluorescent saturation for the detector on the ABI 3130, so that a higher input would no longer produce a linear increase in signal.<sup>24</sup> A graph of mean peak height RFU versus amount of input DNA was plotted, using the loci from each dye channel for the respective STR kits. The samples

consisted of triplicate serially diluted sets of control samples with each amplification kit. Control samples used in the dynamic range study were the same as those used in the sensitivity study.

## **STOCHASTIC STUDIES**

These studies were completed to assess the performance of each thermal cycler with the STR amplification kits used in three areas: stutter percentage, heterozygous peak imbalance, and allelic dropout/drop-in. Stutter peaks are reproducible artifacts of PCR, where an allele that is one repeat more or one repeat less than the true DNA allele is amplified, and has a corresponding RFU reading of approximately 15% of that of the true DNA allele.<sup>23</sup> The stutter percentage was calculated by dividing the stutter allele peak height RFU by the true allele peak height RFU, for triplicate ranges of template DNA amounts for each respective thermal cycler. An example of a stutter peak is provided in Appendix A. This study was done to determine whether there was any statistically significant difference between the stutter percentages produced with each thermal cycler, and also to compare the stutter percentages observed with Philisa® to those published by Applied Biosystems for the GeneAmp® PCR System 9700.

Heterozygous Peak Imbalance is a ratio of the peak height RFU of sister alleles. Sister alleles are two alleles appearing together (heterozygous pair) at any locus in the genetic profile. An example of sister alleles at the TH01 locus in Identifiler™ can be found in Appendix B. The ratio is calculated by dividing the peak height RFU from the larger allele by the peak height RFU of the smaller allele of the pair. This study was done to show whether sister allele peak heights from template DNA amplified on Philisa® were significantly different from those produced from

input DNA that was amplified with the traditional 9700 thermal cycler. Moreover, the template DNA mass corresponding to a severe peak height imbalance resulting in the loss of one allele of the pair was noted.

Allelic dropout is an assessment of the complete loss of one, or several alleles from the genetic profile. Drop-in is the appearance of an allele with peak height RFU above 150 that is not part of the known control DNA profile. The mass of template DNA where allelic dropout occurred was noted. Also, the number of dropout alleles was calculated. Allelic drop-in was calculated as the number of times extra peaks with 150RFU or more appeared throughout each electropherogram, for each thermal cycling platform. The mass of template DNA corresponding to these allelic drop in events was also noted.

## **DNA AMPLIFICATION**

### **Philisa® Rapid Thermal Cycler**

Control template DNA samples for the respective STR kits were prepared for Philisa® using a serial dilution from 5ng/μL -313pg/μL. A final sample using deionized DNase, RNase-free water was included as a negative control.

The Master mix for each STR kit was prepared according to the manufacturer recommended protocol, using 25μL final reaction volumes, with specific modifications made according to the kit being studied. A half-reaction volume of 12.5μL was also assessed for each STR kit. The Master mix 25μL preparation modifications are described in Table 1.

**Table 1.** Master mix set-up for three STR kits and Q-TAT™ for use with Philisa™ (Streck Inc., Omaha NE).

Quantity in $\mu\text{L}$ per reaction	Identifiler™	Yfiler™	MiniFiler™	Q-TAT™
10X Fast Buffer II	5.5	5.1	5.5	1.25
dNTP mixture	5.0	4.1	4.0	1.0
Primer Mix <sup>a</sup>	5.5	5.0	5.0	1.25
SpeedSTAR HS™	0.5	0.8	0.5	0.0625
pRL (1ng/ $\mu\text{L}$ )	-	-	-	2.0
diH <sub>2</sub> O	-	-	-	5.9
Template DNA	10	10	10	1.0

<sup>a</sup> The primer mix used for each reaction was provided with each respective assay.

The Hot Start Q-TAT™ PCR Setup, a multiplex DNA quantification method using end-point PCR described by Allen and Fuller,<sup>25</sup> and later modified to distinguish between male and female nuclear DNA by Wilson et al,<sup>26</sup> was optimized for use with this thermal cycler. For Q-TAT™ a standard curve needed to be generated. The Q-TAT™ standard curve consisted of 6 samples. Four samples were made from a 100ng/ $\mu\text{L}$  male DNA standard with concentrations of 1000pg/ $\mu\text{L}$ , 333pg/ $\mu\text{L}$ , 111 pg/ $\mu\text{L}$ , and 37 pg/ $\mu\text{L}$ . A DNase, RNase-free water control, served as a negative control and a 100pg/ $\mu\text{L}$  9947A female control DNA sample from the Identifiler™ kit was added as a positive control sample. Dilutions were prepared using

nuclease free water purchased from Teknova Corporation, Hollister, CA.

Three different PCR methods were used to amplify the DNA samples for the three STR amplifications, and Q-TAT™ methods. A 2-step method was used with Identifiler™ and Yfiler™, while a 3-step method was used with MiniFiler™. A special 3-step method was optimized for the Q-TAT™ reactions.

The 2-step PCR method consisted a denaturation step and a combined annealing/elongation step. Cycling occurred from step 1. The 3-step method separated the annealing and elongation steps, with cycling occurring from steps 1-3. The special 3-step method optimized for use with Q-TAT™ reactions had the three PCR steps separated, however, cycling occurred only between the annealing and elongation steps.

The 2-step method used for Identifiler™ was chosen because it was the validated PCR protocol established by Halsell, Choquette and Kelly for use with Philisa®.<sup>11</sup> The 3-step method used with MiniFiler™ was recommended by the manufacturers of the SpeedSTAR™ HS polymerase.<sup>27</sup> The optimized method for Q-TAT™ reactions was self-developed based on a combination of the previous models. This method produced the highest RFU for the amplicons in the Q-TAT™ assay of several variations attempted. The denaturation hold was extended to 30 seconds to facilitate the unwinding of the pRL plasmid. The annealing temperature was elevated but still within the heat-stable range of the polymerase. The extension temperature was slightly lowered and the hold lengthened, to increase primer-binding specificity, and to eliminate the amplification of non-specific fragments. These respective PCR parameters for Philisa® (Streck Inc., Omaha NE) were as described in Table 2.

**Table 2.** Two- step and three-step PCR parameters used on Philisa® (Streck Inc., Omaha NE) for the respective amplification reactions.

2- step (28 cycles)	Temperature (°C)	Time (s)
	95	5
	65	25
3-step (30 cycles)	Temperature (°C)	Time (s)
	98	5
	55	10
	72	5
3-step (28 cycles <sup>b</sup> )	Temperature (°C)	Time (s)
	98	30
	92	10
	70	20
<sup>b</sup> The 28-cycle repeat was from Steps 2-3.		

### GeneAmp® PCR System 9700

The studies were repeated with the GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA), using final reaction volumes of 25µL. The PCR cycling parameters used for each STR kit were those described by the manufacturer. Five serial dilutions of concentrations of 1 ng/µL, 500pg/µL, 250pg/µL, 125pg/ µL, and 63pg/µL were made using the control DNA standards provided with each STR kit. A final sample with DNase, RNase-free water was added

as a negative control. The Hot Start Q-TAT™ PCR Setup described by Allen and Fuller,<sup>25</sup> with the PCR cycling parameters for the improved multiplex assay that was developmentally-validated by Jonathan Wilson,<sup>26</sup> was also used with the GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA). The master mix for Q-TAT™ amplification on this instrument was prepared as described by Wilson<sup>26</sup> using Hot Start GoTaq® Colorless Mastermix (Promega Corporation, Madison, WI) and a final reaction volume of 12.5µL.

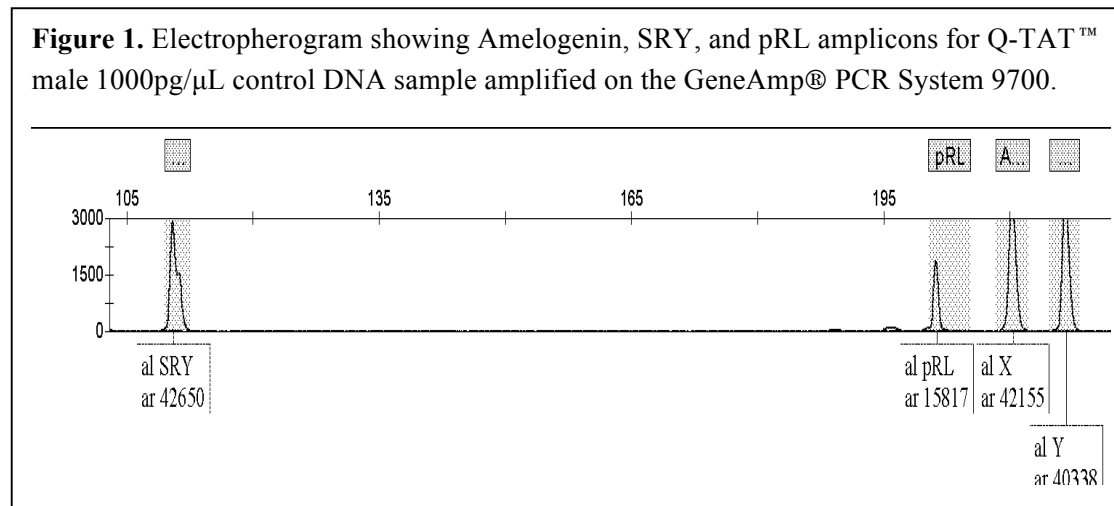
The QTAT™ standard curve on the 9700 was prepared in the same way, as had been done for the rapid thermal cycler. Four serial dilutions were made of concentrations 1000pg/µL – 37pg/ µL of the male DNA standard. The female reference standard used was the 9947A sample with a concentration of 0.1ng/µL. A water control sample was added to assess contamination. The average of three replicates of standard curve runs was subsequently used to determine the unknown concentrations of the forensic sample extracts.

## **GENETIC ANALYSIS OF PCR PRODUCT**

All amplified samples were prepared for capillary electrophoresis on an ABI 3130® Genetic Analyzer (Applied Biosystems, Foster City, CA) 4-capillary system with POP-4 polymer, using 1µL of amplified sample mixed with 15µL HiDi Formamide (Applied Biosystems, Foster City, CA) and 0.7µL GS-500 LIZ Size Standard (Applied Biosystems, Foster City, CA). The samples were then electro-kinetically injected for ten seconds for Philisa® samples, and five seconds for GeneAmp® PCR System 9700 samples, and allowed to electrophorese according to the parameters described by the manufacturer. The Q-TAT™ samples were allowed to



electrophoresis according to the parameters described by Wilson.<sup>26</sup> The fluorescence was collected using a PC-based ABI 3130 Data Collection Software with 5-dye collection. The peaks were analyzed using the GeneMapper™ ID version 3.2 software program (Applied Biosystems, Foster City, CA) with manufacturer-recommended specifications for each STR kit. A macro developed and described by Wilson<sup>26</sup> was used for the Q-TAT™ samples. Figure 1 depicts an electropherogram showing the X, Y, SRY and pRL amplicons collected for the 1 ng/μL male DNA sample amplified on the GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA) with Q-TAT™.



### Generation of A Standard Curve

The Q-TAT™ samples were amplified using the PCR cycling parameters described previously for the rapid and traditional thermal cyclers respectively. The ABI 3130xl detected and collected the fluorescence from five amplicons in each sample, namely HPX, SRY, pRL, and Amelogenin X and Y; of these, the HPX amplicon was not relevant to this study and was not used, because it

was not needed to generate the total human standard curve. GeneMapper™ ID v. 3.2 software analyzed the peak area for each amplicon. A total human standard curve was generated by plotting the known DNA concentration of each all-male control sample against the average peak area for the Amelogenin X and Y amplicons of three replicates.

**Figure 2.** Graph of Q-TAT™ Total Human DNA Standard Curve plotting known DNA concentrations versus average total X+Y peak areas generated by GeneAmp® PCR System 9700.

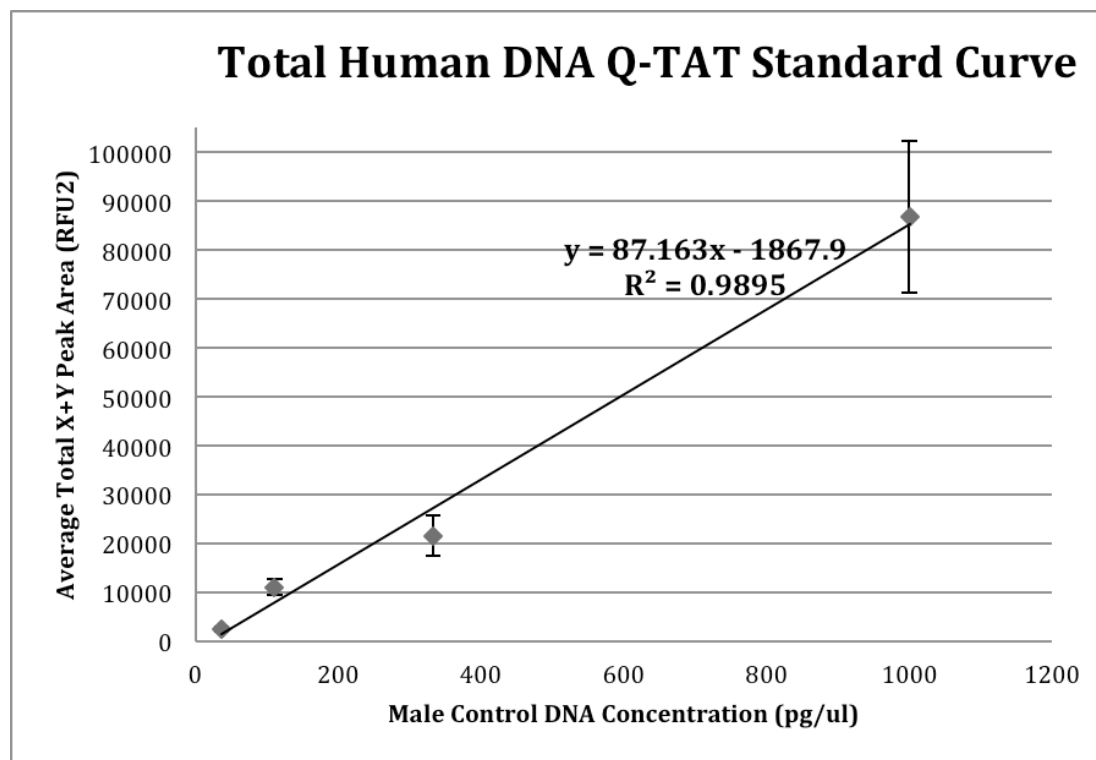


Figure 2 shows an example of a total human DNA standard curve generated from the GeneAmp® PCR System 9700. A similar standard curve was attempted for control DNA samples amplified with the rapid thermal cycler but could not be generated because of non-amplification of the internal positive control, pRL. Analysis methods, described by Wilson<sup>26</sup>, were followed to

determine the unknown concentrations of the forensically-relevant samples using the standard curve generated from the GeneAmp® 9700.

### **Human Identification Profiling of Forensically Relevant Samples**

The forensically relevant samples consisted of eight total samples; five buccal swabs, one blood sample from a male donor, one blood sample from a female donor, and an extraction blank. The DNA from each sample was extracted using the phenol-chloroform extraction procedure. The extracts were purified and concentrated with the Zymo™ Clean and Concentrate system, resulting in approximately 30µL of DNA eluent.

Once quantification of these samples was achieved with the Q-TAT™ PCR method and standard curve, three male:female mixture samples of ratios 1:10, 1:1, and 10:1 were made. These samples were prepared by targeting 100pg/µL for amplification on the GeneAmp® 9700 thermal cycler, and 500pg/µL for amplification with Philisa®, prior to the samples being mixed. A 1:0 male:female sample, as well as the 0:1 male:female sample were also amplified with the mixture samples so as to obtain full single-source profiles for each contributor to the mixtures. This information was used to assess the ability of each thermal cycler to resolve the mixtures, by comparing each single-source profile to the profiles obtained from the mixture samples.

The ABI Identifiler™ kit was used, targeting 15 genetic markers plus Amelogenin, along with the MiniFiler™ kit, with eight target genetic markers plus the sex marker Amelogenin. Each amplified sample was diluted so that 100pg/µL of template DNA was targeted for profiling on the 9700 (Applied Biosystems Inc., Foster City, CA), while 500pg/µL of template DNA were targeted for use with Philisa®. A 12.5µL AmpFℓSTR® Identifiler™/MiniFiler™/Yfiler™ PCR

amplification kit reaction volume was used on each system, using the Master mix setup and PCR parameters that were optimized during these studies.

The HID-amplified products were analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using 1µL of amplified sample mixed with 15µL HiDi Formamide (Applied Biosystems, Foster City, CA) and 0.7µL GeneScan®-500 LIZ Size Standard (Applied Biosystems, Foster City, CA) for the Identifiler™ and MiniFiler™ samples. The Yfiler™ samples were mixed with 20 µL HiDi Formamide and 0.7 µL GeneScan®-500 LIZ Size Standard. The samples were electro-kinetically injected for the times previously stated. The samples then electrophoresed for 28 minutes, on a five-dye platform. Data was collected with the PC-based ABI 3130 Collection Software, and the sizes for all amplified products were estimated and labeled using GeneMapper™ ID v.3.2 software. Examples of the DNA profiles produced from the MiniFiler™ and Yfiler™ kits on the two thermal cycling platforms are contained in Appendices C, D, E, and F.. Electropherograms from the Identifiler™ kit on each platform are found in Figures 7 and 8. The profiling results were also tabulated to record the sixteen loci from the Identifiler™ multiplex, and the nine loci from the MiniFiler™ multiplex kit. A dash was used for any locus where no alleles were size-called throughout the electropherogram. Brackets were used to denote any alleles with relative fluorescence units (RFU) less than 150.

The genetic profiles from these forensically relevant DNA samples amplified with each thermal cycler were assessed for stutter, heterozygote peak imbalances, and mixture resolution.

## **STATISTICAL ANALYSIS**

Statistical analyses of the results from each of the studies done for each thermal cycler were carried out using GraphPad Prism® 6 statistics software (GraphPad Software Inc., CA) on a Windows-based computer. Performance comparisons in stutter and heterozygous peak imbalances were made with a one-way ANOVA and Tukey multiple comparison tests at a 95% confidence level, to determine whether there were any significant differences between the thermal cyclers.

Nonlinear regression analyses were used to determine the dynamic range of each thermal cycler. The curves for each dye were compared against one another within each system and between systems using Tukey multiple comparison tests, assuming no Gaussian relationship.

## CHAPTER IV

### FINDINGS

The two thermal cyclers were compared in several ways, namely set-up costs, total PCR run time, dynamic range, sensitivity, percentage stutter, heterozygous peak imbalance and percentage allele dropout or drop-in. These comparisons were made using known control DNA samples, as well as mock forensic samples that would typically be encountered in the context of the forensic DNA laboratory. Complete studies were successful with the Identifiler™ and MiniFiler™ STR amplification kits on the two thermal cyclers. Results from those comparisons and those from the forensic sample work were subjected to subsequent statistical analyses. Some difficulties were encountered with the Q-TAT™ method and the AmpFlSTR Yfiler™ STR amplification kit on Philisa®; therefore, studies with these two assays were incomplete. These will be discussed later on in this chapter. As a result, no comparisons were drawn for these two methods.

### COST ANALYSIS

The Philisa® rapid thermal cycler conventional system 8-well instrument costs \$5,625.00.<sup>28</sup> The cost of the Philisa® Computer and Accessories, included with the system is \$6,000.00.<sup>28</sup> An extended warranty is offered for \$800.00.<sup>28</sup> Additionally, special PCR tubes sold at 100 tubes per container for \$45.00<sup>28</sup> need to be purchased for use with this machine. Sterile gel-loading pipette tips are also required for use with the tubes, and those were purchased commercially for about \$120.00.<sup>29</sup>

GeneAmp® PCR System 9700 96-well instruments are sold with silver- or gold-plated blocks, with prices ranging from \$8,730 to \$9,010 for new instruments.<sup>30</sup> A refurbished 96-well aluminium- or silver-plated block thermal cycler can be bought for \$2,750 or \$2,950 respectively.<sup>31</sup> The 9700-system is self-contained; therefore no additional computer/accessories are required. Sterile 0.2 ml MicroAmp® Reaction tubes with caps can be purchased for use with this thermal cycler from Life Technologies at \$120/1000 tubes.<sup>20</sup> Filtered pipette tips, already being used in the forensic DNA laboratory, can be used with the 9700 thermal cycling systems. No special tips need to be purchased. Table 3 shows an overall set-up cost comparison for each thermal cycler. It shows a slightly cheaper initial investment with the setup of Philisa®.

**Table 3.** A cost comparison for the setup of Philisa® and the GeneAmp® PCR System 9700.

Cost of Component	Philisa® - silver block	GeneAmp® 9700 – silver block
Thermal Cycler (\$)	6,000	8,730
PCR Tubes (\$)	45/100	120/1000
Pipette Tips (\$)	118.63/576 gel-loading tips	120.38/576 aerosol-filtered tips
Total (\$)	6,163.63	8,970.38
Number of wells	8	96

## PCR RUN TIME

Table 4 provides a comparison of the average PCR run times for the two thermal cycling systems specific to the analyses performed on each machine.

<b>Table 4.</b> A comparison of PCR run times for Philisa® and the GeneAmp® PCR System 9700 with three STR kits and Q-TAT™ quantification method.				
Thermal Cycler	Identifiler™	MiniFiler™	Q-TAT™	Yfiler™
Philisa®	00:16:40	00:14:05	00:16:31	00:17:57
	00:16:39	00:14:04	00:17:35	00:17:52
<b>Average run time</b>	<b>00:16:39.5±1s</b>	<b>00:14:04.5±1s</b>	<b>00:17:06±45s</b>	<b>00:17:54±4s</b>
ABI 9700®	03:15:00	03:15:00	02:02:00	03:41:00
	03:21:00	03:23:00	02:05:00	03:42:00
<b>Average run time</b>	<b>03:18:00±4min</b>	<b>03:19:00±6min</b>	<b>02:03:00±2min</b>	<b>03:41:00±1min</b>

These results show considerable time-savings for each assay used with Philisa®. The largest time difference was with the Yfiler™ kit, saving over 3 hours and 20 minutes per 8-sample run. If, however, the 9700 is used to capacity, the rapid thermal cycler would have to be re-loaded 12 times to achieve the same throughput. The amount of time saved dramatically decreases to 17 minutes. The smallest time difference was observed with the Q-TAT™ assay, at 2

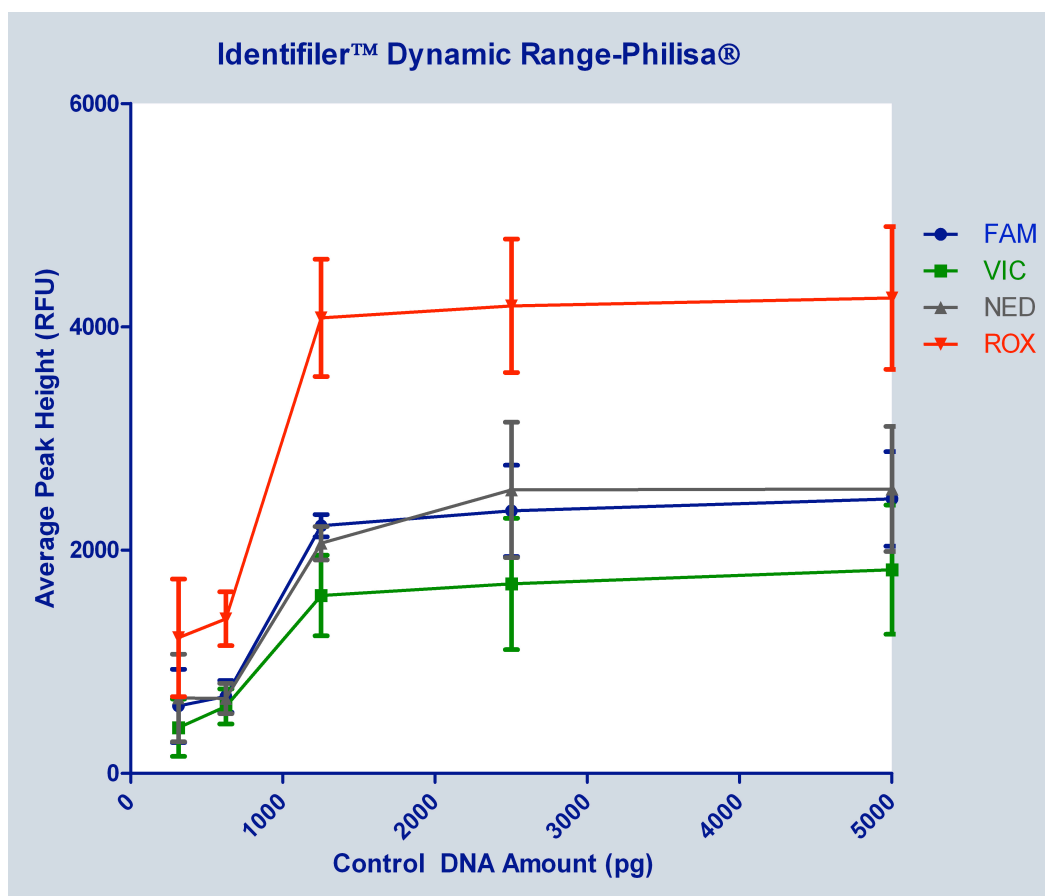


hours and 46 minutes. Again, if the 9700 is ran at full capacity, it would take 3 hours and 24 minutes to run the same 96 samples on Philisa®, resulting in roughly one extra hour and 20 minutes PCR run time.

### **DYNAMIC RANGE STUDIES**

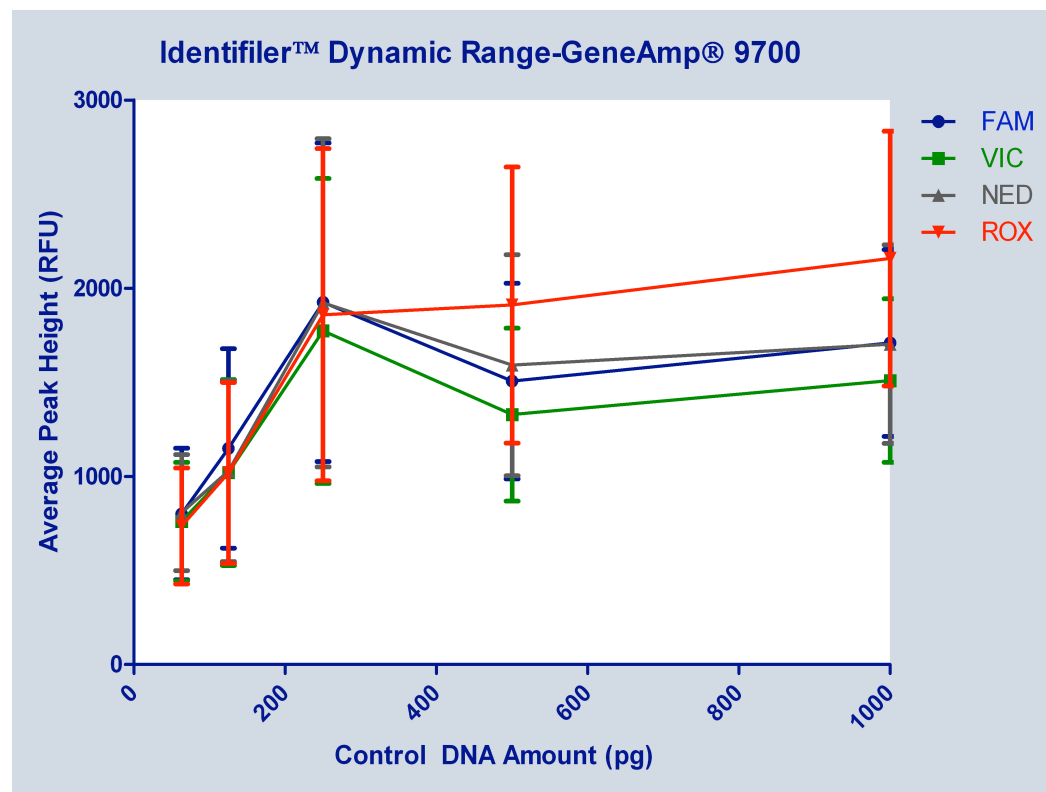
Figure 3 shows the dynamic range of 25µL Identifiler™ amplification determined from the averages of three replicates of serially diluted 9947A DNA control sample, ranging from 5ng to 313pg for Philisa®.

**Figure 3.** Graph showing the dynamic range for Philisa® based on triplicate 25µL amplifications with Identifiler™ for four dye channels.



This graph indicates a dynamic range for Philisa® with Identifiler™ of 313pg – 1250pg. There is an upward trend from 313pg to 1250pg, after which the curve reaches plateau, which is indicative of detector saturation. No further increase in RFU was observed with increased amounts of input DNA. Figure 4 shows the dynamic range of 25µL Identifiler™ amplification determined from the averages of triplicate serially diluted 9947A DNA control samples, ranging from 1ng – 63pg for GeneAmp® PCR System 9700.

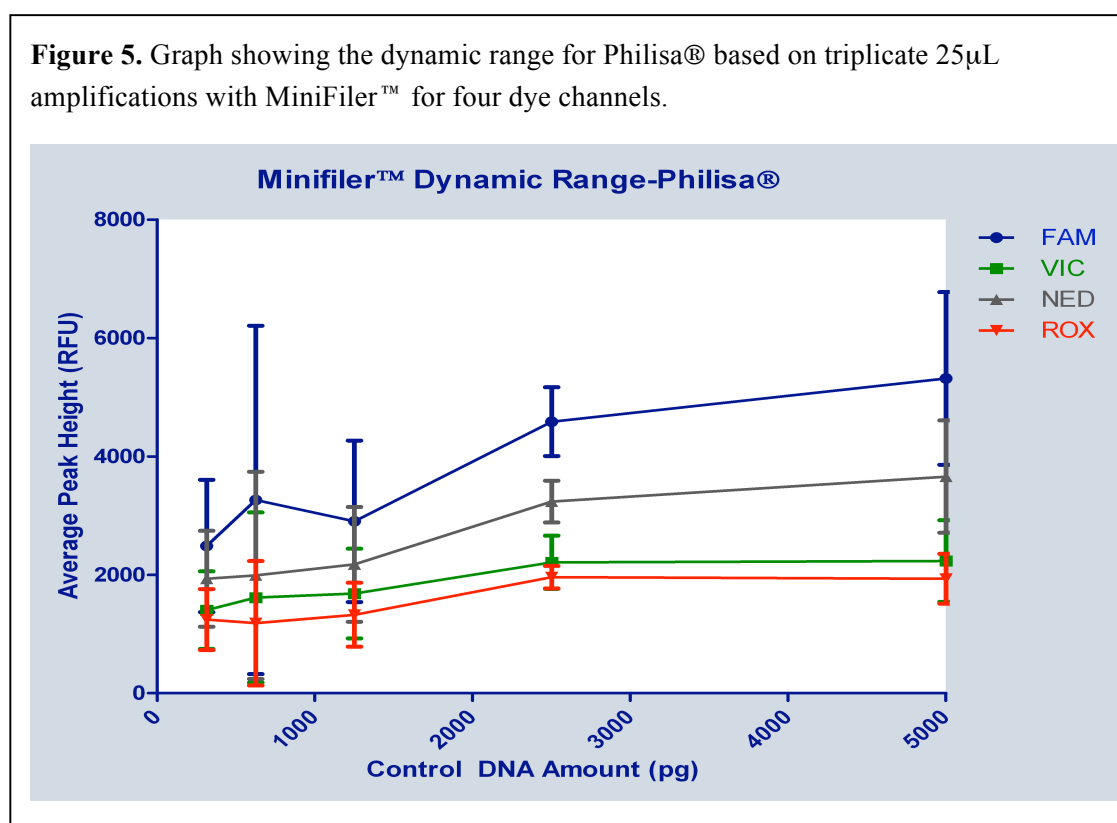
**Figure 4.** Graph showing the dynamic range for the GeneAmp® 9700 based on triplicate 25µL amplifications with Identifiler™ for four dye channels.



The dynamic range for this kit with the 9700 was 63pg – 500pg. Here, there is a marked increase in signal from 63pg-250pg, with a further increase in the ROX dye to 500pg. Generally, the signal shows an upward trend, indicating the potential for a further increase in RFU with more input DNA. There is a marked increase in the standard deviation at the higher input amounts (250pg-1ng), due to the preferential amplification of the smaller loci over the larger ones.<sup>24</sup> This difference is usually observed when too much template is available for replication.

The dynamic range for the MiniFiler™ kit with each thermal cycler was similarly determined.

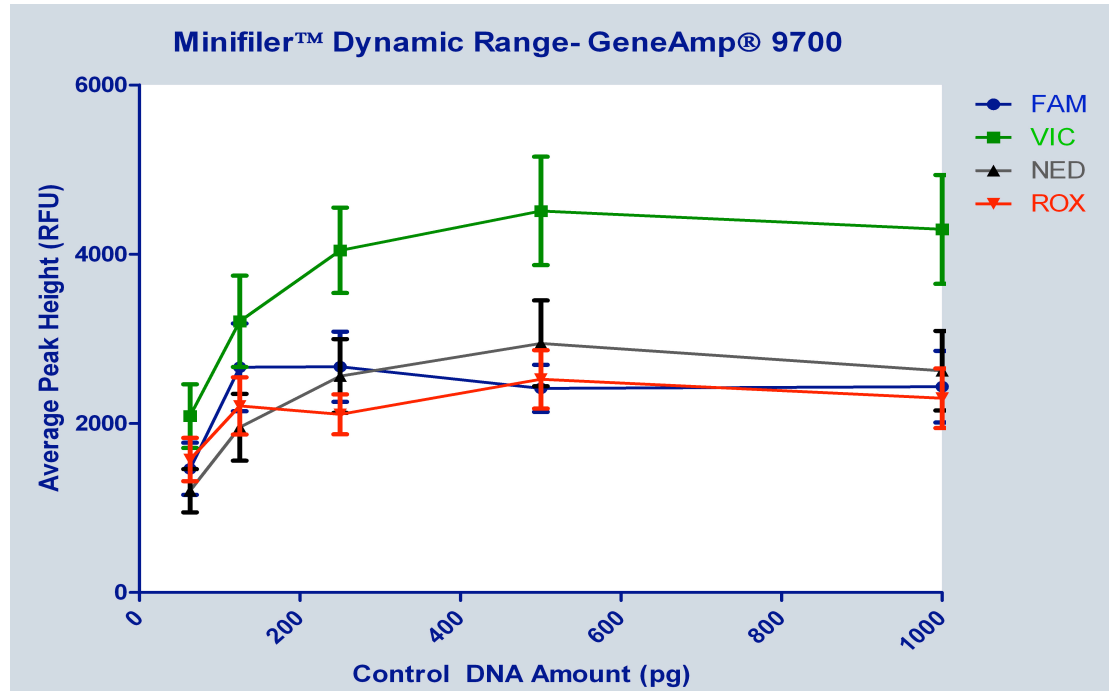
Figure 5 shows the dynamic range of 25µL MiniFiler® amplification from the averages of three replicates of serially diluted 9947A DNA control sample, ranging from 5ng to 313pg for Philisa®.



There is a general upward trend in accumulated fluorescence from 313pg to 2500pg. The ROX and VIC dyes reach detector saturation levels after the 2500pg amount; however, the FAM and NED dyes show the potential for increased signal if more input DNA was added.

Figure 6 shows the dynamic range of 25µL MiniFiler® amplification from the averages of three replicates of serially diluted 9947A DNA control sample, ranging from 1ng to 63pg for GeneAmp® PCR System 9700 thermal cycler.

**Figure 6.** Graph showing the dynamic range for GeneAmp® 9700 based on triplicate 25µL amplifications with MiniFiler™ for four dye channels.

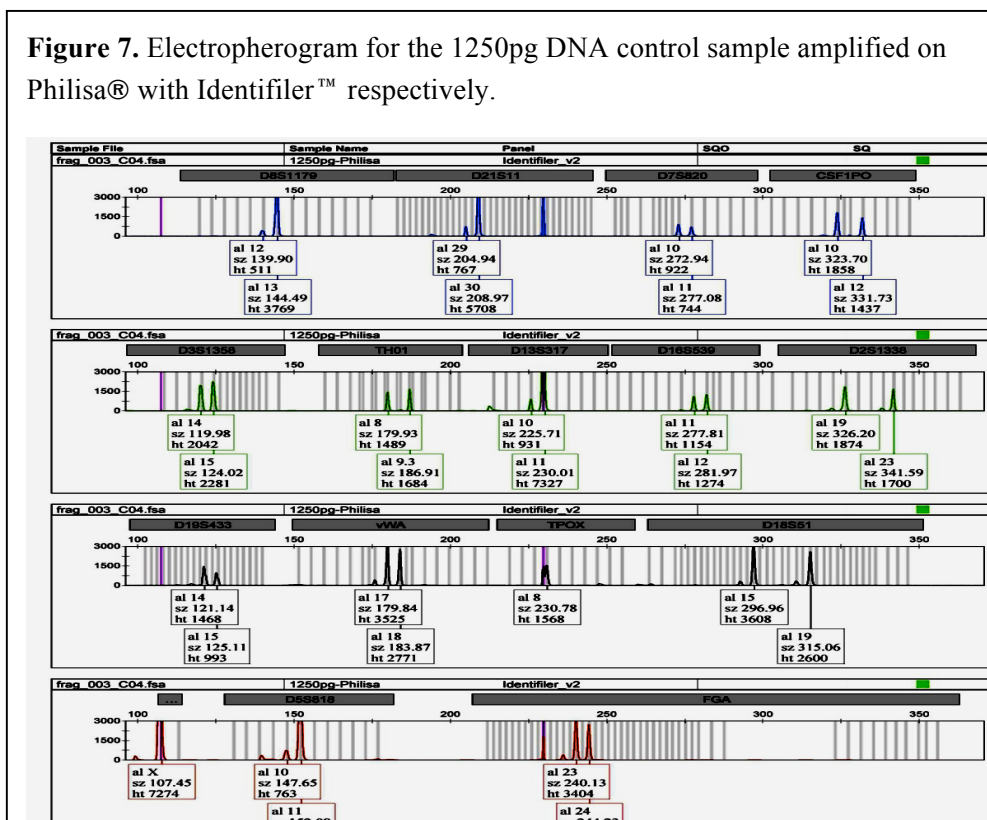


The dye channels in this figure achieve detector saturation after 500pg of input DNA. The best performing dye was VIC. The other three dyes performed similarly to one another with no statistically significant differences between them ( $p > 0.05$ ).

## SENSITIVITY STUDIES

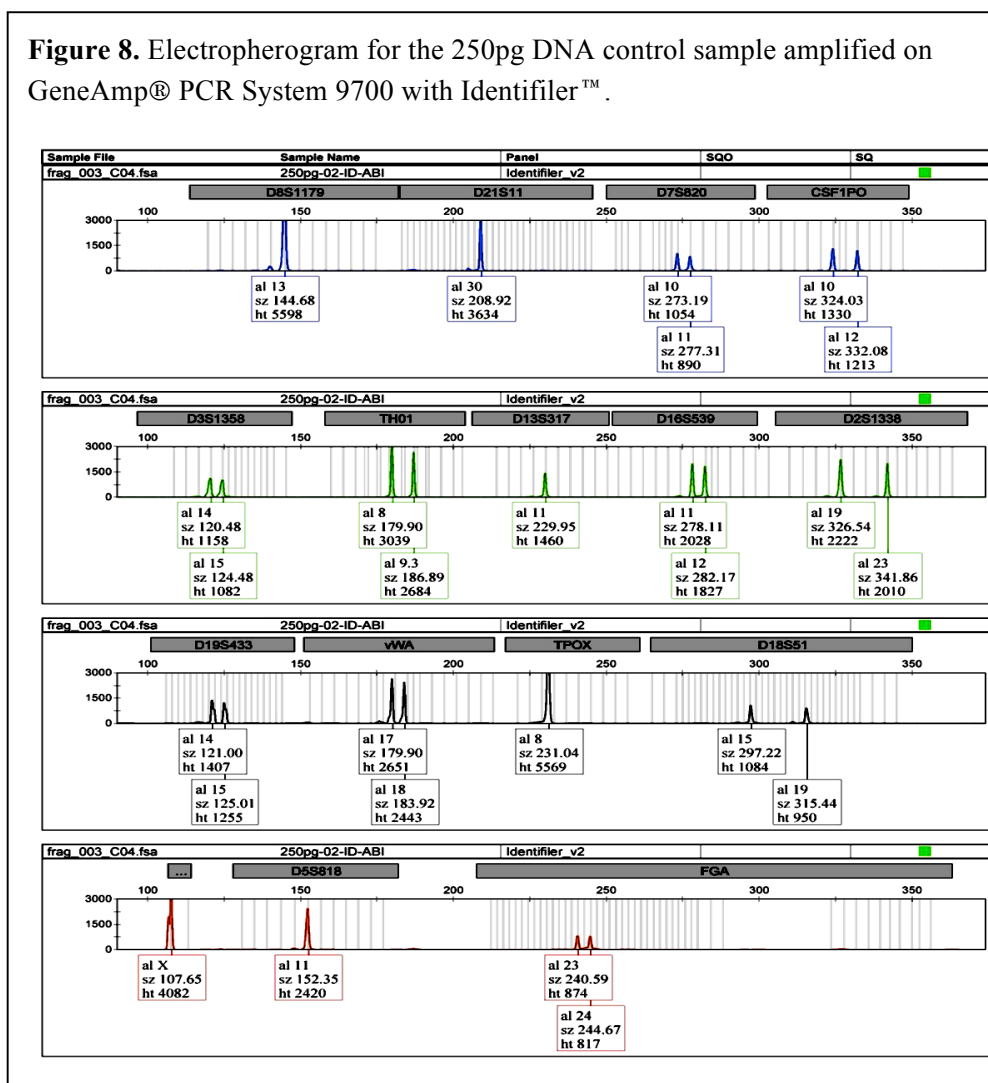
From the sensitivity studies conducted on Philisa®, the lowest amount of template DNA that can reproducibly produce a complete DNA profile, with allele peak height RFUs above 150 was 1250pg. Comparatively, the lowest amount of template DNA to produce a full profile with allele peak height RFU above 150 on the GeneAmp® 9700 was 125pg. From the sensitivity studies, the optimal target input DNA amount for Philisa® was 250pg. This optimal target input DNA amount was determined as the mass of template corresponding to the electropherogram that featured the least number of PCR artifacts, with all allele peak heights above 150RFU. Electropherograms from DNA inputs in excess of 1250pg exhibited considerable stutter and pull-up peaks. Electropherograms from DNA inputs of 625pg and below showed alleles with peak heights below 150RFU. Figure 7 depicts an Identifiler™ electropherogram for amplification of the 1250pg control DNA sample from Philisa®.

**Figure 7.** Electropherogram for the 1250pg DNA control sample amplified on Philisa® with Identifiler™ respectively.



The allele peak heights from the electropherogram at 625pg bordered 150RFU, which may have been a result of poor mixing of serial dilution prior to PCR. Therefore, it was more prudent to use the 1250pg amount because the RFUs spanned 744 to 7113, which were at least three standard deviations from the statistical threshold of 150RFU.

**Figure 8.** Electropherogram for the 250pg DNA control sample amplified on GeneAmp® PCR System 9700 with Identifiler™.



For the GeneAmp® PCR System 9700, the optimal target input DNA concentration was 250pg

Figure 8 shows an electropherogram from Identifiler™ for the 250pg DNA control sample amplified with the GeneAmp® PCR System 9700. Here, there are no artifacts recorded in the electropherogram and the allele peak heights span 817 to 4082, well above three standard deviations higher than the 150RFU threshold. Notwithstanding a few outliers, both thermal



cyclers generally produced the same amount of amplified product (similar RFUs) from each template DNA amount.

## STUTTER ANALYSIS

The stutter percentages were calculated for each locus for the serially diluted control samples amplified on Philisa® or the GeneAmp® 9700 with the MiniFiler™ STR kit. Stutter was calculated by dividing the peak height RFU of the stutter allele by that of the true allele. The highest percentages at each locus were as outlined in Table 5. These were compared with published stutter percentages for the amplification kit<sup>32</sup> used in conjunction with the ABI 9700 instrument. It is noteworthy that stutter was observed at every input DNA amount on Philisa®.

<b>Table 5.</b> Highest stutter percentages for MiniFiler™ on Philisa® and the GeneAmp® 9700 compared with published stutter percentages for MiniFiler™.			
Locus	Stutter Percentage (%) - Philisa®	Stutter Percentage (%) - GeneAmp® 9700	Published Stutter filter percentages (9700) <sup>32</sup>
D13S317	28	5	14
D7S820	2	0	11
D2S1338	0	0	18
D21S11	15	9	16
D16S539	0	0	15
D18S51	0	5	18
CSF1PO	23	0	14
FGA	0	0	15

There was a higher incidence of stutter observed with Philisa® than with the GeneAmp® 9700. The large percentages at D13S317 and CSF1P0 for Philisa were attributable to dye artifacts (pull-up), which made the stutter peak seem considerably taller than it truly was, hence recording a higher peak height RFU. Notwithstanding those outliers, it is interesting to note that both instruments produced profiles with lower stutter percentages than the values published by Applied Biosystems with their instruction manuals for the multiplex kits.

The stutter percentages were also calculated for each locus of the serially diluted control samples amplified with the Identifiler™ STR kit on Philisa® and the GeneAmp® 9700. The highest stutter percentages at each locus were as outlined in Table 6. These were also compared to published highest stutter percentages for the Identifiler™ kit on the 9700.<sup>33</sup>

**Table 6.** Highest stutter percentages for Identifiler™ on Philisa® and the GeneAmp® 9700 compared with published stutter percentages for Identifiler™.

Locus	Highest Stutter Percentage (%)- Philisa®	Highest Stutter Percentage (%) GeneAmp® 9700	Published Stutter Percentage (GeneAmp® 9700) <sup>33</sup>
D8S1179	14	0	8.2
D21S11	14	0	9.4
D7S820	0	7	8.2
CSF1PO	0	0	9.2
D3S1358	13	0	10.7
TH01	0	0	5.1
D13S317	19	0	8.0
D16S539	0	0	10.4
D2S1338	16	0	11.1
D19S433	0	0	13.3
vWA	14	0	12.6
TPOX	0	0	4.8
D18S51	0	0	17.0
D5S818	16	0	6.8
FGA	0	0	14.7

In this case, Philisa® did not perform as well as the GeneAmp® 9700 platform, and the stutter was significantly higher for many loci, as revealed by a one-way ANOVA with Tukey multiple comparison tests ( $p < 0.0001$ ). The principal difference between the MiniFiler™ and Identifiler™ kits is the size of the PCR product produced. The MiniFiler™ kit uses primers that

are closer to the tandem repeat within each allele, thereby reducing the opportunity for polymerase slippage during replication.

## ALLELIC DROP OUT

### MiniFiler™ STR Amplification Kit

There was allelic dropout observed for the Philisa® thermal cycler with MiniFiler™ at the 625pg template DNA amount. There were five dropout events occurring at four loci, namely Amelogenin, D21S11, D18S51, and FGA. No allelic dropout was observed with the GeneAmp® PCR System 9700 for MiniFiler™.

### Identifiler™ STR Amplification Kit

There also was no allelic dropout observed on the GeneAmp® 9700 thermal cycler with Identifiler™, but with Philisa®, there was one allelic dropout event at the D7S820 locus of the 313pg template DNA amount. These allelic dropout results are displayed in Table 7.

<b>Table 7.</b> Allelic dropout events for Philisa® and GeneAmp® 9700 for control DNA samples amplified with MiniFiler™ and Identifiler™.		
Amplification Kit	Philisa®	GeneAmp® PCR System 9700
MiniFiler™ (n=162 loci)	5	0
Identifiler™ (n=288 loci)	1	0

## ALLELIC DROP-IN

The number of times drop-in alleles appeared in the electropherograms was noted for each thermal cycler with each STR amplification kit.

### MiniFiler™ STR Amplification Kit

There were six drop-in alleles observed for Philisa® with this kit. They occurred at the 5000pg, 2500pg, and 625pg template DNA amounts. The loci exhibiting drop-in were D13S317 with 1 event, D2S1338 with 4 separate events, and D16S539 had 1 drop in event. There were three drop-in alleles observed for the GeneAmp® 9700 system, with this kit, occurring at the 1000pg and 500pg template DNA amounts. One allele appeared at D13S317, and the same allele appeared at D16S539 for two template DNA amounts.

### Identifiler™ STR Amplification Kit

There was no allelic drop-in observed on the GeneAmp® 9700 thermal cycler with Identifiler™, but with Philisa®, there was a single drop-in event at D19S433. These results were as displayed in Table 8 below.

<b>Table 8.</b> Allelic drop-in events for Philisa® and GeneAmp® 9700 for control DNA samples amplified with MiniFiler™ and Identifiler™.		
Amplification Kit	Philisa®	GeneAmp® PCR System 9700
MiniFiler™ (n=162 loci)	D13 (7), D2(17), (29),(29), (37.2), D16 (11)	D13 (7), D16 (5), D16 (5)
Identifiler™ (n=288 loci)	D19 (17)	0

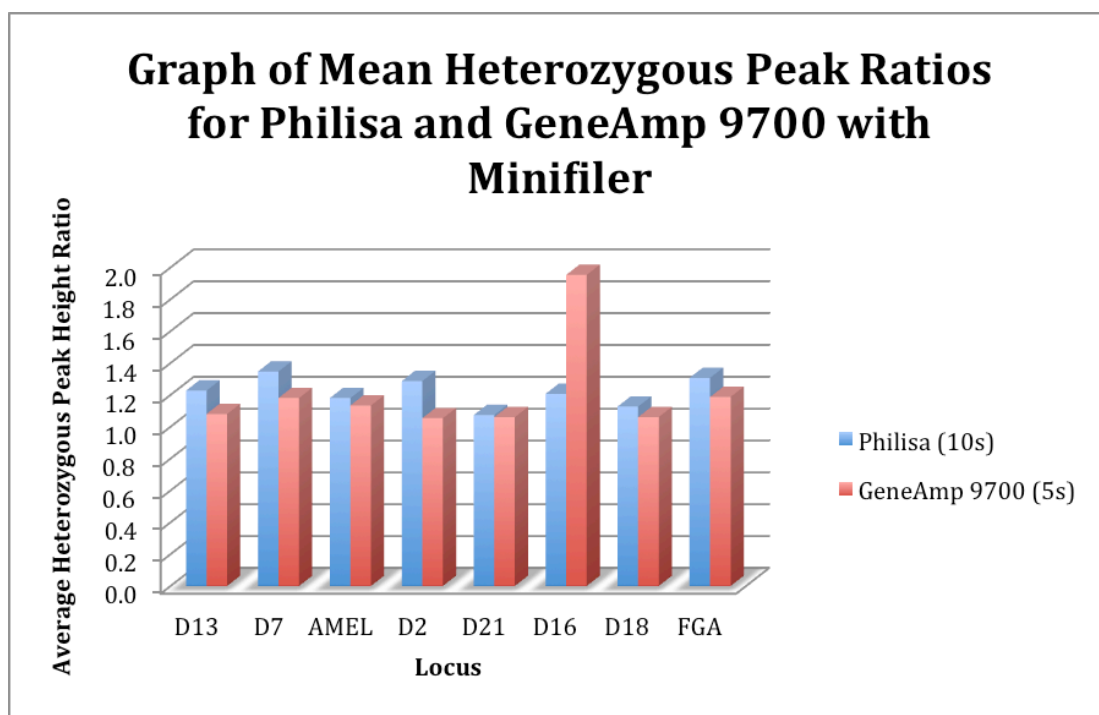
Generally there were more drop-in alleles recorded for Philisa® than for the GeneAmp® 9700, especially with MiniFiler™.

## HETEROZYGOUS PEAK IMBALANCE STUDIES

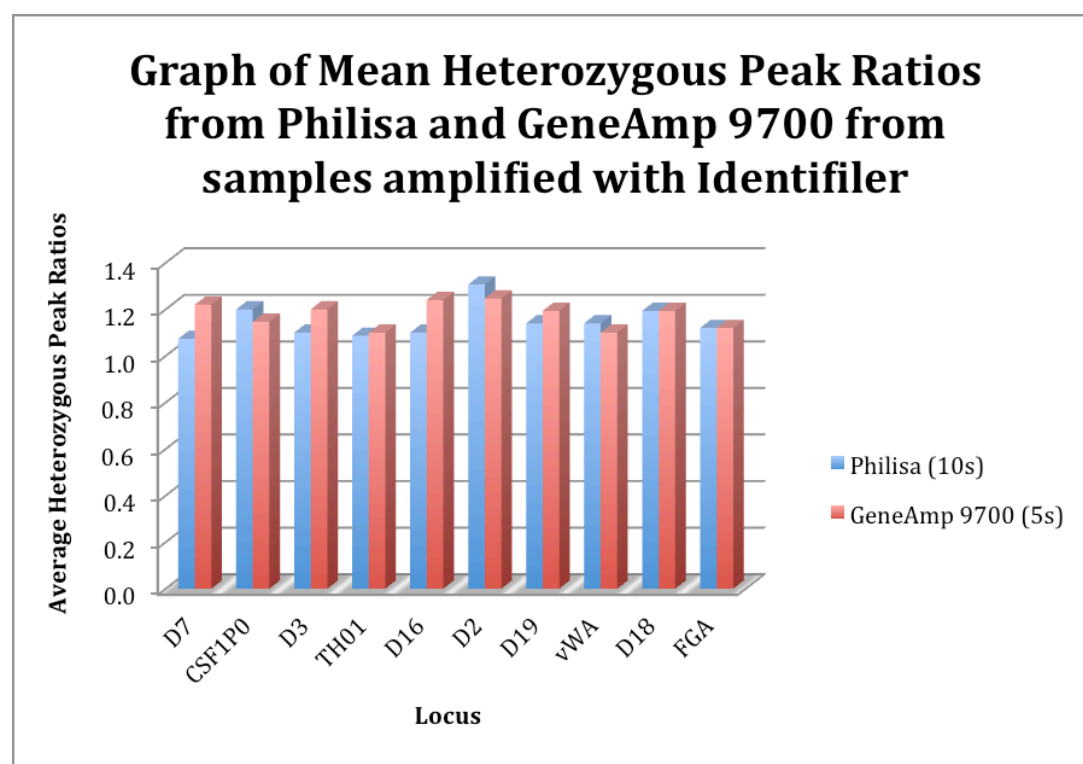
### Control Samples

The ratio of peak heights for sister alleles at heterozygous loci for each STR profiling kit was calculated for each kit and thermal cycling platform. The average ratios for each heterozygous locus from triplicate serially diluted control DNA samples are compared in Figures 9 and 10.

**Figure 9.** Comparison of Heterozygous Peak Ratios for the control DNA samples amplified with MiniFiler™ on the two thermal cycling platforms (full reaction volume in triplicate).



**Figure 10.** Comparison of Heterozygous Peak Ratios for the control DNA samples amplified with Identifiler™ on the two thermal cycling platforms (full reaction volume in triplicate).



Statistical analysis using a 1-way ANOVA, with a 95% confidence level, and a post Tukey multiple comparison test was carried out on the heterozygous peak height ratios from the two thermal cyclers with the MiniFiler™ kit. The results indicated no significant difference in the performance of the two machines ( $p=0.445$ ). Similarly, comparison of the two thermal cyclers with the Identifiler™ kit also indicated no statistically significant difference between the ratios generated from the two instruments ( $p>0.05$ ).

**Figure 11.** Summary of the average heterozygous peak ratios for control DNA samples amplified on Philisa® and the GeneAmp® 9700 with MiniFiler™ and Identifiler™ kits.

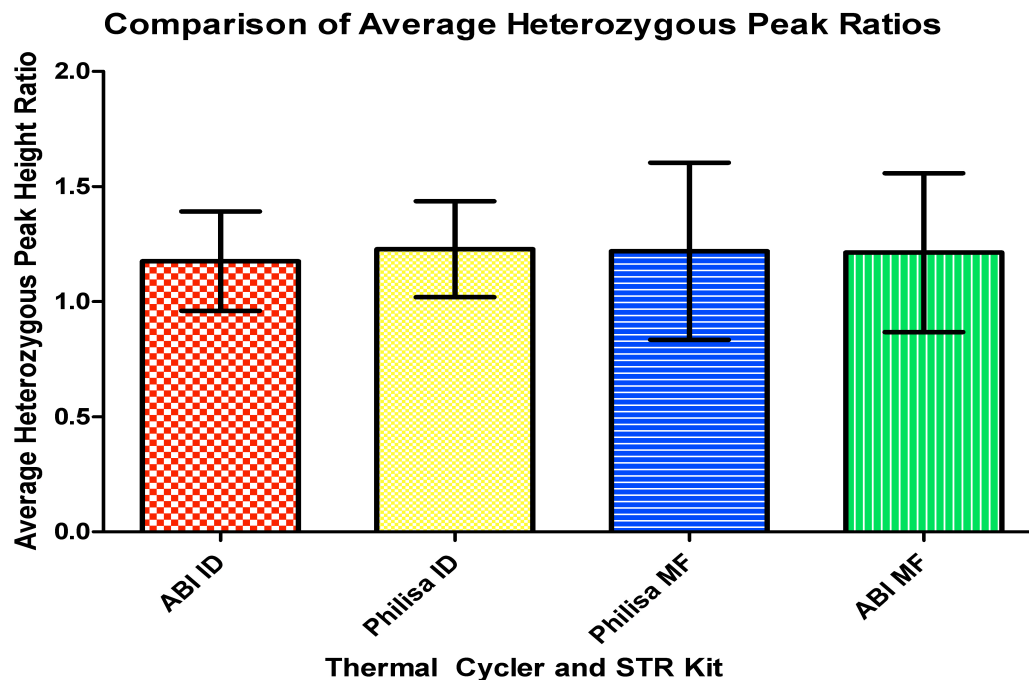


Figure 11 portrays the mean heterozygous peak ratios for each instrument with the two STR amplification kits. In this illustration, the average heterozygous ratios for each kit with the respective thermal cyclers are very similar. The ideal heterozygous peak ratio is 1.0, and the system and kit with a ratio closest to this standard, was the Identifiler™ kit used with the GeneAmp® PCR System 9700.

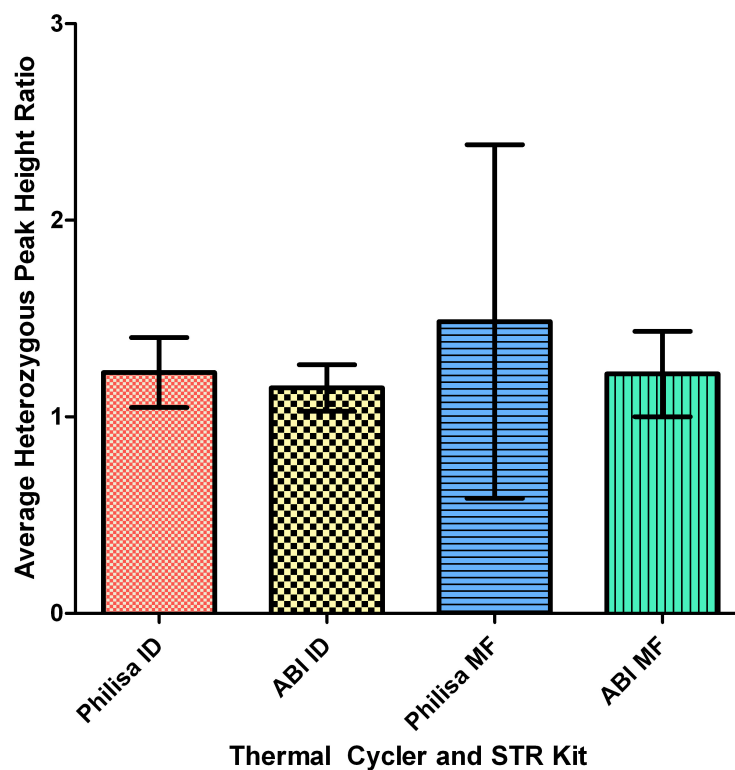


## FORENSIC SAMPLES

The ratio of the peak heights of sister alleles at heterozygous loci for Samples 1-5 10, and 11 of the forensically relevant samples, amplified with each of the STR amplification kits, was also calculated for the respective thermal cycling platforms. The results were compared for the two systems below in Figure 12.

**Figure 12.** Comparison of Average Heterozygous Peak Ratios for seven forensic samples amplified with Minifiler™ and Identifiler™ on the respective thermal cyclers.

### Average Heterozygous Peak Ratios for Forensic Samples Combined



A one-way ANOVA and Tukey post test at the 95% confidence level, to determine whether there was any difference in the performance of the two thermal cyclers with the respective STR amplification kits revealed no difference in the performance of the two thermal cyclers with the Identifiler™ kit ( $p>0.05$ ). However, there was a significant difference between them with MiniFiler™ ( $p=0.0017$ ). With both the control samples and the forensic samples, the two thermal cyclers performed equally well with Identifiler. However, the GeneAmp® 9700 produced more ratios closer to 1.0 than Philisa® did with MiniFiler™.

## **MIXTURE STUDIES**

The mixture samples, numbers 7-9, were evaluated according to the quality of the electropherograms produced from each ratio. Samples 1-5, 10 and 11 were single source non-probative forensic DNA samples. Sample 6 was the extraction blank. Each mixture ratio was assessed to determine whether the minor contributor's DNA was amplified, and appeared in the profile. Major peaks were assigned as those with peak heights of at least twice that of the smaller alleles (minor peaks). Major/minor peak height ratios were calculated to determine the potential of the mixture to be de-convoluted. In some instances this calculation was not feasible because of masking by shared alleles, or the disappearance of one allele in a heterozygous pair. The results were as follows:

## **Sample 7**

This DNA sample was a mixture of a male: and female in a ratio of 1:10. The MiniFiler™ electropherograms generated from amplification of this sample on the GeneAmp® 9700 showed a predominantly female profile. Minor alleles appeared at AMEL, D21S11 and D18S51 loci, with 2 of those loci having alleles with peak height RFUs above 150. The ratio of X:Y was 41:1. Because this ratio is so high, it would be very difficult to obtain DNA information from the minor contributor to the mixture. Any statistical weight assigned to the minor component would be small because of such little useful DNA information above the threshold of 150RFU. Similarly, the electropherogram for this sample after amplification on Philisa® showed a predominantly female profile, with no amplification of Amelogenin Y. One or more alleles from the minor component appeared at D13S317, D7S820, D2S1338, D16S539 and D18S51. Only those alleles at D16S539 and D18S51 fell below 150RFU. However, there was no Y-allele found at AMEL, suggesting that this sample could have been a mixture of two female individuals, had the mixture contributors not been previously known.

When this sample was amplified with Identifiler™ on the GeneAmp® 9700 and Philisa®, both EPs showed a predominantly female single source profile. There was a minor Y-allele found at AMEL that amplified in the GeneAmp® 9700 instrument, but the peak height was below 150RFU. Conversely, amplification of this sample on Philisa® showed more evidence of a minor contributor. There was a small AMEL Y-allele, and six loci had more than 2 alleles present. Those minor alleles were found at D8S1179, D21S11, D3S1358, vWA, TPOX, and D5S8181. However, only the allele at D8S1179 had a peak height above 150RFU. Again, the

X:Y ratio was large, 118:1, therefore not much statistical weight could be assigned to the minor component.

### **Sample 8**

This DNA sample was an equal mixture of male and female DNA. The MiniFiler™ electropherograms generated from the GeneAmp® 9700 and Philisa® did not produce alleles with equal peak heights. The major component of the mixture was attributable to the female contributor while the minor component was attributable to the male contributor. There were at least 3, but no more than 4 alleles at each locus. The male and female shared an allele at those loci with 3 alleles. There was no Y-allele in the genetic profile from Philisa®. The ratio of X:Y on the 9700 was 5:1. All minor alleles appearing in the profiles had peak heights above 150RFU. The minor component resolved by the Philisa® thermal cyclers would prove difficult to determine the gender of that contributor, with the Y-allele missing, especially when the contributors to the mixture are unknown. There could still be considerable statistical weight assigned to that minor component, even without knowing the gender.

When this sample was amplified with Identifiler™ on the GeneAmp® 9700 and Philisa®, a major/minor profile again resulted. There were at least 3 (when an allele was shared), but no more than 4 alleles at each locus. The female was the major component, with the male as the minor component of the mixture. Both thermal cyclers produced X:Y ratios of 9:1. All minor alleles had peak heights above 150RFU, except from the Philisa®-generated profile. Six loci, D7S820, D13S317, D2S1338, TPOX, D18S51, and FGA had minor alleles with less than 150 RFU. This may have been due to the sizes of the loci. Those six loci are among the largest of their

respective dye channels. Because of the short hold time for the denaturation step of the 'fast' PCR process, those fragments may not have had sufficient time to completely unwind before the primers and polymerase annealed, resulting in less amplified product. Nonetheless, even with those six loci excluded from interpretation, nine useful sites remain for comparison and statistical weight assignment.

### **Sample 9**

This sample was a male and female DNA mixture in the ratio 10:1. When this sample was amplified with MiniFiler™ on each thermal cycler, the resulting electropherogram exhibited a clear major/minor mixture. There were at least 3, but no more than 4 alleles at every locus. The major component of the mixture was attributable to the male contributor, while the minor component of the mixture was attributable to the female contributor. All of the peak heights were above the 150RFU threshold. The ratio of X:Y was 1.4:1.

When this sample was amplified with Identifiler™ on the GeneAmp® 9700 and Philisa®, both electropherograms again showed clear major/minor mixture profiles. There were at least 3, but no more than 4 alleles at each locus. The male was the major contributor, while the female was the minor contributor to the mixture profiles. The ratio of X:Y from each profile was 2:1. All minor alleles had peak heights above 150RFU, except those at D7S820 and TPOX on Philisa®, with RFUs below 150. The electropherograms produced from this sample on both machines with each STR kit supplied information that could readily be used to de-convolute the mixture and assign distinct profile to a major and minor contributor. The GeneAmp® 9700

produced greater RFUs than Philisa® and would be more useful to determine the statistical weight of each component. They performed equally well with MiniFiler™.

### **Material Modification**

Amplification of the control DNA samples with each STR kit on both thermal cyclers was repeated using a 12.5µL total reaction volume. Complete genetic profiles were obtained from each thermal cycler, with allele peak heights between 500 and 4000RFU. This showed that a half-reaction volume could be used successfully with both instruments.

### **Contamination**

The negative controls and blanks used in this study were injected from various places on the 96-well ABI 3130 plate. There were no allele peaks observed in any of the blanks or water control samples. Of the 36 samples ran between the two thermal cyclers, there were no instances of drop-in observed.

## **DISCUSSION OF FINDINGS**

### **Q-TAT™ and Yfiler™ Studies**

Studies with the Q-TAT™ and Yfiler™ assays were incomplete due to issues with the amplification of the pRL internal positive control for Q-TAT™, and the DYS389II locus for Yfiler™ with the Philisa® thermal cycler. Several efforts to amplify pRL were attempted. The PCR method was changed to have a longer denaturation time and a longer annealing time, in separate instances, to give the pRL template a longer hold at each respective step. It was hoped that a longer denaturation time would allow the plasmid more time to unwind before exposure to the primers and polymerase. A longer annealing time should have improved the primer-binding.<sup>27</sup> Both experiments were unsuccessful. Additionally, the pRL plasmid was subjected to endonuclease digestion with EcoRI in an attempt to linearize the circular configuration, and improve amplification. This was also unsuccessful. Future studies should be carried out to optimize the fast PCR parameters, possibly lowering the annealing temperature, or increasing the hold times at each step of PCR.

With the AmpFℓSTR® Yfiler™ amplification kit, there was successful amplification of all the loci except the DYS389II locus. An example of a Yfiler™ profile from Philisa® can be found in Appendix D. In most cases the DYS389II locus would not amplify, it would be incorrectly size-called, or it would have two off-ladder peaks. DYS389II, although not the largest locus in the assay, is the largest locus of the FAM dye channel. Large loci are more susceptible to degradation, thereby affecting amplification. Nonetheless, all the other loci in the assay were successfully amplified with RFUs above 150. Therefore, useful information can still be obtained

from the other 14 loci in this assay on Philisa®. If a forensic DNA laboratory performs studies and one or more loci consistently fail to amplify, the laboratory should consider establishing a written policy that delineates how Yfiler™ results will be interpreted and reported. Other laboratories may be more successful with the locus and might not need to have a policy that eliminates it.

### **Dynamic Range Studies**

#### *Identifiler™*

One-way ANOVA comparing the two thermal cyclers with the Identifiler™ STR kit revealed that there was no significant difference between the performances of the four dye channels on each instrument ( $p > 0.05$ ).

For the GeneAmp® 9700 thermal cycler, the dynamic range was 63pg – 500pg with Identifiler™. There was no apparent flat-line for any dye channel, which indicated that the detector was not completely saturated. Therefore, a further increase in input DNA might still produce a linear increase in signal. A one-way ANOVA of the 4 curves from the different dye channels indicated that there was no significant difference between the curves ( $p = 0.874$ ). Moreover, a non-linear fit analysis revealed that it was possible to have one curve for all the data sets (dye channels). The best performing dye channel was ROX, while VIC was the poorest performer, with average RFU of 1278 compared to 1537 for ROX.



By comparison, the dynamic range was 313pg-1250pg for the Philisa® instrument. There was a clear indication of detector saturation after 1250pg. A one-way ANOVA for the four dye channels revealed no statistically significant difference between them; however, a non-linear fit analysis indicated that it was not possible to have one curve for all data sets ( $p=0.0002$ ). The best performing dye channel was ROX, while VIC was the dye with the lowest average RFU of 1225, compared to an average of 3026 for ROX.

#### *MiniFiler™*

A one-way ANOVA comparing the performance of the dye channels from the two thermal cyclers with this STR kit revealed that there was a statistically significant difference between them in the VIC dye channel ( $p=0.0002$ ). The GeneAmp® 9700 thermal cycler had larger peak heights than the Philisa® cycler in this dye channel.

For the GeneAmp® 9700 thermal cycler, the dynamic range was also 63pg – 500pg with MiniFiler™, with apparent saturation of each dye channel after the 500pg amount. A one-way ANOVA of the 4 curves from the different dye channels indicated that there was a significant difference between the FAM vs. VIC, VIC vs. NED and VIC vs. ROX channels ( $p=0.01$ ). Also, a non-linear fit analysis revealed that it was not possible to have one curve for all the data sets (dye channels), since they each performed so differently. The best performing dye channel was VIC, while ROX was the poorest performer, having the lowest average RFU of 2141 compared to an average of 3628 for the VIC dye channel..

In comparison to the 9700, the Philisa® thermal cycler performed about the same. There was a larger dynamic range observed with MiniFiler™ on Philisa® than with Identifiler™, ranging from 313pg – 2500pg. A one-way ANOVA for the four dye channels revealed a statistically significant difference in the FAM vs. VIC, and FAM vs ROX channels ( $p=0.0017$ ). The FAM dye channel outperformed all the dyes with this instrument, while the ROX dye recorded the lowest average peak height RFU of 1529, compared to an average of 3712 for the FAM dye channel. . Also, a non-linear fit analysis of each curve indicated that they were each different ( $p=0.0002$ ); therefore it was not possible to have one curve for all the data sets (dye channels). This improvement in performance of Philisa® with the Minifiler™ kit may relate to the smaller size range of the STR alleles amplified.

### **Stutter Analysis**

Overall, the rapid thermal cycler had significantly more stutter than the traditional thermal cycler ( $p<0.05$ ) with the Identifiler™ kit. There was no significant difference in the highest stutter percentages of the two instruments with the MiniFiler™ kit studies ( $p=0.3174$ ). The Philisa® thermal cycler generally had more stutter at the smaller loci with each STR kit. This may have been because it requires less energy to unwind a smaller template DNA fragments, in the short hold times at the denaturation step of the rapid PCR process. Again, amplification could be favored for the smaller loci, creating more opportunity for polymerase slippage resulting in increased reverse stutter. Reverse stutter is an allele that is one repeat less than the true DNA allele. There was very little stutter recorded for the GeneAmp® 9700, which is indicative of the robustness and reliability of the instrument.

### *Identifiler™*

The loci that showed the most stutter with Philisa® were D13S317 (19%), D2S1338 (16%), and D5S818 (16%). A one- way ANOVA of the highest stutter percentages with the Philisa thermal cycler indicated a significant difference between the stutter percentages at those loci ( $p < 0.0001$ ). This predicts a high degree of variability with the expected results from Philisa® with this STR kit. Conversely, there was very little stutter observed with the GeneAmp® 9700; however, the locus with the highest stutter percentage was D7 (7%). This percentage was lower than the filter percentage published by Applied Biosystems for that locus.

### *MiniFiler™*

Both thermal cyclers revealed no significant difference in the highest stutter percentages among the eight loci ( $p > 0.05$ ). The loci with the highest stutter percentages on Philisa® were: D13S317 (28%), CSF1P0 (23%), and D2S111 (15%). The marked increase at D13S317 and CSF1P0 were attributable to pull-up artifacts, which cause the peaks to record higher RFUs than the true peak height. On the GeneAmp® 9700, the D16S539 (9%) and D21S11 (9%) loci recorded the highest stutter percentages. With consideration of the pull-up in the stutter on the rapid thermal cycler, Philisa® generally had less stutter than the GeneAmp® 9700 thermal cycler with the MiniFiler™ STR kit. Again, the marked improvement with stutter on Philisa® may be related to the sizes of the STR amplicons with this kit.

### **Allelic Dropout/Drop-in**

There was no dropout observed with the GeneAmp® 9700. This is again, a reflection of the high sensitivity of the instrument to amplify small amounts of template DNA. Despite having greater DNA input amounts, there was still dropout of the larger loci from the Philisa® amplifications. This may be attributable to the fast PCR process that may be less efficient with amplification of larger loci due to the short hold times at the different stages of the process. A possible remedy may be to extend denaturation and annealing holds by 5-10 seconds to improve the efficiency of PCR.

With allelic drop-in, each of the peaks recorded were not part of the expected genetic profile, with peak heights above 150. Each peak was assessed to ensure that it could not be classified as a dye artifact such as pull-up or dye blob. Each peak displayed good peak morphology. Drop-in poses a challenge to DNA analysis and interpretation because it is often difficult to determine whether the drop-in is an artifact or a true allele peak. Extra peaks in the electropherogram might be interpreted as a low-level mixture. This is one of the reasons why validation studies are important. Expected results parameters can then be established, documented, and incorporated into a laboratory's overall quality assurance plan. This plan should always be known and readily available.

### **Heterozygous Peak Ratio Studies**

Statistical analysis of the heterozygous peak ratios (one-way ANOVA, 95% confidence level) from the two instruments for the respective kits indicated no significant difference between them ( $p=0.446$ ). The system with a mean ratio closest to 1.0 was the GeneAmp® 9700 with the

Identifiler™ kit. It is important to have heterozygous peak ratios near 1.0 because it is an indication that both alleles in a heterozygous pair are equally amplified. This is a reflection of the robustness and reliability of the thermal cycler to produce well-balanced profiles. In addition, with well balanced sister alleles in heterozygous profiles, it is much easier to distinguish between single source samples and mixtures when only two alleles are visible.

With the forensic samples, there was also no difference between ratios between peak height ratios for each thermal cycler with the Identifiler kit™ ( $p>0.05$ ); however, overall the GeneAmp® 9700 had less peak height imbalance than the rapid thermal cycler with the MiniFiler™ assay. This was largely traceable to large imbalances at the AMEL locus in one sample amplified with the Philisa® thermal cycler. Accounting for this outlier, the peak height ratios from the two instruments were relatively similar.

### **Other PCR Artifacts**

An additional PCR artifact that was characteristic of rapid thermal cycling, from the literature, was minus A peaks. These are split peaks due to the incomplete adenylation of the newly synthesized DNA fragments during the extension step.<sup>16</sup> An example can be seen in Appendix C. From the studies on each thermal cycler, minus A peaks were not as prevalent as expected from the citations in the literature. With the MiniFiler™ kit, minus A peaks were noted at the D16S539, CSF1P0, and FGA loci. Minus A peaks were only observed at the AMEL locus with the Identifiler™ kit and Philisa®.

## **Mixture Studies**

From this study, each thermal cycler exhibited equally low resolution of the mixture profiles. Despite having quantified the samples prior to mixing, it was evident that they were not of equal concentration in the 1:1 mixture, since those electropherograms produced clear major/minor components. This affected the amplification of the samples. Nonetheless, for the most part, the number of contributors to the mixture could be determined.<sup>34</sup>

Also, at most loci, the ratio of minor alleles to major alleles was between 50% and 60%, which are preferred ratios for mixture interpretation.<sup>23</sup> Moreover, when minor alleles appeared in the profile, the peak heights were above 150 RFU, which would allow them to be used statistically to report a probability of the minor profile in the population.<sup>35</sup> Overall, amplification of the minor component with Philisa® produced higher peak height RFUs than on the GeneAmp® 9700. For example for Identifiler™ with the 1:1 mixture, a range of 163-4269 RFU on Philisa® versus 177-1147 RFU from the GeneAmp® 9700. This difference may be attributable to the higher amounts of template DNA used with the rapid thermal cycler.

## CHAPTER V

### CONCLUSIONS

From the sensitivity studies conducted on the two thermal cyclers, the GeneAmp® 9700 was the more sensitive instrument, having a lower input DNA mass which could produce a full genetic profile with allele peak heights above 150RFU.

For the GeneAmp® 9700 thermal cycler, the dynamic range was 63pg – 500pg with Identifiler™. The best performing dye channel was ROX, while VIC was the poorest performer. By comparison, the dynamic range was 313pg-1250pg for the Philisa® instrument. Similar to the GeneAmp® 9700, the best performing dye channel was ROX, while VIC was the dye with the lowest average RFU.

For the GeneAmp® 9700 thermal cycler, the dynamic range was also 63pg – 500pg with MiniFiler™. The best performing dye channel was VIC, while ROX was the poorest performer. The dynamic range observed with MiniFiler™ on Philisa® was between 313pg and 2500pg. The FAM dye channel outperformed all the dyes with this instrument, while the ROX dye recorded the lowest average peak height RFU.

Overall, the rapid thermal cycler had significantly more stutter than the traditional thermal cycler ( $p < 0.05$ ) with the Identifiler™ kit. There was very little stutter recorded for the GeneAmp® 9700. The loci with the highest stutter percentages on Philisa® were: D13S317

(28%), CSF1P0 (23%), and D2S111 (15%). On the GeneAmp® 9700, the D16S539 (9%) and D21S11 (9%) loci recorded the highest stutter percentages.

There was no dropout observed with the GeneAmp® 9700 for either STR kits. There was dropout of the larger loci from the Philisa® amplifications. There were very few incidences of drop in on either instrument.

There was no indication of a significant difference in the heterozygous peak ratios between the two thermal cyclers ( $p=0.446$ ). With the forensic samples, there was also no difference between ratios between peak height ratios for each thermal cycler with the Identifiler kit™ ( $p>0.05$ ); however, overall the GeneAmp® 9700 had less peak height imbalance than the rapid thermal cycler with the MiniFiler™ assay.

With the MiniFiler™ kit, minus A peaks were noted at the D16S539, CSF1P0, and FGA loci. Minus A peaks were only observed at the AMEL locus with the Identifiler™ kit and Philisa®. Split peaks were observed at the D7S820, D2S1338, D16S539, CSF1P0, and FGA loci. No minus A peaks were observed with the Identifiler™ kit.

Results from the mixture study, indicated that each thermal cycler exhibited equally low resolution of the mixture profiles. Nonetheless, for the most part, the number of contributors to the mixture could be determined.<sup>34</sup>

The statistical analyses performed from the various studies on each thermal cycler indicated comparable performance between the two systems with the Identifiler™, MiniFiler™ and Yfiler™ STR amplification kits, and the Q-TAT™ method. A marked difference was with the



stutter percentages, where Philisa® exhibited significantly more stutter than the GeneAmp® 9700. Also, Philisa® was unable to amplify the DYS389II locus in Yfiler™, and the pRL amplicon in Q-TAT™. The GeneAmp® 9700 was more sensitive, and was shown to be a robust, reliable thermal cycler with every comparison study performed; albeit having higher setup costs. Philisa® was considerably faster, saving over 3 hours, 23 minutes of PCR run time, and performed as reliably as the GeneAmp® PCR System 9700 with heterozygous peak ratios and mixture resolution. However, with only 8 wells, additional analyst time must be factored in for the repetitive sample handling required getting through a casework batch of more than eight samples. The time-savings with Philisa® are more advantageous with small batch sizes.

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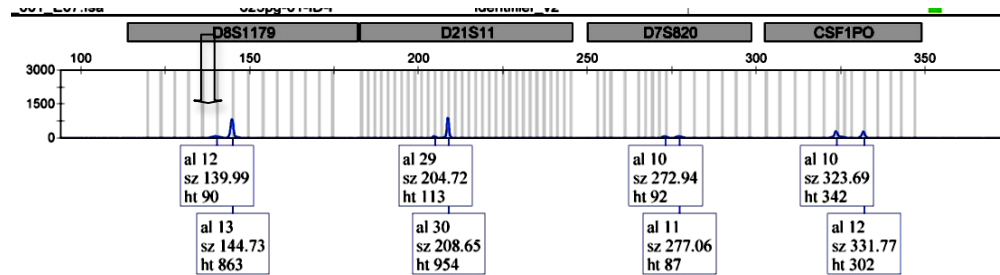
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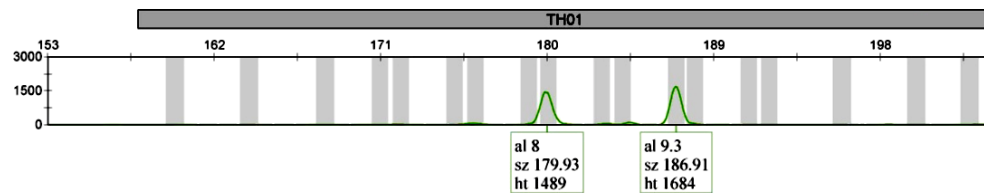
## APPENDICES

Appendix A. Electropherogram showing a stutter allele.



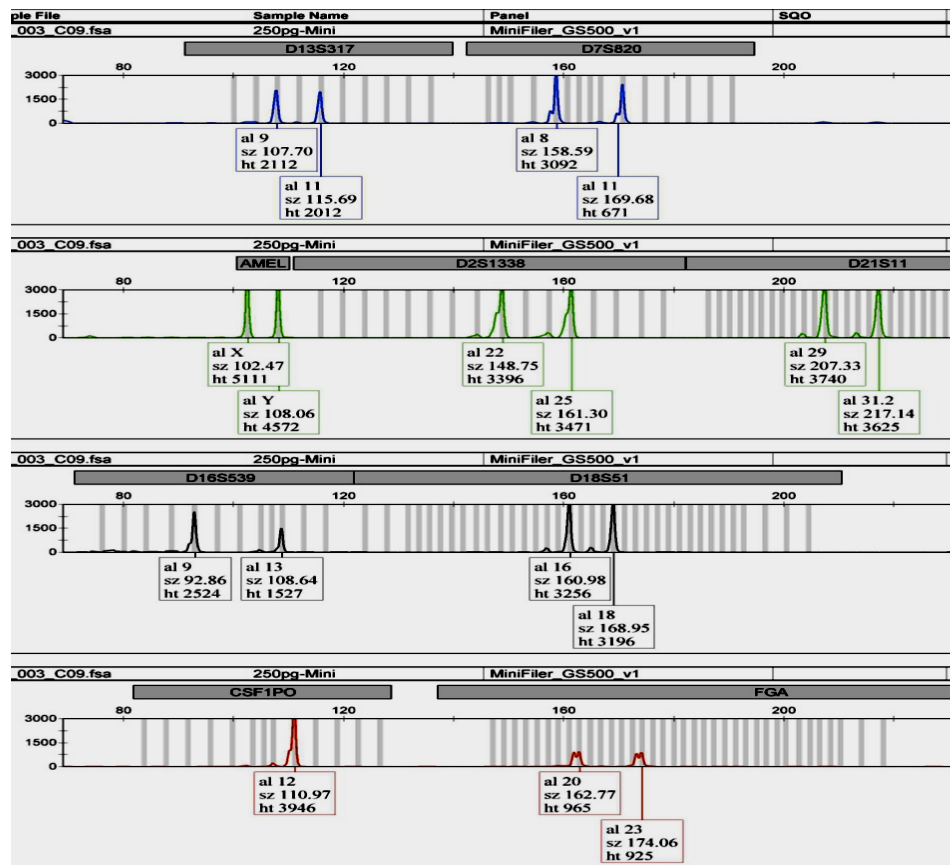
The arrow indicates the stutter allele at the D8S1179 locus. The stutter allele is 12 and the true allele is 13. The stutter percentage of 12 is  $90/863 = 10\%$ .

Appendix B. Electropherogram showing two sister alleles at the TH01 locus.



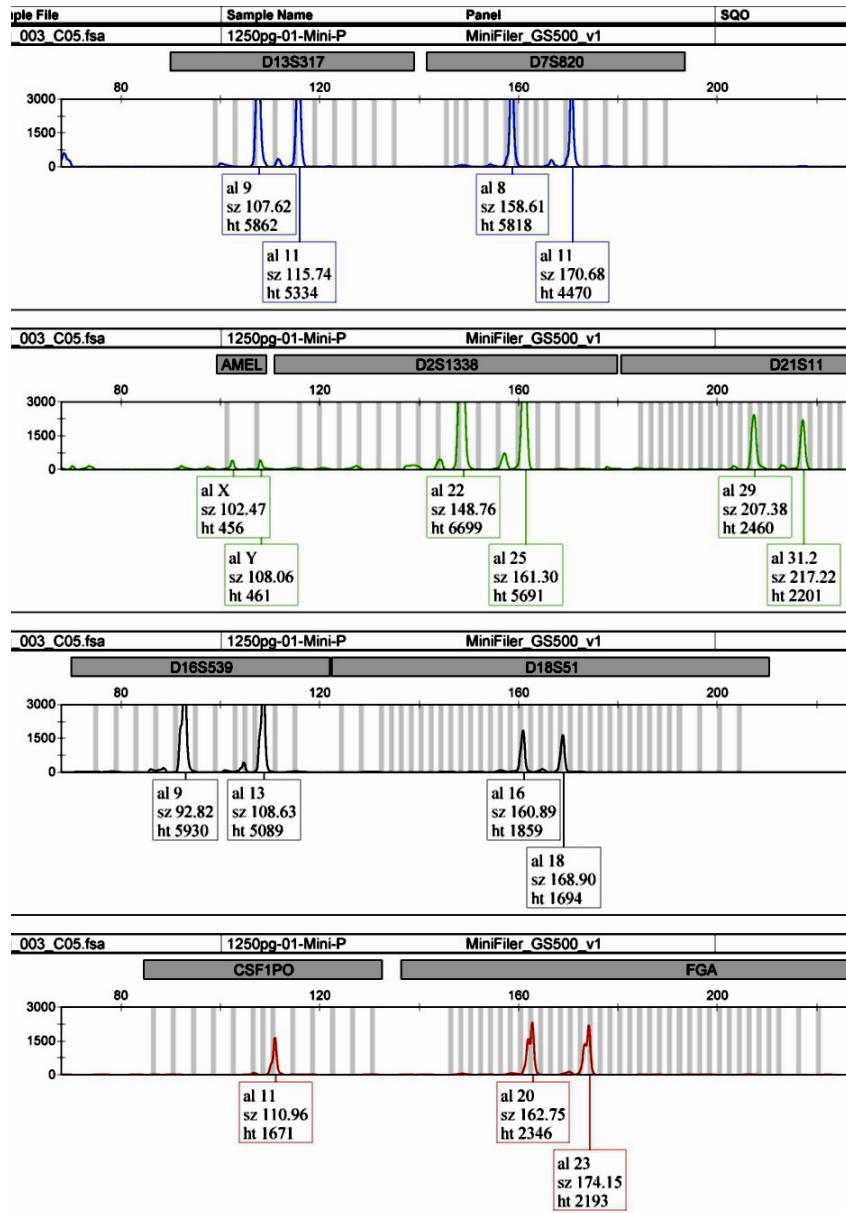
The heterozygous peak ratio of these two alleles is calculated as:  $1684/1489 = 1.1$ .

Appendix C. Electropherogram showing a genetic profile of 2800M control DNA generated by the GeneAmp® 9700 with MiniFiler™.



Minus A peaks can be seen at D7S820, D2S1338, D16S539, CSF1P0 and FGA.

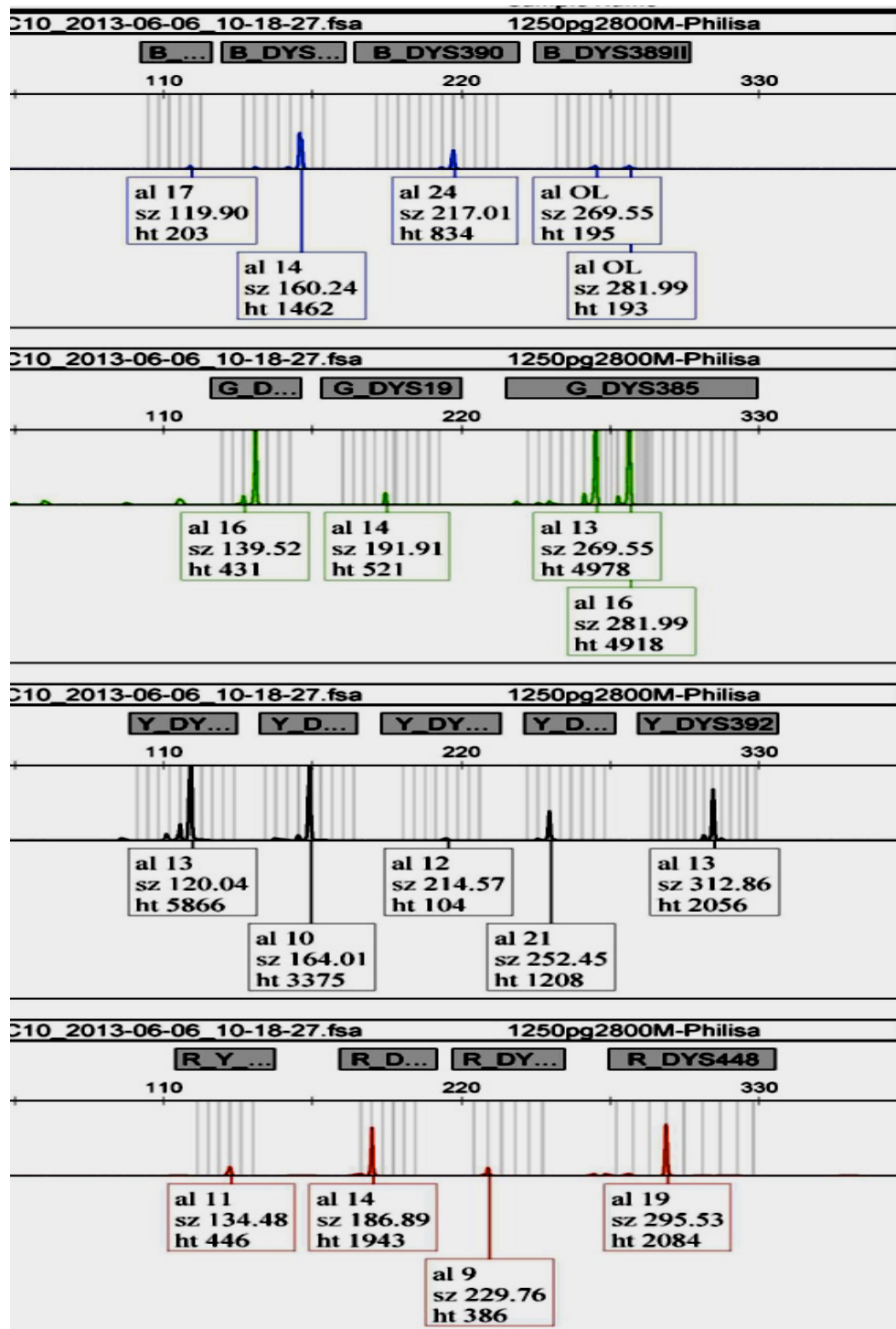
Appendix D. Electropherogram showing a genetic profile of 2800M control DNA generated by Philisa® with MiniFiler™.



This electropherogram shows minus A peaks at the D16S539 and FGA loci.

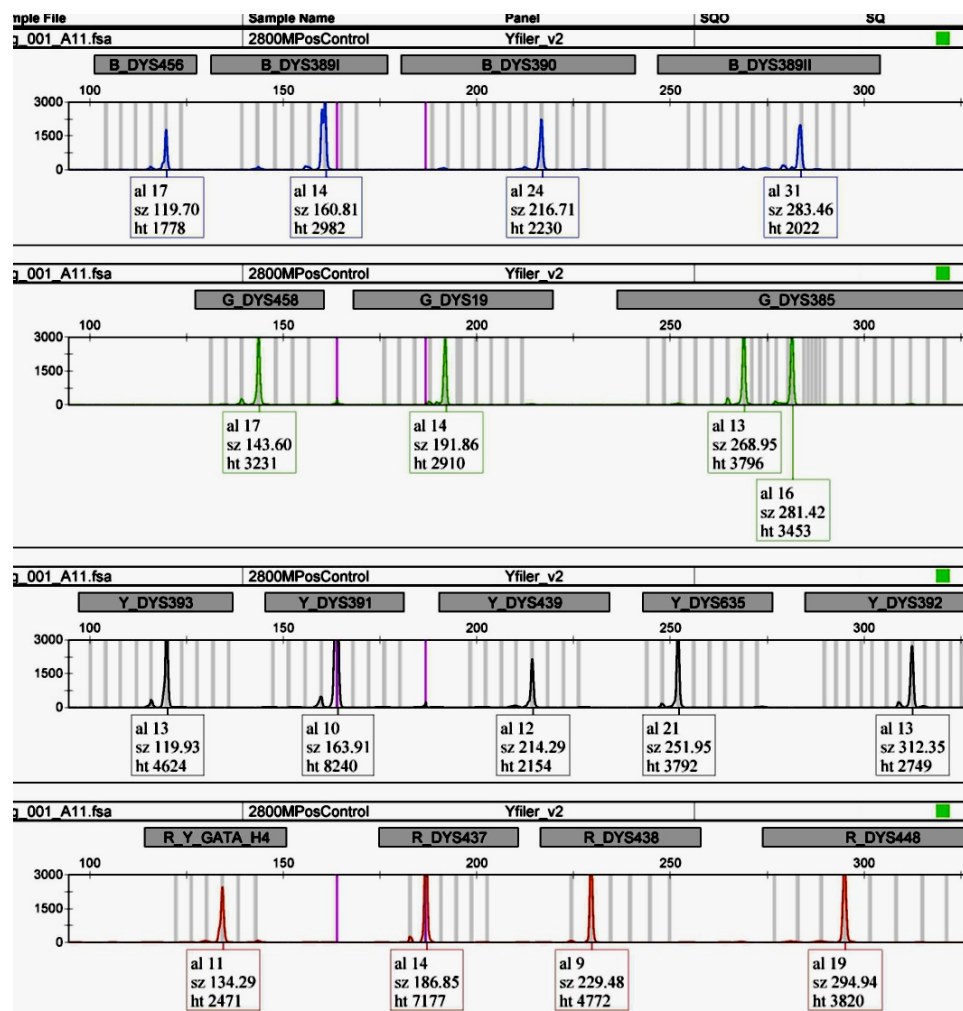


Appendix E. Electropherogram showing a genetic profile of 2800M control DNA generated by Philisa® with Yfiler™.



This electropherogram shows the double off-ladder (OL) peaks at the DYS389II locus.

Appendix F. Electropherogram showing a complete genetic profile of the 2800M control DNA sample amplified with Yfiler™ on the GeneAmp® 9700 thermal cycler.



## VITA

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Master of Science

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