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VALIDATION OF THE POWERPLEX 16 STR SYSTEM AT THE

HARRIS COUNTY MEDICAL EXAMINERS OFFICE

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VALIDATION OF THE POWERPLEX 16 STR SYSTEM AT THE HARRIS COUNTY MEDICAL EXAMINERS OFFICE

INTERNSHIP PRACTICUM REPORT

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CHAPTER I

INTRODUCTION

The amplification and detection of extracted DNA are essential steps in the processing of forensic case work for DNA typing. Over the years the forensic community has strived to improve the techniques used for DNA typing. The methods used for DNA typing have advanced significantly due to the use of the Polymerase Chain Reaction (PCR) technique developed in 1985 by Kary Mullis [1]. This technique provides the ability to amplify minute amounts of DNA at specific regions of interest called short tandem repeats (STR's). The PCR reaction is well adapted for DNA amplification because it is sensitive, rapid, and has the potential to analyze degraded samples. The evolution of DNA typing methods has progressed from our ability to analyze one DNA region of interest at a time with the restriction fragment length polymorphism (RFLP) procedure to typing several STR markers using multiplexing PCR methods. A total of 13 specific STR markers were selected by the FBI in order to standardize analysis of DNA for use in a nation wide database [2]. The Combined DNA Index System (CODIS) is the database developed to provide the comparison between crime scene evidentiary samples and known samples from previously convicted criminals. Commercial kits are available which provide forensic laboratories the ability to amplify the 13 core STR loci required for the CODIS database. One such amplification system is the AmpFℓSTR® Profiler Plus™ PCR amplification kit (Applied Biosystems, Foster City, CA). The AmpFℓSTR® Profiler Plus™ kit amplifies 9 of the 13 CODIS loci plus the Amelogenin sex typing marker. This kit is used in conjunction with a sister kit, the AmpFℓSTR® COfiler™ PCR

amplification kit which amplifies the additional 4 core loci along with 2 overlapping loci (D3S1358 and D7S820) and Amelogenin. Promega Corporation (Madison, WI) has developed the PowerPlex® 16 PCR multiplex system which amplifies the 13 core STR loci plus two additional STR systems and Amelogenin in a single PCR reaction. The Harris County Medical Examiners (HCME) Office located in Houston, Texas has validated the AmpFℓSTR® Profiler Plus™ and COfiler™ PCR Amplification system for use in their DNA laboratory. The HCME DNA laboratory was interested in incorporating the newly improved PowerPlex® 16 PCR system (Promega Corp., Madison, WI) for DNA amplification and detection purposes. The use of the PowerPlex® 16 system would provide a single amplification system while maintaining the sensitivity and robustness required for forensic DNA testing. Prior to implementation in casework, the HCME laboratory was required to perform internal validation experiments to assess the performance and limitations of the system in the laboratory. The study undertaken at HCME was designed to fulfill the requirements mandated by national standards issued by the Director of the FBI and guidelines issued by the Technical Working Group on DNA Analysis Methods (TWGDAM).

CHAPTER II

BACKGROUND

Polymerase Chain Reaction

The use of the polymerase chain reaction (PCR) is routinely utilized by forensic laboratories for the identification of human remains and the analysis of evidentiary samples [3], [4]. Limited amounts of DNA extracted from evidentiary samples are amplified using the PCR process to produce over 1 billion copies of the specific DNA regions of interest [5]. The PCR amplifies DNA by combining the double stranded (ds) DNA segment to be amplified (template DNA) along with forward and reverse primers which are short single stranded (ss) oligonucleotides complementary to short sequences on the template DNA. The previous components are combined with dinucleotide triphosphates and DNA polymerase which is an enzyme that propels the extension of the new DNA strand [2]. Exponential amplification of the STR marker occurs through a repetitive process of denaturation, annealing and extension which constitutes a cycle. Typically about 25-40 thermal cycles are required to amplify DNA in the PCR reaction. Development of the PowerPlex® 16 PCR system (Promega Corp., Madison, WI) has shown that a total of 32 cycles for an input of 0.5ng - 1.0ng of amplification is required to generate a profile with DNA templates [6], [7].

Degraded fragments of DNA of only a few hundred base pairs in length can still serve as effective templates for amplification [2], [8]. Simultaneous amplification of multiple STR markers is possible with multiplex PCR reactions. Through the use of highly specific human primers, contamination from exogenous DNA originating from

fungal or bacterial sources does not interfere or produce foreign PCR amplification products [2].

Multiplexing PCR Reactions

A Multiplex PCR reaction requires the stringent optimization of all components in order to perform the simultaneous amplification of two or more regions of DNA. The use of commercially available multiplex PCR amplification kits provides several significant advantages over the older single-plex reactions. Multiplex systems reduce the amount of template required for the analysis of the core 13 loci resulting in smaller sample consummation. Multiplex PCR kits provide the ability to generate more genetic information in a shorter span of time. When multiple loci are amplified in one reaction, data collection time can be reduced while producing an increased power of discrimination. In addition multiplex kits minimize the amount of labor time for forensic analysts in the lab, providing more time for data analysis [6], [7].

The quality of primers used in the multiplex reactions is extremely important because they control which set of sequences or STR's get amplified. The primers used in the PCR reaction must be complementary to a specific sequence which assures that only human or higher primate DNA is amplified. If non specific binding occurs, the DNA at that location will be amplified and nonspecific products will be produced which could complicate the interpretation of the results [2].

Application of Short Tandem Repeats

The discovery of short tandem repeats, STR's, occurred in 1993 [1]. STR's are regions in DNA contain a repeat sequence that is between 2-7bp in length. The number of repeat units found at these STR markers allows the individualization of one human from the next making these loci highly effective for human identification testing. STR polymorphisms have become the standard genetic markers used throughout the world for development of forensic databases and casework. Currently the STR loci that are being analyzed at the HCME contain tetranucleotide repeats in which there are 4 bases per STR repeat unit. The PowerPlex® 16 PCR system (Promega Corp., Madison, WI) amplifies two loci containing pentanucleotide Penta D and Penta E, which contain a pentanucleotide (5bp repeat) [6]. The PowerPlex® 16 PCR system was the first commercially available multiplex STR system to co-amplify 16 loci, including the 13 core CODIS STR loci and the sex marker Amelogenin in a single amplification.

Progression of DNA typing

Prior to PCR which was developed by Kary B. Mullis in 1985 [1], the process of DNA identification was carried out by a process referred as the restriction fragment length polymorphism analysis (RFLP) [3],[6]. The RFLP method was slow and laborious compared to STR analysis. The analysis of low copy number and degraded samples was not possible using the RFLP method. The PCR process allows the analysis of STR alleles ranging from 100-400bp, were as the RFLP procedure analyzes fragments from 400-20,000bp [3][6].

Internal Validation

When new technologies are developed in forensic science, there is usually a period where the new procedures and protocols overlap the old techniques. Validation of the new methods is crucial to maintaining a laboratories high standard for quality results. Before a manufacturer releases a product or procedure into the forensic community for commercial use, it must undergo developmental validation. Developmental validation provides a level of assurance to forensic laboratories that the procedure has been evaluated for its reliability. The manufacturer will conduct numerous experiments in order to determine the optimum reaction conditions to assure the most accurate and reliable results. In addition the manufacturer should provide data demonstrating the limitations of their product. Before Promega Corporation implemented the PowerPlex® 16 PCR system (Promega Corp., Madison, WI) a series of validation studies were done that met the requirements of the Technical Working Group on the DNA Analysis Methods, TWGDAM, and DNA Advisory Board, DAB, guidelines. These studies demonstrated the reproducibility and accuracy of the system, and served to aid the forensic community in addressing anticipated court challenges [9].

Prior to implementation of any commercial product a laboratory must collect data utilizing their own equipment to demonstrate that the established methods and procedures performed as expected in their hands. This process is called an internal validation, and must be done for every new protocol used within the laboratory. According to the TWGDAM revised validation guidelines, the internal validation process should include the following specific studies that encompass a total of at least 50 samples [10].

3.1 Known and nonprobative evidence samples: The method must be evaluated and tested using known samples and, when possible, authentic case samples; otherwise, simulated case samples should be used. DNA profiles obtained from questioned items should be compared to those from reference samples. When previous typing results are available, consistency as to the inclusion or exclusion of suspects or victims within the limits of the respective assays should be assessed.

3.2 Reproducibility and precision: The laboratory must document the reproducibility and precision of the procedure using an appropriate control(s).

3.3 Match criteria: For procedures that entail separation of DNA molecules based on size, precision of sizing must be determined by repetitive analyses of appropriate samples to establish criteria for matching or allele designation.

3.4 Sensitivity and stochastic studies: The laboratory must conduct studies that ensure the reliability and integrity of results. For PCR-based assays, studies must address stochastic effects and sensitivity levels.

3.5 Mixture studies: When appropriate, forensic casework laboratories must define and mimic the range of detectable mixture ratios, including detection of major and minor components. Studies should be conducted using samples that mimic those typically encountered in casework (e.g., postcoital vaginal swabs).

3.6 Contamination: The laboratory must demonstrate that its procedures minimize contamination that would compromise the integrity of the results. A laboratory should employ appropriate controls and implement quality practices to assess contamination and demonstrate that its procedure minimizes contamination.

3.7 Qualifying test: The method must be tested using a qualifying test. This may be accomplished through the use of proficiency test samples or types of samples that mimic those that the laboratory routinely analyzes. This qualifying test may be administered internally, externally, or collaboratively [9], [10].

CHAPTER III

MATERIALS AND METHODS

Optimal Input DNA for Amplification

A total of 6 tests were set up with varying amounts of DNA. Variable amounts of 9947A DNA were used ranging from 0.5ng to 1.0ng in increments of 0.1ng of DNA. Each sample contained 2.5ul of Goldstar10X buffer, 2.5ul of 10X PowerPlex Primer-pair set, 0.8ul of Ampli Taq Gold DNA Polymerase, and TE-4 buffer for a total volume of 25ul per reaction. The Gene Amp 9700 thermocycler was used to amplify the samples for 32 cycles. The cycling profile was 95°C for 11 minutes (initial incubation), 96°C for 1 minute followed by 10 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C,and extension for 45 seconds at 70°C and then 22 cycles of denaturation for 30 seconds at 90°C, annealing for 30 seconds, and extension for 45 seconds at 70°C. This was followed by a final elongation step of 30 minutes at 60°C. At the end of the PCR reaction, the temperature was kept at 4°C.

Detection of the amplified fragments was done using the ABI PRISM® 3100-Avant with Data Collection Software, Version 2. A loading cocktail was prepared using 0.5ul of Internal Lane Standard (ILS) 600 and 9.5ul Hi-Di™ formamide for each sample tested. 1ul of amplified sample was added to 10ul of the loading cocktail and loaded on to the analyzer. The typical 3 minute denaturing at 95°C followed by a 3 minute cooling on ice step was not done due to a previous validation experiments at the HCME.

Mixture Analysis #1

For this study 10 DNA samples were provided by HCME that had been previously extracted and quantitated using the Applied Biosystems 7000 Real Time quantification system. Each of the 10 samples were diluted down to 100pg/ul from there previous concentrations. As shown in Table 1, five pairs of mixtures were made from the 10 samples containing a fixed input of 600pg. Each pair was mixed in the ratios of 1:5, 1:2, 2:1, and 5:1. Interpretation threshold was set at 60 RFU's.

Table1: Mixture Experiment. The table below describes the different DNA mixture combinations amplified at varying ratios. The identification in parentheses signifies the different donors used. Each letter (e.g., A, B, C, ...) designates the different mixture combinations. The combinations in black have a total input of 600pg DNA; the combinations in red have a total input of 1000pg DNA.

Ratios Used for Mixture Experiment			
1:5	5:1	1:2	2:1
A 100pg(VD10):500pg(EB53) 166pg(VD10):833pg(EB53)	B 500pg(VD10):100pg(EB53) 833pg(VD10):166pg(EB53)	C 200pg(VD10):400pg(EB53) 333pg(VD10):666pg(EB53)	D 400pg(VD10):200pg(EB53) 666pg(VD10):333pg(EB53)
E 100pg(VD1):500pg(VD12) 166pg(VD1):833pg(VD12)	F 500pg(VD1):100pg(VD12) 833pg(VD1):166pg(VD12)	G 200pg(VD1):400pg(VD12) 333pg(VD1):666pg(VD12)	H 400pg(VD1):200pg(VD12) 666pg(VD1):333pg(VD12)
I 100pg(VD13):500pg(VD42) 166pg(VD13):833pg(VD42)	J 500pg(VD13):100pg(VD42) 833pg(VD13):166pg(VD42)	K 200pg(VD13):400pg(VD42) 333pg(VD13):666pg(VD42)	L 400pg(VD13):100pg(VD42) 666pg(VD13):333pg(VD42)
M 100pg(EB45):500pg(EB47) 166pg(EB45):833pg(EB47)	N 500pg(EB45):100pg(EB47) 833pg(EB45):166pg(EB47)	O 200pg(EB45):400pg(EB47) 333pg(EB45):666pg(EB47)	P 400pg(EB45):200pg(EB47) 666pg(EB45):333pg(EB47)
Q 100pg(EB49):500pg(VD9) 166pg(EB49):833pg(VD9)	R 500pg(EB49):100pg(VD9) 833pg(EB49):166pg(VD9)	S 200pg(EB49):400pg(VD9) 333pg(EB49):666pg(VD9)	T 400pg(EB49):200pg(VD9) 666pg(EB49):333pg(VD9)

Amplification and detection of the mixture samples were done using the parameters previously described.

Mixture Study with correct ramping

Samples were mixed together to compare the consistency of peak height ratios between the PowerPlex® 16 and the Profiler Plus™ and COfiler™ systems. These samples were analyzed to assess if the changes made to thermocycler ramping speeds were effective in improving the detection of all loci in the PowerPlex® 16 system. For this study two mixtures in ratios of 1:5 and 1:2 were run 10 times each. Samples were provided by HCME that had been previously extracted and quantitated using the Applied Biosystems 7000 Real Time quantification system. The samples were diluted to 0.1ng/ul of DNA and were amplified at 1ng of DNA using 10ul of each mixture with the PowerPlex® 16 multiplex system. In addition for comparison purposes 10ul of the same samples were amplified according to the manufacturers recommended conditions. The samples were amplified using the Gene Amp 9700 with parameters of 95°C for 11 minutes (initial incubation), 96°C for 1 minute followed by 10 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 45 seconds at 70°C and then 22 cycles of denaturation for 30 seconds at 90°C, annealing for 30 seconds, and extension for 45 seconds at 70°C. This was followed by a final elongation step of 30 minutes at 60°C. At the end of the PCR reaction, the temperature was held at 4°C. Ramping time between denaturing and annealing and between annealing and extension was adjusted to 29% and 23% respectively for the 32 cycles.

Samples were prepared for detection with the addition of the post-amplification mix previously described. Samples were not heat denatured and snap cooled prior to injection. Samples were injected electrokinetically for 5 sec using the 3100-Avant with

Data Collection Software, Version 2. Fragments were sized and the peak heights in Relative Fluorescent Units (RFU) were determined automatically using GeneMapper™ ID Software.

Sensitivity Experiment #1

To compare the differences in sensitivity between amplification systems, a serial dilution was prepared from 2ng down to 7.8pg. Aliquots from the dilution series were amplified using the PowerPlex® 16 and AmpFℓSTR® Profiler Plus™ and COfiler™ systems. The sample used for the serial dilution was the Promega 9947A DNA (10ng/ul). The injection time was 5 seconds for all samples run on the 3100-Avant. The minimum allele threshold detection was set at 60 relative fluorescent units (RFU). All thermocycler parameters were set the correct manufacturers instructions.

Sensitivity Experiment #2

A second sensitivity test was run as previously described, however, this time Applied Biosystems 9947A DNA (0.1ng/ul) was used. Aliquots from the dilution series were amplified as before, and additional aliquots from the 1.0ng and 0.5ng were amplified 10 times each. All equipment settings were the same as in the sensitivity experiment #1.

Precision Study

In order to assess the sizing precision of the Applied Biosystems 3100-Avant genetic analyzer the PowerPlex® 16 ladder and Promega 9947A DNA were run 10 times each. Base pair size was collected and used to calculate the mean base pair and standard deviation (SD) for each allele.

CHAPTER IV

RESULTS & DISCUSSION

Optimal Input DNA for Amplification

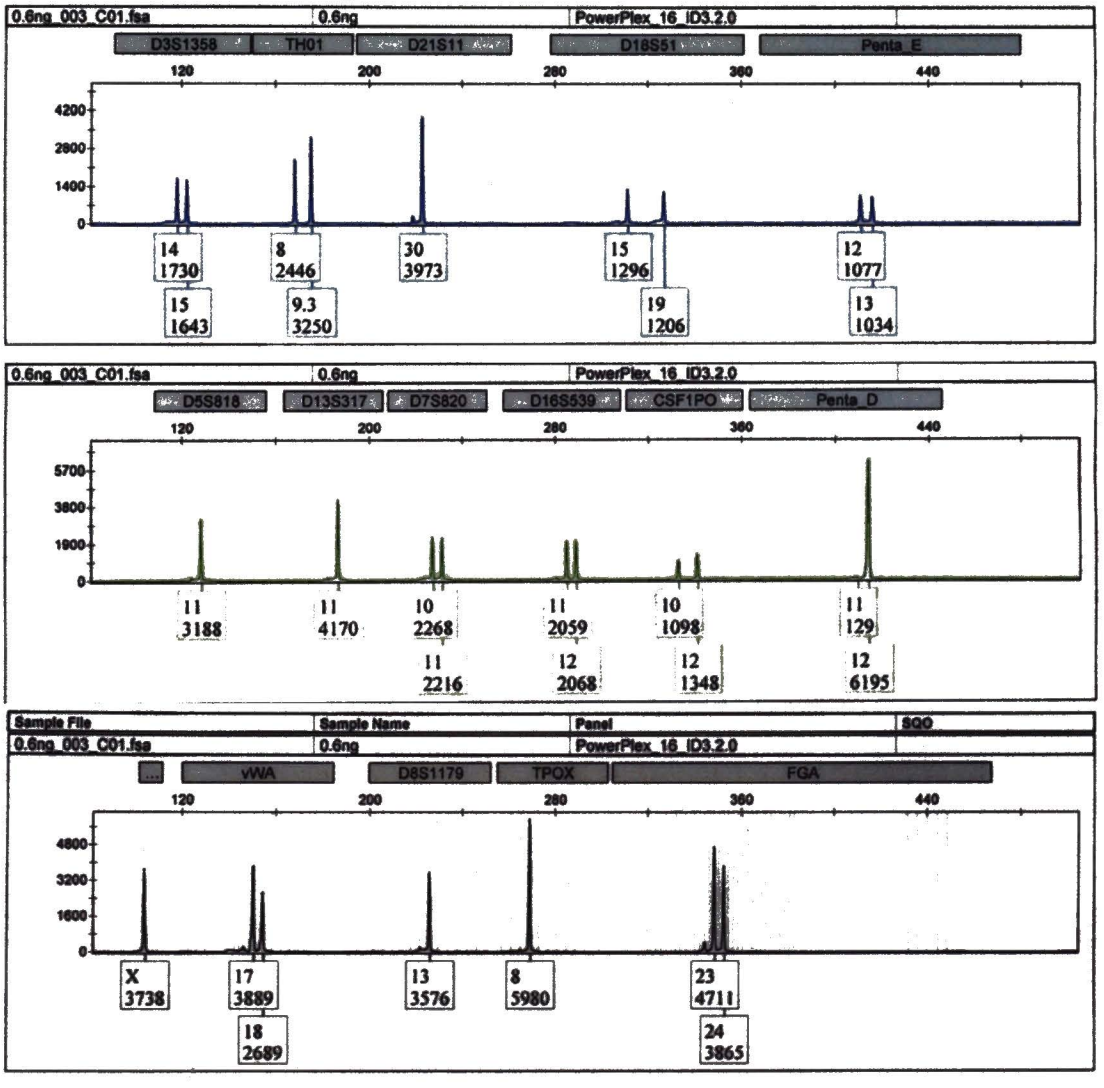
The purpose for this procedure was to determine the optimal input DNA amount to use for testing with the PowerPlex® 16 PCR system (Promega Corp., Madison, WI). The use of too little DNA may result in allelic dropout, and the use of too much DNA may result in off-scale or split peaks [2]. In this study the optimal input amounts of DNA were assessed based upon allelic dropout, locus to locus balance, peak height ratios at 60% or better, and the amount of stutter and incomplete 3` adenosine addition (–A) artifacts. The optimal input amount of DNA in the PCR reaction amplified was 0.6ng to 0.7ng. As shown below in Figure 1a, the 0.6ng sample showed no sign of allelic dropout. Average peak heights for each allele averaged between 1000 and 3000 RFU. The peak height ratios of all heterozygous loci amplified from the 0.6ng samples were better than 60% and artifacts such as stutter and –A artifacts were not observed. No evidence of a poor spectral calibration such as pull-up was observed in the 0.6ng sample.

An input of 0.7ng of DNA showed similar characteristics to the 0.6ng sample. The 0.7ng sample had a few more artifacts present compared to the 0.6ng sample. As shown in Figure 1b, the 0.7ng sample was observed to have few artifacts present. Figure 1b shows off-ladder alleles that can be seen at loci D16S539 and vWA with drop-in seen at the Penta D locus. These artifacts are stutter and are generally one full repeat unit

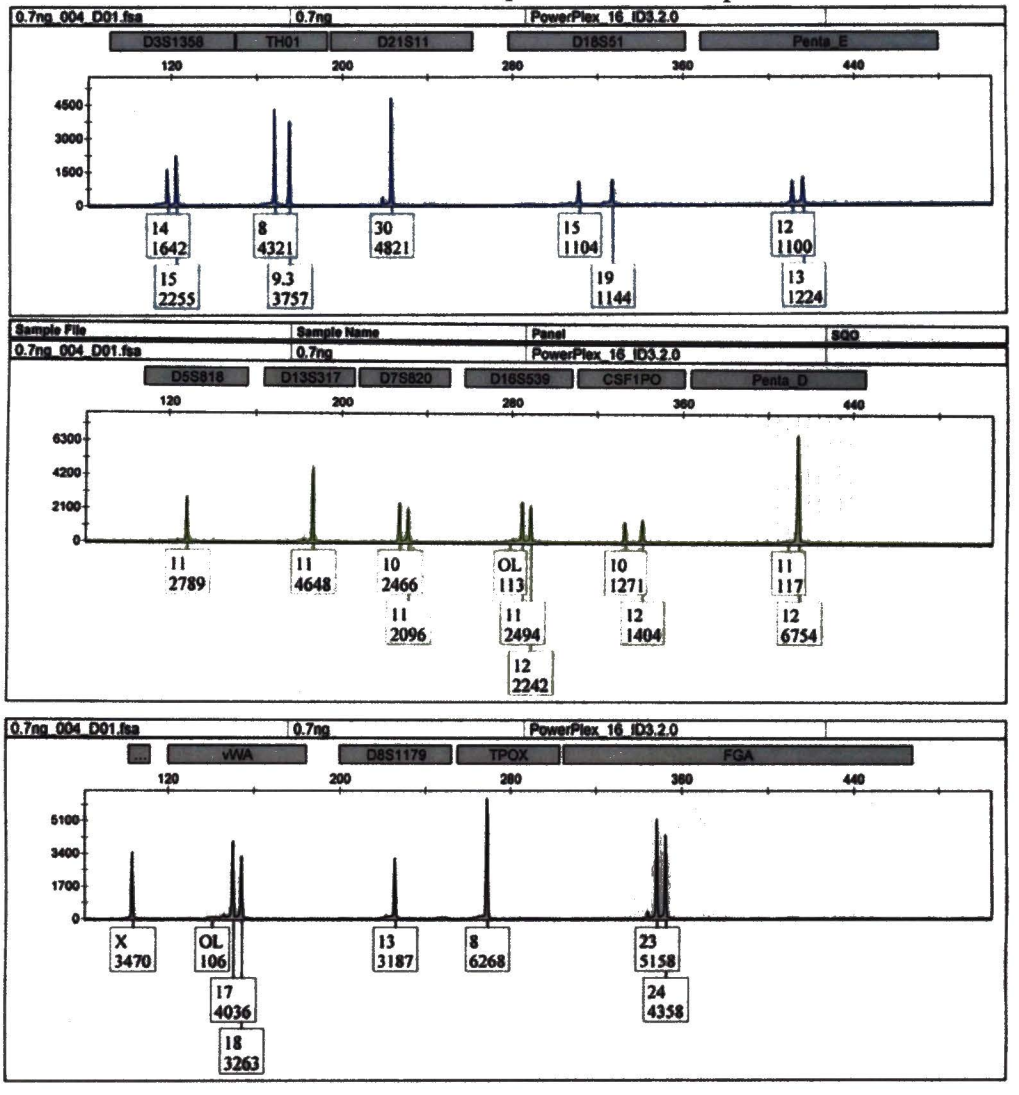
smaller than the actual size of the true allele [16]. Stutter can also be seen in Figure 1b at locus FGA; however the height of the stutter peak is under the detection threshold limit.

Figure 1: Optimal Input DNA. Electropherograms of control DNA 9947A amplified with the PowerPlex® 16 kit.

a) 0.6ng 9947A. Incorrect ramp time set at 100%.



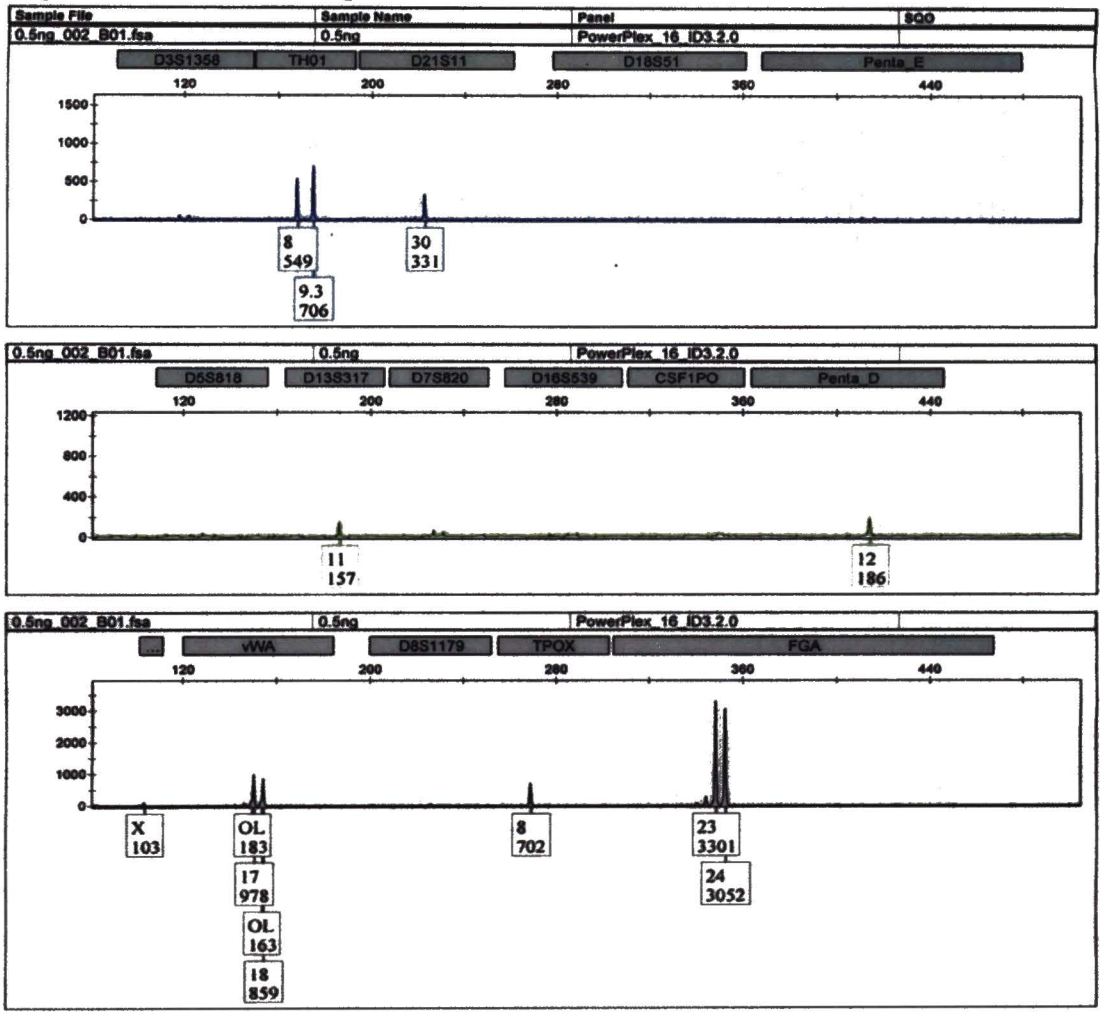
b) 0.7ng 9947A. The two off-ladder (OL) alleles are due to high baseline and do not interfere with the allele calls for this sample. Incorrect ramp time set at 100%.



The amplification of the 0.5ng sample resulted in the dropout of several loci. The few loci that are present in the 0.5ng sample also show unbalanced peak heights. The 0.5ng sample should not have resulted in the allelic dropout seen in Figure 2. According to the

Promega Corporation the optimal target amount for amplification is from 0.5-1.0ng of DNA [6]. These results indicate a problem that will be discussed shortly. The samples containing 0.9 or 1ng show increased stutter and –A artifacts. These observations suggested that optimum results were obtained with an input target of 0.6 to 0.7ng of DNA and that this target amount should be used in further analyses.

Figure 2: Incorrect Ramp Time Set at 100%. Example of 0.5ng control DNA 9947A amplified with PowerPlex® 16 with incorrect ramp times. With 100% ramp time, allelic dropout is observed at multiple loci.

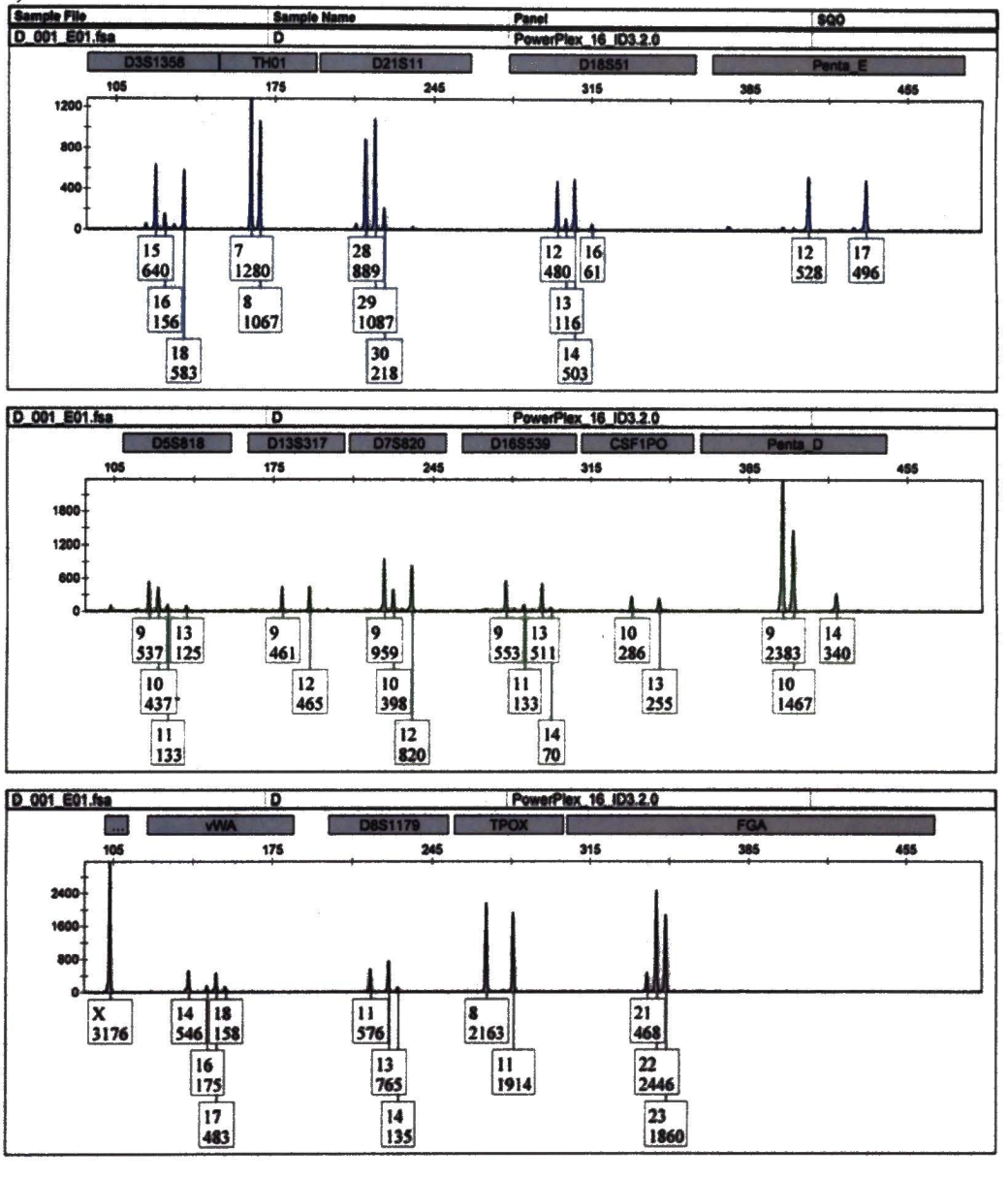


Mixture Analysis #1

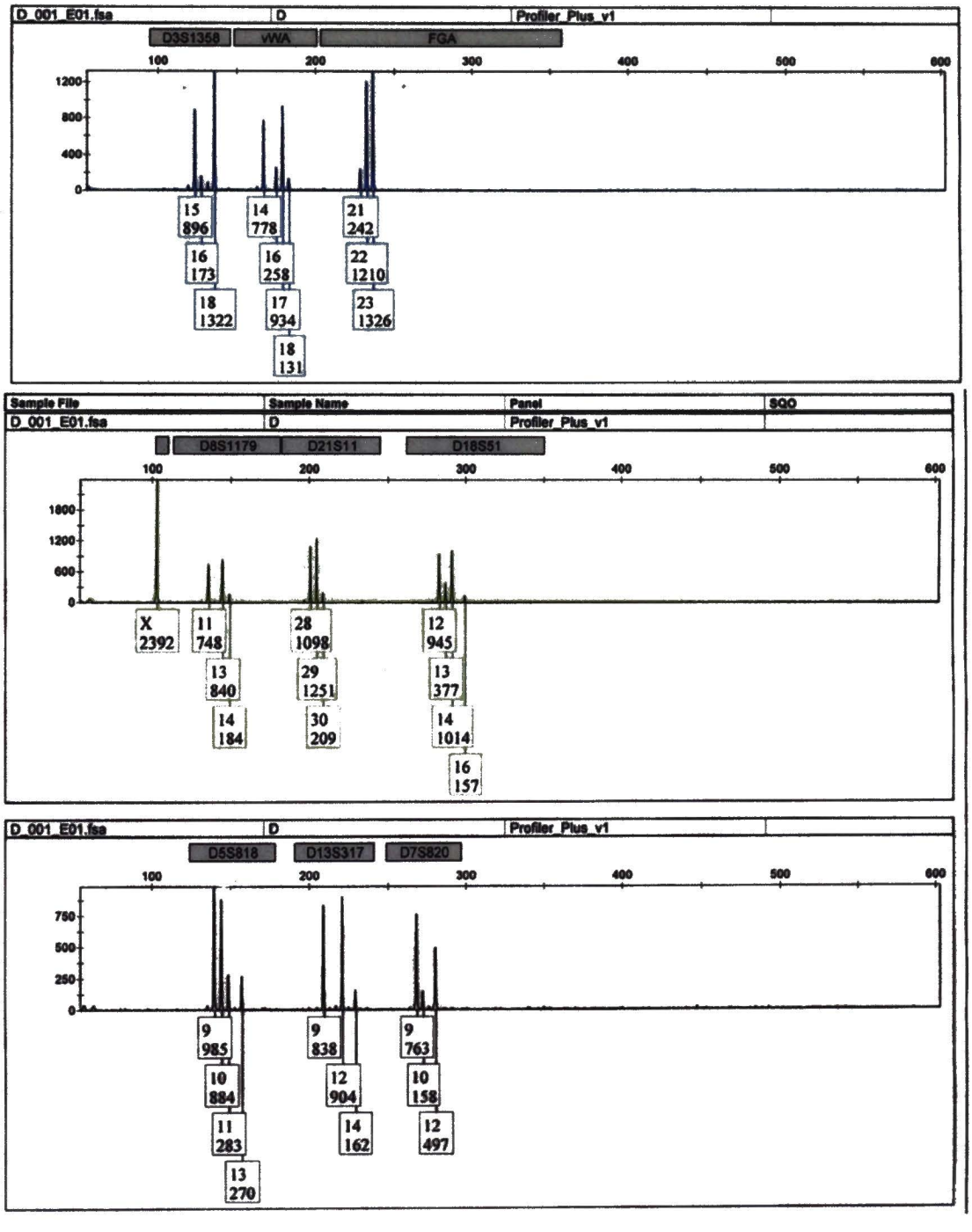
The purpose of the mixture analysis # 1 was to assess the behavior of the PowerPlex® 16 kit when analyzing mixture samples. Mixture samples are usually discerned by the presence of two or more peaks per STR loci. Multiple peaks at a locus may not be caused by a mixture sample only, but could be the result of artifacts [11]. Several criteria have been applied for the assessment of mixture samples (1) more than two peaks at more than one locus and/or significantly imbalanced peaks in heterozygous genotypes; (2) close examination of possible stutter peaks and other artifacts (pull up, off-ladder, $n/n+1$ peaks); (3) check on possible contamination from staff or from other samples analyzed in the same batch (DNA extraction, PCR, electrophoretic run [11]. A total of twenty mixture samples were analyzed in order to determine the ratio at which the minor component of a mixture would be detected. The following parameters were used to evaluate the mixtures: 1) presence of all expected alleles; 2) ratio at which alleles from the minor contributor dropped out; 3) peak height ratios within the heterozygous loci of the minor contributor. A comparison of Figure 3a with 3b and 3c shows concordant allele calls between the two amplification systems.

Figure 3: Amplification of Sample D. Sample D (2:1 mixture of two females with a total input of 600pg DNA) was amplified with three commercial kits. The results obtained with each kit are similar; however differences can be seen in peak heights and one allele call.

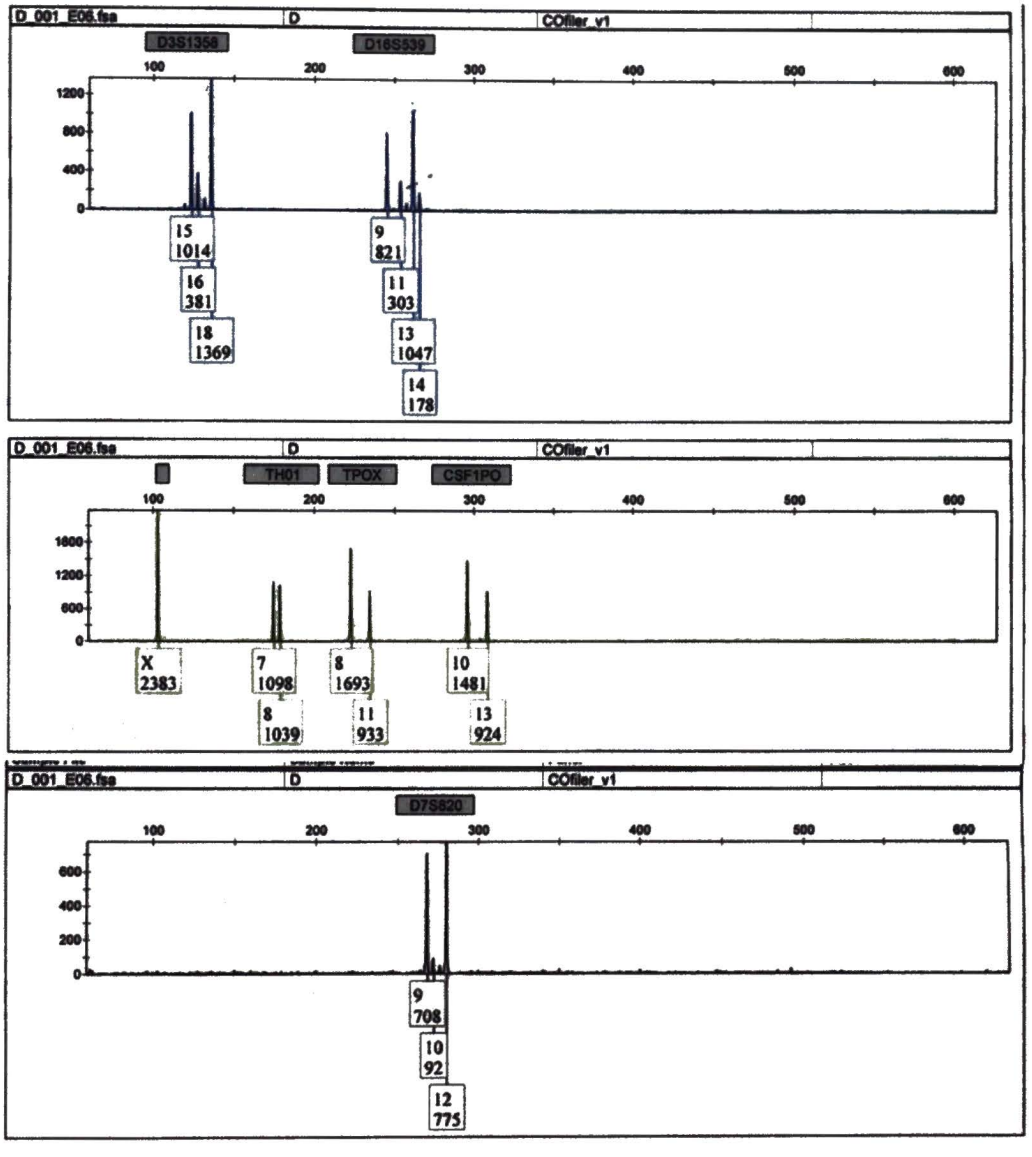
a) PowerPlex® 16.



b) AmpFℓSTR® Profiler Plus™. Profiler Plus™ detected one allele from the minor contributor that PowerPlex® 16 did not detect.



c) AmpFℓSTR® COfiler™.



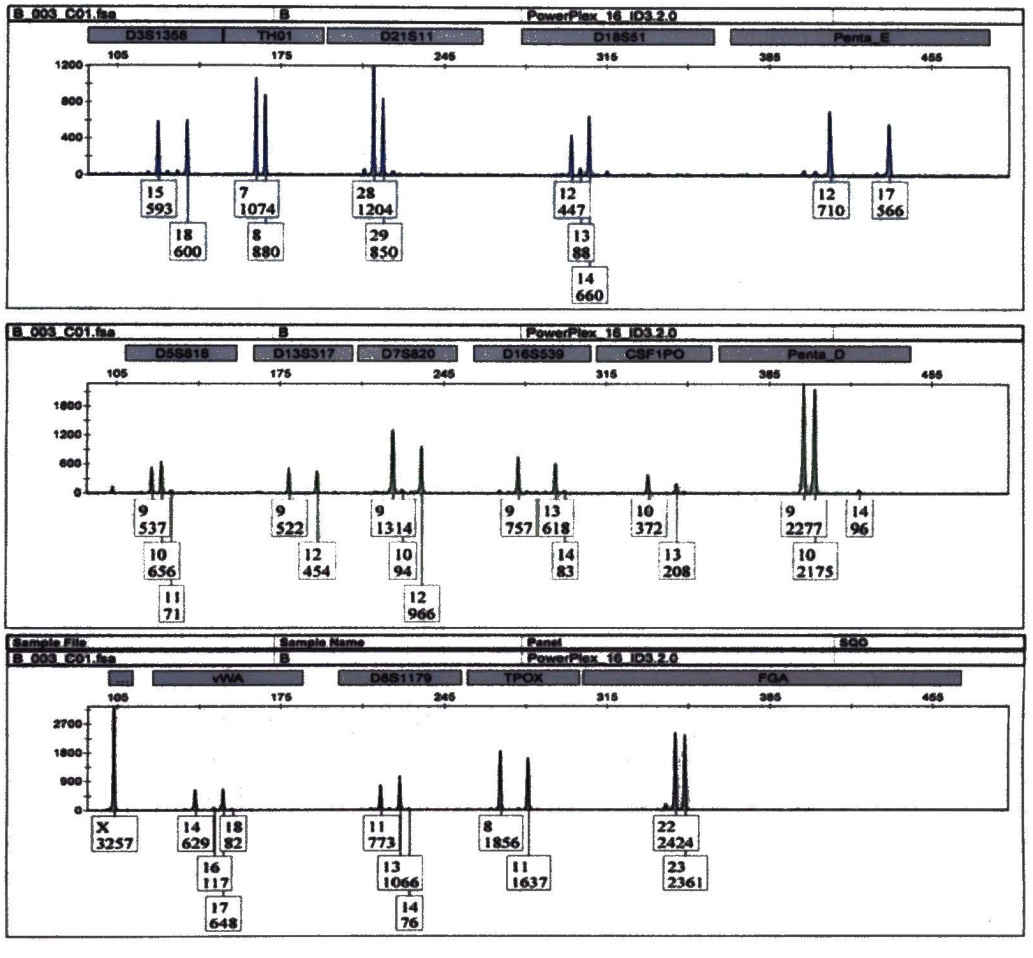
Sample D consists of a 2:1 ratio of a mixture of two females. Four alleles are detected at the D18S51 locus. The genotypes (12,14) and (13,16) represent the major and minor contributors respectively. The peak height ratios at the D18S51 locus for the minor contributors are 0.54 (Figure 3a) and 0.42 (Figure 3b). The peak height ratios for the major contributor are consistent showing 0.95 (Figure 3a) and 0.93 (Figure 3b). The peak

height ratios for the minor contributors are low due to stochastic effects occurring during the early cycles of the PCR reaction.

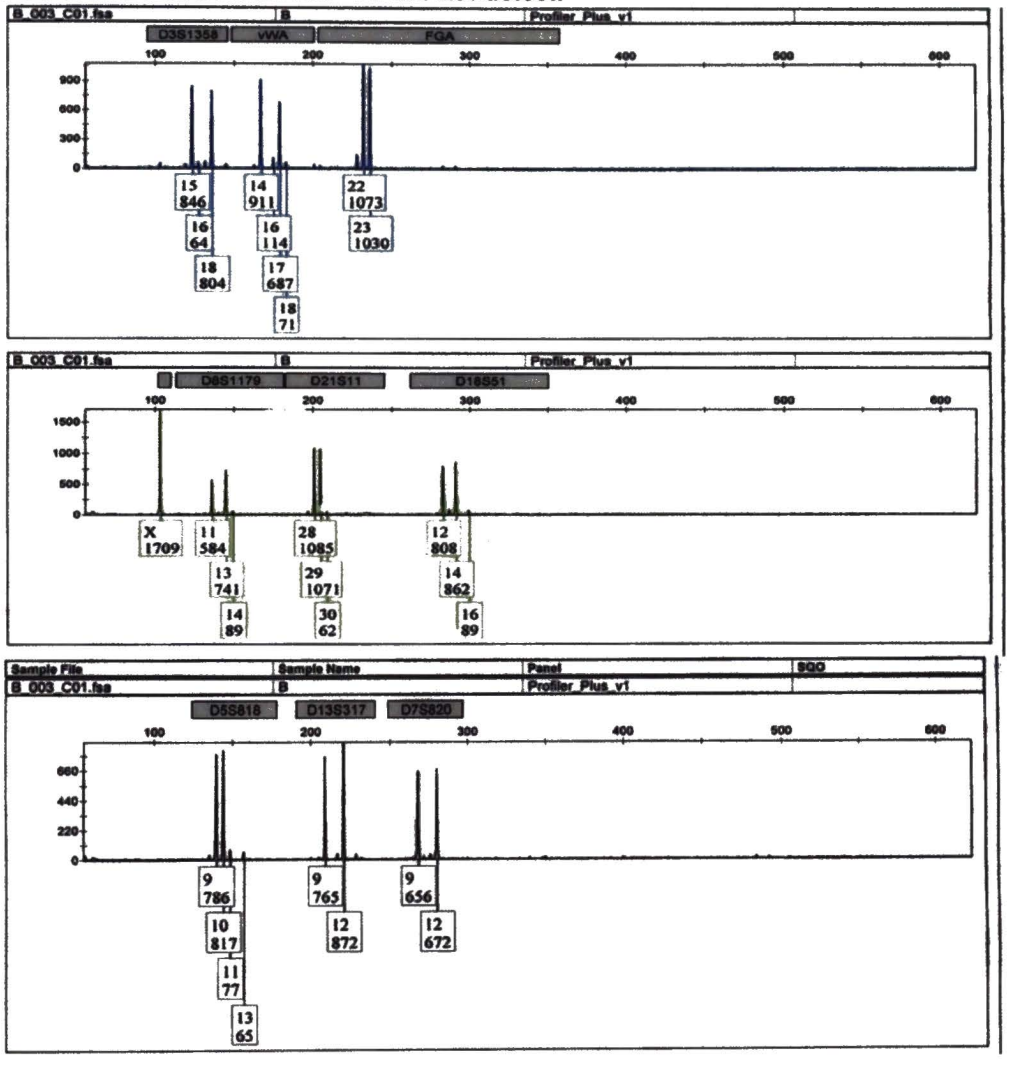
Dropout was predominantly seen in the 5:1 and 1:5 mixtures. This dropout is due to the low concentration of DNA for the minor contributor, and is a result of stochastic effects. Dropout can be seen in sample B which was a 5:1 mixture (500pg:100pg). As indicated in Figure 4a, 4b, and 4c are instances of dropout for both the PowerPlex® 16 and AmpFℓSTR® Profiler Plus™ and COfiler™ systems. Dropout can be seen in the PowerPlex® 16 system, at loci D3S1358, D5S818, D16S539 and D21S11 (Figure 4a). For sample B, the Profiler Plus™ and COfiler™ systems did not show dropout at the D3S1358, D5S818, and D21S11 loci (Figure 4b and 4c).

Figure 4: Amplification of Sample B. Sample B (5:1 mixture of two females with a total input of 600pg DNA) was amplified with three commercial kits and exhibit minor differences as follows: 1) allele calls due to allelic dropout; 2) filtering of alleles due to stutter position; and 3) alleles not detected due to low peak heights below threshold settings.

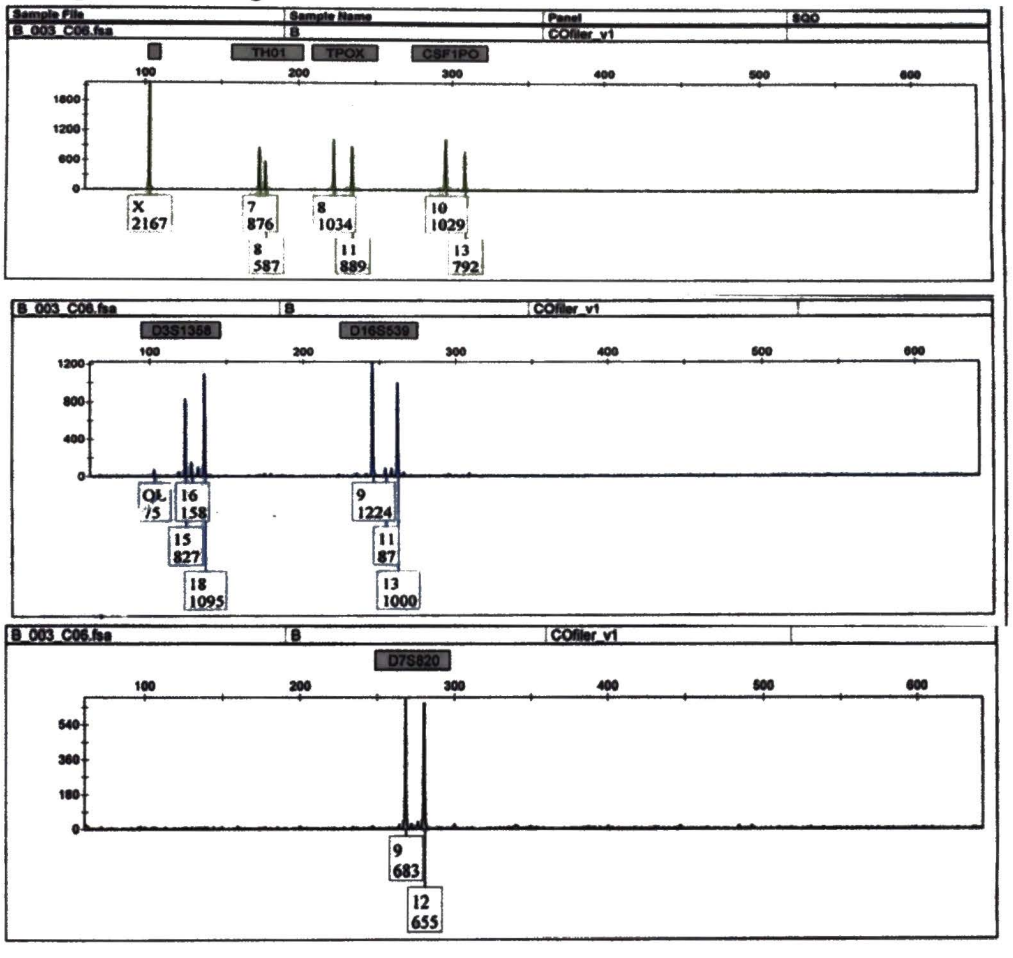
a) PowerPlex® 16.



b) AmpFℓSTR® Profiler Plus™. Profiler Plus™ detected three alleles from the minor contributor that PowerPlex® 16 did not detect.



c) AmpFℓSTR® COfiler™. COfiler™ detected one allele from the minor contributor that PowerPlex® 16 did not detect. The one off-ladder (OL) allele in this sample is due to pull-up from Amelogenin.



Thermocycler Parameter Problem

During the initial validation experiments it was determined that the amplification parameters for the Gene Amp 9700 thermocycler were not properly set. As a result of the incorrect thermocycling parameters, numerous loci including both CSF1PO and D16S539 demonstrated significant allelic dropout. In consultation with representatives from Promega it was determined that incorrect ramp speeds had been set for the Gene Amp

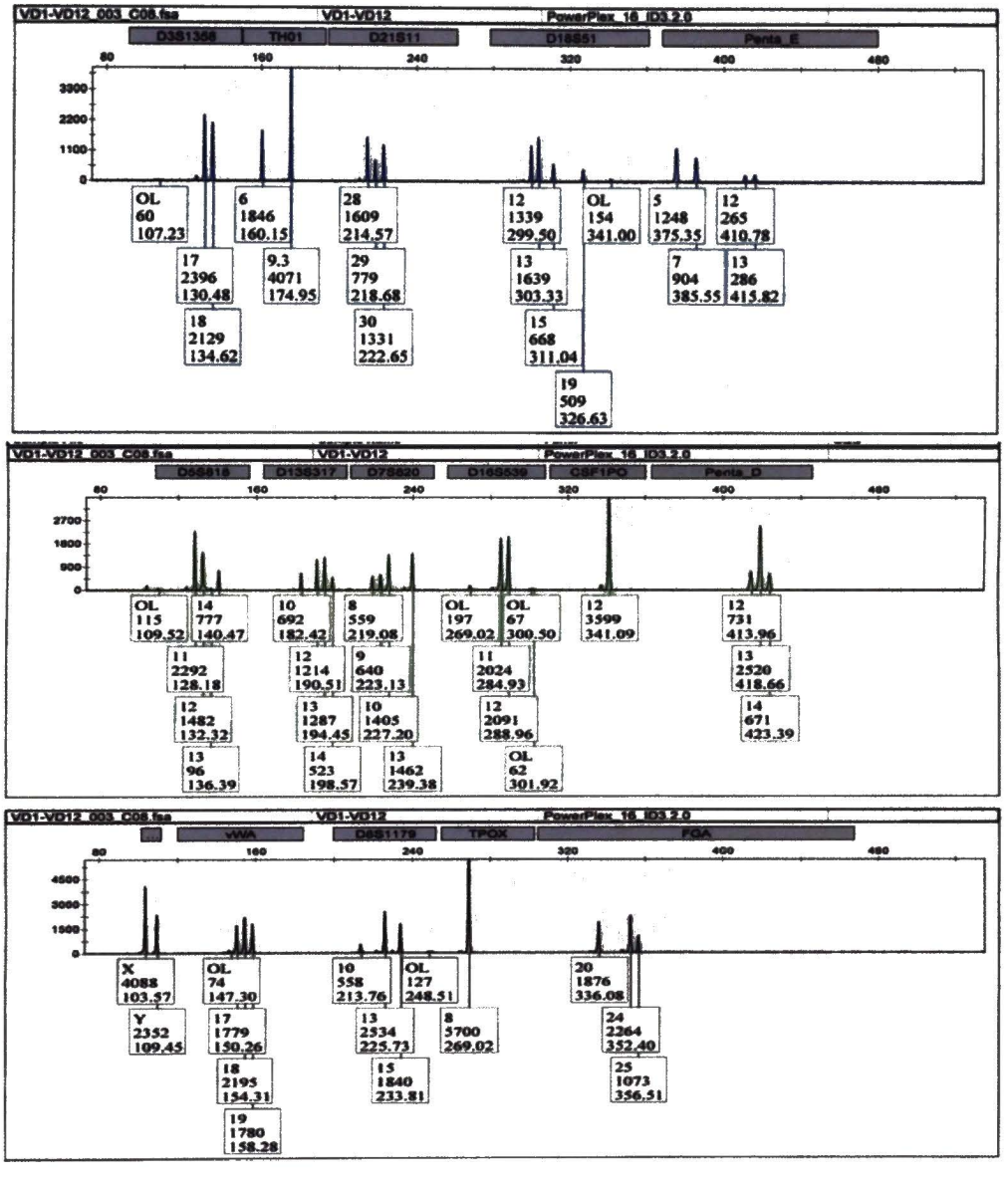
9700 Thermocycler. The ramp speed (which is set at default of 100%) regulates how fast the temperature changes from one cycle to the next.

Promega suggests for optimum results with the PowerPlex® 16 multiplex system, the ramp speed from the denaturing stage to the annealing stage should have been set to 29% and from annealing to extension stage should have been 23%. These speeds are necessary to allow the multiple STR primers and enzymes to function with optimal efficiency. The results obtained with the incorrect ramp parameters could result in improper binding of primer sets during the annealing stage causing a reduced efficiency and allelic dropout at select loci. Further studies where run with the correct thermocycler settings and produced more reliable results.

Mixture Study with Correct Ramping

Both the 1:5 and 1:2 mixture samples gave concordant allele calls between the PowerPlex® 16 and the AmpFℓSTR® Profiler Plus™ and COfiler™ systems. None of the systems demonstrated allelic dropout. The expected major and minor peaks of the mixture samples were present in each of the systems. The results for PowerPlex® 16 can be seen in Figure 5. The majority of off-ladder alleles shown in Figure 5 result from pull up from the major contributor due to poor spectral separation.

Figure 5: Sample E (1:5 mixture of a male and female with a total input of 1ng DNA). No allelic dropout was observed. All off-ladder (OL) alleles are due to incomplete color separation. The allele 13 in D5S818 exhibits increased stutter and was not filtered by the software.



The 10 samples run at each mixture ratio were averaged together and compared to the opposing kit to check for consistency. At each locus, shown in Table 2, similar average peak height ratios were observed for the alleles from the major contributors. A chi square analysis of the data from this mixture study indicates that there was no statistical difference between the peak height ratios for heterozygous loci between the 3 kits (p=0.05).

Table 2: Average Peak Height Ratios for Heterozygous Loci. Each value is an average peak height ratio from 10 amplifications; the average peak height ratios are from the major contributor's heterozygous loci independent of the minor contributor.

A

Sample A (1ng)	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D21S11	TH01	vWA
PP/CO	0.74	0.91	0.80	0.84	0.79	0.90	0.84	0.86	0.85	0.83
PP16	0.80	0.87	0.77	0.89	0.83	0.77	0.81	0.78	0.87	0.88
Chi square	0.92326									

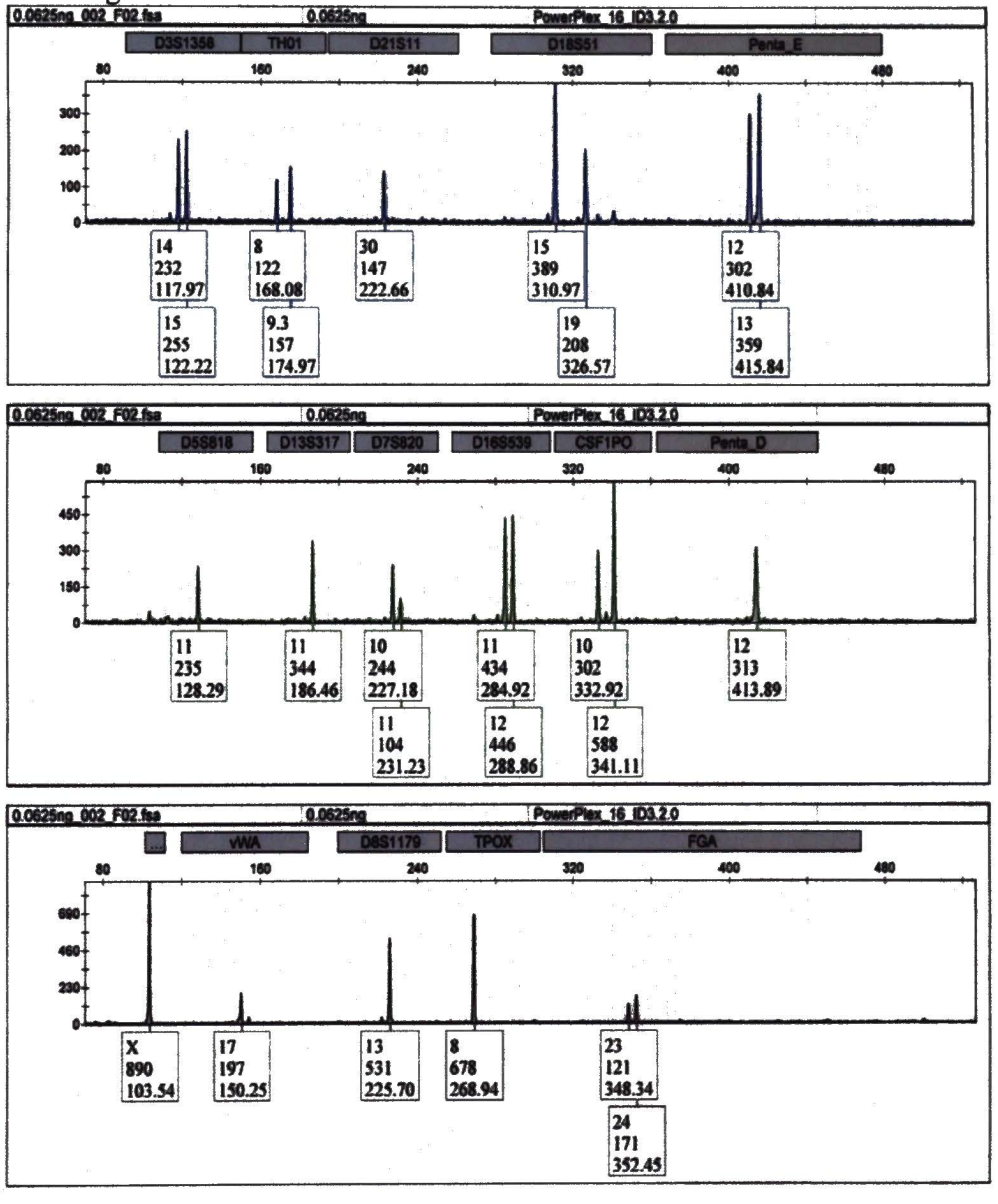
B

Sample G (1ng)	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D21S11	FGA	vWA
PP/CO	0.89	0.80	0.89	0.88	0.88	0.88	0.83	0.74	0.74	0.86
PP16	0.89	0.79	0.92	0.82	0.90	0.87	0.85	0.81	0.78	0.89
Chi square	0.997164									

Sensitivity Experiment #1

The purpose of this study was to determine the lower limit of detection for the PowerPlex® 16 multiplex system. For each input amount of DNA, the overall quality of the profile was assessed including the presence of allelic dropout and peak height imbalance at heterozygous loci. The results from the PowerPlex® 16 were compared to those obtained for the Profiler Plus™ and COfiler™ systems. The PowerPlex® 16 system showed complete profiles from 2ng down to 0.125ng. At 0.0625ng a single allele at the vWA locus dropped out (Figure 6). Input amounts below 0.0625ng of DNA showed more significant allelic dropout. At 0.0313ng of DNA 10 of the 27 (37%) alleles observed dropped-out, and for dilution 0.015625ng, there was 78% allele dropout. The 0.5ng sample demonstrated the optimal quantity. The input of 0.5ng of DNA displays minimum artifacts such as stutter and –A.

Figure 6: Sensitivity Study Dropout. Allelic dropout is first observed for 0.0625ng of 9947A at the vWA locus in the PowerPlex® 16 kit; the 18 allele can be seen but is below the calling threshold.



A comparison of the sensitivity of the PowerPlex® 16 system with that of the AmpFℓSTR® Profiler Plus™ and COfiler™ systems was done. The results indicate that the PowerPlex® 16 system provided greater sensitivity when used in conjunction with the ABI 3100-Avant. Tables 3a and 3b demonstrate that allelic dropout occurs at lower input amounts of DNA with the AmpFℓSTR® Profiler Plus™ and COfiler™ system. For the AmpFℓSTR® Profiler Plus™ and COfiler™ system, dropout was first seen at in the 0.25ng sample. At 250pg one allele dropped out of the 26 alleles observed. Dropout was more prevalent with the AmpFℓSTR® Profiler Plus™ and COfiler™ system samples below 0.0625ng where only a few alleles were detected.

Table 3a: Sensitivity Experiment with PowerPlex® 16. The sensitivity study consisted of a series of amplifications varying the total input of Promega 9947A control DNA. Full profiles are observed from 2ng to 0.125ng. Allelic dropout is first observed for 0.0625ng of DNA.

	2ng		1ng		0.5ng		0.25ng		0.125ng		0.0625ng		0.03125ng		0.015625ng		0.0078125ng	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
D3S1358													X		X	X	X	X
TH01														X	X	X		X
D21S11															X	X	X	X
D18S51																		
Penta E																		
D8S818																		
D13S317															X	X		
D7S820													X	X	X	X		X
D16S539													X		X	X		
CSF1PO															X	X	X	X
Penta D																		
Amelogenin															X	X		
vWA												X	X		X	X		
D8S1179															X	X		
TPOX													X	X			X	X
FGA													X	X	X	X	X	

X= denotes dropout

Table 3b: Sensitivity Experiment with Profiler Plus™ and COfiler™. The sensitivity study consists of amplifications of Promega 9947A control DNA. Full profiles are observed from 2ng to 0.5ng. Allelic dropout is first observed for 0.25ng of DNA. D3S1358 and D7S820 results are from Profiler Plus™.

	2ng		1ng		0.5ng		0.25ng		0.125ng		0.0625ng		0.03125ng		0.015625ng		0.0078125ng	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
D3S1358									X			X		X	X	X	X	X
TH01							X			X	X	X	X	X	X	X	X	X
D21S11											X	X			X	X		
D18S51									X		X	X	X	X	X	X	X	X
D8S1179											X	X	X	X	X	X	X	X
D13S317											X	X	X	X	X	X	X	X
D7S820											X	X	X	X	X	X	X	X
D16S539									X		X	X	X	X	X	X	X	X
CSF1PO											X	X	X	X	X	X	X	X
Amelogenin											X	X			X	X	X	X
vWA											X	X	X	X	X	X	X	X
D8S1179											X	X			X	X	X	X
TPOX											X	X	X	X	X	X	X	X
FGA												X	X	X	X	X	X	X

X=denotes dropout

The PowerPlex ® 16 system not only identifies more alleles at low concentration, but is seen to have a full profile at lower concentrations compared to the AmpFℓSTR® Profiler Plus™ and COfiler™ systems. A human diploid cell carries about 6pg of DNA, and a haploid cell carries about half of that. The PowerPlex ® 16 system shows that it can pick up a full profile when there are about 15 diploid cells present. This study demonstrates that the PowerPlex® 16 system is far more sensitive than the AmpFℓSTR® Profiler Plus™ and COfiler™ system which identifies a full profile when about 50 cells are present. The sensitivity test of both systems reveals that the PowerPlex ® 16 system is more resilient for small input samples.

The concentrations that showed full profiles for both systems were compared to demonstrate any peak height ratios that dropped below 60% for heterozygous loci. The AmpFℓSTR® Profiler Plus™ and COfiler™ system only had three dilutions that had full

profiles to the five from the PowerPlex® 16 system. The dilutions compared were the 2ng, 1ng, and 0.5ng samples and are shown in Table 4.

Table 4: Peak Height Ratios for Sensitivity Experiment. Peak height ratios for all overlapping heterozygous loci with 9947A for the three kits. Only one peak height ratio was below 60%, D16S539 in COfiler™.

PowerPlex® 16.

	D3S1358	TH01	D18S51	D7S820	D16S539	CSF1PO	vWA	FGA
0.5ng	0.71	0.8	0.76	0.9	0.99	0.63	0.82	0.95
1ng	0.94	0.79	0.74	0.63	0.92	0.9	0.91	0.94
2ng	0.99	0.91	0.91	0.95	0.92	0.95	0.9	0.98

Profiler Plus™ and COfiler™.

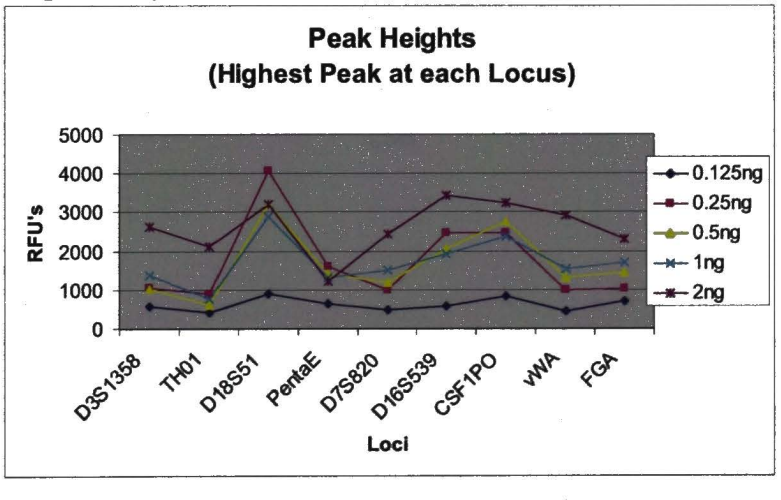
	D3S1358	TH01	D18S51	D7S820	D16S539	CSF1PO	vWA	FGA
0.5ng	0.98	0.97	0.86	0.75	0.58	0.63	0.87	0.72
1ng	0.96	0.87	0.93	0.65	0.88	0.93	0.94	0.94
2ng	0.86	0.89	0.81	0.79	0.92	0.99	0.73	0.99

None of the 8 heterozygous loci assessed for peak height ratios less than 60% were below 60% for the 2ng input sample amplified with the PowerPlex® 16 kit. The minimum peak height ratio for the 2ng sample was 90%. The same results were seen at that amount with the Profiler Plus™ and COfiler™ system; however the minimum peak height ratio observed was 71%. The PowerPlex® 16 system showed no peak height ratios less than 60% for the 1ng concentration, and a minimum peak height ratio of 63%. For the same concentration the AmpFℓSTR® Profiler Plus™ and COfiler™ system also showed no peak height ratios under 60%, and the minimum ratio was 65%. The 0.5ng input amount for the PowerPlex® 16 system showed no peak height ratios under 60%

with the minimum at 63% while the AmpFℓSTR® Profiler Plus™ and COfiler™ system showed one of eight peak height ratios dropped below 60% with a value of 58%.

The peak with the highest RFU value at each locus was compared for each concentration shown in Figure 7. The graph shows unexpected RFU values for peak heights between dilutions at several of the loci. PowerPlex® 16 was observed to have higher peak heights at some of the dilutions with smaller concentrations compared to the samples with higher concentrations. For instance, at locus D3S1358 the 0.5ng sample has a peak height of 1021 RFU's compared to the 0.25ng sample with a peak height of 1046 RFU's. The unexpected values can also be seen at locus D18S51 where the 0.25ng sample has a RFU value of 4083 which is higher than the 2ng, 1ng, and 0.5ng sample.

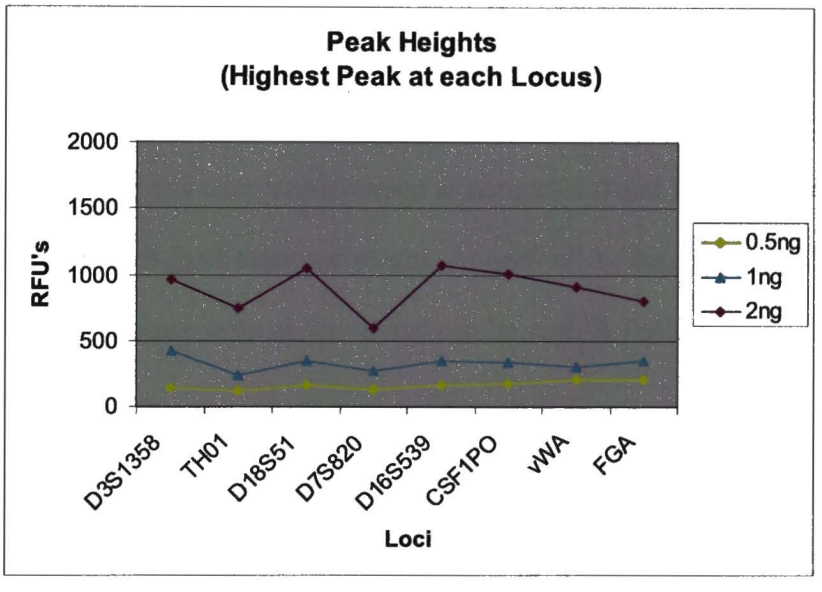
Figure 7: Sensitivity Experiment with PowerPlex® 16. The graph shows the highest peak for each heterozygous locus in PowerPlex® 16 at the defined quantity of DNA. Serial dilutions were performed; the results for the 0.25ng DNA are higher than expected when compared to the peak heights of the other input amounts of DNA.



There were only three concentrations that had full profiles from the AmpFℓSTR® Profiler Plus™ and COfiler™ system that could be compared this way, and they can be

seen in Figure 8. The AmpFℓSTR® Profiler Plus™ and COfiler™ system showed that there were no inconsistencies for any of the loci examined. The graph shows that the peak heights are correspondingly higher at each heterozygous locus for each input DNA amount. This graph represents normal linear progression of peak heights when the amount of DNA is increased.

Figure 8: Sensitivity Experiment with Profiler Plus™ and COfiler™. The graph shows the highest peak for each heterozygous locus in Profiler Plus™ and COfiler™ at the defined quantity of DNA.



The height of the highest peaks shown in Figure 8 demonstrates inconsistencies that should be considered when dealing with mixed samples or samples with very low DNA concentrations. When samples of low DNA concentration are analyzed stochastic fluctuations may occur at heterozygous loci. In this instance an unequal amount of alleles are being represented and in some cases will be seen as homozygous loci. Since there is

such a small amount of DNA in the lower dilutions, an unequal amount of alleles are present for PCR reaction thereby causing some alleles to amplify preferentially to the alleles of low concentration. The alleles represented in small concentrations will occasionally dropout completely.

Sensitivity Experiment # 2

A separate sensitivity analysis of PowerPlex® 16 was done to adjust for pipetting errors and any other conditions that may deliver faulty results. Results were similar to that seen in the sensitivity study #1. The PowerPlex® 16 system showed dropout and peak imbalance at 0.0625ng and over amplification at samples 2.0 and 1.0ng. The two samples amplified 10 times each gave expected results. At each of the heterozygous loci the peak heights from each replicate were observed and can be seen in Table 5.

Table 5: Sensitivity Experiment. 10 amplifications were performed for the 1.0ng and 0.5ng total 9947A DNA in PowerPlex® 16. The highest peak for each heterozygous locus except Penta E is present. As expected, 1.0ng input DNA consistently produced higher RFU values than 0.5ng input DNA.

	D3S1358	TH01	D18S51	D7S820	D16S539	CSF1PO	vWA	FGA
0.5ng	1005	657	2395	1407	1707	2259	1305	1462
	1219	819	1849	1268	2019	1841	1952	1704
	1243	1251	2115	1315	1480	1445	1364	1673
	1052	1121	1141	1457	1395	1316	1103	1727
	1569	1485	4240	1764	2760	1946	1673	1899
	800	794	1242	1085	1533	1672	1661	2635
	1053	1382	1725	2135	1999	2461	1708	1134
	862	772	1723	1077	1745	1583	1921	2149
	1096	1201	2397	1333	1523	2339	1788	1651
	763	764	1487	1534	1325	873	1851	1341
1ng	1259	1075	2391	1525	1971	2353	2158	2505
	2336	2116	2607	2413	2532	2379	2424	2201
	2137	1608	3036	2614	2583	2695	2845	4265
	2567	1843	2853	2670	2564	2440	2342	1948
	2389	1473	3415	2842	3479	3682	2954	4719
	2124	1915	3632	3037	2979	3178	2133	2662
	2018	1459	3886	2984	3116	3570	3136	2883
	2160	1930	2519	2463	2590	2274	2016	2426
	1754	1773	2975	2235	2278	2166	2975	3427
	2245	1943	2280	2286	2365	1874	2692	2197
Average 0.5ng	1066	1025	2031	1438	1749	1774	1633	1738
Average 1.0ng	2099	1714	2959	2507	2646	2661	2568	2923
Ratio of 0.5ng/1.0ng	0.51	0.60	0.69	0.57	0.66	0.67	0.64	0.59

As shown in Table 5, some of the peak heights from the lower concentrated samples result in higher RFU's than the samples containing higher concentration. This inconsistency was discussed earlier, however in the previous test the amplified serial dilution was run only once. By running replicates of this sample it is possible to determine if this inconsistency is due to pipetting error. Table 5 shows that apart from a few outliers, the majority of the results show a normal linear progression. It also shows the loci peak heights for each replicate of the 0.5 and 1ng sample. The averages for each

locus can also be seen at the bottom of the table. The expected value for the ratio of 0.5ng to 1.0ng input amount is 50%. As seen in Table 5 the observed ratio value is similar to the expected, and if the few outliers of each group are ignored the ratio becomes closer to the expected value. The most likely cause for this inconsistency seems to be the result of pipetting error. It is extremely possible to have excess DNA on the tip or outside of the pipette tip when setting up amplification of each sample. Some DNA may even remain in the pipette tip after pipetting. All of these factors can affect the amount of DNA that is detected in the analyzer.

Precision Study

Data generated with the PowerPlex® 16 system on the 3100-Avant showed good sizing precision. Generally, at one standard deviation all size determinations should be well within the +/- 0.5 base pair range. The AmpFℓSTR® Profiler Plus™ and COfiler™ precision test required that 1 SD should be 0.15bp or less. Using these same parameters the PowerPlex® 16 precision results show that no alleles sized outside of 3 SD (0.45bp) and that a few sized between 1 and 2 SD. More specifically the alleles that were above 1 SD are highlighted on the following standard deviation pages attached.

The results of the PowerPlex® 16 controls precision test were similar to those above. None of the alleles tested reported higher than 3 SD, however there are several that fall above 2 SD which can be seen on the precision control pages in the appendix. Overall it seems that the PowerPlex 16 kit produces precise and consistent results when sizing alleles on the 3100-Avant, and should have great results in real case work.

CHAPTER V

CONCLUSION

This study has provided preliminary data demonstrating that the PowerPlex® 16 multiplex system can be a reliable and robust kit for the amplification and analysis of DNA from forensic evidentiary samples. The reliability and sensitivity of this system is dependant upon the utilization of the correct ramping parameters during the PCR reaction. Concordant allele calls between the PowerPlex® 16 and the AmpFℓSTR® Profiler Plus™ and COfiler™ systems were seen for all samples run in the mixture study. The PowerPlex® 16 system was able to yield a complete profile for the minor contributor in a mixture sample when it represented only 100pg of the total sample. The sensitivity study indicated that the PowerPlex® 16 system was able to generate a nearly complete profile with as little as 62.5pg of input DNA. At this input amount, only a single allele at the vWA locus dropped out. In comparison with the AmpFℓSTR® Profiler Plus™ and COfiler™ systems, the PowerPlex® 16 system appears to be 2 to 3 times more sensitive. Allelic drop out was observed in the AmpFℓSTR® Profiler Plus™ and COfiler™ system initially at 250pg of DNA and was prominent with 125pg of input DNA. At 62.5 pg of input DNA, none of the 13 Core CODIS STR loci generated a complete profile with the AmpFℓSTR® Profiler Plus™ and COfiler™ systems. In contrast the PowerPlex® 16 system produced a complete profile at 12 of the 13 Core CODIS STR loci.

Unfortunately only one sample of 9947A DNA was utilized for these sensitivity studies. For a thorough validation of the sensitivity and lower detection threshold of the

PowerPlex® 16 system, samples in addition to 9947A DNA should have analyzed. For both the mixture and sensitivity studies, samples should be chosen that contain a broad range of alleles across each of the loci. Testing of these samples would have provided more data about the PowerPlex® 16 kit limitations.

The study designed to evaluate the sizing precision of the PowerPlex 16 kit on the 3100-Avant produced results that were well within the 1 SD range previously established for the Profiler Plus and COfiler systems. The PowerPlex® 16 allelic ladder and the 9947A amplified products were used to assess the sizing precision on the 3100-Avant. The sizing data and allele calls generated by the PowerPlex® 16 system on the HCME 3100-Avant are comparable to the data generated with the AmpFℓSTR® Profiler Plus™ and COfiler™ systems.

The use of the PowerPlex® 16 kit by the HCME will reduce both the amount of input DNA required and the amount of analyst time, since only one amplification per sample is required as opposed to two with the AmpFℓSTR® Profiler Plus™ and COfiler™ kits. The ability of the PowerPlex® 16 kit to amplify the 13 Core CODIS STR plus the Penta D and Penta E loci will provide additional statistical discriminatory power that may be advantageous in some cases.

The experiments conducted did not constitute a complete internal validation study of the PowerPlex® 16 system. In order to provide more data, the HCME should conduct all validation experiments of the PowerPlex® 16 system with additional samples, and all studies should have been run in triplicate. The mixture studies should have included

samples that ranged from the upper limit to the lower limits of detection for the amplification kit. The mixture samples analyzed in this study only tested a narrow input range. Mixed samples with ratios of 19:1, 14:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:14, and 1:19 would have produced more informative results for an internal validation study. These preliminary results have demonstrated the utility of the Promega PowerPlex® 16 system at the HCME office.

APPENDIX

Precision Study (Ladder)

D3S1358	N	Mean(basepair)	Stand. Dev (bp)
12	10	109.65	0.067
13	10	113.86	0.058
14	10	118.17	0.043
15	10	122.22	0.048
16	10	126.35	0.052
17	10	130.60	0.056
18	10	134.70	0.040
19	10	138.78	0.034
20	10	142.60	0.049
TH01			
4	10	152.21	0.063
5	10	156.20	0.038
6	10	160.23	0.073
7	10	164.17	0.146
8	10	168.17	0.044
9	10	172.12	0.042
9.3	10	175.09	0.088
10	10	176.00	0.062
11	10	180.02	0.058
13.3	10	190.98	0.043
D21S11			
24	10	198.62	0.038
24.2	10	200.60	0.059
25	10	202.56	0.068
25.2	10	204.61	0.085
26	10	206.61	0.050
27	10	210.65	0.084
28	10	214.62	0.066
28.2	10	216.64	0.064
29	10	218.66	0.079
29.2	10	220.65	0.072
30	10	222.67	0.076
30.2	10	224.67	0.080
31	10	226.70	0.107
31.2	10	228.74	0.075
32	10	230.77	0.123
32.2	10	232.78	0.070
33	10	234.85	0.058
33.2	10	236.84	0.073
34	10	238.87	0.097

34.2	10	240.88	0.080
35	10	242.95	0.096
35.2	10	244.97	0.096
36	10	246.99	0.066
37	10	251.04	0.083
38	10	255.12	0.085
D18S51			
8	10	284.27	0.076
9	10	288.07	0.030
10	10	291.89	0.082
10.2	10	293.92	0.070
11	10	295.74	0.135
12	10	299.54	0.065
13	10	303.38	0.088
13.2	10	305.48	0.087
14	10	307.19	0.082
15	10	311.14	0.110
16	10	315.05	0.083
17	10	318.87	0.141
18	10	322.78	0.123
19	10	326.65	0.137
20	10	330.58	0.153
21	10	334.49	0.121
22	10	338.40	0.124
23	10	342.30	0.132
24	10	346.27	0.164
25	10	350.11	0.116
26	10	354.05	0.106
27	10	357.96	0.108
D5S818			
7	10	101.68	0.053
8	10	115.89	0.037
9	10	120.09	0.077
10	10	124.24	0.031
11	10	128.34	0.029
12	10	132.47	0.031
13	10	136.58	0.025
14	10	140.69	0.042
15	10	144.83	0.041
16	10	149.01	0.054

D13S317			
7	10	170.66	0.053
8	10	174.64	0.051
9	10	178.62	0.091
10	10	182.57	0.073
11	10	186.65	0.060
12	10	190.64	0.051
13	10	194.69	0.053
14	10	198.67	0.061
15	10	202.70	0.075
D7S820			
6	10	211.10	0.084
7	10	215.10	0.084
8	10	219.10	0.106
9	10	223.15	0.090
10	10	227.20	0.120
11	10	231.26	0.087
12	10	235.28	0.088
13	10	239.38	0.111
14	10	243.40	0.097
D16S539			
5	10	260.69	0.089
8	10	272.85	0.089
9	10	276.92	0.093
10	10	280.89	0.081
11	10	284.91	0.097
12	10	288.85	0.091
13	10	292.91	0.069
14	10	296.89	0.090
15	10	300.91	0.115
CSF1PO			
6	10	316.76	0.097
7	10	320.70	0.125
8	10	324.73	0.205
9	10	328.86	0.115
10	10	332.88	0.155
11	10	336.94	0.164
12	10	340.98	0.119
13	10	345.05	0.130

14	10	349.13	0.132
15	10	353.13	0.194
AMEL		103.55	0.057
X	10	109.47	0.048
Y	10		
vWA			
10	10	122.16	0.049
11	10	126.17	0.031
12	10	130.12	0.037
13	10	134.21	0.047
14	10	138.22	0.043
15	10	142.21	0.042
16	10	146.27	0.031
17	10	150.25	0.041
18	10	154.28	0.040
19	10	158.30	0.061
20	10	162.25	0.046
21	10	166.15	0.043
22	10	170.08	0.042
D8S1179			
7	10	201.73	0.066
8	10	205.72	0.057
9	10	209.68	0.070
10	10	213.65	0.066
11	10	217.64	0.089
12	10	221.66	0.067
13	10	225.63	0.095
14	10	229.63	0.062
15	10	233.68	0.068
16	10	237.74	0.064
17	10	241.80	0.073
18	10	245.86	0.095
TPOX			
6	10	260.90	0.128
7	10	264.96	0.072
8	10	268.89	0.094
9	10	272.93	0.098
10	10	276.91	0.116
11	10	280.83	0.077
12	10	284.81	0.048
13	10	288.69	0.069

FGA			
16	10	319.59	0.124
17	10	323.67	0.128
18	10	327.76	0.135
18.2	10	329.84	0.104
19	10	331.87	0.123
19.2	10	333.93	0.127
20	10	335.96	0.113
20.2	10	338.04	0.115
21	10	340.07	0.130
21.2	10	342.12	0.142
22	10	344.18	0.139
22.2	10	346.20	0.133
23	10	348.24	0.145
23.2	10	350.35	0.154
24	10	352.33	0.141
24.2	10	354.37	0.169
25	10	356.32	0.116
25.2	10	358.40	0.130
26	10	360.35	0.105
27	10	364.38	0.098
28	10	368.37	0.071
29	10	372.46	0.055
30	10	376.47	0.048
31.2	10	382.45	0.068
43.2	10	431.34	0.156
44.2	10	435.39	0.102
45.2	10	439.61	0.118
46.2	10	443.91	0.156

Precision Study (9947A)

D3S1358	N	Mean(basepair)	Stand. Dev (bp)
14	10	118.17	0.032
15	10	122.32	0.044
TH01			
8	10	168.19	0.038
9.3	10	175.11	0.053
D21S11			
30	10	222.51	0.334
D18S51			
15	10	311.19	0.060
19	10	326.74	0.061
D5S818			
11	10	128.39	0.055
D13S317			
11	10	186.69	0.051
D7S820			
10	10	226.93	0.323
11	10	231.07	0.285
D16S539			
11	10	285.04	0.045
12	10	289.00	0.041
CSF1PO			
10	10	333.01	0.067
12	10	341.15	0.045
vWA			
17	10	150.21	0.036
18	10	154.24	0.040
D8S1179			
13	10	225.45	0.326
TPOX			
8	10	269.04	0.044
FGA			
23	10	348.39	0.056
24	10	352.47	0.062

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