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Prior to being implemented into forensic casework at a laboratory, a new method or product used in forensic DNA analysis must be validated internally within that laboratory. An internal validation of the AmpFlSTR® Identifiler™ PCR Amplification Kit at The Forensic Testing Laboratory was conducted, and it consisted of a sensitivity study, mixture study, non-probative case sample study, precision study, and a contamination study. The sensitivity study served to determine the target amount of input DNA to be added to each reaction, and the mixture study demonstrated how sensitive this kit is in detecting mixed source samples. The non-probative case sample study simulated various types of samples that may occur in casework. The results of each study are satisfactory for an internal validation, and the AmpFlSTR® Identifiler™ PCR Amplification Kit may now be used in forensic casework, since the kit was proven to be reliable, robust, and reproducible.

INTERNAL VALIDATION OF THE AMPFLSTR ®IDENTIFILER™ PCR AMPLIFICATION KIT AT THE FORENSIC TESTING LABORATORY, INC.

PRACTICUM REPORT

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For the Degree of

MASTER OF SCIENCE

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CHAPTER I

INTRODUCTION

The focus of this practicum is to conduct an internal validation study of the AmpFlSTR® Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA) at the Forensic Testing Laboratory (FTL) so that this particular kit may be used in forensic casework. The Identifiler™ (Applied Biosystems, Foster City, CA) kit is used in forensic short tandem repeat DNA (STR DNA) testing, to evaluate the specific regions (loci) found on nuclear DNA, in which the highly variable nature of those specific regions increase the discriminatory power between individual DNA profiles (16). The validation of the Identifiler™ (Applied Biosystems, Foster City, CA) kit is important because it will evaluate the reliability, robustness, and the reproducibility of the results, as well as the integrity of the reagents, instruments, and protocols that are all involved in the process of generating a DNA profile that may be used as evidence in court (4). An internal validation is necessary for a laboratory to verify that the established procedure previously examined under a developmental validation will work in that particular laboratory (22). Each forensic laboratory develops standard operating protocols (SOPs) that provide a detailed listing of all the materials and reagents necessary to perform an assay and steps needed to complete an experiment successfully (22). The AmpFlSTR® Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA) has undergone developmental validation prior to it being used in forensic laboratories (12). The Identifiler™ (Applied

Biosystems, Foster City, CA) kit contains all genetic loci that are present in other AmpFlSTR® brand PCR amplification kits, such as Profiler Plus™, COfiler™, and SGM Plus™ kits, and AmpFlSTR® kit primer sequences previously optimized have been maintained (12). The Identifiler™ (Applied Biosystems, Foster City, CA) kit has been developed in accordance with standards of the forensic community as defined by the DNA Advisory Board (12). The polymerase chain reaction, or PCR for short, is an in vitro process that yields millions of copies of a DNA sample through repeated cycling of a reaction involving a DNA polymerase enzyme (21). The DNA polymerase enzyme in the PCR amplification step serves to speed up the synthesis of nucleic acids on a preexisting template, and therefore build DNA from deoxyribonucleotides (21).

Validation studies are needed in forensic laboratories whenever a new procedure, instrument, assay, or reagent is introduced to the standard operating protocol (22). Included with the validation, it is necessary that the analyst at the laboratory be trained in that technique beforehand, and then the analyst should conduct the study to determine the range and reliability of that new technique so that new or modified standard operating protocols with interpretation guidelines can be developed (22). The DNA Advisory Board (DAB) standards state that internal validation studies performed by laboratories must be appropriately documented along with any material modifications (22). Validation studies—developmental or internal—ensure that successful results are obtained at a high percentage. Internal validation studies also use PCR methods that are tested with both simulated and real casework samples, and the laboratory should document the reproducibility and precision of the procedure using human DNA control(s) (24). Reference DNA must be incorporated into any validation study for quality control purposes. Most commercially available STR typing kits use 9947A or 9948 control DNA, which

have known genotype information and are distributed to laboratories worldwide as a means of universal calibration in forensic DNA typing (28).

The validation study of the Identifiler™ (Applied Biosystems, Foster City, CA) kit includes a sensitivity study, a mixture study, a non-probative case sample study, precision study, and a contamination survey. The Forensic Testing Laboratory has previously validated some multiplex kits that are currently used in casework and implemented into laboratory protocols: PowerPlex® 16™ (Promega, Madison, WI), PowerPlex® Y™ (Promega, Madison, WI), and AmpFlSTR® Minifiler™ (Applied Biosystems, Foster City, CA). The validation of the Identifiler™ (Applied Biosystems, Foster City, CA) kit will allow for alternative tests to be used with forensic casework, as well as expand upon the capabilities and casework throughput of the laboratory. Multiplex kits, such as Identifiler™ (Applied Biosystems, Foster City, CA), are beneficial for forensic DNA testing laboratories because discrete alleles—those DNA fragments that may differ by only a single base pair in size—can be differentiated (17). Multiplexing allows for several samples to be pooled at the same time and therefore increasing speed of analysis (21). Such PCR amplification kits also allow for results to be compared easily between laboratories, and STR typing of low amounts of DNA and degraded DNA is possible (17). Multiplex STR kits were developed because STRs are not as polymorphic as VNTRs (variable number tandem repeats), and high throughput systems were developed to overcome this issue (18). With Identifiler™ (Applied Biosystems, Foster City, CA), several STR systems can be detected at the same time without overlapping loci in the same lane (18). Other than Identifiler™ (Applied Biosystems, Foster City, CA), several other commercially available STR kits are manufactured by Applied Biosystems: Profiler Plus™, COfiler™, SGM Plus™, MiniFiler™, Yfiler™, Profiler™, SEfiler™, Green I™, and Blue™ (19). STR multiplexes such

as these all limit consumption of the DNA sample, and are ideal because the probability of identical alleles in two individuals decrease with an increase in the number of polymorphic loci examined (19). Also, the fluorescent labeling of PCR primers in the kit allows for good spectral resolution between overlapping loci with different colored fluorescent dyes (19).

The AmpFlSTR® Identifiler® PCR Amplification kit (Applied Biosystems, Foster City, CA) is used in forensic laboratories across the country. It is a multiplex STR assay that amplifies 15 loci as well as the amelogenin sex marker. Of the 15 loci, 13 of the loci were chosen by the Federal Bureau of Investigation as the core of the Combined DNA Index System (CODIS) for use in comparison against national databases and to ensure that all forensic laboratories establish the same DNA databases and any important information (16). The Identifiler™ kit uses a 5-dye system to label DNA fragments during STR analysis on the ABI PRISM 3130*xl* and the additional dyes in the Identifiler™ Primer Set (Applied Biosystems, Foster City, CA) allow for more loci to be multiplexed in one capillary injection. This particular kit is designed to be more flexible, with amplicon allele sizes ranging between 100 and 360 base pairs (1). The Identifiler™ (Applied Biosystems, Foster City, CA) kit also has identical primer sequences used in all other AmpFlSTR® PCR kits (1). STR multiplex systems, such as Identifiler™ (Applied Biosystems, Foster City, CA), have many advantages in forensic DNA testing. Not only do they have a high rate of heterozygosity (presence of different alleles at one or more loci on homologous chromosomes), but they also have robust amplification and distinguishable alleles (17). The availability of commercially available multiplex kits has simplified the use of STRs in recent history (30). A report by the National Commission on the Future of DNA Evidence stated that STR typing will be a primary means of forensic DNA analysis for the next decade (31).

STR markers are abundant—there are more than 2,000 reliable for genetic mapping studies that have been described in literature (18). Markers are defined as genes of known location on a chromosome, from which a phenotype is used as a point of reference in mapping other loci (21). The analysis of STR markers is optimal because amplified products can easily be distinguished from one another and reject any microvariant alleles (18). DNA markers used to distinguish individuals from one another have been studied since the 1980s with the advent of RFLP (restriction length polymorphism) analysis (18). A restriction length polymorphism is a variation between individuals in DNA fragment sizes cut by specific restriction enzymes (21). Early studies in STR typing involved polyacrylamide gels. However, capillary electrophoresis methods have become more popular since it eliminates the need to pour gels, and it consumes a small portion of the DNA sample per run so that it may be retested if needed (30). Polymorphic sequences used as markers result, caused by a mutation at a cutting site (21). STR DNA is good to use in forensic DNA testing because they produce the most reliable and robust data due to the nature of the tetranucleotide repeats (18). These repeats tend to have a low rate of mutation, minimal stutter artifacts, and minimal microvariants (18). Also, the discrete nature of alleles in STR DNA has allowed for the development of allelic ladders, which contain a collection of most of the common amplified alleles found in a general population (18).

Comparing the PowerPlex® 16™ (Promega, Madison, WI) kit to the Identifiler™ (Applied Biosystems, Foster City, CA) system, both kits have been used by laboratories to type samples; however, PowerPlex® 16™ (Promega, Madison, WI) has been shown to be more sensitive in detection versus Identifiler™ (Applied Biosystems, Foster City, CA) and less sensitive to PCR inhibitors (7). PowerPlex® 16™ (Promega, Madison, WI) amplifications are carried out for 32 cycles, and Identifiler™ (Applied Biosystems, Foster City, CA) amplification

parameters are set for 28 cycles. In another comparison between Identifiler™ (Applied Biosystems, Foster City, CA) and PowerPlex® 16™ (Promega, Madison, WI), it was observed that allele 24 in the FGA locus dropped out in PowerPlex® 16™ (Promega, Madison, WI) but was fine with Identifiler™ (Applied Biosystems, Foster City, CA) (20). Allele dropout occurs when a sequence variation in the flanking region of STR loci cause failed amplification of that allele at that particular locus (20). The thermal cycling parameters for PCR amplification using Identifiler™ (Applied Biosystems, Foster City, CA) are as follows:

- Hold, Initial incubation step – 95C for 11 minutes
- Cycle – 28 cycles
  - *Denature – 94C for 1 minute*
  - *Anneal – 59C for 1 minute*
  - *Extend – 72C for 1 minute*
- Hold, Final extension – 60C for 60 minutes
- Final Hold – 4C (forever)

Both PowerPlex® 16™ (Promega, Madison, WI) and Identifiler™ (Applied Biosystems, Foster City, CA) kits have been shown to generate full DNA profiles ranging from 2ng to 0.25ng of input human genomic DNA (7). In this same study, it was also demonstrated that PowerPlex® 16™ (Promega, Madison, WI) loci tended to drop out first with increasing concentrations of PCR inhibitors, with amelogenin being one of the first to drop out in inhibition experiments (7). Both kits in this comparison study were amplified following the manufacturer's recommended cycling conditions, and were analyzed at the same time using the same ABI PRISM 3130 Genetic Analyzer (7).

One AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) sufficient for 200 reactions contains the following: AmpFlSTR® PCR Reaction Mix (1.1 mL/tube), AmpFlSTR® Identifiler™ Primer Set (1.1 mL), AmpliTaq Gold® DNA Polymerase (0.05 mL/tube), AmpFlSTR® Control DNA 9947A (0.3 mL), and AmpFlSTR® Identifiler™ Allelic Ladder (0.05 mL) (Applied Biosystems, Foster City, CA). To use this kit, it is also imperative to include some materials that are not included within the kit, such as the AmpFlSTR® Identifiler™ PCR Amplification Kit User Guide (Applied Biosystems, Foster City, CA) and the GeneScan™ -500 LIZ® Size Standard reagent for detection by capillary electrophoresis using the ABI PRISM 3130*xl*.

SPECIFIC AIMS

The validation study in its entirety meets several objectives and specific aims in order to test the various parameters and conditions for the kit to be used with casework. A serial dilution test was designed for the sensitivity study in order to determine the optimal concentration of input DNA that should be used with the amplification kit in the future. According to the user's manual for the Identifiler™ (Applied Biosystems, Foster City, CA) kit, the amount of input DNA added to the kit should be between 0.5 and 1.25ng, and the DNA sample should be quantitated prior to amplification (1). The final concentration of DNA should be between 0.05 and 0.125 ng/µL so that 0.5 to 0.25ng of DNA can be added to the PCR reaction in a 10 µL volume (1). The sensitivity study is also needed to determine the minimal amount of DNA that can be used in a PCR amplification reaction, using the Identifiler™ (Applied Biosystems, Foster City, CA) kit, and can still result in a full genetic profile. The minimal amount of DNA that can be used in the PCR amplification is beneficial to know because laboratory costs may be reduced

when less reagents are needed per sample. In the sensitivity study, the recommended detection load volume was compared to an increased detection load volume, and different injection times for appropriate levels of DNA were also compared.

The optimal concentration of DNA determines the conditions under which the most reliable results can be obtained, and it also addresses any stochastic effects associated with varying levels of DNA concentration. The amount of DNA template added strongly influences the polymerase chain reaction. The sensitivity study is done properly before any other aspects of the validation study because it determines the amount of target input DNA to be used in each amplification reaction (mixture study, non-probative case sample study). If any low level DNA samples are present in the sensitivity study, they may be reinjected for a longer injection time, such as eight seconds versus the standard 5 second injection time. The Identifiler® PCR Amplification kit user's manual states that "individual laboratories may find it useful to determine an appropriate minimum peak height threshold based on their own results and instruments using low amounts of input DNA" (1). When there are low level samples, two alleles of a heterozygous individual may have an unbalanced amplification due to a stochastic fluctuation in the ratio of two different alleles (15). An allele is defined as an alternative form of a gene, and heterozygogous individuals have different alleles at one or more loci on homologous chromosomes (21).

Adversely, if there is too much DNA added to the PCR reaction, then the increased amount of PCR product may result in off-scale data and incomplete A nucleotide addition (1). This results in a split peak formation (two peaks instead of one). This could result in a single source sample being mistaken for a multi-source sample.

A mixture study is then conducted in order to determine the sensitivity of the Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA) in detecting mixed DNA of known ratios. Two individuals, both laboratory employees, are selected (one male, one female) for the study. The peak height ratios are examined in order to determine the difference between observing an actual mixed DNA profile of at least two individuals versus observing a single source sample that may have stutter peaks. In the mixture study, the ratio at which a mixture of two individuals cannot be observed is determined, and the imbalance of male and female in amelogenin is observed. In this validation study, a mixture of increased concentrations of female DNA and a constant concentration of male DNA is used to determine the point at which a male cannot be detected using the Identifiler™ (Applied Biosystems, Foster City, CA) kit. Also, a separate mixture of female DNA and male DNA is mixed at varying ratios to be quantitated and diluted to the optimal template concentration for Identifiler™ (Applied Biosystems, Foster City, CA).

Also in the validation study, non-probative case samples are generated and tested within the laboratory in order to simulate real forensic casework and therefore examine the effects of environmental conditions and possible PCR inhibitors. Non-probative case samples include biological material such as blood, semen, or saliva donated by laboratory employees, each deposited on various substrates to observe how the Identifiler™ (Applied Biosystems, Foster City, CA) kit responds to each sample type, as well as observe any PCR inhibition that may occur. Some of the simulated non-probative case samples may be low copy number, single source, or mixed source samples. All non-probative case samples are amplified using the prescribed Identifiler™ (Applied Biosystems, Foster City, CA) thermal cycling parameters (28

cycles) that are used with the kit, and the profiles are evaluated for correct genetic typing and overall quality of the profile.

A precision study is also necessary in an internal validation study in order to demonstrate the reproducibility of the results, the concordance of the test between all samples, and the precision of Identifiler™ (Applied Biosystems, Foster City, CA) on the ABI PRISM 3130*xl* Genetic Analyzer instrument. This is in order to ensure precision in the future and determine if runs with multiple injections should contain multiple allelic ladders. Allelic ladder injections from the sensitivity study, mixture study, and from the non-probative case samples are studied, and the base pair sizes of each ladder are evaluated to calculate the standard deviation of base pair size per allele and the average standard deviation for each of the 15 loci plus amelogenin amplified by using the Identifiler™ (Applied Biosystems, Foster City, CA) kit.

A contamination study is needed to ensure that all samples tested with the Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA) have a minimal risk of contamination. Therefore, the contamination study surveys all of the negative controls and reagent blanks used with each reaction in the entire validation study for the presence of any amplified product. The minimum analysis threshold for the Identifiler™ (Applied Biosystems, Foster City, CA) kit is determined by evaluating the baseline of amplified negative controls. Full reaction chemistry is used in the evaluation of the reagent blanks and negative controls. The contamination study allows the laboratory to determine if dropping the minimum analysis threshold to a certain RFU (relative fluorescence units) level is acceptable for the particular amplification kit being validated.

The Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA) is validated internally within the laboratory so that the scientific community gets the necessary information to assess the ability of the procedure to get reliable and robust results, define any limitations of the kit, and determine the conditions under which those same results can be obtained in the future. The internal validation of any kit, reagent, or instrument in a forensic laboratory specifically aims to provide an in-house demonstration of the reliability as well as limitations of the kit. Any internal validation study of a particular amplification kit consists of a sensitivity study, a mixture study, the generation and processing of non-probative case samples, a precision study, and a contamination study. For the internal validation, there are standard protocols used by the laboratory for each technique employed in the study, and those procedures include the necessary quality control measures.

The Forensic Testing Laboratory in Las Cruces, New Mexico, is a full-service forensic DNA laboratory that is accredited by FQS-I and ISO 17025. The laboratory has separate rooms for biological evidence examination, DNA extraction, mitochondrial DNA analysis work, and an autosomal post-amplification room. Each room has copies of worksheets and protocols for various procedures. In the biological evidence examination room, presumptive and confirmatory tests for the presence of biological fluid are conducted, as well as any kind of sample preparation. In the main extraction lab at the Forensic Testing Laboratory, DNA extractions are conducted. In this particular room, there are two heat blocks for sample incubation, two Qiagen® BioRobot EZ1 instruments for extraction, vortexes, several centrifuges, and two fume hoods—one specifically for work with reagents, and the other for work with DNA that is to be transferred to the post-amplification room for either quantification or PCR amplification and further testing.

The laboratory in its entirety takes up 8,600 square feet of space (Forensic Testing Laboratories). In the post-amplification room at FTL, quantification of DNA is conducted with the use of the ABI 7500 instrument that is linked to a laptop computer with Sequence Detection Software (SDS). There are two Eppendorf® thermal cyclers for PCR amplification. Fragment analysis by capillary electrophoresis is accomplished with the use of the ABI 3130*xl* instrument. The instrument is linked to a computer with GeneMapper® *ID* version 3.2 software (Applied Biosystems, Foster City,CA), and this software is used for STR data analysis and interpretation.

Prior to beginning the validation study, training on the instruments in the laboratory occurred. Laboratory employee reference profiles were obtained by using the PowerPlex® 16™ multiplex kit. Training was necessary to ensure that proper techniques were performed on instruments such as the Qiagen BioRobot® EZ1™ and laboratory protocols were adhered to.

CHAPTER II

MATERIALS AND METHODS

SENSITIVITY STUDY

The sensitivity experiment in the internal validation study was necessary to determine the optimal concentration of input template DNA that is needed to be added to the AmpFlSTR® Identifiler™ (Applied Biosystems, Foster City,CA) reaction. According to the Identifiler™ User's Guide (Applied Biosystems, Foster City, CA), the recommended input DNA added for optimal results should be between 0.5ng and 1.25ng (1). In the sensitivity study, a serial dilution test with a total of eight different concentrations of 9948 control DNA (male DNA standard from PowerPlex® Y™ kit, (Promega, Madison, WI) was designed, ranging from more concentrated to less concentrated. Since 9948 control DNA was used for this experiment, extraction procedures were not necessary. Two separate dilutions were included with the sensitivity test. The 9948 control DNA was diluted from its original concentration of 10 ng/µL down to 1.5 ng/µL with molecular grade sterile water for a total volume of 50 µL. The 9948 control DNA was then serial diluted halfway down to 0.0117 ng/µL. The table below demonstrates the concentrations of DNA used in the serial dilution.

	Concentration ng/µl	DNA (9948) or Diluted DNA Amount	Water µL Amount
A	1.5	7.5 µl of 9948	42.5
B	0.75	25µl of A	25
C	0.375	25µl of B	25
D	0.188	25µl of C	25
E	0.0938	25µl of D	25
F	0.0469	25µl of E	25
G	0.0234	25µl of F	25
H	0.0117	25µl of G	25

Table 1: DNA Concentration Design for Sensitivity Study

Along with the eight samples labeled A through H in the serial dilution series, two additional dilutions were made, labeled I and J. Dilution I had a concentration of 0.25 ng/µL (1.25 µL DNA, 48.75 µL water), and dilution J had a concentration of 0.5 ngµL (2.5 µL DNA, 47.5 µL water). Approximately 2 µL of each sensitivity study sample was then quantified using the Plexor® HY System (Promega, Madison, WI) to evaluate the accuracy of the dilutions. Due to pipetting error, adjustments to the concentrations of DNA added to each well of the Identifiler™ (Applied Biosystems, Foster City, CA) 96 well plate amplification set-up were necessary. After evaluating the quantitation values of the dilutions, the new concentrations for the serial dilution series were the following: 2.931 ngµL (A), 1.232 ng/µL (B), 0.736 ng/µL (C), 0.342 ng/µL (D), 0.1083 ng/µL (E), 0.0681 ng/µL (F), 0.0293 ng/µL (G), and 0.0222 ng/µL (H). The new concentrations for the separate dilutions made were 0.534 ng/µL (I) and 0.846 ng/µL (J).

Next, the sensitivity samples (2 µL of each) were amplified in duplicate at full reaction chemistry according to the AmpFlSTR® Identifiler™ User Guide (Applied Biosystems, Foster City, CA) using the PCR parameters (28 cycles) for the AmpFlSTR® Identifiler™ kit (Applied Biosystems, Foster City, CA). The full reaction chemistry for Identifiler™ (Applied Biosystems, Foster City, CA) is displayed in Table 2 below. The total volume per sample was 25 µL, with 15 µL of master mix and up to 10 µL of sample.

Full Reaction

Master Mix Component	Volume per sample
Reaction Mix	10.5µl
Primers	5.5µl
AmpliTaq Gold DNA Polymerase	0.5µl
Template (Samples and positive control)	2µl of A-J 8µl Sterile Water

Total Volume = 25µl

Table 2: PCR Amplification Design

Samples in the sensitivity study were set up for PCR amplification using a 96-well plate. The grid below shows the location of each sample, positive control (PC), and negative control (NC) on the plate that was placed in the thermal cycler.

Table 3: Identifiler 96 Well Plate Amplification Set Up – 28 Cycles

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	2.931ng	2.931ng	NC	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	PC	1.232ng	1.232ng	NC	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
C	PC	0.736ng	0.736ng	NC	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
D	NC	0.342ng	0.342ng	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
E	0.534ng	0.1083ng	0.1083ng	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
F	0.534ng	0.0681ng	0.0681ng	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
G	0.846ng	0.0293ng	0.0293ng	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
H	0.846ng	0.0222ng	0.0222ng	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

After amplification, the samples were then loaded onto the ABI PRISM 3130*xl* Genetic Analyzer instrument (Applied Biosystems, Foster City, CA) for STR fragment detection and analysis. The samples were loaded at both recommended load volume (0.3 µL GeneScan™ -500 LIZ® Size Standard, 8.7 µL Hi-Di formamide, 1.0 µL amplification product or allelic ladder) and increased load volume (0.5 µL GeneScan™ -500 LIZ® Size Standard, 19.5 µL Hi-Di formamide, 0.8 µL of amplification product or allelic ladder) into each well on the load plate. The grid below displays the 96-well plate load set-up for detection on the 3130*xl* instrument. Six allelic ladders as well as multiple positive and negative controls were included.

Table 4: Identifiler 96 Well Plate Detection (Load) Plate Set Up
(Recommended load volume, increased load volume)

	10µl Vol	10µl Vol	10µl Vol	10µl Vol			20.8µl Vol	20.8µl Vol	20.8µl Vol	20.8µl Vol		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder	2.931ng	2.931ng	NC	Blank	Blank	Ladder	2.931ng	2.931ng	NC	Blank	Blank
B	PC	1.232ng	1.232ng	NC	Blank	Blank	PC	1.232ng	1.232ng	NC	Blank	Blank
C	PC	0.736ng	0.736ng	NC	Blank	Blank	PC	0.736ng	0.736ng	NC	Blank	Blank
D	NC	0.342ng	0.342ng	Ladder	Blank	Blank	NC	0.342ng	0.342ng	Ladder	Blank	Blank
E	0.534ng	0.1083ng	0.1083ng	Ladder	Blank	Blank	0.534ng	0.1083ng	0.1083ng	Ladder	Blank	Blank
F	0.534ng	0.0681ng	0.0681ng	Blank	Blank	Blank	0.534ng	0.0681ng	0.0681ng	Blank	Blank	Blank
G	0.846ng	0.0293ng	0.0293ng	Blank	Blank	Blank	0.846ng	0.0293ng	0.0293ng	Blank	Blank	Blank
H	0.846ng	0.0222ng	0.0222ng	Blank	Blank	Blank	0.846ng	0.0222ng	0.0222ng	Blank	Blank	Blank

After the fragment analysis of the sensitivity study samples, the data was evaluated to determine whether or not reinjection of any of the samples was necessary. If samples were considered low level or low quality (alleles below 100RFU threshold), those samples were reloaded and reinjected on the 3130*xl* for more time (8 second injection time). If samples contained too much DNA, those were reloaded and reinjected for less time (3 second injection time).

The standard injection parameters for the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA) are listed in Table 5 below.

Variable	Identifiler Setting
Oven Temperature	60 °C
Poly Fill Vol	6500 steps
Current Stability	5.0 µAmps
PreRun Voltage	15.0 kVolts
PreRun Time	180 sec
Injection Voltage	3.0 kVolts
Injection Time	5 sec
Voltage Number of Steps	40 nk
Voltage Step Interval	15 sec
Data Delay Time	1 sec
Run Voltage	15.0 kVolts
Run Time	2800 sec

Table 5: ABI Prism 3130*xl* Genetic Analyzer Injection Parameters

The sensitivity study evaluated the limitations of the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) by interpreting the point at which no profile was obtained (no allele calls above the minimum 100RFU threshold). According to The Forensic Laboratory protocols, the alleles are considered within optimal range when the peak heights are between 800RFU and 2,000RFU (relative fluorescence units).

MIXTURE STUDY

In the mixture study, the sensitivity in detecting mixed DNA of known ratios was determined. Two individuals, both laboratory employees (male = A, female = H) were selected for the mixture. Buccal swabs from the selected individuals were extracted by using the BioRobot® EZ1™ instrument. The samples were each quantified by using the Plexor® HY System™ (Promega, Madison, WI) to determine the extract DNA yield, and then those extracts were diluted to 1 ng/µL. The reference profiles from the male (A) and the female (H) are displayed in Table 6 below.

Locus	A (Male)	H (Female)
D8S1179	11,13	14,15
D21S11	28,31	28,29
D7S820	10,13	11,12
CSF1PO	10	10,12
D3S1358	14,15	14,17
TH01	7,9	9.3
D13S317	12,13	8,12
D16S539	9,10	12,13
D2S1338	19,23	17,19
D19S433	13,14	13.2,16
vWA	14	14,18
TPOX	8	8
D18S51	12,18	15,16
D5S818	11,13	12
FGA	20,24	19,23
AMEL	X,Y	X

Table 6: Reference Profiles for Mixture Study

Two separate experiments were included with the mixture study. Samples in each study were run in duplicate. In the first experiment, diluted extracts of female DNA and male DNA were mixed at increased concentrations of female DNA to constant concentration of male DNA at the following ratios (Female:Male):

Ratio	µl of DNA
1:1	(10+10)
5:1	(16+4)
10:1	(18+2)
15:1	(28+2)
20:1	(38+2)
25:1	(48+2)
50:1	(98+2)
100:1	(198+2)

Table 7: Mixture Study Design – Experiment 1

In the second mixture experiment, diluted extracts (1 ng/µL) of male (A) DNA and female (H) DNA were mixed at the following ratios (Male:Female):

Ratio	µl of DNA
20:1	(19+1)
10:1	(18+2)
3:1	(12+6)
1:1	(10+10)
1:3	(6+12)
1:10	(2+18)
1:20	(1+19)

Table 8: Mixture Study Design – Experiment 2

After the dilutions for each mixture experiment were prepared, each sample was diluted to optimal template concentration for AmpFlSTR® Identifiler™ (Applied Biosystems, Foster City, CA) at 0.75 ng/µL (according to the results of the sensitivity study), and then amplified in duplicate at full reaction chemistry (25 µL reaction – 15 µL master mix, up to 10 µL sample). The PCR amplification design for the mixture study is identical to that of the sensitivity study (refer to Table 2). The amplification plate set-up grid showing the location of each sample, positive control (PC), and negative control (NC) on the 96-well plate is displayed below.

Table 9:

Identifiler Well Plate **Amplification Plate Set Up** – 28 Cycles

Increased concentrations of female DNA mixed with constant male DNA

Male:Female Mixture

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	1:1	1:1	20:1	20:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	5:1	5:1	10:1	10:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
C	PC	10:1	10:1	3:1	3:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
D	PC	15:1	15:1	1:1	1:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
E	NC	20:1	20:1	1:3	1:3	Blank	Blank	Blank	Blank	Blank	Blank	Blank
F	RBK	25:1	25:1	1:10	1:10	Blank	Blank	Blank	Blank	Blank	Blank	Blank
G	Blank	50:1	50:1	1:20	1:20	Blank	Blank	Blank	Blank	Blank	Blank	Blank
H	Blank	100:1	100:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

After the PCR amplification step, samples from both mixture experiments were loaded onto the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City,CA) for fragment detection and analysis at increased volume per sample. The grid below displays the detection (load) plate set-up for the mixture study. Four allelic ladders were included in the set-up, along with a reagent blank, several positive controls (PC), and negative controls (NC).

Table 10:

Identifiler Well Plate **Detection (Load) Plate Set Up**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder	1:1	1:1	20:1	20:1	Ladder	Blank	Blank	Blank	Blank	Blank	Blank
B	Ladder	5:1	5:1	10:1	10:1	Ladder	Blank	Blank	Blank	Blank	Blank	Blank
C	PC	10:1	10:1	3:1	3:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
D	PC	15:1	15:1	1:1	1:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank
E	NC	20:1	20:1	1:3	1:3	Blank	Blank	Blank	Blank	Blank	Blank	Blank
F	RBK	25:1	25:1	1:10	1:10	Blank	Blank	Blank	Blank	Blank	Blank	Blank
G	Blank	50:1	50:1	1:20	1:20	Blank	Blank	Blank	Blank	Blank	Blank	Blank
H	Blank	100:1	100:1	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

NON-PROBATIVE CASE SAMPLE STUDY

In the non-probative case sample study, the samples were intended to simulate forensic casework scenarios that may arise, and observe how the AmpFlSTR® Identifiler™ kit (Applied Biosystems, Foster City, CA) responds to different types of samples. These 20 samples included blood, saliva, semen, and epithelial cells donated by laboratory employees. Some of the samples were low copy number, some single-source, and some were mixed source samples. Each was deposited on various substrates. The item numbers with the letters “N” or “S” next to them implies that the particular sample contained seminal material and therefore required a differential extraction, which separated the epithelial or non-sperm (N) fraction from the sperm (S) fraction of DNA. Samples containing seminal material were purified with the Differex® System

(Promega, Madison, WI) prior to placing those samples on the Qiagen BioRobot® EZ1™ instrument for extraction.

The Differex™ System (Promega, Madison, WI) is needed for the purification of sperm and non-sperm cell DNA fractions. The Differex™ System (Promega, Madison, WI) is the standard differential DNA purification system used by The Forensic Testing Laboratory. This procedure allows the non-sperm fraction cells to be digested prior to the collection of sperm cells in the sample. Once the non-sperm fraction was transferred to a separate tube, the sperm fraction was separately purified using the Differex™ System (Promega, Madison, WI). The Differex™ System (Promega, Madison, WI) separates sperm fraction DNA from non-sperm fraction DNA by combining both phase separation and differential centrifugation.

The table below (Table 11) describes all non-probative case samples in the study, along with their assigned item numbers.

Table 11: Non-Probative Case Samples

Sample Description	Item Number
Saliva on filter paper	1
10µl Blood on filter paper	2
10µl Semen on filter paper	3N 3S
2.5µl Blood + 5.0µl Semen on filter paper	4N 4S
Saliva on styrofoam cup	5
Piece of chewing gum	6
Licked envelope	7
Fingernail swab	8
Buccal swab + 10µl Blood	9
Buccal swab + 10µl Semen	10N 10S
Neck swab + 10µl Semen	11N 11S
Eyeglass Nosepiece Swabbing	12
Swab from Sweatband of Hat	13
10µl Blood on Twig	14
20µl Blood on khaki shorts	15
10µl Blood Smear on khaki shorts	16
20µl Blood on Denim	19
10µl Blood Smear on Denim	20
10µl Semen on Denim	21N 21S
2.5µl Blood + 5.0µl Semen on Denim	22N 22S

All non-probative case samples were extracted using the Qiagen BioRobot® EZ1™ instrument. The non-probative case samples were quantified using the Plexor® HY System (Promega, Madison, WI), and then necessary dilutions were made to each sample prior to PCR amplification. Non-probative case samples were amplified using the thermal cycling parameters set for Identifiler™ (Applied Biosystems, Foster City, CA) at 28 cycles. Non-probative case samples were then loaded onto the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA) for fragment detection and analysis, and the profiles obtained were evaluated for correct genetic typing and overall quality of the profile. Those samples that produced low quality (partial profiles) results were then reinjected for longer injection time (8 seconds instead of 5 seconds).

PRECISION STUDY

In the precision study, allelic ladder injections from the sensitivity study, mixture study, and non-probative case sample study were pooled together to evaluate the precision of AmpFlSTR® Identifiler™ (Applied Biosystems, Foster City, CA) on the ABI PRISM 3130*xl* Genetic Analyzer. The base pair sizes of each ladder exported from GeneMapper® *ID* version 3.2 software were evaluated and the individual alleles from each ladder were compared to calculate the standard deviation of base pair size per allele. Fourteen injections of the Identifiler™ (Applied Biosystems, Foster City, CA) allelic ladder over the course of four weeks were compared to one another at each allele and the standard deviation of base pair size per allele was calculated. The average standard deviation for each locus was also calculated.

The precision study serves to determine the extent to which a given set of measurements of the same sample (allelic ladder) agree with their mean, and to the extent to which those measurements match the actual values being measured (32).

CONTAMINATION STUDY

In the contamination study, all negative controls and reagent blanks from the sensitivity study, mixture study, and non-probative case sample study were analyzed for the presence of any amplified PCR product using the AmpFlSTR® Identifiler™ kit (Applied Biosystems, Foster City, CA). The minimum analysis threshold for this kit was determined by evaluating the noise level in the negative controls that were amplified in the validation study. Full reaction chemistry was used in the evaluation of the reagent blanks and negative controls.

The reagent blanks (TE-4 buffer) were amplified as well as the negative amplification controls (sterile water) and detected on the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). The baseline from each of the negative amplification controls was analyzed at 10RFU to determine the noise ratio typical for the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). Five percent of the data around the mean was trimmed to account for any outliers.

The contamination study served to demonstrate that when following correct laboratory procedures, the potential for contamination is small when amplifying the AmpFlSTR® Identifiler™ kit (Applied Biosystems, Foster City, CA).

GENERAL EXPERIMENTAL CONDITIONS

The DNA samples tested throughout the validation study, including those from the sensitivity and mixture studies and non-probative case samples, were processed according to The Forensic Testing Laboratory Forensic Procedure Manual and all procedures followed protocols within that manual for each step of forensic DNA analysis—extraction, quantitation, PCR amplification, and STR fragment analysis. Any deviations from the protocols provided were authorized by the Technical Manager at the laboratory. Those deviations were necessary for the validation of the Identifiler™ (Applied Biosystems, Foster City, CA) kit since the protocols provided described processing of DNA samples with the PowerPlex® 16™ kit (Promega, Madison, WI).

Samples that were prepared from buccal swabs or prepared from non-probative case samples were each placed in a 1.5mL sterile tube. Those requiring extraction (mixture samples, non-probative case samples) followed the laboratory protocol for DNA extraction and purification unless they contained seminal material, in which case they followed a separate laboratory protocol for differential extractions. Samples to be extracted were treated with Qiagen BioRobot® EZ1™ G2 Digestion Buffer, which was supplemented with Proteinase K and DTT. The G2 Digestion Buffer was diluted 1:1 with sterile water.

For each extraction performed, a reagent blank was run alongside the samples. The reagent blank used in each extraction was a 1.5mL tube containing only reagents and no sample. Each tube was vortexed and quick spun, and then incubated at 56°C for at least one hour but not more than three hours. After this incubation, the tubes were then transferred to a 95°C environment for another five minutes, then vortexed and quick spun again. Any samples that included material (cotton, denim) were centrifuged with the use of a spin basket. All remaining

liquid effluent from each sample was transferred to its own 2 mL extraction tube, and those tubes were placed on the Qiagen BioRobot® EZ1™ instrument for extraction.

Samples in the validation study were quantified using the Plexor® HY System (Promega, Madison, WI) in conjunction with the ABI 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). Plexor® HY Male Genomic DNA Standard (Promega, Madison, WI) at a 50 ng/µL initial concentration was used with each quantitation, where the standard DNA was serially diluted. Each dilution of the standard was vortexed briefly between aliquots. The standard dilutions used for each quantitation in the validation study were prepared by adding 10 µL of the previous aliquot to a fresh tube, along with 40 µL of TE-4 buffer, so that a total of seven concentrations were used in the standard dilution series (50 ng/µL, 10 ng/µL, 2 ng/µL, 0.4 ng/µL, 0.08 ng/µL, 0.016 ng/µL, and 0.0032 ng/µL). Standards were quantified in duplicate, and a no-template control (NTC) reaction was included (2 µL of TE-4 buffer). After determining the number of reactions to be set up in each quantitation, reaction mix was prepared by adding 10 µL of Plexor® HY 2X Master Mix, 7 µL of amplification grade water, and 1 µL of Plexor® HY 20X Primer/IPC Mix for a total volume of 18 µL. Samples (2 µL of each) along with the standard dilution series and NTC reactions were distributed to a 96-well optical plate and then covered with an optical adhesive cover. Once the plate was centrifuged, it was transferred to the ABI 7500 (Applied Biosystems, Foster City, CA) linked to a computer with SDS software for real-time PCR, and then after the run on the ABI 7500 (Applied Biosystems, Foster City, CA) the raw data was analyzed using the SDS software. After analysis, the amplification data as well as the melt/dissociation data was exported to a USB drive so that it could be imported into the associated Plexor® Analysis software (Promega, Madison, WI). The data was then used to generate a standard curve by the calculation of the equation for the best-fit line for the linear

regression to the standard dilution series data. The standard curve, per laboratory protocol, should have an average slope between -3.1 and -3.7, and an R2 value greater than or equal to 0.990. After quantitation, samples were prepared for PCR amplification and thermal cycling using the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA). PCR amplification cycling parameters were previously described in the sensitivity study.

Fragment detection and analysis was performed on the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). Run parameters used were those recommended by the Applied Biosystems User's Manual for the 3130*xl* Genetic Analyzer. GeneMapper® *ID* version 3.2 software was used to determine base sizing and allele designations.

CHAPTER III

RESULTS

SENSITIVITY STUDY

In the sensitivity study, complete profiles were obtained at the 0.534ng, 0.846ng, 0.736ng, and 1.232ng amounts of input 9948 control DNA. Profiles were obtained with 1.232ng and 2.931ng of input DNA demonstrated increased artifacts. Incomplete nucleotide addition resulting in split peak formation was found at the D7S820 locus, and there was spectral pull-up from the dyes observed at the FGA and D18S51 loci. Complete profiles were obtained down to 0.342ng of DNA. Allele drop-out was observed at almost every single locus at the 0.1083ng amount or less DNA. No interpretable data was obtained from 0.0293ng or less DNA. Therefore, it was determined that Identifiler™ (Applied Biosystems, Foster City, CA) samples should be amplified at full reaction chemistry, using 28 cycles for amplification, and that those samples should target approximately 0.75ng of input template DNA for optimal quality genetic profiles. Observed stutter was in line with reported observed stutter, and therefore manufacturer's recommendations for percent stutter will be used.

Quantitation of the sensitivity study samples produced acceptable autosomal and Y standard curve slopes and R2 values within range. The autosomal standard curve slope was approximately -3.5, and the R2 value was 0.990. The Y standard curve slope was

approximately -3.43, and the R2 value was 0.993. Screenshots of the PCR curves as well as standard curves from the Plexor® HY System Analysis software are displayed below (Tables 12 and 13).

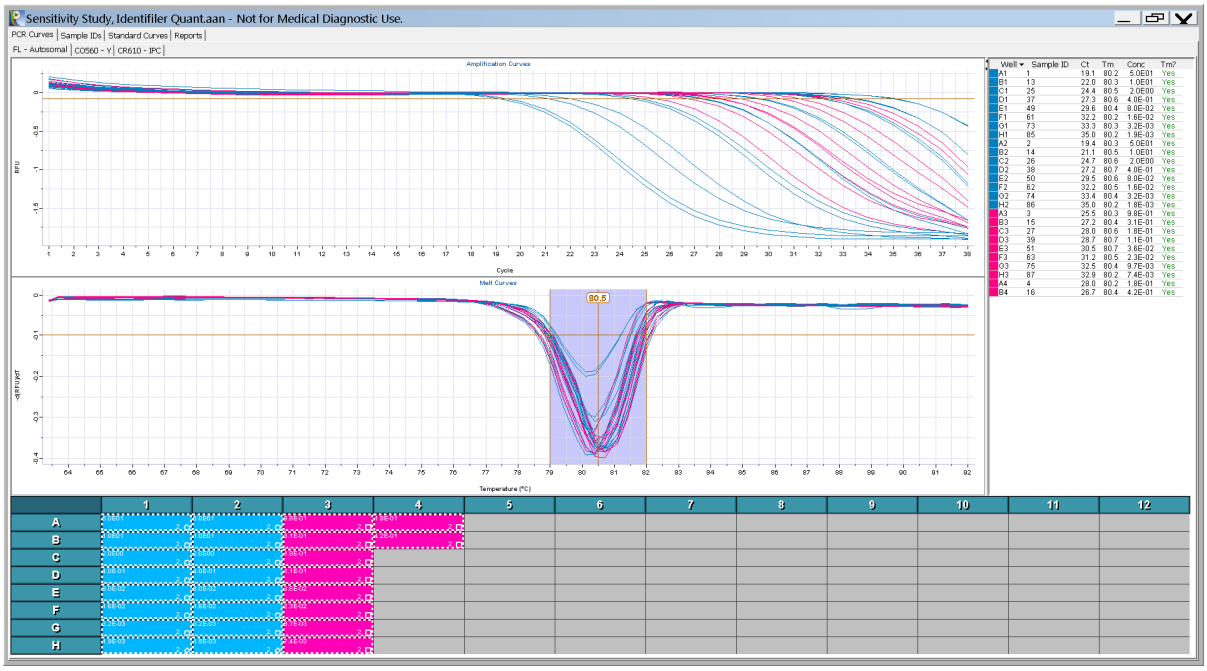

Table 12: Sensitivity Study PCR Curve

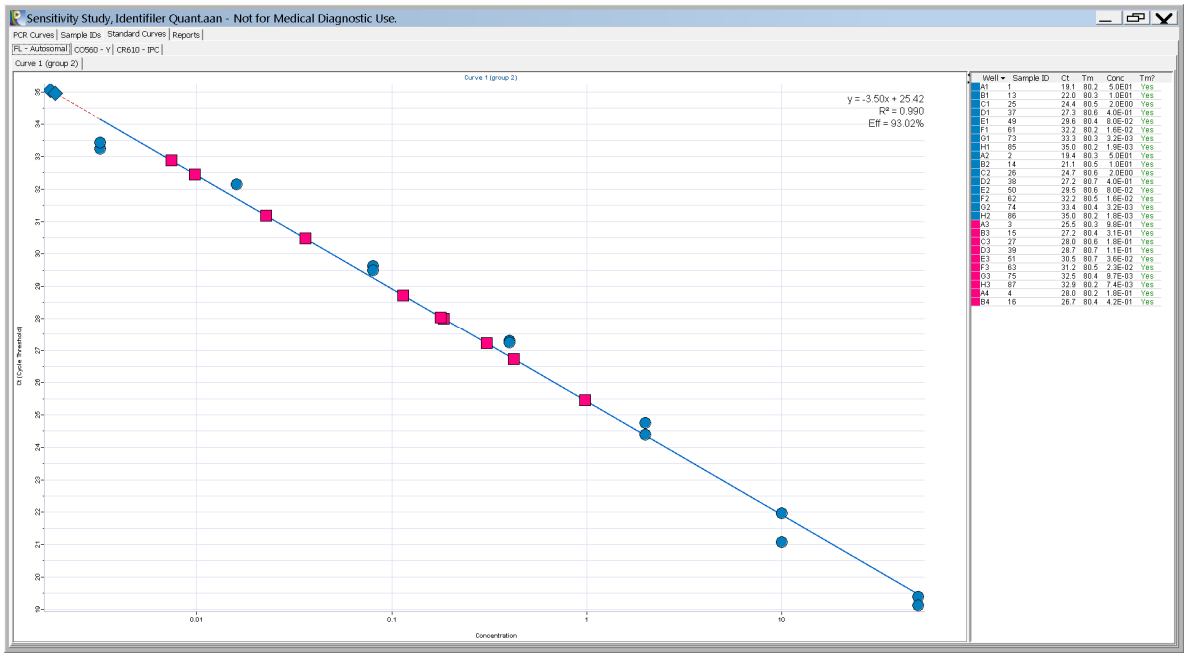

Table 13: Sensitivity Study Standard Curve

The electropherogram data below displays some of the genetic profile observed when 0.736ng of input DNA is added to the sample. A complete profile was identified at this amount, and all of the allele calls were well above the minimum 100RFU threshold. For allele detection, the majority of the alleles called were within the optimal RFU range of 800RFU to 2,000RFU according to the laboratory interpretation guidelines. To be conservative, the 0.736ng amount of DNA was rounded up to 0.75ng. This amount produced optimal quality profiles and will therefore be used as the optimal target amount of DNA for all samples.

Table 14: Sensitivity Study Electropherogram (0.736ng)

Comparing the recommended (0.3µL LIZ® size standard, 8.7µL Hi-Di formamide, 1.0µL amplification product or ladder) versus the increased (0.5µL LIZ® size standard, 19.5µL Hi-Di formamide, 0.8µL amplification product or ladder) load volumes, both showed virtually equivalent quality and RFU values for all samples. Even though they were both relatively similar and both are acceptable practice, it was determined that the Identifiler™ (Applied Biosystems, Foster City, CA) samples be loaded at the increased volume. While complete profiles were identified in the sensitivity study through the 0.342ng sample, heterozygote peak height was optimal (800RFU to 2,000RFU) for the amplification results ranging between 1.232ng and 0.534ng.

The sensitivity study samples that were reinjected for either more or less injection time produced results as expected. By reinjecting the low level samples (0.342ng, 0.1083ng, 0.0681ng, 0.0293ng, 0.0222ng respectively) for more time at 8 seconds rather than the standard 5 seconds, the RFU values were effectively increased. The largest RFU difference was observed in locus CSF1PO at a 169.2% difference, and the smallest RFU difference was in locus D8S1179 at 7.2%. The overall average RFU difference between the 8 second and 5 second injection time changes was 73.4%. Therefore, it was determined that samples tested with the Identifiler™ kit that have data below threshold can be reinjected for 8 seconds to increase the RFU values above threshold (100RFU).

The electropherogram data below displays an example of the difference in peak heights between those low level samples—in this case 0.1083ng—that were injected at the recommended 5 second injection time versus the extended 8 second injection time. The peak heights for all of the alleles in the 8 second injection are clearly higher than those of the 5 second injection time. Also, it is observed that some of the peaks that were not called at all in the 5

second injection are called at the 8 second injection time. As stated above, the overall average RFU difference was 73.4%.

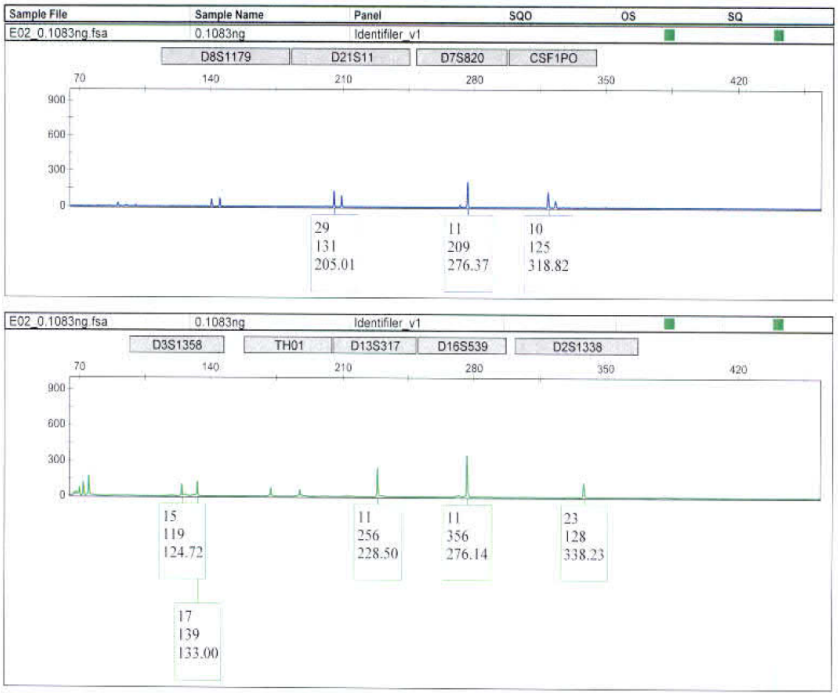

Table 15: 0.1083ng, 5 second injection time

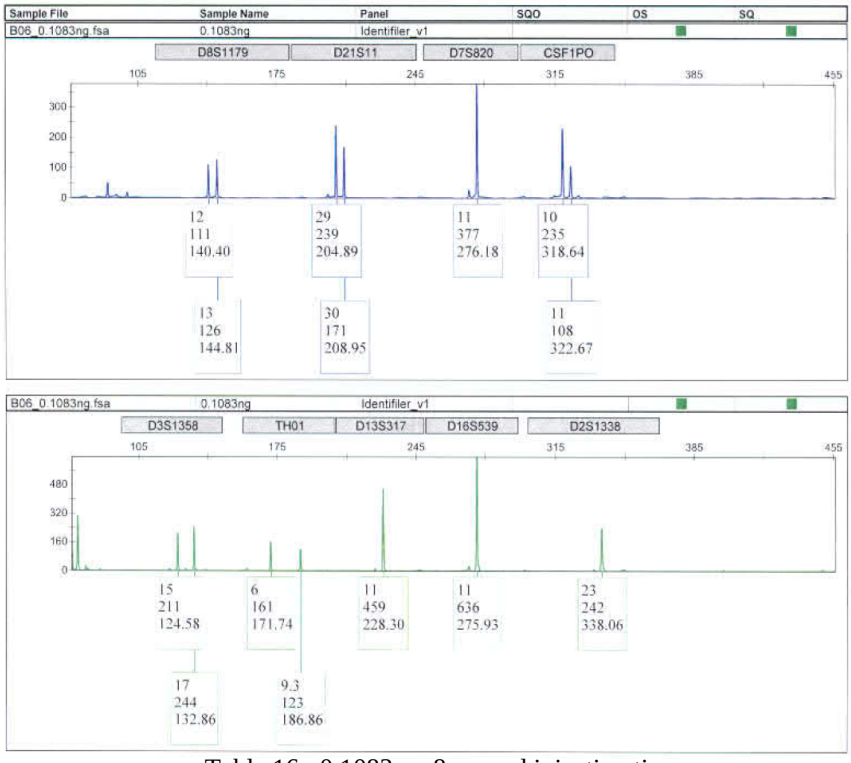

Table 16: 0.1083ng, 8 second injection time

Samples with too much input DNA (2.931ng, 1.232ng) that were reinjected for less time at 3 seconds resulted in an overall average RFU difference of 51.58%. The largest RFU difference between the 3 second and 5 second injections was observed in the FGA locus at 53.7% RFU difference. The smallest RFU difference was observed in locus D19S433 at 49.15%. Therefore it was determined that off-scale samples tested with the Identifiler™ kit can be reinjected for 3 seconds to effectively reduce RFU values and decrease the presence of spectral pull-up and stutter artifacts. Below is an example of electropherogram data (Tables 17 and 18) from the same amount of input DNA (2.931ng) injected at the recommended 5 second injection time (1) versus the decreased 3 second injection time. Note the presence of off-scale data in the run at 5 second injection time.

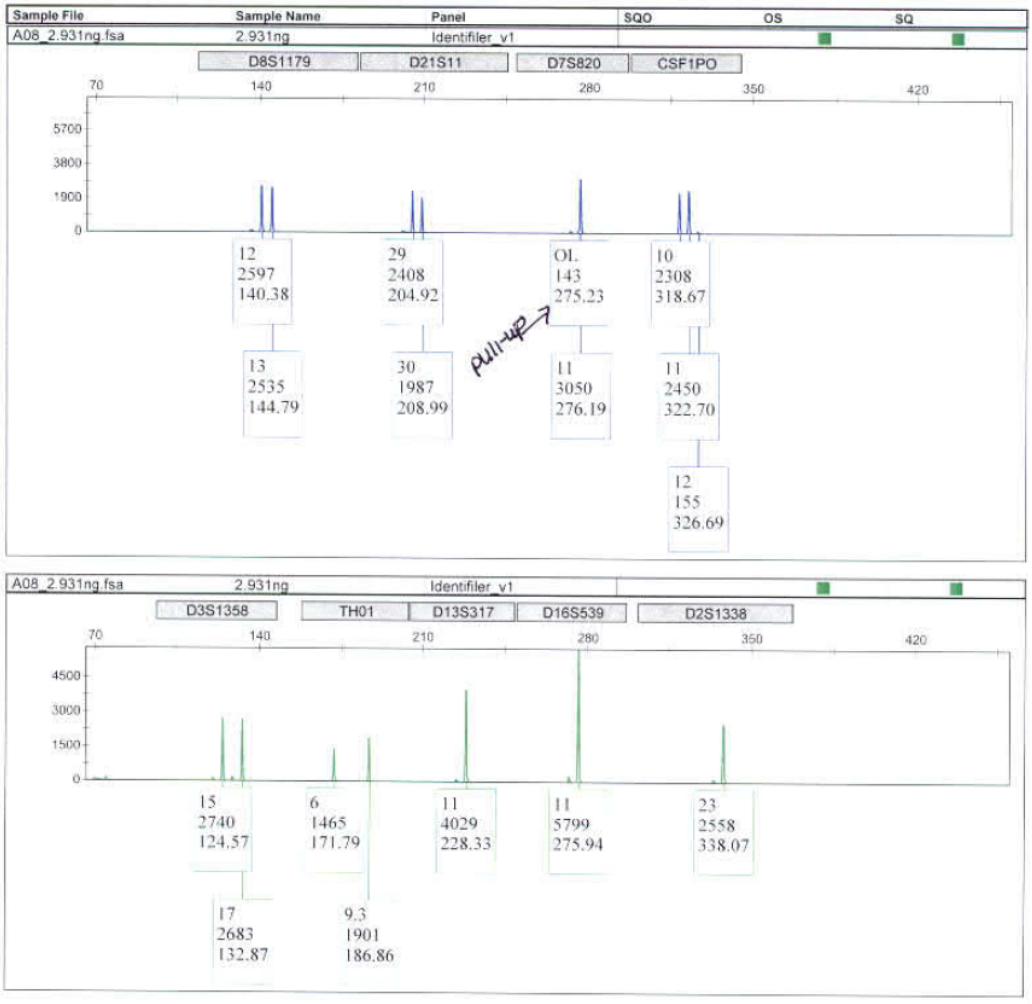

Table 17: 2.931ng, 5 second injection

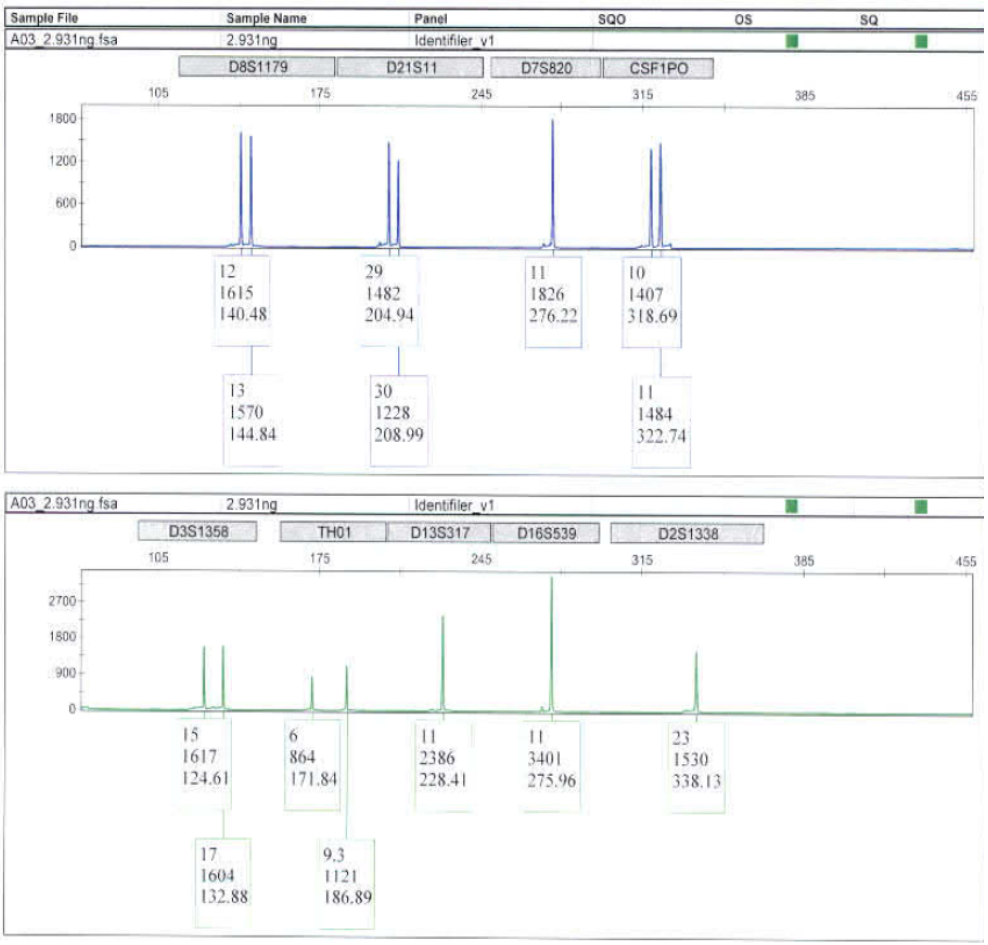

Table 18: 2.931ng, 8 second injection

It was observed that allele 12 of the CSF1PO locus tended to drop out the most often. This allele was maintained in the profiles with 2.931ng input DNA at recommended and increased load volumes. Initial observation of RFU levels below the 100RFU minimum analysis threshold began at the 0.1083ng amount of input DNA. At 0.1083ng of input DNA to the sample at recommended load volume, the majority of the alleles began to drop out, specifically alleles 12 and 13 at D8S1179, allele 30 at D21S11, alleles 11 and 12 at CSF1PO, alleles 6 and 9.3 at TH01, TPOX alleles 8 and 9, D18S51 alleles 15 and 18, D5S818 alleles 11 and 13, FGA alleles 24 and 26, D19S433 allele 14, and both X and Y alleles at amelogenin. Similar results were obtained from the same 0.1083ng amount of input DNA at the increased load volume. At 0.0681ng of input DNA at both recommended and increased load volumes, almost all alleles in

the genetic profile dropped out completely except for the 12 and 13 alleles at locus D8S1179, allele 10 at CSF1PO, allele 11 at D16S539, and alleles 15 and 17 at locus D3S1358. At 0.0293ng of input DNA, all alleles in the profile dropped out except for allele 11 at the D13S317 locus.

MIXTURE STUDY

The purpose of the mixture study is to investigate how the Identifiler™ kit responds to mixed DNA samples at recommended full reaction chemistry (25µL reaction volume). Two separate mixture studies were completed. The study involving mixture ratios of increasing female DNA to constant male DNA showed that very minor male peaks below threshold were detected at the female to male 25:1 ratio. As the ratio increased to 50:1 and 100:1, no significant amount of male DNA was detected as most alleles dropped out. This shows that the Identifiler™ kit at full reaction shows sensitivity to male DNA when mixed with higher amounts of female DNA but the male DNA starting at the 25:1 ratio cannot be interpreted.

In the second part of the mixture study, male DNA to female DNA at ratios ranging from 20:1 (male:female) to 1:20 were studied, and it was determined that no mixture was detected at the 20:1 ratio. However, the 1:20 ratio exhibited a few alleles below threshold. Major profiles were determined between the 10:1 and 1:10 ratios, and partial minor profile determination was also possible. Major and minor profiles were obtained at the 3:1 and 1:3 ratios. No significant minor DNA was observed at the 20:1 and 1:20 ratios.

The table below (Table 19) demonstrates that mixed DNA profiles are best observed between 3:1 and 1:3 ratios. In the table, peak height ratios were calculated for each allele at each locus when there was a mixture detected. The average peak height ratio per mixture ratio was

calculated as well. For those loci where no mixture was detected (indicated by ^), the male and female profiles were identical as well as homozygous at that locus.

Table 19: Mixture Study Peak Height Ratios

		20:1		10:1		3:1		1:1		1:3		1:10		1:20		
Major Profile	High to Low A >	Minor/ Major A04	Minor/ Major A05	Minor/ Major B04	Minor/ Major B05	Minor/ Major C04	Minor/ Major C05	Minor/ Major D04	Minor/ Major D05	Minor/ Major E04	Minor/ Major E05	Minor/ Major F04	Minor/ Major F05	Minor/ Major G04	Minor/ Major G05	High to Low <-- H
D8S1179	11,13	^	^	^	^	26%	^	50%	73%	57%	87%	19%	22%	*	^	14,15
D21S11	28,31	18%	13%	12%	26%	16%	44%	45%	31%	82%	77%	56%	37%	*	^	28,29
D7S820	10,13	^	^	^	^	34%	^	71%	71%	94%	50%	42%	^	*	^	11,12
CSF1PO	10	^	^	^	^	90%	^	72%	67%	79%	77%	94%	37%	*	14%	10,12
D3S1358	14,15	2%	15%	^	^	22%	27%	53%	58%	90%	97%	18%	30%	*	52%	14,17
TH01	7,9	14%	^	20%	25%	84%	68%	44%	58%	38%	39%	11%	^	*	^	9.3
D13S317	12,13	^	^	^	^	^	^	29%	27%	74%	70%	66%	^	*	^	8,12
D16S539	9,10	^	^	12%	^	38%	^	61%	48%	95%	65%	^	^	*	^	12,13
D2S1338	19,23	^	^	^	^	22%	^	59%	87%	47%	47%	^	^	*	^	17,19
D19S433	13,14	^	^	^	^	26%	^	76%	60%	98%	55%	^	26%	*	^	13.2,16
vWA	14	^	^	^	^	89%	^	86%	80%	55%	54%	45%	^	*	28%	14,18
TPOX	8	^	^	^	^	^	^	^	^	^	^	^	^	*	^	8
D18S51	12,18	^	^	^	^	35%	^	58%	71%	92%	88%	^	^	*	^	15,16
D5S818	11,13	^	^	^	^	^	^	60%	65%	95%	84%	31%	^	*	^	12
FGA	20,24	^	^	^	^	36%	^	47%	^	62%	71%	^	^	*	^	19, 23
AMEL	X,Y	1%	10%	33%	27%	7%	64%	75%	46%	72%	82%	^	75%	*	^	X
	AVG:	9%	13%	19%	26%	40%	51%	59%	60%	75%	70%	42%	38%		31%	

* = RFU data below 100

^ = No mixture detected

The mixed source genetic profile at the 1:1 ratio of male to female DNA is displayed below (Table 20). Note that all male and female alleles are present in the sample.

Table 20: Mixture Study Electropherogram, 1:1 Ratio

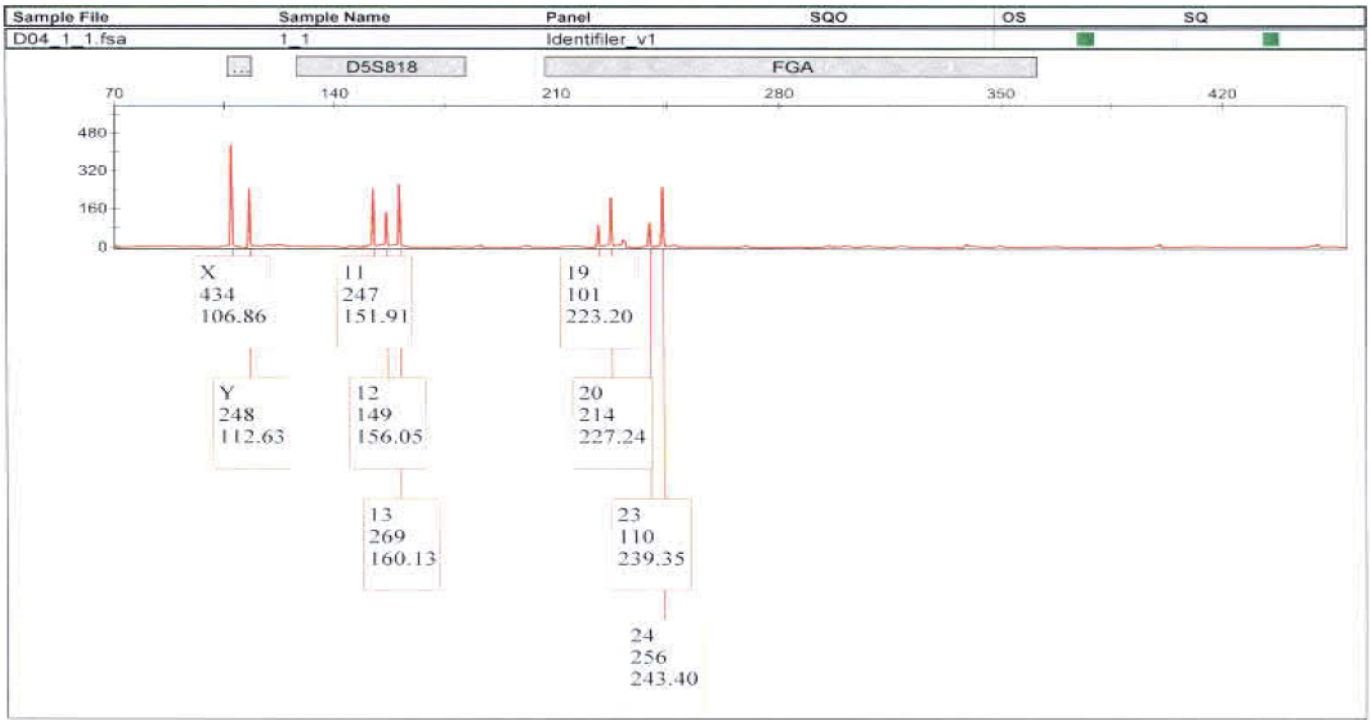

Table 20: Mixture Study Electropherogram, 1:1 Ratio

NON-PROBATIVE CASE SAMPLE STUDY

The purpose of the non-probative case sample study is to investigate how the Identifiler™ kit responds to simulated non-probative samples that resemble typical examples of forensic casework. Of the twenty samples in this study, all produced acceptable genetic profiles and the profiles were consistent with expected results. Therefore, it was determined that the Identifiler™ chemistry is a robust method for typical forensic casework samples. The autosomal and Y quantitation results obtained also correlated with the quality of each profile obtained. Listed below are the non-probative case samples (Table 21) along with their item numbers and quantitation results. A key is also provided (Table 22), which lists the donors of biological material to each non-probative case sample.

Sample Description	Item Number	Auto (ng/µl)	Y (ng/µl)
Saliva on filter paper	1	0.981	N/A
10µl Blood on filter paper	2	2.56	4.10
10µl Semen on filter paper	3N 3S	3.84 0.156	3.56 0.186
2.5µl Blood + 5.0µl Semen on filter paper	4N 4S	1.10 0.0008	1.35 0.00231
Saliva on styrofoam cup	5	0.405	N/A
Piece of chewing gum	6	0.398	N/A
Licked envelope	7	0.24	N/A
Fingernail swab	8	0.107	0.000556
Buccal swab + 10µl Blood	9	9.74	2.10
Buccal swab + 10µl Semen	10N 10S	48.4 4.16	19.2 4.27
Neck swab + 10µl Semen	11N 11S	30.2 0.0356	25.0 0.0509
Eyeglass Nosepiece Swabbing	12	0.124	N/A
Swab from Sweatband of Hat	13	0.102	0.0166
10µl Blood on Twig	14	1.66	2.44
20µl Blood on khaki shorts	15	7.6	10
10µl Blood Smear on khaki shorts	16	0.349	0.321
20µl Blood on Denim	19	0.295	0.307
10µl Blood Smear on Denim	20	0.023	0.0164
10µl Semen on Denim	21N 21S	17.0 0.52	18.0 0.650
2.5µl Blood + 5.0µl Semen on Denim	22N 22S	0.0275 0.014	0.0375 0.0209

N = Nonsperm fraction
S = Sperm fraction

Table 21: Quantitation Results of Non-Probative Case Samples

Table 22: Non-Probative Case Sample Donor Key

Sample Description	Biological Material Donors
Saliva on filter paper	J2
10µl Blood on filter paper	A
10µl Semen on filter paper	Semen X
2.5µl Blood + 5.0µl Semen on filter paper	A + Semen X
Saliva on styrofoam cup	C
Piece of chewing gum	P
Licked envelope	P
Fingernail swab	J2
Buccal swab + 10µl Blood	J2 + A
Buccal swab + 10µl Semen	J2 + Semen X
Neck swab + 10µl Semen	J2 + Semen X
Eyeglass Nosepiece Swabbing	L
Swab from Sweatband of Hat	J1
10µl Blood on Twig	A
20µl Blood on khaki shorts	A
10µl Blood Smear on khaki shorts	A
20µl Blood on Denim	A
10µl Blood Smear on Denim	A
10µl Semen on Denim	Semen X
2.5µl Blood + 5.0µl Semen on Denim	A + Semen X

Using Plexor® HY Analysis software, a PCR curve as well as a standard curve was also generated for the non-probative case samples. The standard curve properties were all within range and acceptable for further testing of the samples. The autosomal R2 value was 0.999, and the observed Y value R2 was 0.995. The autosomal slope was -3.88 and the Y slope was -3.56. Shown below are screenshots (Tables 23 and 24) of the autosomal PCR curve and the autosomal standard curve graphs from the Plexor® HY Analysis software.

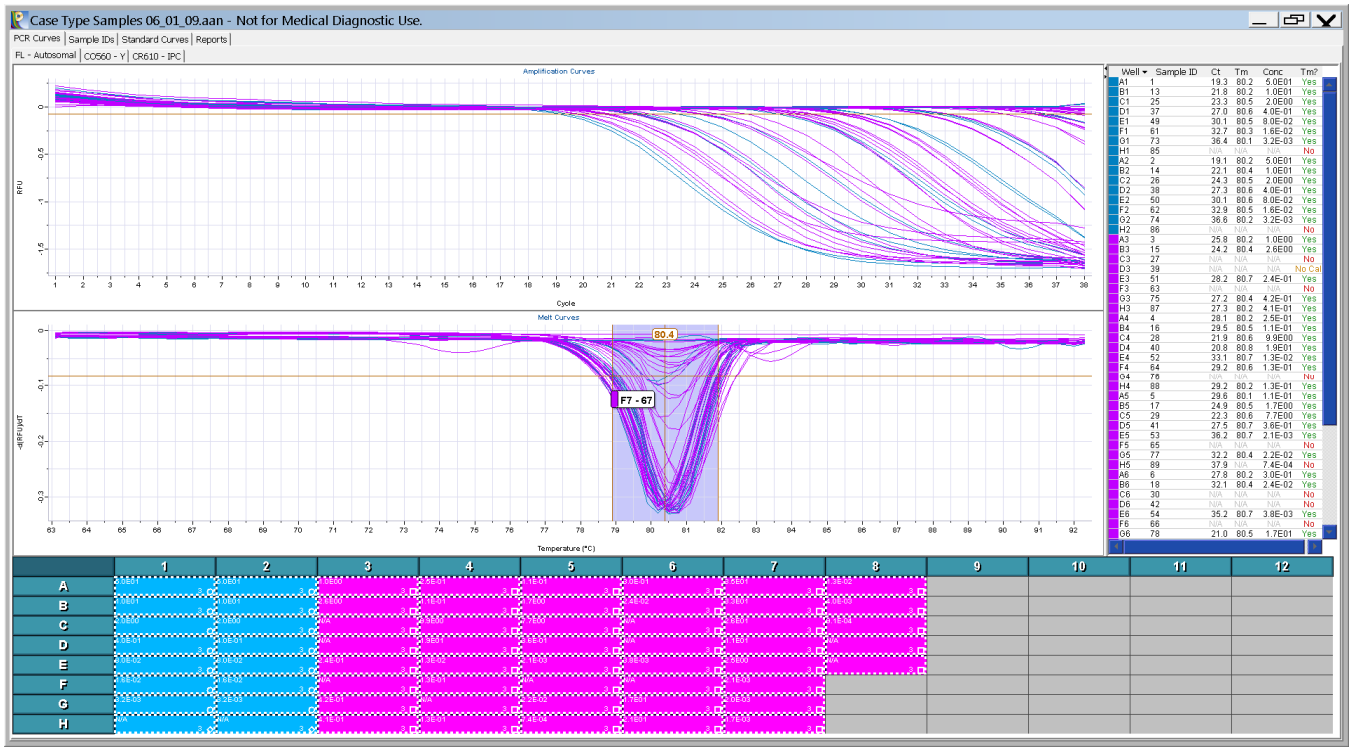

Table 23: Non-Probative Case Sample Study - PCR Curve

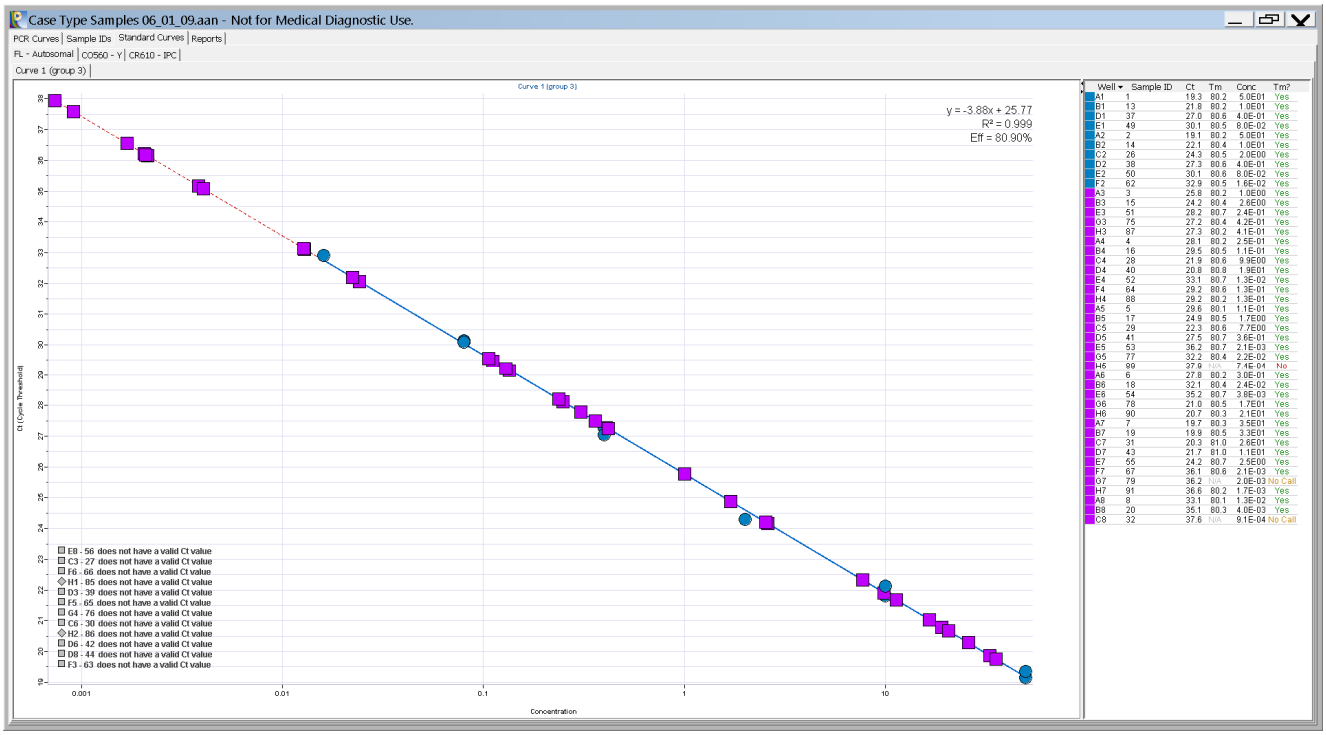

Table 24: Non-Probative Case Sample Study - Autosomal Standard Curve

Reference profile DNA from laboratory employees were compared against the profiles generated from the non-probative case samples, and alleles corresponded between profiles as expected. Below, tables (Table 25 and 26) comparing the reference profile from biological donor "A" and the genetic profile obtained from non-probative case sample #19 (20µL blood on denim) are provided.

A	
D8S1179	11,13
D21S11	28,31
D7S820	10,13
CSF1PO	10
D3S1358	14,15
TH01	7,9
D13S317	12,13
D16S539	9,10
D2S1338	19,23
D19S433	13,14
vWA	14
TPOX	8
D18S51	12,18
AMEL	X,Y
D5S818	11,13
FGA	20,24

Table 25: Reference Profile from donor "A"

Sample 19	
D8S1179	11,13
D21S11	28,31
D7S820	10,13
CSF1PO	10
D3S1358	14,15
TH01	7,9
D13S317	12,13
D16S539	9,10
D2S1338	19,23
D19S433	13,14
vWA	14
TPOX	8
D18S51	12,18
AMEL	X,Y
D5S818	11,13
FGA	20,24

Table 26: Profile obtained from sample (19)

Some of the samples that required differential extractions had to be re-created, re-extracted, and re-quantified: samples 3, 4, 10, 11, 21, and 22. The new quantification values for these were still within range, with an autosomal R2 value of 0.997 and autosomal standard curve slope of -3.66. The observed Y values were also acceptable, with an R2 of 0.994 and standard curve slope of -3.56. An example electropherogram of one of the non-probative case samples is displayed below. This sample is saliva on filter paper, and the profile obtained is consistent with the profile obtained from donor J2.

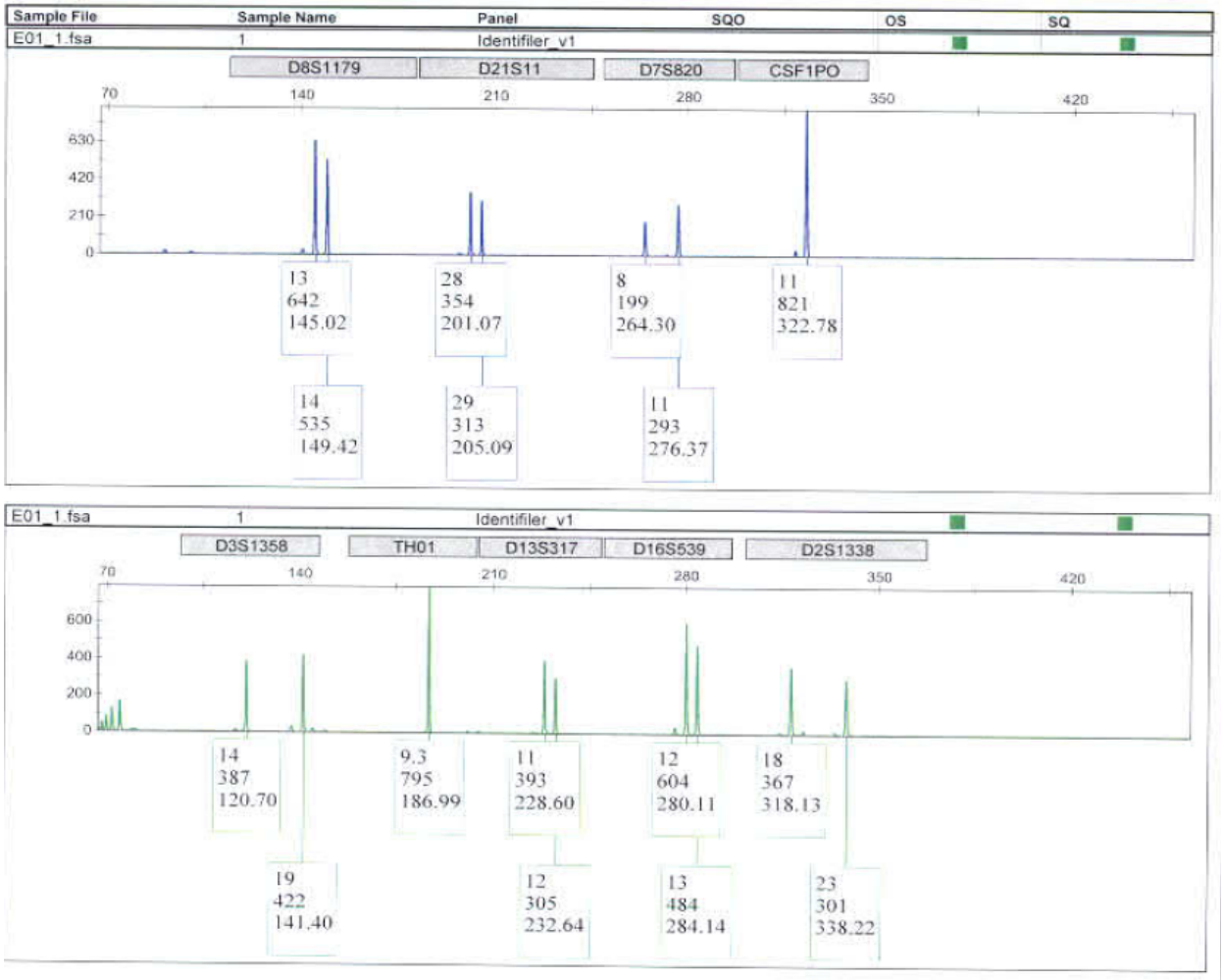

Table 27: Example genetic profile, Non-Probative Case Sample (1) (saliva on filter paper)

Most of the non-probative case samples were chosen to be reinjected on the ABI Prism 3130*xl* for longer time at 8 seconds instead of the recommended 5 second injection time. These samples were run on the same 96-well load plate as the sensitivity study samples that were reinjected for 8 seconds. Differences in peak heights were observed, and when injected for more time, some of the low copy number DNA samples had allele calls where there were none in the original 5 second injection. A comparison of non-probative case sample #5 (saliva on rim of Styrofoam cup) demonstrates the difference in peak heights between the 5 second injection time and the extended 8 second injection time.

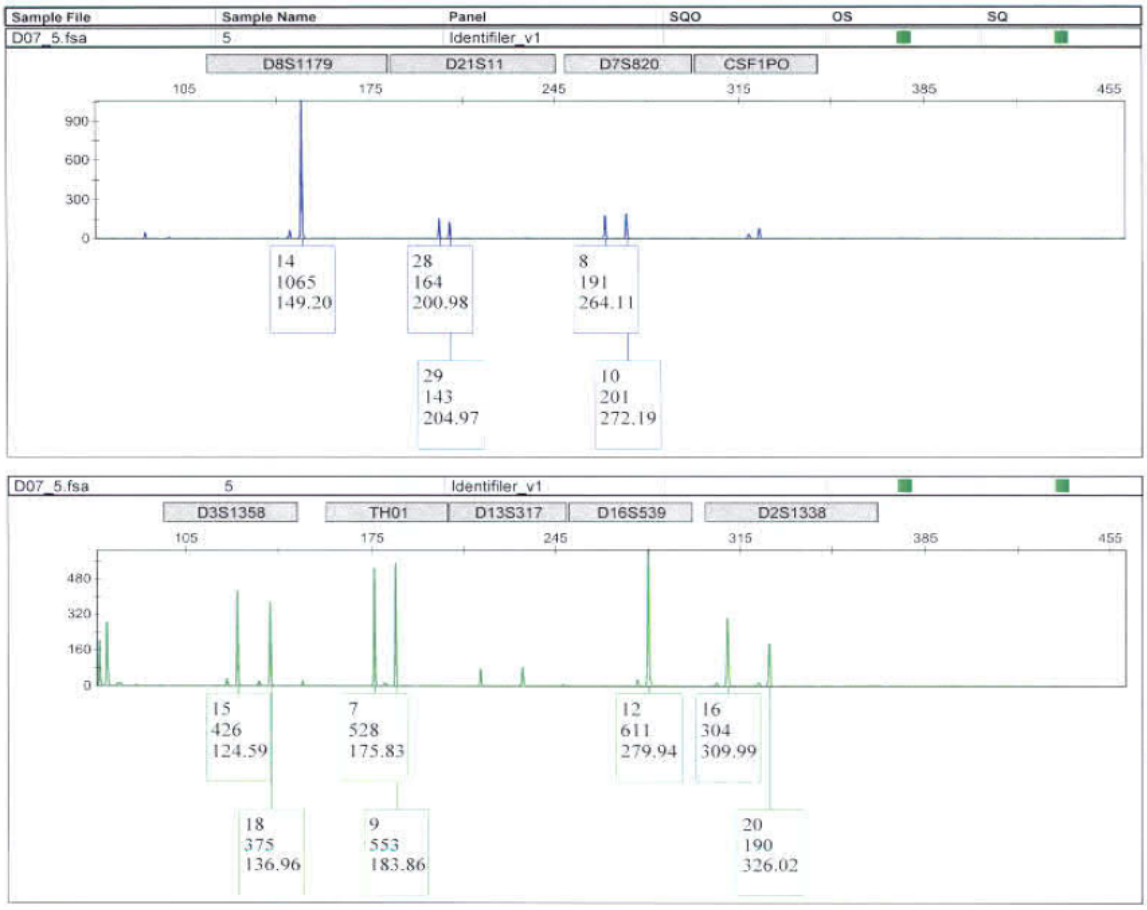

Table 28: Non-Probative Case Sample (5) - 5 second injection time

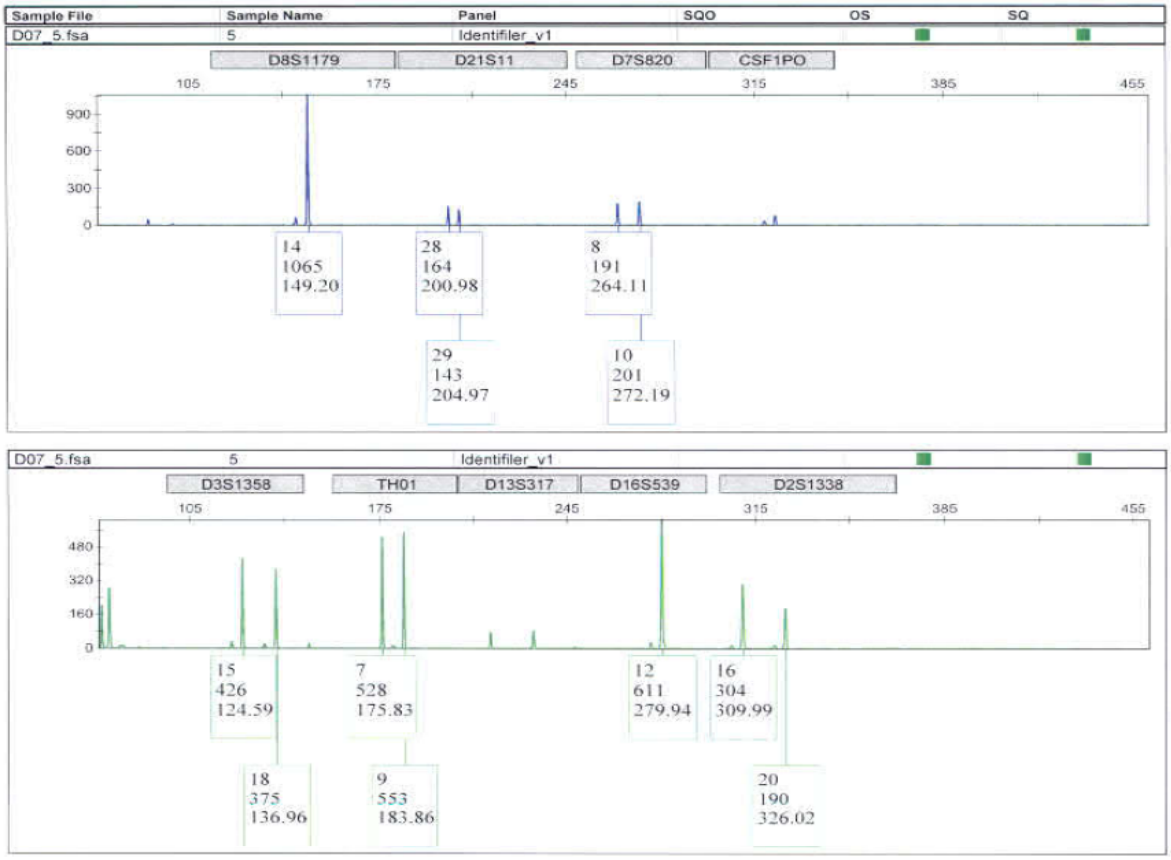

Table 29: Non-Probative Case Sample (5) - 8 second injection time

In the examples above, only partial profiles were identified for both injection times. However, alleles that were not called at all in the original 5 second injection were called once the time was extended another 3 seconds. In the 5 second injection, no alleles were above callable threshold at D21S11, D7S820, CSF1PO, D13S317, vWA, D18S51, FGA, and amelogenin. In the 8 second injection time change, alleles were called at D21S11, D7S820, vWA, D18S51, and gender was determinable at amelogenin whereas it was not in the 5 second injection. Non-probative case samples reinjected for 8 seconds were the following (by item number): 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 19, 20, 21, and 22. Overall, the injection time change aided in effectively increasing the peak heights of most alleles. However, some allele dropout remained.

PRECISION STUDY

The precision study serves to investigate the precision of the Identifiler™ kit on the ABI Prism 3130*xl* instrument by studying the standard deviation of base pair sizes between allelic ladder injections throughout the validation study. All runs throughout the validation study contained multiple allelic ladders to ensure precision and accuracy as well as err on the side of caution. The precision data is used to help in making allele assignments for outlier and variant alleles (26). In this precision study, the maximum standard deviation observed for any single allele was approximately 0.1317 base pairs. This was observed in the FGA locus. The FGA locus had an overall average standard deviation of 0.1055 base pairs. The locus with the lowest average standard deviation was D19S433 at 0.0313 base pairs. The standard deviation for all remaining loci fell between these two values. The precision observed for the four weeks of allelic ladder data showed a maximum standard deviation of 0.1055 base pairs. This is well within the required precision of 0.5 base pairs. This indicates the run conditions on the ABI Prism 3130*xl* are very consistent, and Identifiler™ can be run with excellent precision. To ensure precision in the future, runs with multiple injections will also contain multiple allelic ladders. The precision data showed the extent to which the base pair sizes of the multiple injections of the same Identifiler™ allelic ladder agreed with their mean value, and the extent to which the standard deviations matched the actual values being measured.

CONTAMINATION STUDY

The contamination study overall showed no contamination in any of the results throughout the course of the validation study. The reagent blanks and negative controls were evaluated and the minimum threshold analysis parameters were determined by analyzing the

baseline of the amplified negative controls. It was determined that when following correct laboratory procedures, the potential for contamination is small when the Identifiler™ PCR Amplification Kit is used in the laboratory.

Negative amplification controls were analyzed at 10 RFU and peak heights larger than 10 RFU were used in the evaluation of the baseline. 5% of the data around the mean was trimmed to account for any outliers. The trimmed mean RFU was 14.87 RFU for the full reaction. It was determined that five times the mean noise RFU would be a conservative minimal RFU analysis threshold. The trimmed mean multiplied by five was 74.34 RFU for the full reaction chemistry using Identifiler™. The routine analysis threshold for Identifiler™ (all controls and samples) is typically 100 RFU. However, it was determined in the contamination study that it is acceptable to drop the threshold to 75 RFU for the Identifiler™ kit. No analysis should be performed below the 75 RFU threshold except for possible contamination investigation purposes.

CHAPTER IV

DISCUSSION

The results from each experiment in the internal validation study at the Forensic Testing Laboratory are acceptable and successful, with profiles obtained at a high percentage with few samples needing to be repeated. This study demonstrates that AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) is a robust, reliable, and reproducible method in the hands of the personnel performing the test in the laboratory while following the standard operating protocols and documenting the entire processing of the sample(s). With Identifiler™ successfully validated at The Forensic Testing Laboratory, the laboratory can now create new standard operating protocols (SOPs) with interpretation guidelines based on the validation study, and training of other personnel on the technique may begin.

The data obtained from the non-probative case sample study reveals that reportable profiles may be obtained from a variety of samples on different substrates, and those results showed no background reaction with any of the substrates. For instance, the denim that was used in a few of the non-probative case samples did not appear to inhibit PCR, since quality profiles were obtained from those samples. The study of non-probative case samples and the

evaluation of their profiles confirms that the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) is reliable to use with forensic casework under the standard operating protocols established by The Forensic Testing Laboratory.

The precision experiment demonstrates the reproducibility and repeatability of the method. It indicates what kind of variability may be expected when Identifiler™ (Applied Biosystems, Foster City, CA) is used by a single analyst on one instrument, such as the ABI Prism 3130*xl*, over a short time frame, such as a six-week duration (23). The precision study also demonstrates the sort of variability to be expected between results when a sample is analyzed in duplicate (23). The precision experiment in this validation study shows that the precision of size determination is satisfactory for discrimination between alleles.

After the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) is validated, The Forensic Testing Laboratory can establish interpretation guidelines and modify protocols for use with this particular kit. Now that the optimal template DNA concentration needed for Identifiler™ has been found to be 0.75 ng/μL in the sensitivity study, personnel in the future know how much DNA is required for an optimal profile in forensic casework. The same applies to the mixture analysis study. The laboratory can establish interpretation guidelines for mixtures, differentiate between single source and multi-source DNA samples, and differentiate between multi-source samples and single-source samples that may have a high prevalence of stutter peaks.

The results of the non-probative case sample study can be used to establish interpretation guidelines for various types of forensic casework, when biological material containing DNA may be on different types of substrates that may or may not inhibit PCR amplification.

Partial profiles or complete profiles were obtained for almost all of the non-probative case samples with the use of the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA), implying that the Identifiler™ kit responds well to a variety of samples, from blood on denim to low copy number samples such as sweat from the nosepiece of eyeglasses.

Overall, the internal validation study for the use of the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) will help The Forensic Testing Laboratory to establish interpretation guidelines and revise standard operating protocols as needed. With the addition of this kit to the other multiplex kits (such as PowerPlex® 16™ from Promega) already validated at this laboratory, personnel can now be trained using new methodology and new techniques and therefore further expand upon the capabilities of the laboratory and increase throughput of DNA samples.

Based on the results of the sensitivity study, I would recommend that alleles may be called with confidence when an input amount of DNA ranges between 0.5ng and 1ng, and optimal profiles may be interpreted with the 0.75ng amount of input template DNA. Low copy number samples with the majority of the peaks between 100RFU and and 200RFU, or with peaks that fall below the 100RFU threshold, may be reinjected for more time to bring those alleles up into an interpretable range. Single source profiles that should not have more than two alleles per locus, and alleles that fall below 100RFU should be approached with caution. Based on the calculations from the precision study, I would recommend that the 100RFU threshold be lowered to 75RFU to aid in interpretation of such alleles. Injection times for samples amplified with the Identifiler™ kit produce confident and reproducible allele calls should be ranging between 5 and

8 seconds. I would recommend that multiple allelic ladders be injected at the beginning and end of every set of 10 samples.

As for mixture interpretation guidelines based on the results of the mixture study using the Identifiler™ kit, I would recommend that the average peak height ratio optimal for interpreting mixed source samples be between 50% and 70%. 3:1, 1:1, and 1:3 ratios of male DNA to female DNA appeared to produce the best examples of mixed source profiles. Low copy number samples that may be mixed source should be interpreted with caution, since a sister allele below the 100RFU threshold may be mistaken for a stutter artifact. Such samples may be reinjected for 8 seconds rather than 5 seconds to bring these alleles into an interpretable range, and GeneMapper® *ID* version 3.2 software thresholds may be lowered to 75RFU to aid in interpretation.

No incidences of contamination occurred throughout the duration of the validation study. The risk of contamination was minimized in the laboratory. Gloves were worn at all times, pipette tips were replaced as needed during any transfer of liquids, separate fume hoods were utilized for PCR reagents and DNA samples, and amplification and STR analysis of samples was contained to a separate laboratory room. Reagent blanks were incorporated into every extraction, and positive and negative controls were used throughout the validation study. If contamination were to occur, corrective action would take place after finding the source of the contamination. For instance, if a reagent blank had any PCR products in its profile, then re-extraction of the sample would be necessary. If contamination occurred in a negative control, the sample would need to be amplified again.

CHAPTER V

CONCLUSIONS

The study of short tandem repeat DNA is becoming more prevalent in the forensic science community across the world, since tetranucleotide repeats are amplified with better reliability than dinucleotide repeats, and they produce robust results (17). The internal validation of Identifiler™ (Applied Biosystems, Foster City, CA) is critical in order for it to be used in forensic casework. Since the validation study has been completed, The Forensic Testing Laboratory (FTL) now has the ability to develop interpretation guidelines based on the results of the sensitivity study, mixture study, and so forth. A minimum interpretation threshold, in RFU, may be adopted based on the results of the validation study.

Each experiment (sensitivity study, mixture study, non-probative case sample study, precision study, contamination study) is concordant with SWGDAM guidelines for internal validation of a new technique, instrument, or kit used in a forensic DNA laboratory (32). Developmental and internal validation studies are necessary for PCR-based forensic laboratory testing to not only explore new, more effective methods, but also to reinforce the quality of an already established method. Validation studies, in general, exist to prove whether or not a procedure that follows scientific method is actually following scientific method.

Auditors must review anything in a PCR-based forensic lab that has been recently validated. Audits, certification, and accreditation are all important components of forensic lab quality assurance procedures because they join forces to preserve and maintain the integrity of casework evidence and the environment(s) they are stored and analyzed in. Based on the results of this internal validation study, the AmpFlSTR® Identifiler™ (Applied Biosystems, Foster City, CA) is a reliable product to use with forensic casework at The Forensic Testing Laboratory.

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