A Validation of Promega's PowerPlex® 16 HS System Testing the Strengths and Limitations

Megan A. Forbes

University of Central Oklahoma

Forensic Science Institute

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By: Megan Annsley Forbes

A THESIS

APPROVED FOR THE FORENSIC SCIENCE INSTITUTE

April 2011

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Dr. Dwight E. Adams Committee Chair

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Dr. Wayne D. Lord Committee Member

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Dr. Michelle Haynie Committee Member

Abstract

An internal validation study was conducted using the PowerPlex® 16 HS system to ensure proper performance on the Applied Biosystems 3130 Genetic Analyzer in the University of Central Oklahoma laboratory. Manual extraction with the DNA IQ™ system was performed. The Quantifiler™ Human Quantification kit was used to quantify the samples. Promega Corporation's PowerPlex® 16 HS system was used to amplify DNA samples on a GeneAmp® PCR System 9700 thermal cycler. Separation occurred through capillary electrophoresis on an Applied Biosystems 3130 Genetic Analyzer. Following parameters established through the validation, an environmental study was conducted to simulate casework samples. The environmental study included ultraviolet treatment, tannic acid, humic acid, and hematin. The results support the multiplexing system is capable of handling DNA samples.

Keywords: Validation, PowerPlex® 16 HS, Environmental Inhibitors

Table of Contents

Introduction

As technology evolves, deoxyribonucleic acid (DNA) analysis procedures have become routine forensic tests that are performed in laboratories. Forensic DNA testing involves the evaluation of biological material within evidence using DNA technologies and methodologies (DNA Advisory Board, 2000). DNA analysis evaluates biological evidence, which consists of semen, blood, saliva, vaginal secretions, urine, and feces.

Different environmental contaminants degrade biological material and inhibit the analysis process, which may prevent genetic profiles from being obtained. The mission of manufacturers is to produce a commercialized amplification system that can handle forensic evidence. An amplification system's performance is vital when performing successful analysis of degraded and minute amounts of DNA. Standards are set through the DNA Advisory Boards (DAB) guidelines to ensure quality results are achieved for inclusion into a national database. The different multiplexing systems used to analyze casework are regulated by the National DNA Index System (NDIS) and must be tested and evaluated on the individual laboratory's equipment.

This study consisted of the examination of the PowerPlex® 16 HS system (Promega Corporation, Madison, WI, USA, #DC2100), particularly testing the strengths and limitations through the analysis of single source, mixed, and environmentally insulted blood samples. An internal validation study was conducted to ensure proper functioning of the equipment present in the laboratory with the PowerPlex® 16 HS system. Insight gained through the validation was then applied to all samples that were analyzed with the PowerPlex® 16 HS system. At the conclusion of the manufacturers research, it was determined that the PowerPlex® 16 HS system had the ability to generate a profile from insulted samples (Promega Corporation, 2011). An environmental study was conducted to evaluate the effect of known inhibitors to the

amplification process. This more accurately depicts samples and results that would be obtained from casework DNA samples. The study included samples that were insulted with ultraviolet treatment, tannic acid, humic acid, and hematin.

Literature Review

Deoxyribonucleic Acid (DNA)

The human genome.

DNA serves as the molecule of genetic inheritance. The functional unit of DNA is the nucleotide monomer, which includes a 2'-deoxyribose, phosphoric acid, and a nitrogenous base, as seen in Figure 1.1 (Watson & Crick, 1953). The sugar is a pentose compound, which contains five carbon atoms, and a 3' hydroxyl group that is specific to DNA. The phosphate group is bound to the 5' carbon of the 2'-deoxyribose sugar molecule via a phosphoester bond. The phosphate is negatively charged, which imparts an overall negative charge to the DNA molecule. Nucleotides are composed of the four bases, guanine, adenine, cytosine, and thymine, which form a glycosidic bond to the 1' carbon of the 2'-deoxyribose (Watson & Crick, 1953). The four bases are displayed in Figure 1.1. Individual nucleotides polymerize to one another through the formation of phosphodiester bonds.

The double helix structure of DNA is formed when complementary and anti-parallel chains of nucleotides anneal to one another, as shown in Figure 1.2. The annealing of complementary bases is regulated and made stable through the formation of multiple hydrogen bonds (Watson & Crick, 1953). Complementary bases cytosine and guanine pair to form three hydrogen bonds, whereas adenine and thymine result in the formation of two hydrogen bonds (Watson & Crick, 1953).

The human genome is comprised of 23 pairs of chromosomes. Located on each of these chromosomes are two distinct regions of DNA, coding regions and non-coding regions. Coding regions consist of DNA that is transcribed and translated, and the regions are often referred to as genes. Genes are stretches of DNA that are responsible for the production of a specific protein

product (National Research Council, 1996). While a gene is composed of DNA, not all DNA serves as a gene. Non-coding regions of DNA are not transcribed and translated; therefore, they do not code for protein sequences. The majority of the genome, approximately 98%, is comprised of introns and non-coding sequences, which offer a large amount of genetic diversity and regulation in gene expression.

Figure 1.1. Nucleotide Structure and Base Structures. (Brown, 2007)

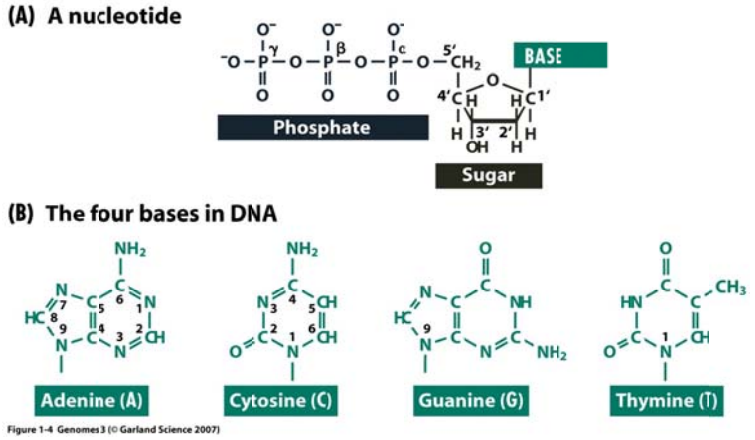

Figure 1.1. Depicts the functional unit of DNA, which is the nucleotide. The nucleotide is composed of a phosphate group, a 2'-deoxyribose, and a nitrogenous base. The four bases present within DNA are displayed at the bottom of the figure.

Figure 1.2. The DNA Double Helix Structure. (Brown, 2007)

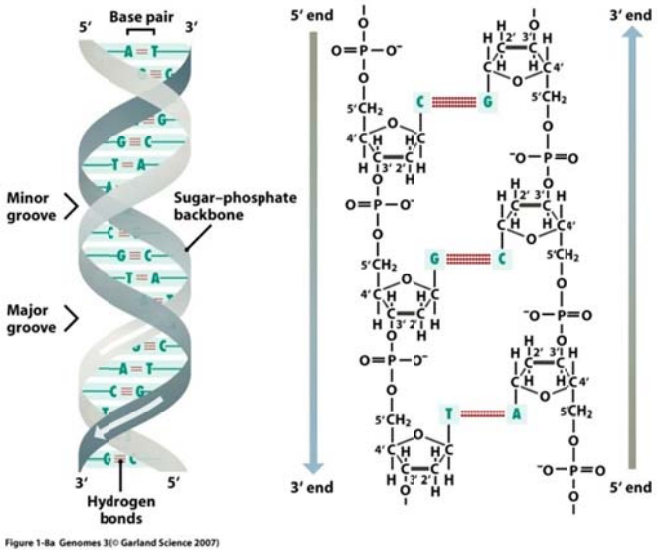

Figure 1.2. Displays the double helix structure of the DNA molecule. The anti-parallel chains are seen with hydrogen bonds located between the complementary bases and a phosphodiester backbone.

The beginning of forensic science.

Forensic DNA analysis plays an important role in the ability of the legal system to resolve criminal cases. The science of forensic DNA analysis has not always been instituted as it is today. In the 1980's, sequences called variable number of tandem repeats (VNTRs) were used to analyze genotypes (Nakamura et al., 1987). VNTRs are also known as minisatellites, a tandemly repeated region of DNA. VNTRs are 10 to 100 base pair (bp) long repeated units of DNA that ranged in size from 0.5 to 40 kb. Dr. Alec Jeffrey's proposed the idea that numerous different sequences and patterns of VNTRs could be used to identify an individual's specific genomic DNA (Jeffreys, Wilson, & Thein, 1985).

The analysis of VNTRs required the use of restriction enzymes and a technique called restriction fragment length polymorphism (RFLP) (Saiki et al., 1985). RFLP utilizes a restriction

enzyme to break high quality genomic DNA in numerous locations called restriction sites. Gel electrophoresis was used to separate the fragments of genomic DNA according to molecular weight. The DNA fragments were placed on a membrane and hybridized to a probe to determine the length of the fragment. The varying lengths were used as identifiers of individuals. VNTRs are highly variable, which made them ideal for use in differentiation between individuals. The time and labor intensive nature of VNTRs analysis were quickly replaced with more rapid forms of DNA analysis. Over time, the methodology used by scientist shifted from VNTRs to short tandem repeats due to the new technologies developed, which has enabled improved analysis of samples (U.S. Department of Justice, 2000).

Short tandem repeats (STRs).

Several groups of tandemly repeated regions of DNA exist with varying patterns of base pairs. Microsatellites, or short tandem repeats (STRs), usually contain 2 to 10 bp repeats. STRs are useful genetic markers in a forensic setting that can be amplified by the polymerase chain reaction (PCR) (Ellegren, 2004). Many STRs are located throughout the human genome; however, after extensive investigations only a few have been selected for use in forensic testing (Butler & Reeder, 2010).

Thirteen STRs were chosen by the Federal Bureau of Investigation (FBI) to be utilized in a national database that serves as a library for genetic profiles. CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 are referred to as the thirteen Combined DNA Index System (CODIS) Core STR Loci in the United States (Budowle, Moretti, Niezgoda, & Brown, 1998). STR loci are unlinked, found on different chromosomes, or located on distant regions of the same chromosome. An advantageous characteristic that makes STRs useful within forensic analysis is their high discrimination factor,

which provides a differentiation ability with individuals in the population through distinguishable alleles (Butler & Reeder, 2010). STRs are compatible with multiplexing, which allows the amplification of all thirteen DNA targets in one PCR tube. Low stutter and mutation rates exist with STR loci. Most STRs utilized in forensic testing are tetrameric (Bacher et al., 1998). Tetrameric STRs have been proven to efficiently identify individuals with low levels of mutations present (Caskey, Chakraborty, Edwards, Hammond, & Jin, 1992). STRs have high heterozygosity levels that present genetic variability throughout the human population. STRs offer ideal markers for forensic DNA testing.

DNA Analysis

The methodology for the forensic analysis of DNA samples begins with DNA extraction. The goal of extraction is to separate genomic DNA from a biological sample located on a substrate. The sample must then be quantified. During the quantification step, DNA is measured to estimate the amount of usable DNA that exists within a sample. Information that is obtained from DNA quantitation will be utilized during the amplification phase of analysis. The PCR process exponentially copies DNA to generate quality DNA amplicons. Genetic analysis of the amplicons is performed through capillary electrophoresis (CE), a form of chromatography. The resulting data are obtained and analyzed as a genetic profile.

DNA Extraction and the DNA IQ™ System

The DNA IQ™ system is an extraction method available from Promega Corporation that utilizes paramagnetic beads to extract and purify DNA from cellular components, PCR inhibitors, and other problematic material commonly associated with forensic casework samples (Promega Corporation, 2009). The paramagnetic resins that are used to attract and hold DNA

are positively charged. The negative charge of DNA allows a strong interaction to occur, which binds the phosphate backbone of DNA to the resin throughout the extraction process.

Extraction process.

The DNA IQ™ system extraction process incorporates numerous chemistries designed to break the cell membrane, purify the DNA, and stabilize the DNA throughout the protocol. The DNA IQ™ system lysis buffer functions to liberate the DNA from the cells located on the item of evidence. Proteinase K, sodium dodecyl sulfate (SDS), and dithiothreitol (DTT) within the lysis buffer help to free cells from a sample by breaking down the proteins, lysing the cellular membranes, and disrupting the disulfide bonds that bond the cell membrane together respectively. Paramagnetic resin is used to bind the DNA located within the sample. Wash buffer is used to clean the sample of any components that may interfere with the PCR process. Elution buffer is used to neutralize the charge of DNA, which releases the bond of the resin and DNA.

The DNA IQ™ system is advantageous for use in forensic casework due to a lack of hazardous chemicals involved throughout the process and reduced occurrences of contamination (Promega Corporation, 2009). The main concern with the DNA IQ™ system lies with the amount of DNA that is extracted from the sample. The limit exists with the amount of DNA the paramagnetic resin can bind. The binding affinity of the resin for FTA blood-card punches is 50 to 100 ng of DNA, liquid whole blood is 50 to 200 ng of DNA, and buccal swabs is 100 to 500 ng of DNA (Promega Corporation, 2009).

DNA Quantification and the Quantifiler™ Human Quantification Kit

DNA quantification serves to identify the concentration of amplifiable human DNA in a given sample (Nicklas & Buel, 2003). This process is required by the DAB in casework extracts

using a standard method, which is specific for human nuclear DNA (DNA Advisory Board, 2000). The Quantifiler™ Human DNA Quantification kit is an upper primate specific nuclear DNA quantitation assay manufactured by Applied Biosystems, a Life Technologies company, using real-time PCR methods.

The Quantifiler™ human quantification assay utilizes two 5' nuclease assays, a human specific assay and an internal PCR control assay (Applied Biosystems, 2010). The human telomerase reverse transcriptase (hTERT) gene on chromosome 5p15.33 amplifies a non-translated region of DNA (Applied Biosystems, 2010). The internal PCR control components include synthetic template, two primers, and a TaqMan probe used to detect the amplified DNA (Applied Biosystems, 2010).

TaqMan probe.

The TaqMan probe utilizes a reporter dye linked to the 5' end of the probe, a minor groove binder linked to the 3' end of the probe, and a non-fluorescent quencher at the 3' end of the probe (Applied Biosystems, 2010). During real-time PCR, the TaqMan probe anneals to the complementary sequence between the primers, and the reporter dye and quencher dye are suppressed on the probe (Applied Biosystems, 2010). As the DNA polymerase travels from the 5' end toward the 3' end, the reporter dye is cleaved and separated from the quencher resulting in a fluorescence signal released (Applied Biosystems, 2010). The TaqMan probe method is depicted in Figure 1.3.

Figure 1.3. Quantifiler™ Human DNA Quantification kit graphic. (Applied Biosystems, 2010)

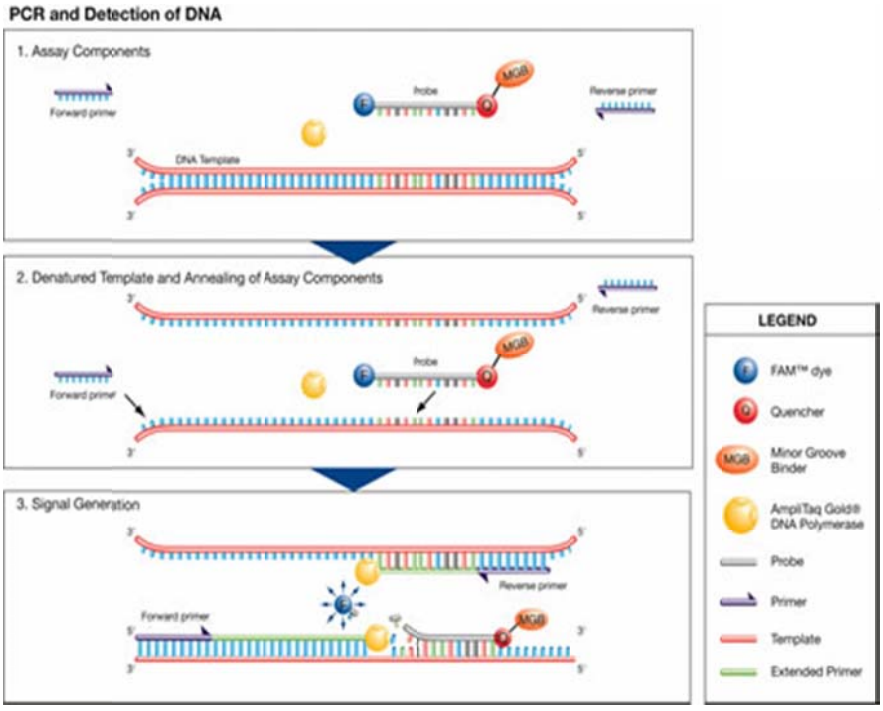

Figure 1.3. The figure shows the process of double stranded DNA denaturing followed by the TaqMan Probe annealing to the single stranded DNA. The DNA polymerase releases the reporter dye, which emits a fluorescent signal.

DNA Amplification and Polymerase Chain Reaction (PCR)

Amplification serves as the process of "increasing the number of copies of a DNA region, usually by PCR" (National Research Council, 1996). PCR exponentially copies portions of DNA to increase the amount of the targeted region that is present. PCR is utilized to examine genetic polymorphisms (Mullis & Faloona, 1987).

The PCR process contains thermal cycles, which heat and cool the template DNA in phases. Denaturation is the process of breaking double stranded DNA through disruption of the hydrogen bonds linking nucleotides. This process occurs at approximately 94 °C (Saiki et al., 1988). The annealing phase binds primers to the single stranded DNA at a temperature range of

50 to 60 °C (Saiki et al., 1988). PCR is concluded by elongation conducted at 72 °C, the optimum temperature for Taq DNA polymerase (Saiki et al., 1988). Elongation synthesizes the DNA strands (Saiki et al., 1988). Taq polymerase is a heat stable DNA polymerase, which is beneficial for PCR. Figure 1.4 depicts the phases of PCR.

Several components and reagents are used to perform PCR, which include DNA polymerase, dNTPs, primers, DNA template, buffer, divalent cations, and water (Butler, 2005). DNA polymerase is the enzyme that synthesizes the nucleotides of the new DNA strand to the 3' end. Nucleotides, also called dNTP's, are the building blocks of DNA. Primers or oligonucleotides are short DNA sequences that flank the target region that will be copied, and they are necessary because DNA polymerase cannot synthesize a new strand without having starting material. Buffer maintains and controls the pH of the reaction. Divalent cations are used to help the DNA polymerase facilitate the reaction. Water is utilized to bring the overall mix to its final volume.

Commercialized manufactured systems combine most components that are necessary for the PCR process into a master mix. The master mix includes everything except the DNA template, and it is used to avoid human mistakes that could occur in pipetting small amounts of materials (Butler, 2005).

A major component for amplifying a DNA strand is the DNA polymerase, which synthesizes the new strand. PCR utilizes Taq DNA polymerase, which originates from the bacterium *Thermus aquaticus*. *Thermus aquaticus* is a prokaryote that can survive at extremely high temperatures, and allows for a quick and direct method to amplify DNA (Innis, Myambo, Gelfand, & Brow, 1988); (Saiki et al., 1988).

PCR is a crucial step in the analysis of DNA evidence in forensic science, and numerous advantages exist with the process. PCR requires very little time and labor from the analyst, and it does not require large quantities of DNA. PCR works on degraded DNA samples, which are present at the majority of crime scenes (Butler, 2005). Human specific primers prevent non-human contaminants, such as bacteria and fungi, from interfering with amplification (Dieffenbach, Lowe, & Dveksler, 1993). Finally, multiplexing is possible, which is the simultaneous amplification of two or more regions of DNA (Edwards & Gibbs, 1994). One major disadvantage with PCR and STRs is the ease in which contamination can occur by the analyst and environment (Butler, 2005).

Figure 1.4. Steps in the PCR Process. (Brown, 2007)

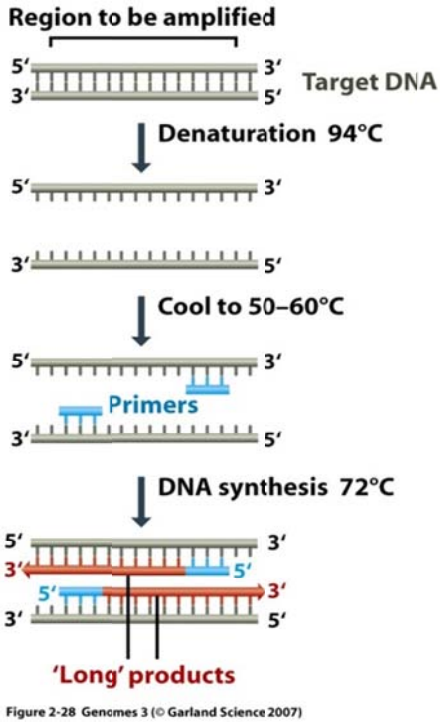

Figure 1.4. The steps in the PCR process are shown. Denaturation occurs at 94 °C. The primers anneal to the single stranded DNA within a range of 50 to 60 °C. Elongation occurs at 72 °C to synthesize new DNA strands.

Capillary Electrophoresis

Capillary Electrophoresis (CE) is a form of chromatography that is used to separate various materials. CE separates DNA through movement of a mobile phase in a stationary phase evaluated by molecular weight and shape.

CE identifies fluorescently labeled DNA products of the PCR process. Various sized products move through the capillary based on the fragment size and charge. In genetic analysis, the stationary phase within the capillary is composed of Performance Optimized Polymer 4 (POP-4) (Lazaruk et al., 1998). The mobile phase consists of DNA fragments moving through the POP-4 within capillaries. POP-4 has the capability to separate microsatellites, specifically separate DNA fragments that differ in size from 1 to 250 nucleotides (Lazaruk et al., 1998). The polymer used in forensics coats the inside of the capillary to control the electro-osmotic flow of DNA (Lazaruk et al., 1998). Movement is based on the electrical current that runs from the anode to the cathode, which drives the DNA to migrate through the capillary (Department of Justice, 2004). The shortest fragments of DNA move through the capillary with more ease than the larger fragments thus they move more quickly through the polymer. A laser is used to identify the color of light that is given off through the 5' fluorescent tag attached to the single stranded DNA (Department of Justice, 2004). The burst of light is captured by the charge-coupled device (CCD) camera. The bursts are reported as peaks on an electropherogram for an analyst to interpret (Department of Justice, 2004).

Forensic analysis.

The steps for forensic labeling are: data collection, peak recognition, color separation, peak sizing, allelic ladder comparison, and allele assignment (Butler, Buel, Crivellente, & McCord, 2004). The peak is sized by comparison to an internal size standard that is run concurrently with

all samples. An allelic ladder is run with every batch of samples, and it represents common known allele sizes at a specific locus (Butler et al., 2004).

Numerous characteristics have been determined to be necessary for a separation and detection technique utilized in forensic casework which are: "reliable sizing over a 75 to 500 bp size range, high run-to-run precision between processed samples to permit comparison of allelic ladders to sequentially processed STR samples, effective color separations of different dye sets used to avoid bleed through between four or five different colors, and finally, resolution of at least one base-pair to approximately 350 bp to permit reliable detection of microvariant alleles" (Butler et al., 2004).

Capillary Electrophoresis instruments, like the Applied Biosystems 310 Genetic Analyzer, 3100 Genetic Analyzer, and 3130 Genetic Analyzer are common in forensic laboratories that analyze STRs (Department of Justice, 2004).

Numerous advantages exist with little preparation time needed when performing CE. CE is fully automated, and easy to use with little time necessary for set up and preparation (Butler et al., 2004). CE has better reproducibility due to the polymer type used and the little affect bubbles have on the process (Butler et al., 2004). Greater resolution is due to effective heat dissipation (Butler et al., 2004). Due to the numerous wash and rinse steps, cross-contamination between samples is significantly reduced (Butler et al., 2004). Finally, small quantities are required to perform CE.

Promega's PowerPlex® 16 HS System

The PowerPlex® 16 HS system is a second generation multiplexing system that is new to forensic DNA testing. The PowerPlex® 16 HS system amplifies sixteen locations in the human

genome, 15 STR loci and Amelogenin, in a three color detection system (Promega Corporation, 2011).

The PowerPlex® 16 HS loci consist of: Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317, and D5S818 (Promega Corporation, 2011). Three different fluorescently labeled dyes are used to label the primers. The fluorescein (FL) dye labels D3S1317, TH01, D21S11, D18S51, and Penta E (Promega Corporation, 2011). Carboxy-tetramethylrhodamine (TMR) is used to label FGA, TPOX, D8S1179, vWA, and Amelogenin; and 6-carboxy-4',5'-dichloro-2'7'-dimethoxy-fluorescein (JOE) is used to label Penta D, CSF1PO, D16S539, D7S820, D13S317, and D5S818 (Promega Corporation, 2011). The internal lane standard 600 is labeled with carboxy-X-rhodamine (Promega Corporation, 2011). Figure 1.5 depicts the dye colors and 16 loci examined.

Second generation system.

The PowerPlex® 16 HS system has a newly designed master mix, which includes a hot start Taq polymerase. A monoclonal antibody blocks the polymerase activity of *Thermus aquaticus* (Kellogg et al., 1994). The antibody prevents Taq polymerase from beginning amplification when it is added to the PCR reaction at room temperature (Kellogg et al., 1994). During the initial denaturation step, the antibody is denatured to regain full enzyme activity (Genesis Biotech Inc.).

"The ILS 600 contains 22 fragments of 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, and 600 bases in length" (Promega Corporation, 2011). The allelic ladder contains common alleles observed within the population that is run with each electrophoretic injection as a reference standard (Sajantila, Puomilahti,

Johnsson, & Ehnholm, 1992). The PowerPlex® 16 HS Allelic Ladder was analyzed by Promega on an Applied Biosystems 3130 Genetic Analyzer utilizing the 3 kV and 5 seconds parameter (Promega Corporation, 2011).

The loci and primers used were selected to limit artifacts such as repeat slippage and terminal nucleotide addition present when used with Taq DNA polymerase. The primers for the 15 STR loci specific to Promega Corporation's multiplexing system are located in Table 1.6.

The PowerPlex® 16 HS system amplifies DNA in two sets of cycles during amplification. The first set is 10 cycles long, which is followed by a set of 22 cycles. This change allows the system after ten rounds of amplification to switch from using the genomic DNA as the template to the amplicon (Lyons, 2010). This slight change allows the melting temperature to drop to 90 °C in order to save the half-life of the Taq (Lyons, 2010). Essentially, this is performed to reduce the heat damage endured during the first ten cycles at 94 °C.

Figure 1.5. The PowerPlex® 16 Loci and Four Dye Channels (Butler & Reeder, 2010)

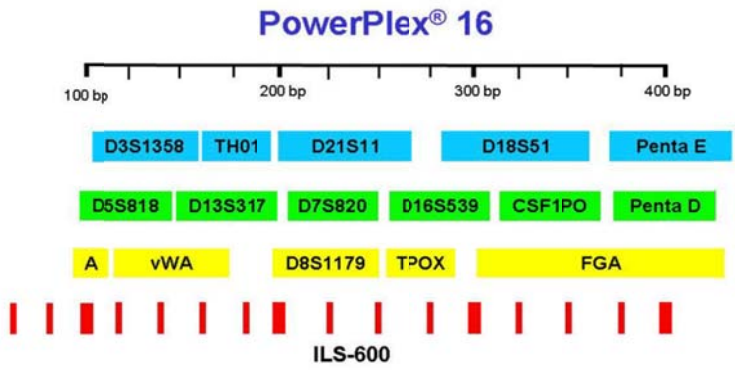

Figure 1.5. The figure displays the four dye channels analyzed by PowerPlex® 16 HS System. The loci are shown in the position a genetic profile would be viewed.

Table 1.6. Fifteen STR Primer Sequences Specific to the PowerPlex® 16 HS System. (Butler & Reeder, 2010)

STR Locus	Promega's Primer Sequence	Chromosome Location
CSF1PO	5'-[JOE]-CCGGAGGTAAAGGTGTCTTAAAGT-3' 5'-ATTTCCTGTGTCAGACCCTGTT-3'	5q33.1
FGA	5'-[TMR]-GGCTGCAGGGCATAACATTA-3' 5'-ATTCTATGACTTTGCGCTTCAGGA-3'	4q28
TH01	5'-[FL]-GTGATTCCCATTGGCCTGTTC-3' 5'-ATTCCTGTGGGCTGAAAAGCTC-3'	11p15.5
TPOX	5'-GCACAGAACAGGCACTTAGG-3' 5'-[TMR]-CGCTCAAACGTGAGGTTG-3'	2p25.3
vWA	5'-GCCCTAGTGGATGATAAGAATAATCAGTATGTG-3' 5'-[TMR]-GGACAGATGATAAATACATAGGATGGATGG-3'	12p13.31
PentaD	5'-[JOE]-GAAGGTCGAAGCTGAAGTG-3' 5'-ATTAGAATTCTTTAATCTGGACACAAG-3'	21q22.3
PentaE	5'-ATTACCAACATGAAAGGGTACCAATA-3' 5'-[FL]-TGGGTTATTAATTGAGAAAACTCCTTACAATTT-3'	15q26.2
D3S1358	5'-ACTGCAGTCCAATCTGGGT-3' 5'-[FL]-ATGAAATCAACAGAGGCTTGC-3'	3p21.31
D5S818	5'-GGTGATTTTCCTCTTTGGTATCC-3' 5'-[JOE]-AGCCACAGTTTACAACATTTGTATCT-3'	5q23.2
D7S820	5'-[JOE]-ATGTTGGTCAGGCTGACTATG-3' 5'-GATTCCACATTTATCCTCATTGAC-3'	7q21.11
D8S1179	5'-ATTGCAACTTATATGTATTTTTGTATTTCATG-3' 5'-[TMR]-ACCAAATTGTGTTCATGAGTATAGTTTC-3'	8q24.13
D13S317	5'-ATTACAGAAGTCTGGGATGTGGAGGA-3' 5'-[JOE]-GGCAGCCCAAAAAGACAGA-3'	13q31.1
D16S539	5'-GGGGGTCTAAGAGCTTGTAAAAAG-3' 5'-[JOE]-GTTTGTGTGTGCATCTGTAAGCATGTATC-3'	16q24.1
D18S51	5'-[FL]-TTCTTGAGCCCAGAAGGTTA-3' 5'-ATTCTACCAGCAACAACACAAATAAAC-3'	18q21.33
D21S11	5'-ATATGTGAGTCAATTCCCCAAG-3' 5'-[FL]-TGTATTAGTCAATGTTCTCCAGAGAC-3'	21q21.1

Data Analysis

Data analysis of DNA encompasses numerous different artifacts and unwarranted peaks within a genetic profile. When DNA is degraded, the sequence of STRs can be interrupted resulting in unsuccessful amplification.

Spurious peaks.

A detection threshold is established to separate baseline noise from a true allele. The threshold value is set by the individual laboratory according to a validation study. Artifacts can

occur within a profile that include dye blobs, spikes, and noise. Artifacts are generally instrument related malfunctions or small chemical problems (Department of Justice, 2004).

A dye blob is the breakdown of a fluorescent dye tag on a primer (Applied Biosystems, 1988). Dye blobs are typically wider peaks with a rounded apex. Spikes are short intense peaks that are thought to be caused by air bubbles within the equipment or fluctuations in the electrical current within the Applied Biosystems 3130 Genetic Analyzer (Department of Justice, 2004). Spikes are not reproducible. Noise is non-reproducible peaks that are caused by many factors such as air bubbles, crystals, contamination, or current fluctuations in the equipment (Department of Justice, 2004). Pull-up is an artifact that occurs when dyes bleed into additional color channel (Department of Justice, 2004). Pull-up results from too much DNA being injected into the analyzer, or the spectral calibration failing to discriminate between dye colors (Department of Justice, 2004).

Stutter is an artifact represented by a peak which is one repeat unit smaller or larger than the principal allele observed (Walsh, Fildes, & Reynolds, 1996). Stutter is a frequent occurrence with the amplification process, and it is a reproducible and predicted artifact. Slipped-strand mispairing is thought to explain how stutter is formed (Walsh et al., 1996). The DNA polymerase becomes detached during synthesis, as it re-anneals a loop occurs that creates a PCR product one repeat smaller (Walsh et al., 1996). Stutter calculations are performed by dividing the peak height value of the stutter peak by the peak height value of the true allele.

Non-template addition is an additional peak that is one base pair longer than the principal allele, usually '+A' or '-A' (Clark, 1988). An extra nucleotide is added to the 3' end of the amplification product. The result occurs because DNA polymerase is not able to finish the

elongation step of PCR for all products. Multiplexing systems are designed to limit the addition of adenine.

Microvariants and off-ladder (OL) alleles occur when an allele is detected that is not present in the allelic ladder. An allelic ladder contains the most prevalent alleles seen in a population. Unique or rare alleles may not be present within the allelic ladder. A microvariant occurs when an allele contains incomplete repeat units. Microvariants may be called an off-ladder allele because it does not size to a known reference bin within the allelic ladder.

DNA imbalance.

Stochastic effects are the unequal sampling of two alleles in an individual who is heterozygous at a particular locus. Stochastic effects often occur in degraded DNA sample, when low quantities of DNA are amplified. The Scientific Working Group on DNA Analysis Methods (SWGDAM) (2010) recommends a 60% value to be used when determining if a heterozygote imbalance occurs. Allelic dropout occurs when an allele is not present within the genetic profile that otherwise should.

Mutations are changes in the DNA sequence. They can occur in primer binding areas or within the STR amplified region (Ellegren, 2004). Null alleles are alleles that are not amplified due to primer binding region mutations. Null alleles are rare due to the elevated level of success that DNA multiplexing systems are manufactured with today (Budowle, 2000).

Validation Process

Validation refers to a forensic laboratory demonstrating procedures that are robust, reliable, and reproducible using specific chemistries on specific instrumentation (Butler & Reeder, 2010). A validation is robust if successful results are obtained consistently with few errors forcing a procedure to be repeated (Butler & Reeder, 2010). Reliability ensures correct

results are gathered from tests, and reproducibility ensures the same results will be obtained each time a sample is tested (Butler & Reeder, 2010). Two forms of a validation are established and required in forensic science, a developmental and internal validation.

Developmental validation.

Developmental validations ensure accuracy, precision, and reproducible results. The developmental validation "is the acquisition of test data and determination of conditions and limitations of a new or novel DNA methodology for use on forensic samples" (DNA Advisory Board, 2000). It is used primarily to test new STR multiplexing systems, STR loci, primer sets, and new innovations (Butler & Reeder, 2010). The requirements of a developmental validation are species specificity, sensitivity, stability, mixture studies conducted, and population distribution data available for use (DNA Advisory Board, 2000).

PowerPlex® 16 HS system developmental validation.

A developmental validation was performed using PowerPlex® 16 HS, which found it to be accurate and consistent when used on forensic samples (Ensenberger et al., 2010). Within the PowerPlex® 16 HS system, the primers used were human specific, it was able to resist inhibitors amplification, reproducible results between numerous laboratories were obtained, it held up against forensic case samples and mixtures, and the protocol for PCR designed by the manufacturer was sufficient in their instruction (Ensenberger et al., 2010).

The results of the developmental validation are an important key to laboratories determining whether to validate the system for internal use. The developmental validation included a species specificity test. Twenty-eight non-human samples were tested, and no peaks were detected in the STR loci. A sensitivity study was conducted, to determine the lowest amount of input DNA the system could withstand when analyzing samples. The study

concluded, when using a 32 cycle amplification protocol, 85% of alleles were called with 31.25 picograms (pg) of DNA (Ensenberger et al., 2010). In a 30 cycle amplification protocol more than 50% of alleles were called with 62.5 pg of DNA (Ensenberger et al., 2010). A reproducibility and concordance study was carried out to ensure alleles were called correctly and consistently when samples were re-analyzed. The results determined complete concordance was displayed (Ensenberger et al., 2010). The peak height ratios were evaluated at heterozygous loci for 313 samples. This examination aided the process to set a stochastic threshold. The average peak height ratio was 0.86 with a standard deviation of 0.10 (Ensenberger et al., 2010). A mixture study was conducted on the following ratios: 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1: 5, 1:9, and 1:19 (Ensenberger et al., 2010). All minor alleles ranging between 5:1 and 1:5 were identified (Ensenberger, et al., 2010). Between 9:1 and 1:9, over 90% of minor alleles were detected and present; however, the ratios 19:1 and 1:19 only detected minor alleles for around 70% of the data (Ensenberger, et al., 2010). The findings of the study concluded that the PowerPlex® 16 HS system is adequately prepared to analyze samples that were comprised of low quality and quantity DNA (Ensenberger, et al., 2010).

A developmental validation focuses on the accuracy of the multiplexing system, whereas an internal validation is performed by the individual laboratories to ensure the multiplexing system works properly on the instrumentation that will run casework samples.

Internal validation.

An internal validation is an "accumulation of test data within the laboratory to demonstrate that established methods and procedures perform as expected in the laboratory" (DNA Advisory Board, 2000). The requirements include testing on known and non-probative evidence, documented reproducibility, and precision for human samples (DNA Advisory Board, 2000).

Numerous different components comprise an internal validation study. A detection threshold study is conducted to determine a threshold above which allele calls are confidently recognized as true alleles. The detection threshold separates baseline noise recorded on the instrument from true allele peaks. A dynamic range and sensitivity study establishes a range of DNA concentrations in which a complete and useable genetic profile is obtained with no allele dropout or off-scale products observed. The stochastic threshold is studied to establish a level that confidently recognizes two alleles as sister alleles if their peak heights exceed the threshold value. Stochastic thresholds play a large role in mixture deconvolution. Precision is studied to determine the accuracy and reproducibility of the CE instrument used in the laboratory. A stutter study is conducted to examine all stutter peaks that occurred when analyzing a sample. Stutter is a known and reproducible artifact; therefore, it can be analyzed and a percentage can be set for every locus examined. A mixture study establishes interpretation guidelines when dealing with two source DNA profiles. A contamination study is run to ensure carry over is not present between capillaries. Finally, a mock case is conducted to ensure proper processing of casework simulated samples by the analyst and instrumentation.

An internal validation of the PowerPlex® 16 HS system was performed by the CeSAAN Laboratory in Venezuela, which concluded this kit was able to produce results when samples contained degraded and small amounts of DNA (Caraballo, Loyo, Sanchez, & Takiff, 2009). Its use has been credited with increasing the types of samples that have been successfully analyzed to identify human genetic markers (Caraballo et al., 2009).

Environmental Inhibitors

Crimes occur in nature; therefore, numerous factors within the environment affect the longevity and life of the evidence deposited in a scene. Due to the standards that forensic

methodologies must withstand to be admitted into a court of law, it must be demonstrated the multiplexing system chemistries have the ability to analyze real-world samples.

Previous studies were performed by the FBI that evaluated environmental insults, different substrates, and contaminants when RFLP analysis was the standard within the field (Adams et al., 1991). Common links between RFLP analysis and PCR analysis include the utilization of probes and primers that label target DNA. Due to the nature of crimes, environmental inhibitors need to continually be evaluated as new technology evolves to analyze DNA samples. The inhibitors that were focused on in this study are ultra violet treatment, tannic acid, humic acid, and hematin.

Ultra Violet (UV) treatment.

UV treatment simulates the effect of sunlight on a DNA sample. UV irradiation effects the structure and break down of DNA (Klouwen, Appelman, & Barendsen, 1962). Figure 1.7 depicts the break in DNA bonds. "UV treatment induces cyclobutane dimmers (CPD), pyrimidine-pyrimidone (6-4) photoproducts and DNA-protein cross-links" (Sgura, Meschini, Antoccia, Palitti, Obe, & Tanzarella, 1996). "CPDs are formed at TT, TC-CT, and CC sequences, with a frequency of 50, 40, and 10% respectively" (Ellision & Childs, 1981). "Structural studies indicate that the presence of CPDs leads to a distortion of the DNA double helix" (Pearlman, Holbrook, Pirkle, & Kim, 1985). CPDs and 6-4PPs induce a bend in DNA (Thoma, 1999). The "structural distortion" seen through these studies is believed to be the reason degradation exists in the DNA double helix (Pang & Cheung, 2007). The DNA polymerase is blocked from synthesizing the strand during PCR by the location of dimmers (Pang & Cheung, 2007). This degradation is efficient in breaking down the DNA molecule when exposed to UV irradiation at 254 nm. Amplification cannot proceed with fragmented

DNA. A degraded profile is represented by a decrease in peak heights as allele size increases in the electropherogram.

In a previous UV treatment experiment, 1 ng of DNA was exposed to 254 nm of UV intensity for 0, 5, 10, 20, 40, 60, and 120 seconds (Pang & Cheung, 2007). As the time increased, the peak heights decreased with the most effect being on larger alleles (Pang & Cheung, 2007). At 120 seconds, no peaks were seen on the electropherogram (Pang & Cheung, 2007).

Figure 1.7. The Effect of UV Light on DNA (Allen, 2001).

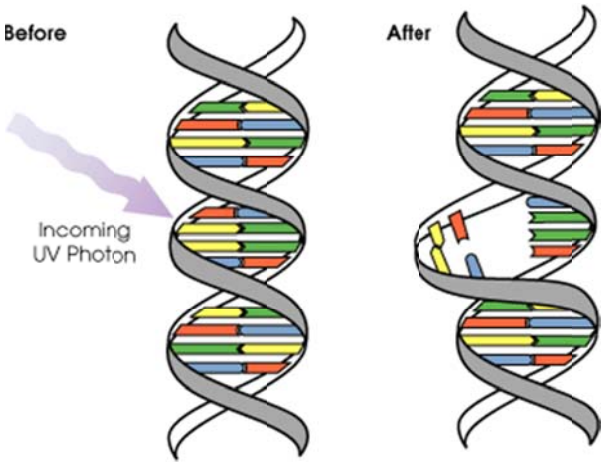

Figure 1.7. The effect UV irradiation on the DNA structure. Known structural distortion occurs to the bonds, which results in fractured DNA strands. The fractured DNA strands prevent amplification from properly occurring.

Tannic acid.

Tannic acid is a PCR inhibitor that is indicative of DNA samples found in soil compositions. "Tannins and other oligomeric compounds with free phenolic groups oxidize to form quinines, which covalently bond to and inactivate Taq DNA polymerase" (Kontanis & Reed, 2006).

A study of soil inhibition was conducted using RFLP technology. Stains were prepared with 50 µL of blood and 0.1 g of air dried soil (Adams et al., 1991). The stains were air dried and analyzed. The results revealed "components of the soil physically inhibited DNA extraction rather than that DNA was degraded by enzymes present in the soil" (Adams et al., 1991).

Kontanis and Reed (2006) tested the ability of real-time PCR to withstand DNA inhibited with tannic acid. "Tannic acid powder was serially diluted (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ng/µl) in water to act as the inhibitory agent" (Kontanis & Reed, 2006). Inhibition was directly related to the amount of tannins in the reaction (Kontanis & Reed, 2006). Complete inhibition of real-time PCR occurred with 1.4 ng per 25 µl reaction (Kontanis & Reed, 2006).

Humic acid.

Humic acid is a PCR inhibitor that is frequently associated with samples found in soil. Humic acid is a known PCR inhibitor, affecting the annealing of primers to template DNA, and preventing Taq DNA Polymerase from synthesizing DNA strands.

Humic acid "can inhibit the polymerase activities or binding of primers and reduce the sensitivity of detection" (Tsai & Olson, 1992). Humic substances are found in high organic contents within soil (Young, Burghoff, Keim, Minak-Bernero, Lute, & Hinton, 1993). "Humic acids with phenolic groups that denature biological molecules by bonding to N-substituted amides or proteins" inhibit PCR (Young et al., 1993). Dilution can alleviate the effect of humic acid on a PCR reaction; however, the detection limit will decrease (Tsai & Olson, 1992).

Tsai and Olson (1992) stated that, "as little as 1 µl of undiluted humic-acid-like extract from high-CEC sediments is sufficient to completely inhibit PCR regardless of the amount of DNA present in the 100 µl reaction mixture".

In a study conducted by Applied Biosystems during the validation of AmpFℓSTR® Identifiler® Plus PCR Amplification Kit, humic acid was examined. The concentrations used were 0, 50, 100, and 150 ng/µl (Applied Biosystems, 2010). All of the concentrations yielded full genetic profiles following analysis.

Hematin.

Hematin, a derivative of hemoglobin, is a PCR inhibitor that is found in blood. Many inhibitors within blood affect PCR such as heme, leukocyte DNA, EDTA, and heparin (Al-Soud & Radstrom, 2001). Hemoglobin contains iron, which inhibits PCR through the ability to release iron ions (Al-Soud & Radstrom, 2001). Hemin regulates DNA polymerase activity by resembling $MgCl_2$, and it is competitive with the DNA template (Al-Soud & Radstrom, 2001). Hematin inhibits Taq DNA polymerase activity in PCR by weakening the binding of any ligand to iron heme (Akane, Kazuo, Nakamura, Takahashi, & Kimura, 1994). In a study conducted by Akane et al. (1994), 0.25 µM of alkaline or acid hematin inhibited PCR amplification.

In a previous study conducted during the validation study of Applied Biosystems AmpFℓSTR® Identifiler® Plus PCR Amplification Kit, a hematin study was performed (Applied Biosystems, 2010). The concentrations used were 0, 100, 200, and 300 µM of hematin (Applied Biosystems, 2010). All concentrations failed to inhibit the samples.

Objectives

The objective of this study was to conduct an internal validation of the PowerPlex® 16 HS system within the University of Central Oklahoma forensic science laboratory. This would allow the implementation of this system when examining forensic samples. A second objective encompassed testing the overall strengths and limitations of the PowerPlex® 16 HS system. Through testing different quantities and qualities of DNA that were subjected to environmental

factors the limitations of the kit were observed. The environmental factors that were analyzed were UV treatment, tannic acid, humic acid, and hematin. Overall, a thorough review of the PowerPlex® 16 HS system was performed to analyze the data obtained when completing the DNA analysis process.

Materials and Methods

DNA testing occurred in Edmond, Oklahoma at the University of Central Oklahoma's biology laboratory in Howell Hall. Single source biological material, specifically blood, was obtained from Innovative Research (Novi, MI, USA). One single source sample was used to conduct all phases of this research except the mixture study. Two single source blood samples were used to fulfill the mixture study.

Procedures

All DNA samples used during this research were manually extracted with the Promega DNA IQ™ system (Promega Corporation, Madison, WI, USA, #DC6700). The Quantifiler™ Human DNA Quantification kit (Applied Biosystems, Carlsbad, CA, USA, #4343895) was used on an Applied Biosystems 7500 Real-Time PCR System (Serial No.275001373) for human identification to quantify all DNA samples. Amplification of all DNA samples was conducted using the PowerPlex® 16 HS system chemistry (Promega Corporation, #DC2100) on an Applied Biosystems GeneAmp® PCR System 9700 thermal cycler (Serial No.805S8201803). An Applied Biosystems 3130 Genetic Analyzer (Serial No.21364-025) was employed to separate all DNA samples for this research. Data obtained from the Genetic Analyzer was interpreted using the Applied Biosystems GeneMapper® Software version 3.2. All methods were guided by the DNA Advisory Boards (DAB) guidelines Standard 8.1 in accordance with performing a validation study (DNA Advisory Board, 2000). An attempt was made to follow all guidelines set forth for DNA analysts working in a laboratory; however, due to the public location that the tests were conducted, control of all variables was not possible.

DNA IQ™ System Protocol

A cutting or portion of a sample was taken from the evidence and placed in a Seal Rite 2.0 milliliter (ml) Natural Microcentrifuge tube (USA Scientific, #1620-2700). As per DAB guidelines, a reagent blank control sample was created at the time of extraction. The reagent blank was treated just like a sample; however, it did not contain an actual DNA sample. It was used to measure potential contamination that could occur throughout the procedure, through contaminated reagents, environmental contamination, or analyst contamination.

Samples were exposed to a specified amount of the provided lysis buffer. The amount of lysis buffer was dependent on the substrate the sample was located on. All samples were incubated on a Dri-bath (Thermolyne Type 16500, #229920807548) at 70 °C for 30 minutes to aid cellular membrane denaturation. The tubes were removed from the heat source, the substrate the samples were collected on transferred to individual spin baskets (Promega Corporation, #V1221) and seated in the same 2.0 ml microcentrifuge tube. The spin basket and tube apparatus for each sample was centrifuged at room temperature for 2 minutes. The spin baskets were removed and discarded. Seven microliters (µl) of DNA IQ™ Resin was added to each tube. For 5 minutes, the resin, lysis buffer, and sample mixtures were vortexed and incubated at room temperature. The sample tubes were placed on a manufacture provided magnetic stand where the magnetic resin separated from the lysis buffer almost instantaneously. The lysis buffer from each sample was removed and discarded. The magnetic resin and DNA pellets of each sample were not disturbed. An additional 100 µl of lysis buffer was added to the tubes DNA and resin pellet. The tubes were removed from the magnetic stand, vortexed for 2 seconds, returned to the magnetic stand, and the lysis buffers were discarded following separation. One-hundred microliters of 1X Wash Buffer, provided in the kit, was added to the samples. The tubes were

removed from the magnetic stand, vortexed for 2 seconds, returned to the magnetic stand, and the wash buffer from each sample was discarded following separation. This process was repeated for a total of three wash steps for each tube. Following the last wash buffer discard step, the tubes were left open on the magnetic stands to allow the resin to air dry for 5 minutes. Caution was paid to all samples at this point so that cross-contamination did not occur. Elution buffer was added to the tubes ranging between 25 µl and 100 µl dependent on the amount of biological material used in each sample. The tubes were vortexed for 2 seconds, and incubated at 65 °C for 5 minutes. Subsequent to removal from the heat source, the tubes were vortexed and returned to the magnetic stands. The DNA is no longer fixed to the resin because it is now located within the elution buffer. The elution buffer and DNA was transferred to another tube for each sample extracted (Promega Corporation, 2009).

Quantifiler™ Human Quantification Kit Protocol

The following procedure was performed for all quantification reactions. Eight standards were concurrently run with every real-time PCR procedure. A serial dilution was created to achieve the following DNA standard concentrations: 50.000 ng/µl (Standard 1), 16.700 ng/µl, 5.560 ng/µl, 1.850 ng/µl, 0.620 ng/µl, 0.210 ng/µl, 0.068 ng/µl, and 0.023 ng/µl (Standard 8) (Applied Biosystems, 2010). Standard one is composed of 10 µl of 200 ng/µl stock and 30 µl of sterile water.

Two components were used to form a master mix, which was added to every well that contained a standard, DNA sample, or reagent blank. A volume of 10.5 µl Quantifiler Human Primer Mix and 12.5 µl Quantifiler PCR Reaction Mix was added to each reaction. The primer mix was thawed completely and vortexed for 3 to 5 seconds. The Quantifiler PCR reaction mix was swirled gently. The appropriate volumes of both components were pipetted into a 2.0 ml

microcentrifuge tube. The mixture was vortexed, and centrifuged. Twenty-three microliters of the mix was dispensed into each reaction well. Two microliters of DNA sample or standard was added to each reaction well. The MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems, #N801-0560) was covered with an MicroAmp® Optical Adhesive Film (Applied Biosystems, #4311971) and placed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, 2010).

PowerPlex® 16 HS System Amplification Protocol

Amplification set-up.

PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix were added to each PCR reaction. The number of reactions was determined, which included all samples to be amplified, a positive control, a negative control, and a reagent blank associated with the extracted and quantified samples. The reaction volume of each amplification was determined by the following ratios: 5.0 µL PowerPlex HS 5X Master Mix, 2.5 µL PowerPlex HS 10X Primer Pair Mix, up to 17.5 µL Template DNA (range of 0.5-1.0 ng), and water to a final volume of 25.0 µL. The PCR amplification mix of PowerPlex® HS 5X Master Mix and PowerPlex® 16 10X Primer Pair Mix were distributed into each tube followed by template DNA into each tube. A PCR tube for a positive control and negative control were made. The positive control contained known 9947A DNA. The negative control contained 5.0 µl Master Mix, 2.5 µl Primer Mix, and 17.5 µl of sterile amp grade water. The PCR tubes were placed on the GeneAmp® PCR System 9700 thermal cycler (Promega Corporation, 2011).

Thermal cycling parameters.

The GeneAmp® PCR System 9700 thermal cycler was set to emulate the operation mode of the GeneAmp® PCR System 9600 thermal cycler for all reactions performed. The

manufacture recommended the use of a 32 cycle protocol and described the method as optimal for the PowerPlex® 16 HS system. Figure 2.1 and 2.2 depict the protocols followed when using the GeneAmp® PCR System 9700 thermal cycler.

Figure 2.1. Protocol for the GeneAmp® PCR System 9600 & 9700 Thermal Cycler. (Promega Corporation, 2011)

Protocol for the GeneAmp® PCR System 9600 Thermal Cycler	Protocol for the GeneAmp® PCR System 9700 Thermal Cycler[1]
96°C for 2 minutes, then:	96°C for 2 minutes, then:
94°C for 30 seconds ramp 68 seconds to 60°C (hold for 30 seconds) ramp 50 seconds to 70°C (hold for 45 seconds) for 10 cycles, then:	ramp 100% to 94°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 10 cycles, then:
90°C for 30 seconds ramp 60 seconds to 60°C (hold for 30 seconds) ramp 50 seconds to 70°C (hold for 45 seconds) for 22 cycles, then:	ramp 100% to 90°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 22 cycles, then:
60°C for 30 minutes	60°C for 30 minutes
4°C soak	4°C soak

Figure 2.1. The step-by-step procedures for amplification of DNA samples with the PowerPlex® 16 HS system on a GeneAmp® PCR System 9700 thermal cycler is described. This research utilized a 9700 Thermal Cycler with a 9600 emulation mode.

Figure 2.2. The ramp rates for thermal cycler protocols. (Promega Corporation, 2011)

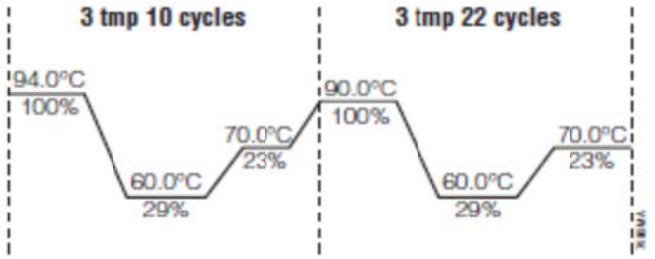

Figure 2. The ramp rates for the GeneAmp® PCR System 9700 thermal cycler.

Figure 2.2. The specific temperatures for the PCR cycles are shown for the PowerPlex® 16 HS system that were utilized. The ramp rates are shown for the PowerPlex® 16 HS system.

PowerPlex® 16 HS System Genetic Analyzer Protocol

The Applied Biosystems 3130 Genetic Analyzer was used to obtain genetic profiles through capillary electrophoresis. A loading cocktail was prepared by combining Internal Lane Standard (ILS) 600 and formamide at the ratio: [(0.5µl ILS 600) × (# injections)] + [(9.5µl formamide) × (# injections)] (Promega Corporation, 2011). Ten microliters of the formamide and ILS mix was deposited into each well. One microliter of amplified sample was added to each designated well. The plate was denatured at 95 °C for 3 minutes and then immediately snap cooled for 3 minutes on ice. The plate was loaded onto the Applied Biosystems 3130 Genetic Analyzer (Promega Corporation, 2011).

STR Data Analysis

To interpret the data obtained, GeneMapper® ID software version 3.2 was used for fragment sizing and allele calling. Peaks represent DNA fragments following electrophoresis, which are sized based on the internal lane standard run simultaneously. STR fragments are sized based on the Local Southern Method, using the ILS 600 peaks surrounding the unknown fragment to determine an allele call.

Electropherograms for all analyzed samples were obtained, which contained the genetic profile represented by peaks within the 15 STR loci's examined bins and Amelogenin. This software was used to interpret each sample for artifacts and amplified DNA products.

Validation Protocol

To determine the dynamic range, sensitivity, stochastic threshold, precision of capillary electrophoresis, reproducibility, and stutter ratios, the following quantities of DNA were amplified and analyzed with the protocols previously described: 5.00 ng, 2.50 ng, 1.25 ng, 0.63 ng, 0.31 ng, 0.16 ng, 0.08 ng, 0.04 ng, 0.02 ng, and 0.01 ng. DNA was extracted and quantified

prior to the amplification and separation process. Five replicated samples of each concentration were analyzed and evaluated.

All data was amplified using Promega's PowerPlex® 16 HS system, with 32 cycles on a GeneAmp® PCR System 9700 for 5 seconds at 3 kV. Capillary electrophoresis was performed on an Applied Biosystems 3130 Genetic Analyzer, 36 cm 4-capillary array (Applied Biosystems, #4333464) with POP-4 (Applied Biosystems, #4352755).

Detection threshold.

To establish the detection threshold, or analytical threshold, for the Applied Biosystems 3130 Genetic Analyzer, six negative controls were analyzed under various parameters. Instrument sensitivity can vary between laboratories, and Promega Corporation recommends an injection time range of 3 to 22 seconds and an injection voltage range of 1 to 3 kV (Promega Corporation, 2011). The six negative control samples were amplified and then electrophoresed at the following conditions: 1 kV for 5 seconds, 1 kV for 11 seconds, 1 kV for 22 seconds, 2 kV for 5 seconds, 2 kV for 11 seconds, 2 kV for 22 seconds, 3 kV for 5 seconds, 3 kV for 11 seconds, and 3 kV for 22 seconds.

Each set of samples were analyzed using the threshold of 1 RFU. The highest peak height observed in each sample at each injection condition in all dye channels was recorded. The data collected was used to determine a detection threshold on the instrumentation in conjunction with the PowerPlex® 16 HS system.

Dynamic range and sensitivity.

The previously listed DNA concentrations were also analyzed to determine the range of DNA concentrations that resulted in an interpretable and useable genetic profile. To determine the upper limit of the dynamic range, the highest DNA concentration that resulted in a complete

profile was recorded after accounting for extra alleles and artifacts. Numerous peaks such as OL alleles, shouldering, stutter, pull-up, minus-a artifacts, and extra peaks not related to amplification were accounted for and documented. The lower limit was determined through analysis of low level DNA concentrations that did not result in allelic drop-out. The sensitivity of the PowerPlex® 16 HS system is equivalent to the dynamic range lower limit.

Stochastic threshold.

To determine a stochastic threshold, peak height ratios were compared at heterozygous loci for the previously listed DNA samples. The stochastic threshold represented a minimum peak height at which an analyst could confidently call two alleles as sister alleles. This was extremely important for mixture interpretations.

SWGDAM recommends a general peak height ratio of < 60% is used to determine whether "two alleles at a heterozygous locus exhibit considerably different peak heights or an allele fails to amplify" (SWGDAM, 2010). Peak height ratios were calculated by dividing the peak height of the lesser allele by the peak height of the greater allele and multiplied by 100.

The stochastic threshold was determined by "plotting the peak height ratio of sister alleles for the sample replicates versus the lower peak height for the allelic pair at those heterozygous loci" (Promega Corporation, 2006). The stochastic threshold was the peak height (RFU value) where a rapid drop-off in peak height ratios was observed (Promega Corporation, 2006). This was a subjective value and should include a statistically significant portion of the data analyzed.

Precision of capillary electrophoresis and reproducibility.

The precision of the capillary electrophoresis instrument was determined by comparing base pair calls for a given allele. The reproducibility was determined through comparison of allele calls for a particular peak's replicated samples.

The reproducibility study was conducted by ensuring the same peak, following numerous amplification procedures and electrophoretic processes, was consistently and correctly called. This study was conducted by examining the electropherograms produced.

The precision of capillary electrophoresis was conducted utilizing two different methods. First, the base pair call of every allele for each replicated sample of all concentrations was recorded. The precision was analyzed by comparing every individual allele call of the five duplicated samples at each concentration. The standard deviation was calculated for the five values at every allele within the genetic profile. Second, the precision was calculated through comparison of the individual allele's base pair (bp) call to the allelic ladder's bp call. The standard deviation was calculated between two bp calls. Standards within the field accept a three standard deviation value less than 0.5 bp.

Stutter rate calculations.

Stutter was identified as one repeat unit smaller or larger than the true allele call. Stutter percentage values were determined by dividing the peak height of the stutter peak by the peak height of the true allele. The analysis parameters within GeneMapper were altered to account for all potential stutter peaks. Within the Analysis Method Editor, in the tab "Allele," the "Minus Stutter Distance" in the "Tetra" and "Penta" columns under normal analysis conditions is "Tetra" from 3.25 to 4.75 and "Penta" from 3.75 to 5.75. Both sets of data were changed to the values 0.00 and to 0.00, which allowed all potential stutter peaks that the software cancels out to be seen and assessed. The detection threshold was lowered to 25 RFU to allow stutter that may be masked by the threshold to be analyzed.

The average percent of stutter at each locus was calculated. The highest stutter peak observed was recorded. A locus-by-locus stutter percentage was set due to documented fluctuations between the fifteen loci.

Simulated mixture study.

Two single source DNA samples were extracted, then combined to create the mixture ratios used. The following ratios were amplified and analyzed in duplicate: 1:0, 19:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:19, and 0:1.

Utilizing electropherograms, the peak height ratios were calculated for all true alleles. Major and minor contributors were determined. The peak height ratio was calculated as before. The mixture ratio was denoted as (Minor : Major). The calculation used to determine the mixture ratio was the following:

$$1 : \frac{sum\ of\ Major\ allele\ peak\ heights}{sum\ of\ Minor\ allele\ peak\ heights}$$

The minor proportions and major proportions of mixtures were calculated as the following:

$$Minor\ Proportion = \frac{sum\ of\ Minor\ peak\ heights}{sum\ of\ All\ peak\ heights}$$

$$Major\ Proportion = 1 - Minor\ Proportion$$

Mixture interpretation guidelines were established according to the results produced.

Cross-contamination.

A contamination study was performed to ensure no unexpected peaks were obtained on the instrumentation. An injection plate was created containing a checkerboard pattern of samples and blanks as seen in Figures 2.3 and 2.4. A checkerboard plate was repeated twice with an opposing pattern in the plate to ensure every well was analyzed for unexpected peaks that could

occur. This ensures there was an absence of contamination between the samples on the Applied Biosystems 3130 instrument as well as the analyst and procedural methods.

The blank wells contained the appropriate volume of ILS and formamide, and the sample wells contained a known amplified DNA sample. The blank wells were examined for unexpected allele calls. If an unexpected peak occurred, it was documented.

Figure 2.3. Checkerboard plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A		5		13		21		29		37		45
B	1		9		17		25		33		41	
C		6		14		22		30		38		46
D	2		10		18		26		34		42	
E		7		15		23		31		39		47
F	3		11		19		27		35		43	
G		8		16		24		32		40		48
H	4		12		20		28		36		44	

Figure 2.4. Checkerboard plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1		9		17		25		33		41	
B		5		13		21		29		37		45
C	2		10		18		26		34		42	
D		6		14		22		30		38		46
E	3		11		19		27		35		43	
F		7		15		23		31		39		47
G	4		12		20		28		36		44	
H		8		16		24		32		40		48

Figure 2.3 and 2.4 represent the opposing checkerboard patterns that were used to fulfill the requirement of a contamination study. The red wells represent the blank wells that contained formamide and ILS 600. The white wells represent known amplified DNA samples.

Mock case.

The mock case test was conducted to ensure the protocols and instrumentation were functioning properly and that the correct genotypic profile was obtained. Three samples were analyzed that originated from Collaborative Testing Services, Inc. (CTS) exam, test no. 10-573, for forensic biology. The scenario for the test was the following:

> The police are investigating a residential burglary. The victim provided investigators with a detailed description of the individual, which led them to a suspect. The suspect is a teenager that lives in the neighborhood. The police recovered one questioned stain from the floor of the victim's home. The investigators are submitting the stains from the victim's floor along with reference blood samples from the victim and suspect.

Item 1: Known blood from the Victim

Item 2: Known blood from the Suspect

Item 3: Question stain from the home

The questioned sample was analyzed first, followed by the known samples at a separate time and place. At the amplification step, a positive control, negative control, and individual reagent blank was run for both the questioned sample and the known samples. The questioned sample electropherogram was examined for unexplainable peaks observed. Once stutter was filtered out of the profile, and artifacts were recognized, the allele calls were determined for each loci and recorded. The known reference samples electropherograms were examined following completion of the questioned sample. The known genotypes were compared to the genotype obtained from the questioned sample. Known results exist for the specific case, and the results obtained following electrophoresis were compared for accuracy with published results by an independent and known reviewer.

Environmental Insults

Ultra Violet treatment samples.

Five replicated DNA samples were exposed to 30, 60, 100, 200, and 300 seconds of 254 nm ultraviolet (UV) light utilizing an AirClean 600 PCR Combination Workstation (USA Scientific, Ocala, FL, USA, #AC648LFUVC-43352). This study was designed to achieve failure in obtaining a genetic profile. Previous studies were examined to determine the exposure limit and to extend the exposed time period further than PowerPlex® 16 HS system was designed to endure.

The AirClean 600 PCR Combination Workstation chamber was designed for sterilization of laboratory equipment, and it allowed for easy cleaning between amplification set up to limit contamination. Therefore, it presented an ideal environment for the degradation of DNA samples in an effort to replicate degradation via sunlight through UV irradiation.

DNA was extracted, quantified, amplified, and analyzed according to the protocols previously described. Following extraction and quantification, DNA samples were placed in Seal-Rite 0.2 ml Indiv. Thin Wall PCR tubes with attached Dome Cap (USA Scientific, #1602-4300). Each tube contained 3 µl of 1 ng DNA. Each tube was left open and exposed to the UV bulb for the previously stated time periods. The tubes were located approximately 100 mm directly below the UV bulb on a built-in shelf within the chamber. Figure 2.5 and 2.6 depict the set-up for the experiment.

Following DNA degradation, 1.0 µl of exposed DNA samples, which were previously quantified at 1.0 ng, were amplified with PowerPlex® 16 HS system on a GeneAmp® PCR System 9700. Capillary electrophoresis was utilized to capture the PCR fragments on an Applied Biosystems 3130 Genetic Analyzer.

Figure 2.5. The UV Irradiation Hood.

Figure 2.5 shows the Airclean 600 PCR Combination Workstation that was used to degrade DNA samples. The overall set up of the UV chamber to carry out the environmental insult experiment is displayed in Figure 2.5.

Figure 2.6. UV Light Experiment Set-up.

Figure 2.6. A close-up view of the UV bulb approximately 100 mm from the DNA samples within the 0.2 ml PCR tubes is shown. Five tubes were placed in the position depicted for each UV exposure time that was tested.

Soil acid samples (tannic acid and humic acid).

Samples were subjected to tannic and humic acid to simulate DNA samples potentially contaminated with PCR inhibiting soil components. This study was designed to test the level of inhibition the PowerPlex® 16 HS system would withstand when analyzing a genetic profile. Therefore, previous studies were examined to determine the limit and to extend the concentrations further than the multiplexing system was designed to endure.

One nanogram of DNA was amplified in the presence of 0 ng/µL, 50 ng/µL, 100 ng/µL, 200 ng/µL, and 400 ng/µL concentrations of tannic and humic acids. Each concentration for both potential inhibitors was repeated five times. One microliter of humic acid and tannic acid for each concentration was added directly to the PCR tube. The amount of water added to the PCR tube decreased by 1 µl to account for the addition. The PCR tube was vortexed for 10 to 15 seconds to ensure adequate mixing of the template DNA, PCR components, and potential inhibitor.

A pipette and scientific balance (Sartorius, Goettingen, Germany) was used to weigh out tannic acid (Fisher Science Education, Hanover Park, IL, USA, #1401-55-4) and humic acid (Alfa Aesar, Ward Hill, MA, USA, # 1415-93-6) to construct the above mentioned concentrations. Tannic acid (approximately 0.01 g) was added to 1 mL of sterile water (Fisher Scientific, #1609-47-8). Humic acid (approximately 0.01 g) was added to 1 mL of sterile water. The tubes were vortexed for 10 to 15 seconds to ensure the solid was dissolved into the liquid. Both measurements formulated the highest concentration, 400 ng/µl, of the respective acids used to potentially simulate inhibition. A serial dilution was made for both acids from the stock to formulate 200 ng/µl, 100 ng/µl, and 50 ng/µl.

Hematin inhibition.

Samples were subjected to hematin to mirror inhibition of components found naturally in blood. This study was designed to test the level of inhibition the PowerPlex® 16 HS system could withstand when analyzing a genetic profile. Therefore, previous studies were examined to determine the limit and to extend the concentrations further than the multiplexing system was designed to endure.

One nanogram of DNA was inhibited with the following concentrations of hematin: 0 µM, 125 µM, 250 µM, 500 µM, and 1000 µM. Each concentration was repeated five times. One microliter of the specified hematin concentration was added directly to the appropriate PCR tube. The sterilized water incorporated into the PCR reaction decreased by 1 µl. Once all components of the PCR process had been added, the tubes were vortexed for 10 to 15 seconds to ensure adequate mixing of the template DNA, PCR components, and inhibitor.

To formulate the above listed hematin concentrations a pipette and scientific balance was used to weight out hematin porcine (MP Biomedicals, LLC, Solon, OH, USA, #198969). Hematin porcine (approximately 0.0634 g) was dissolved into 1 mL of 0.1 Normality (N) NaOH (Chung, 2004). The 0.1 N NaOH was formulated by dissolving approximately 0.02 g NaOH (Sigma-Aldrich Inc., St.Louis, MO, USA, #221465-500G) into 5 mL of sterile water. The initial stock of hematin was diluted twice to create the first concentration desired for potential inhibition. Serial dilutions were made to achieve the concentrations of the desired hematin amounts in 0.1 N NaOH.

Results

Internal Validation

Limit of detection threshold.

Six negative controls were subjected to nine varying electrophoretic conditions to analyze the baseline noise present on the instrumentation when using the PowerPlex® 16 HS system. The negative controls were analyzed under the nine conditions to ensure sufficient samples were evaluated to determine a minimum height that noise peaks were observed for numerous injection parameters.

The raw data for the negative controls at all electrophoretic conditions are located in Table 3.1. Table 3.1 represents the highest detected noise peak for each replicated sample within the nine conditions. As the time component increased, an increase in observable noise peak heights was seen. As the injection voltage increased, an increase in observable noise peak heights occurred. Table 3.2 depicts statistical information that was useful in determining a detection threshold effectively high enough to filter out detected noise peaks within the nine conditions. The data depicted in Tables 3.1 and 3.2 can be used to formulate a detection threshold at a second injection condition if it is deemed necessary. SWGDAM recommends a scientific method be utilized to determine a threshold. The average baseline noise plus three standard deviations was compared to doubling the highest peak to achieve the most conservative threshold.

Table 3.1. Highest noise peak recorded for each sample in the three dye channels that contained STR loci.

Sample	Blue	Green	Yellow	Sample	Blue	Green	Yellow	Sample	Blue	Green	Yellow
#1 1kV 5s	13	25	14	**#1 2kV 5s**	10	20	20	**#1 3kV 5s**	12	44	26
#2 1kV 5s	11	18	13	**#2 2kV 5s**	12	39	32	**#2 3kV 5s**	15	37	46
#3 1kV 5s	18	20	14	**#3 2kV 5s**	14	17	13	**#3 3kV 5s**	14	27	24
#4 1kV 5s	16	17	17	**#4 2kV 5s**	13	32	27	**#4 3kV 5s**	13	32	30
#5 1kV 5s	11	15	13	**#5 2kV 5s**	12	19	21	**#5 3kV 5s**	14	22	24
#6 1kV 5s	13	18	14	**#6 2kV 5s**	15	20	20	**#6 3kV 5s**	13	40	30
#1 1kV 11s	19	34	23	**#1 2kV 11s**	23	38	37	**#1 3kV 11s**	14	62	53
#2 1kV 11s	12	22	18	**#2 2kV 11s**	12	45	39	**#2 3kV 11s**	15	80	66
#3 1kV 11s	14	17	15	**#3 2kV 11s**	14	33	32	**#3 3kV 11s**	16	49	45
#4 1kV 11s	12	19	19	**#4 2kV 11s**	16	31	54	**#4 3kV 11s**	17	95	82
#5 1kV 11s	11	21	17	**#5 2kV 11s**	12	37	35	**#5 3kV 11s**	15	39	40
#6 1kV 11s	15	18	14	**#6 2kV 11s**	15	35	22	**#6 3kV 11s**	14	38	31
#1 1kV 22s	13	21	29	**#1 2kV 22s**	15	86	59	**#1 3kV 22s**	24	112	92
#2 1kV 22s	11	36	24	**#2 2kV 22s**	16	96	72	**#2 3kV 22s**	14	111	75
#3 1kV 22s	15	29	30	**#3 2kV 22s**	15	56	60	**#3 3kV 22s**	20	83	75
#4 1kV 22s	16	25	18	**#4 2kV 22s**	19	120	115	**#4 3kV 22s**	25	127	116
#5 1kV 22s	13	27	23	**#5 2kV 22s**	16	41	54	**#5 3kV 22s**	21	98	98
#6 1kV 22s	13	29	24	**#6 2kV 22s**	19	59	65	**#6 3kV 22s**	23	71	60

Table 3.2. Statistical calculations for determining the detection threshold for each injection parameters examined.

Injection Condition	Average	Highest Peak	Baseline w/ Average (average+3std dev)	Baseline w/ Highest Peak (highest peak x 2)
1kV 5s				
blue	13.7	18	22.08094	36
green	18.8	25	29.12411	50
yellow	14.2	17	18.58255	34
1kV 11s				
blue	13.8	19	22.61399	38
green	21.8	34	40.56032	68
yellow	17.7	23	27.27916	46
1kV 22s				
blue	13.5	16	18.78205	32
green	27.8	36	42.82333	72
yellow	24.7	30	37.76628	60
2kV 5s				
blue	12.7	15	17.92024	30
green	24.5	39	51.11391	78
yellow	22.2	32	41.83136	64
2kV 11s				
blue	15.3	23	27.58078	46
green	36.5	45	51.16629	90
yellow	36.5	54	67.83528	108
2kV 22s				
blue	16.7	19	22.25236	38
green	76.3	120	164.8138	240
yellow	70.8	115	138.2744	230
3kV 5s				
blue	13.5	15	16.64643	30
green	33.7	44	58.45376	88
yellow	30	46	54.88373	92
3kV 11s				
blue	15.2	17	18.6738	34
green	60.5	95	129.8707	190
yellow	52.8	82	108.5855	164
3kV 22s				
blue	21.2	25	33.07885	50
green	100.3	127	162.2139	254
yellow	86	116	145.97	232

Dynamic range and sensitivity.

Fifty samples were examined to determine the DNA concentrations necessary to obtain a complete and useable profile compared to an incomplete and uninterruptable profile. Following analysis, the samples were analyzed for known and reproducible artifacts and unexplainable peaks within the profile. The dynamic range of the system was established by reviewing fifty known genetic profiles.

Table 3.3 depicts the number of alleles called correctly for each sample that was examined. In the larger concentrations of input DNA, unexplainable off-ladder alleles were present. The 5.0 ng, 2.5 ng, and 1.25 ng DNA concentrations repeatedly contained extreme levels of pull-up, and numerous unaccountable off-ladder alleles. In the lower DNA concentrations, allelic dropout occurred at numerous loci, which affected the ability of the profile to be interpreted correctly. Allelic dropout was first observed at the larger loci analyzed within the 0.08 ng DNA samples. The sensitivity of the system is equivalent to the lower limit of the dynamic range. The data supports a confident dynamic range of 0.63 ng to 0.16 ng of DNA to produce an interpretable and explainable genetic profile. A concentration of 1.0 ng of DNA may be utilized with caution due to the potential for pull-up and additional alleles. The sensitivity of the system is 0.16 ng of DNA. Lower DNA concentrations should be used with caution due to extreme allelic dropout observed.

Table 3.3. Correct number of allele calls for the DNA concentrations examined for the dynamic range and sensitivity studies.

Total Conc. of Input DNA/Sample	5.0ng	2.5ng	1.25ng	.63ng	.31ng	.16ng	.08ng	.04ng	.02ng	.01ng
FTA1	29/29	29/29	29/29	29/29	29/29	29/29	20/29	14/29	4/29	0/29
FTA2	29/29	29/29	29/29	29/29	29/29	29/29	27/29	20/29	5/29	5/29
FTA3	29/29	29/29	29/29	29/29	29/29	29/29	22/29	19/29	0/29	0/29
FTA4	29/29	29/29	29/29	29/29	29/29	29/29	27/29	22/29	3/29	3/29
FTA10	29/29	29/29	29/29	29/29	29/29	29/29	26/29	14/29	0/29	0/29

Note: denotes numerous OL alleles, pull-up, shouldering, minus-a, and stutter artifacts that render the profiles un-interpretable; represented useable and interpretable DNA profiles that were examined; allele dropout was present which affected the ability to interpret the profile.

Stochastic threshold.

The stochastic threshold is "a value above which it is reasonable to assume that allelic dropout has not occurred within a single-source sample" (SWGDAM, 2010). The field has

determined an analyst can confidently interpret two alleles at a heterozygote locus to be sister alleles if their peak height ratio exceeds 60%. SWGDAM interpretation guidelines set a 60% general peak height ratio for sister alleles at heterozygous locus.

During analysis of single source samples, numerous sister alleles at heterozygous loci do not possess a peak height ratio above 60%. The peak height ratios for thirteen heterozygous loci of a single-source sample are displayed in Table 3.4. The percentages observed below the recommended 60% are highlighted in red within Table 3.4. This is problematic due to the assumption that sister alleles at a heterozygous locus in a single-source sample have generally comparable peak heights. A problematic situation for interpretation arises due to sister alleles possessing a 30% peak height ratio.

Figure 3.1 displays the peak height ratio percentage compared to the lowest RFU value associated with the percent. Figure 3.1 was used to determine the potential threshold values and percent of the data included. A threshold was established at a location where the majority of the data was included with respect to a value most data could be analyzed against.

A subjective threshold at which 97.44% of the data was within the threshold. Five outliers were present within the viable range of 195 data points. The stochastic threshold for this system was set at 750 RFU. The stochastic threshold can be increased to 900 RFU, which would include 191 data points out of 195 total data points. This includes 97.95% of the viable range. An analysis of setting the threshold at 1300 RFU, lead to 98.97% of the data incorporated within the viable range.

Table 3.4. Peak Height Ratios

	D3	TH01	D21	D18	PentaE	D5	D13	D7	D16	CSF	PentaD	Amel	vWA	D8	TPOX	FGA
0.63 FTA1	98.24%	81.26%	90.60%	72.72%	80.82%	71.01%	98.34%		86.58%		53.96%		59.94%	92.14%	80.97%	77.60%
0.63 FTA2	77.23%	98.29%	94.96%	76.78%	82.58%	67.14%	96.73%		80.55%		82.61%		66.62%	46.06%	99.10%	80.25%
0.63 FTA3	84.21%	86.07%	82.43%	94.35%	95.14%	92.55%	95.48%		94.88%		81.96%		95.13%	50.22%	69.28%	99.18%
0.63 FTA4	96.13%	99.69%	85.02%	80.81%	92.64%	82.60%	90.96%		82.51%		77.73%		87.59%	75.82%	98.27%	81.79%
0.63 FTA10	82.71%	90.44%	92.75%	85.83%	92.96%	72.83%	87.46%		87.08%		99.85%		83.06%	43.69%	83.62%	95.08%
0.31 FTA1	81.38%	97.91%	89.59%	95.12%	66.79%	72.74%	99.15%		95.33%		94.10%		91.85%	72.23%	78.28%	76.42%
0.31 FTA2	79.38%	77.05%	87.38%	99.57%	69.97%	67.90%	86.07%		84.59%		90.98%		98.49%	59.34%	80.46%	79.31%
0.31 FTA3	72.21%	76.66%	76.19%	61.96%	74.54%	77.36%	73.79%		89.01%		95.05%		94.74%	49.78%	55.74%	100%
0.31 FTA4	96.37%	83.20%	79.73%	47.01%	58.71%	84.92%	83.50%		82.01%		65.79%		96.98%	48.17%	52.38%	78.04%
0.31 FTA10	94.83%	98.46%	97.00%	64.55%	91.26%	91.20%	78.02%		98.66%		78.52%		84.82%	30.31%	60.27%	86.22%
0.16 FTA1	67.97%	73.48%	64.65%	42.11%	65.31%	52.08%	95.14%		86.33%		72.39%		74.92%	66.23%	65.89%	49.34%
0.16 FTA2	66.48%	96.62%	95.73%	92.27%	47.32%	58.08%	61.97%		58.68%		81.48%		56.01%	82.58%	86.17%	65.60%
0.16 FTA3	82.56%	77.98%	51.17%	83.92%	98.76%	49.03%	82.99%		85.67%		58.77%		77.97%	94.68%	59.85%	63.48%
0.16 FTA4	70.47%	74.44%	83.30%	65.17%	53.23%	99.26%	54.63%		62.32%		94.14%		77.20%	90.49%	89.62%	80.55%
0.16 FTA10	75.09%	73.39%	62.23%	61.32%	87.80%	86.53%	91.23%		76.72%		74.51%		82.11%	53.79%	55.54%	84.03%

Figure 3.1. Stochastic Threshold

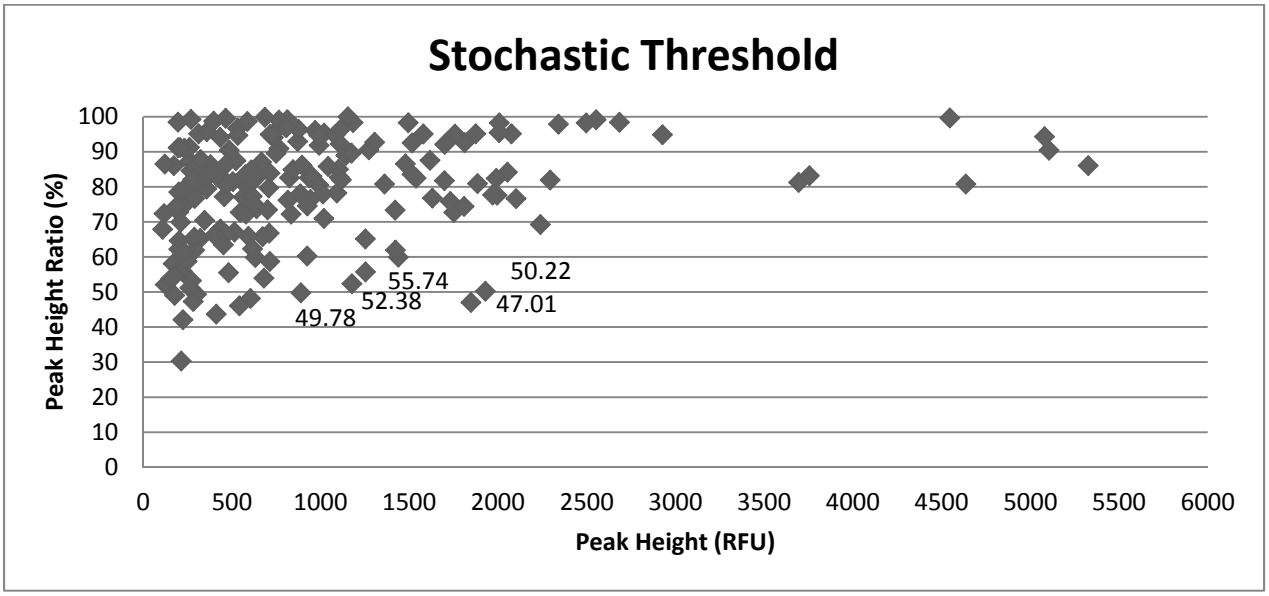

Figure 3.1. The figure represents the peak height ratio of sister alleles at a heterozygous loci on the y-axis and the lowest peak height associated with the ratio on the x-axis (Promega Corporation, 2006). A total of 195 data points were included in this analysis. The five highlighted values represent the outliers that were not included in the 750 RFU stochastic threshold.

Precision of capillary electrophoresis and reproducibility.

The precision and reproducibility study analyzed three concentrations of DNA within the dynamic range to ensure each known profile was called correctly. As DNA fragments move through the capillary they are detected in a bell-curve pattern. The sloping sides of the bell curve represent $\pm$ 3 standard deviations of the DNA. Reproducibility ensures that every time a sample is analyzed it is called the same in a genetic profile. Precision examines whether the base pair calls are within the 0.5 base pair bin in the allelic ladder.

The five replicated samples base pair calls for every allele within the dynamic range were examined. Two methods were used to analyze the precision of the instrumentation with

multiplexing systems. The first included comparing the replicated samples to each other. The second compared individual samples to the allelic ladder they were simultaneously analyzed with. To ensure every DNA fragment is within the 0.5 bp allotted bin as they migrate through the capillary, three standard deviations of the replicated samples were calculated and compared to each other. The standard deviation of the five replicated samples for each allele at the loci examined is depicted in Table 3.5. One allele did not meet the required value of < 0.5 bp, which is highlighted in red in Table 3.5. The standard deviation was multiplied by three and analyzed against the 0.5 bp value set as a standard for precision of an instrument. This was problematic for the precision study; however, the allele in question was reported as the correct allele every time it was analyzed.

The second method included comparing an individual allele call to the allelic ladder. When the five replicates of the problematic allele were individually compared to the concurrent allelic ladders they were analyzed with, each allele was significantly lower than 0.5 bp value required. Table 3.6 shows the statistical data for comparison of the allele that did not produce a precision bp value < 0.5 bp when compared to its replicates. When the individual samples were compared to the simultaneously analyzed allelic ladders, each sample was less than 0.5 bp as seen in Table 3.6. Various studies have utilized both methods to examine precision.

Through all phases of the validation study the DNA profiles were compared to known profiles for the corresponding sample. The reproducibility of each sample proved consistent throughout the entire experiment.

Table 3.5. Standard Deviation of Base Pair Calls

		D3S1358		TH01		D21S11		D18S51		PentaE		D5S818		D13S317		D7S820
Std Dev of 0.63ng	15	0.14007	7	0.11261	29	0.14467	18	0.11349	7	0.14107	10	0.14967	12	0.12341	12	0.12582
	16	0.13353	8	0.12194	32.2	0.14673	20	0.11925	13	0.13882	11	0.16407	13	0.12872		
Std Dev of 0.31ng	15	0.14755	7	0.11023	29	0.15116	18	0.11	7	0.13038	10	0.1589	12	0.11437	12	0.09407
	16	0.15515	8	0.12116	32.2	0.11653	20	0.11971	13	0.08468	11	0.18158	13	0.10334		
Std Dev of 0.16ng	15	0.13134	7	0.11502	29	0.14822	18	0.1161	7	0.11212	10	0.15073	12	0.139	12	0.11887
	16	0.1333	8	0.12317	32.2	0.13502	20	0.11189	13	0.10035	11	0.14923	13	0.10502		

		D16S539		CSF1PO		PentaD		Amel.		vWA		D8S1179		TPOX		FGA
Std Dev of 0.63ng	11	0.10464	10	0.09381	11	0.11987	X	0.00837	15	0.03421	12	0.04159	7	0.03362	21	0.06914
	13	0.09263			12	0.11632			18	0.04266	17	0.06892	8	0.02966	23	0.04899
Std Dev of 0.31ng	11	0.06782	10	0.11874	11	0.11238	X	0.03	15	0.02702	12	0.0658	7	0.02739	21	0.05788
	13	0.08408			12	0.10464			18	0.02702	17	0.04669	8	0.03209	23	0.0497
Std Dev of 0.16ng	11	0.07861	10	0.06245	11	0.1494	X	0.04528	15	0.02074	12	0.06841	7	0.03742	21	0.04879
	13	0.09772			12	0.13198			18	0.04147	17	0.04868	8	0.03834	23	0.04775

Table 3.6. Allele Compared to Allelic Ladder

Sample	Allele Call	Base Pair Call	Allele Call	Std Dev	3*Std Dev
0.31 FTA2	10	123.83	10	0.066833	**0.20049938**
	11	128.03	11	0.044347	**0.13304135**
Allelic Ladder	10	123.98			
	11	128.11			
Allelic Ladder2	10	123.85			
	11	128.01			
Allelic Ladder3	10	123.9			
	11	128.03			
0.31 FTA10	10	123.99	10	0.091924	**0.27577164**
	11	128.28	11	0.021213	**0.06363961**
Allelic Ladder	10	124.12			
	11	128.25			
0.31 FTA4	10	124.15	10	0.055076	**0.16522712**
	11	128.39	11	0.025166	**0.07549834**
Allelic Ladder	10	124.26			
	11	128.37			
Allelic Ladder2	10	124.2			
	11	128.34			
0.31 FTA3	10	123.82	10	0.06994	**0.20982135**
	11	128.03	11	0.044347	**0.13304135**
Allelic Ladder	10	123.98			
	11	128.11			
Allelic Ladder2	10	123.85			
	11	128.01			
Allelic Ladder3	10	123.9			
	11	128.03			
0.31 FTA1	10	123.76	10	0.09215	**0.27645072**
	11	127.98	11	0.055603	**0.16680827**
Allelic Ladder	10	123.98			
	11	128.11			
Allelic Ladder2	10	123.85			
	11	128.01			
Allelic Ladder3	10	123.9			
	11	128.03			

Stutter rate.

A stutter study was conducted to document observed stutter within the dynamic range. Elevated stutter and n+4 stutter was revealed within the dynamic range. Stutter was distinguished as a peak one repeat unit below a true allele, and on occasion one repeat unit above an allele.

Stutter was evaluated on a locus-by-locus condition due to the fluctuation of stutter percentages within the 15 loci examined. Amelogenin was not observed to have stutter. The average stutter plus three standard deviations was compared to the highest recorded stutter value. They were evaluated to formulate a conservative stutter ratio for every locus. Stutter values for tetra-nucleotide repeats are normally less than 10% of the true allele peak (Walsh et al., 1996). After consideration, the highest stutter value was used to formulate the marker specific stutter ratio. The values chosen to represent stutter ratios for each loci are located in Figure 3.2.

When the dynamic range was analyzed for stutter artifacts, loci were observed to possess outlying stutter peaks. The stutter data analyzed is depicted in Table 3.7. Table 3.7 lists the average stutter seen at each locus, the standard deviation of stutter at each locus, the highest stutter percent seen at each locus, and an outlier if present at each locus. The marker specific stutter ratio represent the value stutter will be held to during interpretation of profiles. The outlying stutter peaks were not observed more than once; therefore, it is recognized they did occur but were not detected again. The outlying stutter peaks were not used in determining a marker specific stutter ratio.

Figure 3.2. Marker Specific Stutter Ratios

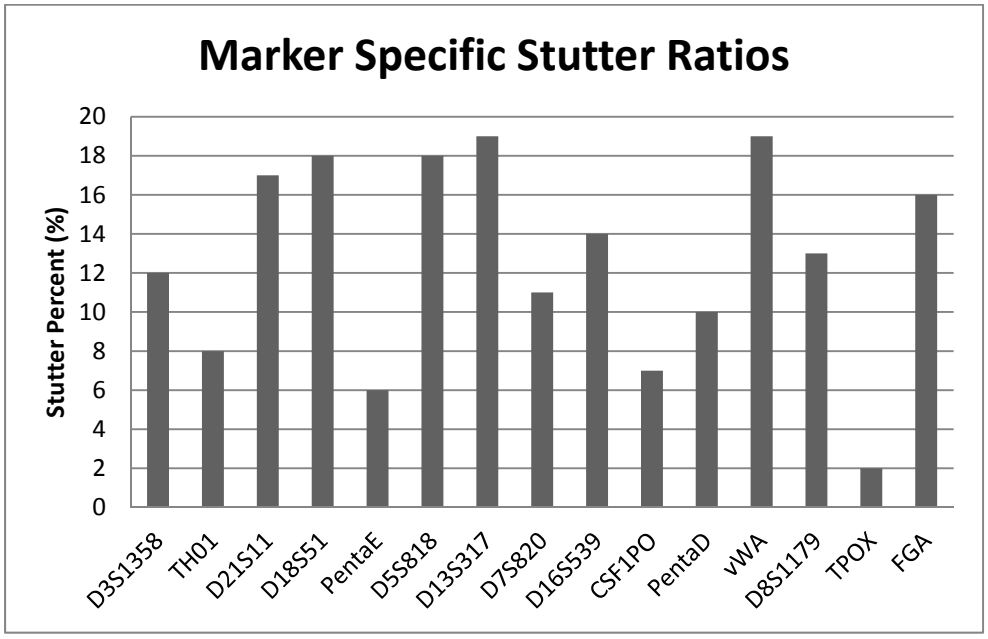

Figure 3.2 indicates stutter percent values that were evaluated and applied to each locus during interpretation.

Table 3.7. Stutter Calculations

	Average (%)	Std Dev	Average + 3Std Dev	Highest Stutter (%)	Outlier (%)	Marker Specific Stutter Ratio (%)
D3S1358	9.45	2.4762875	16.87886	11.6		12
TH01	2.9714286	1.405171	7.186942	7.4		8
D21S11	8.825641	3.1209578	18.188515	16.9		17
D18S51	10.534286	4.4047396	23.748505	17.6		18
PentaE	3.9818182	1.2536492	7.742766	5.3		6
D5S818	8.89	3.8670833	20.49125	17.7		18
D13S317	10.033333	6.1749108	28.55807	18.8	25.9	19
D7S820	5.375	3.2054286	14.99129	10.6		11
D16S539	9.5466667	3.3052449	19.462401	13.2	20.5	14
CSF1PO	4.9	1.4383633	9.21509	6.2		7
PentaD	4.075	3.7579915	15.34897	9.5		10
vWA	10.328125	5.9085086	28.053651	18.9	31.4	19
D8S1179	7.6964286	6.2408144	26.418872	12.5	35.4	13
TPOX	1.6	0.0707107	1.812132	1.7		2
FGA	9.7967742	4.5899516	23.566629	15.6	19.6	16

Simulated mixture study.

A mixture study was performed on two single-source blood samples. Two person mixtures were obtained in every ratio. This was interpreted as at least three alleles present at a locus within the profile.

The complete genetic profile for the major and minor contributor were present within the 1:1 and 4:1 samples. The 1:4 mixture ratio experienced minor allele dropout for the minor contributor. The mixture ratios of 19:1, 1:19, 9:1, and 1:9 saw allele dropout at numerous loci for the minor contributor.

The observable and calculable differences in peak heights at loci were analyzed. Peak height ratios were calculated for all alleles present at a specific locus. The minor contributor's percent was determined at a locus possessing four allele calls. When a contributor's percent was applied to other loci within the genetic profile, wrong allele calls were attributed to the suspected contributor. In a major and minor contribution scenario, the quantity of one contributor should be imbalanced compared to the second contributor. However, when the mixture samples were analyzed with PowerPlex® 16 HS system the result was an indistinguishable mixture. The majority of the peaks presented similar peak heights. Therefore, an allele could not be contributed to a specific individual with any certainty.

Cross-contamination.

A checkerboard pattern was run to ensure contamination did not occur between wells on the Applied Biosystems 3130 Genetic Analyzer. Nine off-ladder (OL) alleles were present within the 96 blank wells that were examined. The OL alleles were not present within the bins for the specific loci. The peak heights and location of the OL alleles are shown in Table 3.8. The OL alleles were not present within a bin located in the allelic ladder. The OL peaks did not

repeat throughout the entire 96-well plate. Therefore, it was determined no potentially harmful carry over existed between the wells.

Table 3.8. Contamination Study Off-ladder Alleles

Sample Name/Plate No.	RFU value, bp call	Location
1MF/Plate 1	108RFU, 399.91bp	middle of FGA
3MF/ Plate 1	97RFU, 299.92bp	between TPOX & FGA
3MF/ Plate 1	112RFU, 399.91bp	middle of FGA
9MF/ Plate 1	99RFU, 399.83bp	middle of FGA
17MF/ Plate 1	95RFU, 299.92bp	between TPOX & FGA
17MF/ Plate 1	98RFU, 399.91bp	middle of FGA
27MF/ Plate 1	96RFU, 399.91bp	middle of FGA
10MF/ Plate 2	175RFU, 258.85bp	between D7 & D16
10MF/ Plate 2	232RFU, 312.51bp	between D16 & CSF1PO

Mock case.

A mock case exam was performed on previously prepared samples to ensure the proper genetic profile was obtained from testing. One questioned sample was tested, followed by two known reference samples. The genotypes obtained are listed in Table 3.9. The positive controls, negative controls, and reagent blanks all performed as expected with no unwarranted peaks. The profiles contained correct allele calls when compared to known genotypes for the samples. The results were confirmed by an independent and known reviewer. The correct results for the mock case exam are displayed in the appendix, Figure A.

Table 3.9. Mock Case Results

Sample tested	D3	TH01	D21	D18	PentaE	D5	D13	D7	D16	CSF1P0	PentaD	Amel	vWA	D8	TPOX	FGA
Q1	17,18	7,8	28,32.2	15	12,16	8,11	11,12	9,11	11,12	12	5,8	X,Y	18,19	13,14	8	19,22
K1	14,17	9.3	29,31.2	12	5,17	10,13	11,12	11	9,12	11,12	8,13	X	16,18	12,15	8	19,23
K2	17,18	7,8	28,32.2	15	12,16	8,11	11,12	9,11	11,12	12	5,8	X,Y	18,19	13,14	8	19,22

Environmental Inhibitors

UV treatment.

A UV treatment study was conducted to test the limit at which DNA would fail amplification. The amount of correctly called alleles decreased as the exposure time of UV light increased as seen in Table 3.10. A full genetic profile was detected with repeated samples exposed to 30 seconds of UV light at a distance of 100 mm. Within in the five replicates exposed to 60 seconds of UV light the peak height values decreased significantly. This pattern was observed throughout the study as exposure time increased. D18S51, Penta E, CSF1P0, Penta D, D8S1179, TPOX, and FGA repeatedly dropped-out of the genetic profile after sustaining 60+ seconds of exposure to UV light. At 200 seconds, seven alleles were called with peak heights ranging from 200 to 500 RFU. Loci D3S1358, D21S11, and vWA were correctly called in every sample at every exposed time period. TH01 and Amelogenin were called correctly for all samples at every exposed time except two. An electropherogram for each UV exposure time is located in the appendix in Figures B-F.

Table 3.10. Correctly called alleles for UV treatment exposure samples

Sample	# of correctly called alleles	Sample	# of correctly called alleles	Sample	# of correctly called alleles
0sec-1	29/29	60sec-1	24/29	200sec-1	8/29
0sec-2	29/29	60sec-2	22/29	200sec-2	9/29
0sec-3	29/29	60sec-3	20/29	200sec-3	8/29
0sec-4	29/29	60sec-4	19/29	200sec-4	10/29
0sec-5	29/29	60sec-5	21/29	200sec-5	8/29
30sec-1	29/29	100sec-1	19/29	300sec-1	8/29
30sec-2	29/29	100sec-2	12/29	300sec-2	7/29
30sec-3	29/29	100sec-3	16/29	300sec-3	7/29
30sec-4	29/29	100sec-4	17/29	300sec-4	7/29
30sec-5	28/29	100sec-5	16/29	300sec-5	7/29

Tannic acid.

A study was conducted to test the limit at which DNA would fail to amplify due to inhibition in the PCR process caused by tannic acid. As the concentration of tannic acid increased, the number of alleles called decreased as seen in Table 3.11. The PowerPlex® 16 HS system was able to withstand relatively high concentrations of tannic acid added directly to the PCR tube. All alleles were called correctly after an inhibition of 50 ng/µl; however, the peak height dropped at every loci compared to peak heights observed with no inhibition. Tolerance for inhibition rapidly dropped off between 50 ng/µl to 100 ng/µl. An electropherogram for each tannic acid concentrations is depicted in the appendix Figures G-J.

Table 3.11 Correctly called alleles for Tannic acid inhibited samples

Sample	# of correctly called alleles	Sample	# of correctly called alleles	Sample	# of correctly called alleles
0ng/µl-1	29/29	100ng/µl-1	4/29	400ng/µl-1	0/29
0ng/µl-2	29/29	100ng/µl-2	4/29	400ng/µl-2	0/29
0ng/µl-3	29/29	100ng/µl-3	6/29	400ng/µl-3	0/29
0ng/µl-4	29/29	100ng/µl-4	4/29	400ng/µl-4	0/29
0ng/µl-5	29/29	100ng/µl-5	4/29	400ng/µl-5	0/29
50ng/µl-1	29/29	200ng/µl-1	0/29		
50ng/µl-2	29/29	200ng/µl-2	0/29		
50ng/µl-3	29/29	200ng/µl-3	0/29		
50ng/µl-4	29/29	200ng/µl-4	0/29		
50ng/µl-5	29/29	200ng/µl-5	0/29		

Humic acid.

A study was conducted to test the limit of humic acid inhibition. As the concentration of humic acid increased within the PCR reaction, fewer alleles were present within the genetic profile as seen in Table 3.12. Intermediate amounts of humic acid led to larger loci dropping out, with smaller loci still amplifying. The PowerPlex® 16 HS system showed tolerance with humic

acid inhibition with numerous alleles amplifying despite 100 ng/µl inhibition. An electropherogram for each inhibited concentration is located in the appendices, Figures K-N.

Table 3.12 Correctly called alleles for Humic acid inhibited samples

Sample	# of correctly called alleles	Sample	# of correctly called alleles	Sample	# of correctly called alleles
0ng/µl-1	29/29	100ng/µl-1	16/29	400ng/µl-1	0/29
0ng/µl-2	29/29	100ng/µl-2	17/29	400ng/µl-2	0/29
0ng/µl-3	29/29	100ng/µl-3	19/29	400ng/µl-3	0/29
0ng/µl-4	29/29	100ng/µl-4	18/29	400ng/µl-4	0/29
0ng/µl-5	29/29	100ng/µl-5	5/29	400ng/µl-5	0/29
50ng/µl-1	29/29	200ng/µl-1	0/29		
50ng/µl-2	29/29	200ng/µl-2	0/29		
50ng/µl-3	29/29	200ng/µl-3	0/29		
50ng/µl-4	29/29	200ng/µl-4	0/29		
50ng/µl-5	29/29	200ng/µl-5	0/29		

Hematin.

A study was conducted to test the limit at which a concentration of hematin completely inhibited the PCR process. The initial concentrations of hematin did not inhibit the profile; however, when an apparent threshold of 1000 µM was added to the PCR process, the entire genetic profile dropped out. PowerPlex® 16 HS system withstood a great deal of inhibition. Table 3.13 depicts the number of correctly called alleles for the various hematin concentrations tested. Electropherograms for the hematin concentrations used to inhibit the PCR process are located in the appendices, Figures O-R.

Table 3.13 Correctly called alleles for Hematin inhibited samples

Sample	# of correctly called alleles	Sample	# of correctly called alleles	Sample	# of correctly called alleles
0µM-1	29/29	250µM-1	29/29	1000µM-1	0/29
0µM-2	29/29	250µM-2	29/29	1000µM-2	0/29
0µM-3	29/29	250µM-3	29/29	1000µM-3	0/29
0µM-4	29/29	250µM-4	29/29	1000µM-4	0/29
0µM-5	29/29	250µM-5	29/29	1000µM-5	0/29
125µM-1	29/29	500µM-1	29/29	2000µM-1	0/29
125µM-2	29/29	500µM-2	29/29	4000µM-1	0/29
125µM-3	29/29	500µM-3	29/29		
125µM-4	29/29	500µM-4	29/29		
125µM-5	29/29	500µM-5	29/29		

Discussion

Internal Validation

Limit of detection threshold.

A conservative detection threshold was set to ensure all background noise would be filtered out of genetic profiles for interpretation. When setting a high detection threshold, the ability to lose allelic data is an important consideration. SWGDAM interpretation guidelines recommend a laboratory "establish an analytical threshold based on signal-to-noise analyses of internally derived empirical data through scientific methods" (SWGDAM, 2010). Promega Corporation recommends 3 kV and 5 seconds as the optimal electrophoretic condition with 0.5 to 1.0 ng of template DNA. Numerous methods were executed in order to evaluate the most conservative threshold value that could be set to reliably distinguish noise from true peaks.

The electrophoretic condition of 3 kV and 5 seconds was chosen for analysis. Within the yellow channel the largest noise peak was observed at 46 RFU. The largest noise peak seen in the blue channel was 15 RFU and in the green channel was 44 RFU. The value of 46 RFU was the largest noise peak observed in all six samples within 3 kV and 5 seconds. To effectively set a conservative threshold, the 46 RFU value was doubled and rounded up to produce a value of 95 RFU. The baseline average plus three standard deviations produced a rounded value of 60 RFU. After comparing the two values, a conservative and subjective detection threshold was established at 95 RFU. As more data is collected and analyzed, the detection threshold can be reexamined and re-established.

Within the developmental validation of the PowerPlex® 16 HS system, a calling threshold of 50 RFU was used (Ensenberger et al., 2010). The value for peak detection threshold usually

ranges from 50-150 RFU (Promega Corporation, 2011). Therefore, an allele call should confidently be recognized as a true allele with a detection threshold set at 95 RFU.

Dynamic range and sensitivity.

The dynamic range and sensitivity studies were conducted to establish a consistent usable range of DNA concentrations for amplification that resulted in the highest quality data output following capillary electrophoresis. Forensic DNA typing utilizes PCR and electrophoretic technology, which may result in data originating from locations other than the sample (SWGDAM, 2010). It is necessary for interpretation procedures to attempt to limit prospective non-allelic peaks from being observed (SWGDAM, 2010).

Non-allelic peaks were observed in the 5.0 ng, 2.5 ng, and 1.25 ng DNA concentrations. In higher concentrations, large amounts of stutter were observed, as well as other artifacts, non-specific amplification product, and instrumental limitations. Most resulted from pull-up due to off-scale data in an adjacent channel; however, some OL alleles could not be explained. The PowerPlex® 16 HS system technical manual states samples may show low level artifacts between particular loci, OL artifacts can be seen in the 690 to 691 bp position, and one or more extra peaks have been observed in certain loci that are not related to amplification (Promega Corporation, 2011). Extra peaks not related to amplification were largely present within the TH01 locus. High amounts of template DNA, or poor quality formamide, polymer, and capillaries can cause the extra unexplained peaks to exist (Promega Corporation, 2011). This causes a problem during interpretation of a genetic profile; therefore, the 5.0 ng, 2.5 ng, and 1.25 ng DNA concentrations were not included in the dynamic range. Extreme caution should be taken when DNA samples of 1.0 ng or higher are used.

Beginning with 0.08 ng of DNA, allelic dropout was seen in the replicated samples at the larger loci. The 0.04 ng, 0.02 ng, and 0.01 ng samples did experience dramatic allelic dropout or complete dropout at all 16 loci analyzed. Allelic dropout hinders the ability to interpret genetic profiles correctly; therefore the 0.08 ng, 0.04 ng, 0.02 ng, and 0.01 ng of DNA concentrations were not included in the dynamic range. The sensitivity was determined to be 0.16 ng of DNA. Extreme caution should be used when concentrations below 0.16 ng are used due to expected allelic dropout.

The dynamic range was determined to be 0.63 ng to 0.16 ng of DNA. The recommended amplification concentration of template DNA is 0.5 ng in a 25 µl reaction volume for 3 kV and 5 second parameters (Promega Corporation, 2011). Promega also states, "with >1 ng of DNA, preferential amplification of smaller loci may occur" (Promega Corporation, 2011). Therefore, the dynamic range that was determined is in accordance with the manufacturer recommended concentrations existing within the range of DNA that was determined applicable on the instrumentation for the established parameter used.

Stochastic threshold.

SWGDAM has set interpretation guidelines that are used by the field. In SWGDAM's guidelines, section three addresses "Interpretation of DNA Typing Results" (SWGDAM, 2010). Under "Application of Peak Height Thresholds to Allelic Peaks" (guideline 3.2), stochastic thresholds are addressed (SWGDAM, 2010). A general peak height ratio of 60% is used to determine whether "two alleles at a heterozygous locus exhibit considerably different peak heights or an allele fails to amplify" (SWGDAM, 2010). A peak height ratio larger than 60% increases the statistical likelihood that the peaks originated from a single-source.

A peak height ratio of 60% was used to determine the stochastic threshold. However, due to the extreme peak height imbalances observed between known sister alleles determining a reasonable stochastic threshold was unlikely. A 750 RFU threshold value was determined to be the most practical stochastic threshold. Five of 195 data points were considered outliers with a 750 RFU threshold. A stochastic threshold of 750 RFU represents 97.44% of the viable range. Due to the extreme level of peak height imbalance, the stochastic threshold is not a reliable tool for use in convoluted interpretations.

The stochastic threshold plays a large role in determining mixture ratios within a genetic profile. Ideally, sister alleles should have balanced peak heights, which allow comparisons to be made in a major/minor situation. Sister alleles do not possess balanced peak heights at heterozygous loci when analyzed with the PowerPlex® 16 HS system. A stochastic threshold set at 750 RFU is problematic for low-template DNA samples. Most low-template DNA samples will not have peak heights larger than 750 RFU; therefore, the loci that do not exceed 750 RFU cannot be interpreted for potential mixture deconvolution. More investigation into this area is needed to explore the effect of various amplification cycles and electrophoretic parameters on peak height ratios and establishment of a stochastic threshold.

The PowerPlex® 16 HS System technical manual addresses ways to troubleshoot peak height imbalance. One cause is the amplification of > 1.0 ng of template DNA. This is resolved by decreasing the template DNA or decreasing the amplification cycles used. The use of FTA® paper, degraded DNA samples, and insufficient template DNA cause peak height imbalance. It should be mentioned, whole blood and blood samples on FTA® cards were extracted and used throughout this research. One did not provide a better balance in peak heights compared to the other. DNA concentrations under 1.0 ng were utilized as well which did not result in peak height

balance. The stochastic threshold should not be the only interpretation guideline that is taken into account when dealing with a convoluted situation.

Precision of capillary electrophoresis and reproducibility.

Precision and reproducibility are a large component within the validation study. The assurance that an allele will be called correctly every time it is analyzed is a large element of withstanding scrutiny in court. This study was analyzed through comparing allele calls and base pair calls of alleles.

Two methods have been established to examine the precision of the instrument. One method compares the individual allele's base pairs to the allelic ladder, while the second compares replicates of the allele's base pairs. An allele is called based on the allelic ladder; however, just because an allele is called correctly, three standard deviations of the DNA base pairs may not fall perfectly within the assigned bin of the allelic ladder. Depending on the electrophoretic conditions, the migrating DNA may not produce a normal bell curve distribution.

Unexpectedly, when both methods were used to examine precision, two different outcomes were observed. One allele out of eighty-seven did not achieve a precision value of less than 0.5 bp that the field has established as the standard when comparing the replicates to one another. However, when the five replicated samples were analyzed to the simultaneous allelic ladders, each allele was less than 0.5 bp.

This has an effect on interpretation of genetic profiles. It was estimated that an allele could potentially fall outside of its allotted window, which would result in an incorrect allele call being made. However, every time this allele was analyzed it was called correctly.

The precision situation that arose cannot be ignored. However, the reproducibility of the entire study did not encounter any issues of mislabeled alleles. No evidence exists within

documented validation studies of the PowerPlex® 16 HS system that indicates this issue has occurred previously. As more samples are collected and more data are analyzed, the precision of the multiplexing system should be revisited.

Stutter rate.

Elevated levels of stutter were present during the study. Parameters were altered to account for all potential stutter peaks that could be observed. Stutter was evaluated on a locus by locus situation. The overall average of stutter present at each locus closely paralleled the average stutter rates that were seen during the developmental validation. A locus specific percentage was used to determine a stutter percentage for each locus analyzed. Due to the variability in stutter percentages, a flat percentage would not adequately benefit the analyst during interpretation.

The marker specific stutter ratio used for each locus represents a conservative percentage. Following an evaluation of three standard deviations plus the average stutter values compared to the highest stutter value, the highest stutter value produced the most cautious values for interpretation support.

Due to outliers present within the data, the stutter percentages should only be used to assist when interpreting genetic profiles. The validation study was performed on 32 cycles of amplification. Thermal cycle procedures affect stutter that is present within genotypes. A validation study is recommended to be performed at 30 amplification cycles in an effort to decrease the amount of stutter that was present within the dynamic range. As more samples are examined and data is collected genetic profiles should continually be examined for high stutter peaks present.

Simulated mixture study.

Numerous methods exist for deconvoluting a mixture. The mixture samples were interpreted for assignment of a major and minor contributor. However, due to imbalanced peak height ratios this became an unachievable task for interpretation procedures. Due to peak height differences observed between sister alleles, interpretation of data past a single-source profile is not recommended. The PowerPlex® 16 HS system does not meet the necessary requirements to interpret mixture profiles to a statistical certainty. The peak height ratio imbalance seen in sister alleles creates a difficult scenario to correctly assign the genotypes at a locus with multiple alleles where all the alleles have similar peak heights.

The classic analytical procedures to interpret DNA mixture samples broke down at equal concentrations of DNA and unequal concentrations of DNA. Mixture ratios of 4:1 and 1:4 should be distinguishable due to unequal representation within the sample. However, the PowerPlex® 16 HS system could not differentiate the two contributing DNA profiles.

Cross-contamination.

The contamination study is intended to prove no carry-over exists between the four capillaries within the Applied Biosystems 3130 Genetic Analyzer. Contamination is not expected to occur due to the numerous wash and rinse steps the machine performs between sample injections. The wash and rinse steps that were performed before, between, and after each samples injection are designed to prevent contamination. The nine OL alleles that were present within the blank wells were not located within the bins designed to call true alleles. It is more likely that due to a lack of consistent performance on the Applied Biosystems 3130 Genetic Analyzer, non-reproducible artifacts such as bubbles or urea crystals built up and were moved through the capillary as injections were run on the instrument. The OL alleles were not present

in every negative control that was analyzed; therefore, it was not a reproducible artifact. Extra peaks may be visible within the green dye color due to contaminated water used to dilute 10X Buffer and fill the wash and rinse buffer reservoir (Promega Corporation, 2011). After analysis, the OL alleles that were present do not pose a threat of contamination within the instrument.

Mock case.

The results were treated in a casework manner. Following analysis of the questioned sample and known samples, the genetic profiles obtained were all correctly identified with known results by an independent reviewer. No unexpected occurrences were encountered during the laboratory testing and interpretation process. All alleles were present following interpretation of known artifacts. All controls performed as expected.

Environmental Inhibitors

The PowerPlex® 16 HS system withstood known environmental inhibitors with a great deal of tolerance. Extreme amounts of inhibitors caused the PCR process to fail and severely degrade DNA fragments. However, intermediate ranges of inhibitors were overcome using the PowerPlex® 16 HS system. The extreme amounts of inhibitors would most likely not be seen in casework samples due their excessive nature. No effort was made to overcome the effect of inhibition in the samples. Bovine serum albumin (BSA) was not added to the samples to help alleviate the inhibitors dramatic effect on the PCR process. The multiplexing system proved it is capable of handling forensic samples that may be subjected to the four inhibitors examined.

UV treatment.

The PowerPlex® 16 HS system endured a great deal of degradation caused by UV irradiation applied directly to the DNA samples. The color of the DNA samples did not change following degradation. Peak heights at all loci examined decreased between 30 seconds of

exposure to 60 seconds of exposure. Due to degradation caused by prolonged exposure to UV irradiation, only small PCR products were able to amplify. The amplicon size of D3S1358, TH01, D21S11, and vWA proved an effective tool at overcoming DNA degradation. No dropout was seen at the previously mentioned loci. Allelic dropout was seen between 332 bp and 415 bp at 60 seconds of exposure, and amongst 289 bp and 415 bp at 100 seconds. Increased allelic dropout occurred from 128 bp to 415 bp at 200 and 300 seconds of degradation. UV irradiation simulates sunlight exposure to DNA samples; however, the intensity and close proximity of the samples to the source of degradation in this research to simulated only the most intense inhibition potentially seen in casework.

Tannic acid and humic acid.

The PowerPlex® 16 HS system demonstrated tolerance with both tannic acid and humic acid. Identical concentrations at each inhibitor were added to the PCR tubes to examine potential inhibition that could occur. Both inhibitors resulted in complete profile drop-out at 200 ng/µl and 400 ng/µl. The coloration of tannic acid was less pronounced compared to humic acid. Tannic acid produced a slightly enhanced yellow tint; whereas, humic acid displayed a black coloration. A photograph of the Tannic Acid inhibition samples is seen in Figure S in the appendix. A photograph of the Humic Acid inhibition samples is seen in Figure T in the appendix.

Following addition directly to the PCR tubes, humic acid was expected to have an effect on the samples amplification process. Tannic acid was suspected to have an effect; however, the severity of inhibition that occurred was unexpected. The extreme nature of discoloration that occurred with directly adding humic acid and tannic acid to the amplification process greatly exaggerates the most likely encounters with these inhibitors in casework samples. The inhibitor

will not be added directly to the amplification process in casework samples. The process of extraction is designed to alleviate the affect of inhibitors, which was not tested in this research.

Hematin.

The PowerPlex® 16 HS system demonstrated a great deal of tolerance within the hematin study. Hematin was added directly to the PCR tube to ensure potential inhibition had the opportunity to occur. The level of inhibition on the multiplexing system most likely outweighs any level of inhibition a casework sample may be presented with. The color of the inhibitor when it was formulated indicated a level of inhibition would be seen. The 1000 µM concentration of hematin produced a completely black substance. A better understanding of the extreme nature of the coloration is evident through the undistinguishable label written in sharpie on the 2.0 ml microcentrifuge tube. A photograph of the Hematin inhibition samples is seen in Figure U in the appendix. As the serial dilutions were made, the tint gradually lightened; however, the final concentration of 125 µM still presented a substance encompassing a dark coloration. The PowerPlex® 16 HS system was able to produce amplification of all 16 loci with 500 µM of hematin added directly to the reaction.

Conclusions

Following the internal validation process, the following parameters were established for all subsequent samples analyzed utilizing the PowerPlex® 16 HS system. The detection threshold was set at 95 RFU. The dynamic range of the system was 0.63 ng – 0.16 ng of input DNA. The sensitivity of the multiplexing system was 0.16 ng. The stochastic threshold was set at 750 RFU. The precision and reproducibility of the system were successful. Marker specific stutter ratios were established for each locus. Cross-contamination was not found within the instrumentation or set-up procedures. The mixture study led to indistinguishable major and

minor contributors for the ratios tested, except 1:19 and 19:1. The mock case was successfully analyzed and confirmed by a known independent reviewer.

At the conclusion of this research, the PowerPlex® 16 HS system is not recommended for forensic analysis of challenging DNA samples. It adequately analyzed single-source DNA samples. However, due to the imbalance in peak heights the system restricts the analyst in interpretation scenarios.

Following the environmental inhibitor studies, the PowerPlex® 16 HS system displayed great tolerance to known amplification inhibitors. The PowerPlex® 16 HS system showed it was capable of analyzing DNA samples through the PCR process with known inhibitors. The PowerPlex® 16 HS system was recommended to analyze inhibited single-source samples.

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Appendix

Figure A. Published Mock Case Results

Collaborative Testing Services, Inc.

FORENSIC TESTING PROGRAM

P.O. Box 650820 Sterling, VA 20165-0820
e-mail: forensics@cts-interlab.com
Telephone: +1-571-434-1925
Fax: +1-571-434-1937 or
Toll-free Fax (US only): 1-866-fax-2cts

Manufacturer's Information

Test No. 10-573: Forensic Biology

Amelogenin and STR Results

Results compiled from predistribution laboratories and a consensus of at least 10 participants.

Item	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D21S11
1	14,17	10,13	11,11	12,15	11,12	9,12	12,12	29,31.2
2	17,18	8,11	9,11	13,14	11,12	11,12	15,15	28,32.2
3	17,18	8,11	9,11	13,14	11,12	11,12	15,15	28,32.2
4-Blood	14,17	10,13	11,11	12,15	11,12	9,12	12,12	29,31.2
4-Semen	15,18	11,12	8,8	8,12	8,11	9,11	14,20	29,30

Item	Amel	CSF1PO	FGA	TH01	TPOX	vWA	Penta D	Penta E
1	X,X	11,12	19,23	9.3,9.3	8,8	16,18	8,13	5,17
2	X,Y	12,12	19,22	7,8	8,8	18,19	5,8	12,16
3	X,Y	12,12	19,22	7,8	8,8	18,19	5,8	12,16
4-Blood	X,X	11,12	19,23	9.3,9.3	8,8	16,18	8,13	5,17
4-Semen	X,Y	11,12	22,23	7,9	8,8	15,17	10,12	7,13

Item	D2S1338	D19S433
1	17,20	14,16.2
2	18,21	14.2,15
3	18,21	14.2,15
4-Blood	17,20	14,16.2
4-Semen	23,24	15,15.2

Figure A depicts the published and correct genotypic information for the mock case exam that was conducted using CTS exam, test no. 10-573, for forensic biology samples. Item 4 was not tested.

Figure B. UV treatment 30 second electropherogram

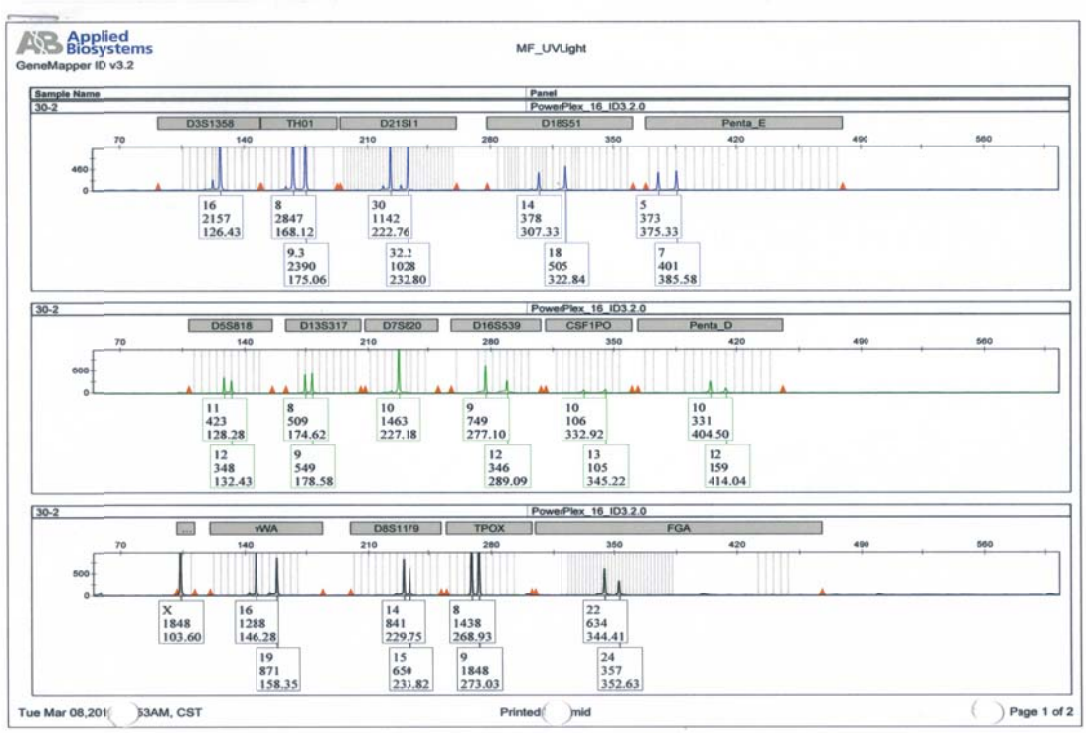

Figure B depicts an electropherogram for a DNA sample following 30 seconds of DNA degradation from UV treatment. All 29 true alleles are present.

Figure C. UV treatment 60 second electropherogram

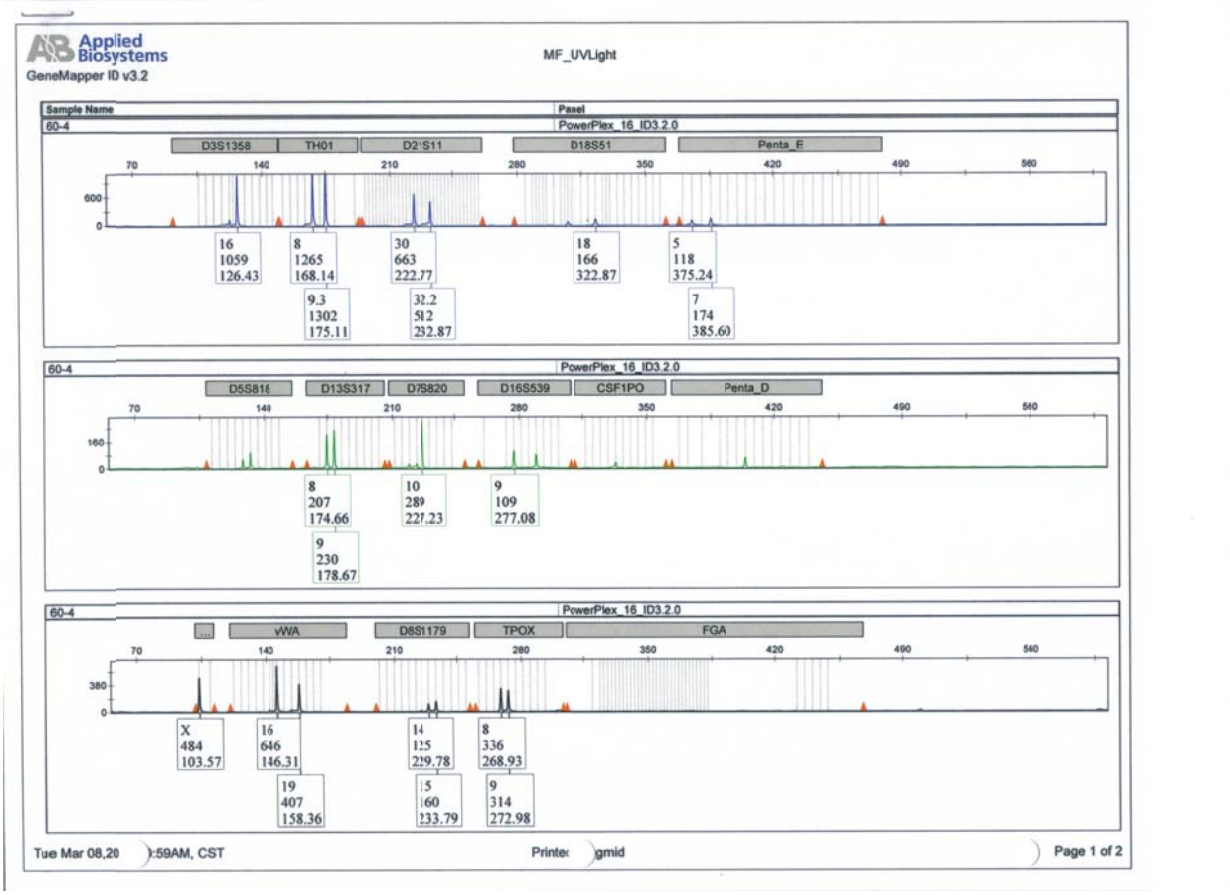

Figure C depicts an electropherogram of a DNA sample following 60 seconds of DNA degradation from UV treatment. Nineteen alleles are present out of 29 true alleles.

Figure D. UV treatment 100 second electropherogram

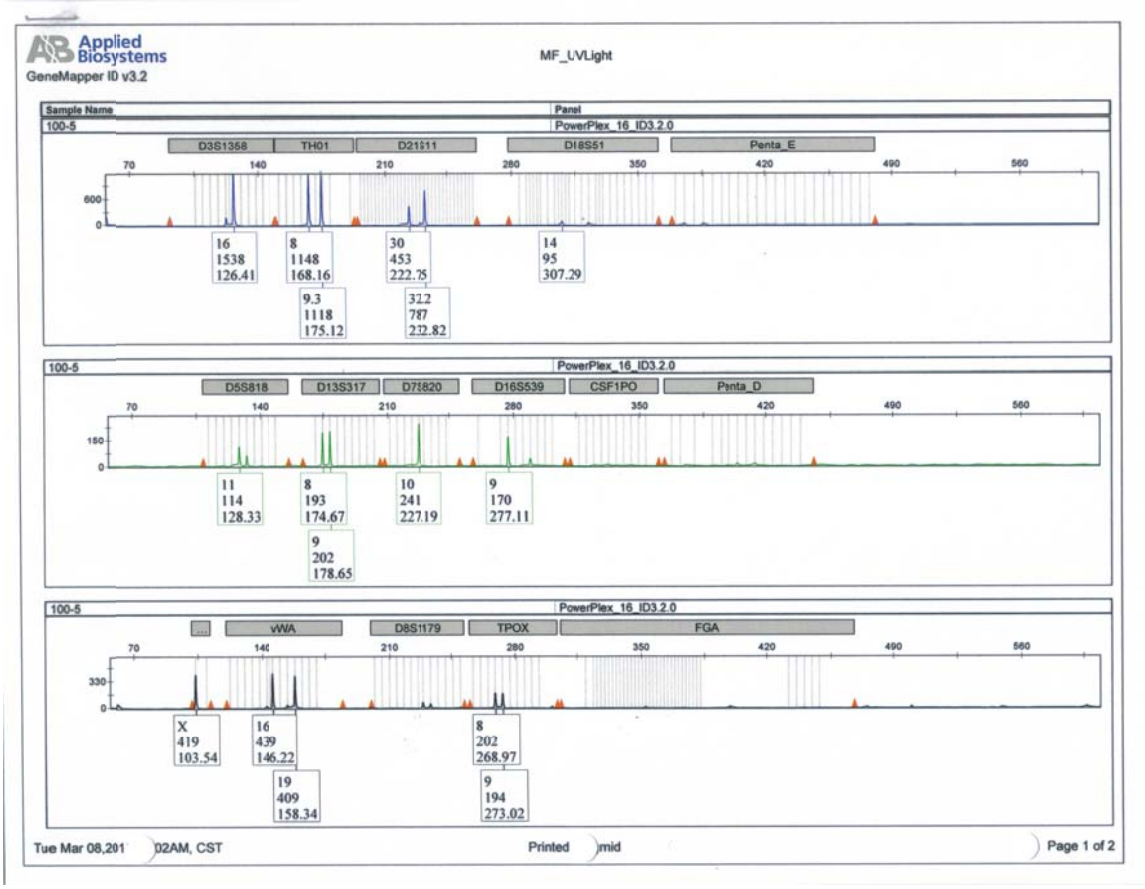

Figure D depicts an electropherogram of a DNA sample following 100 seconds of DNA degradation from UV treatment. Sixteen alleles are present out of 29 true alleles.

Figure E. UV treatment 200 second electropherogram

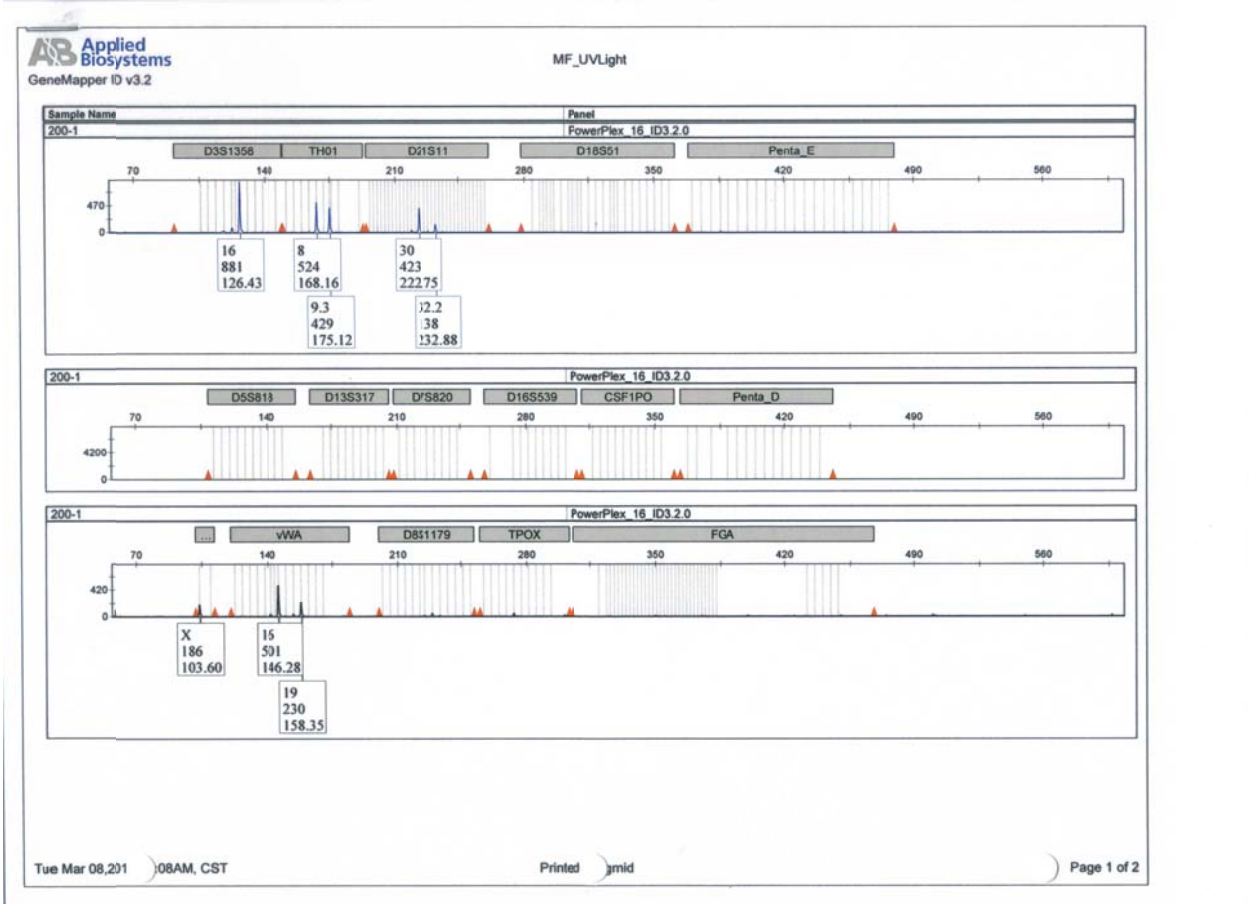

Figure E depicts an electropherogram for a DNA sample following 200 seconds of DNA degradation from UV treatment. Eight alleles are present out of 29 true alleles.

Figure F. UV treatment 300 second electropherogram

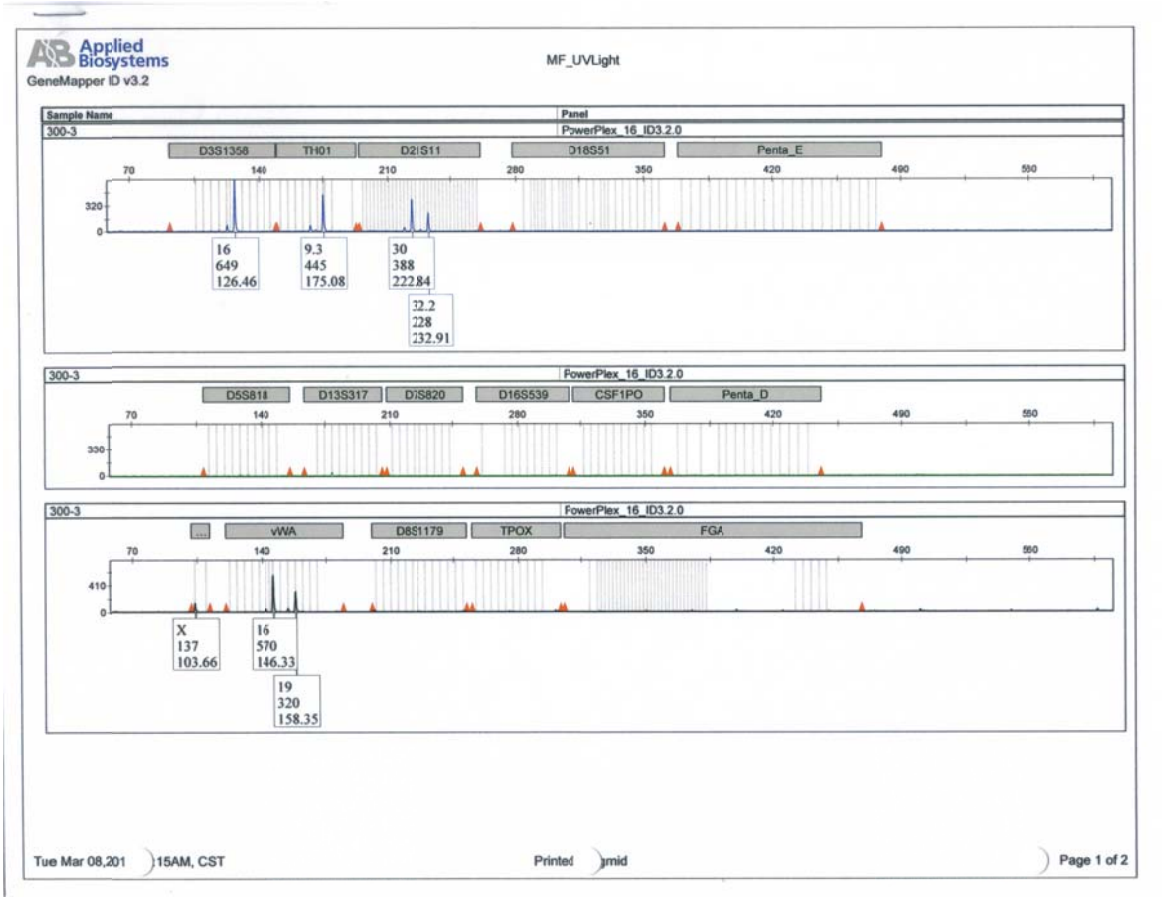

Figure F depicts an electropherogram of a DNA sample following 300 seconds of DNA degradation from UV treatment. Seven alleles are present out of 29 true alleles.

Figure G. Tannic Acid 50 ng/µl electropherogram

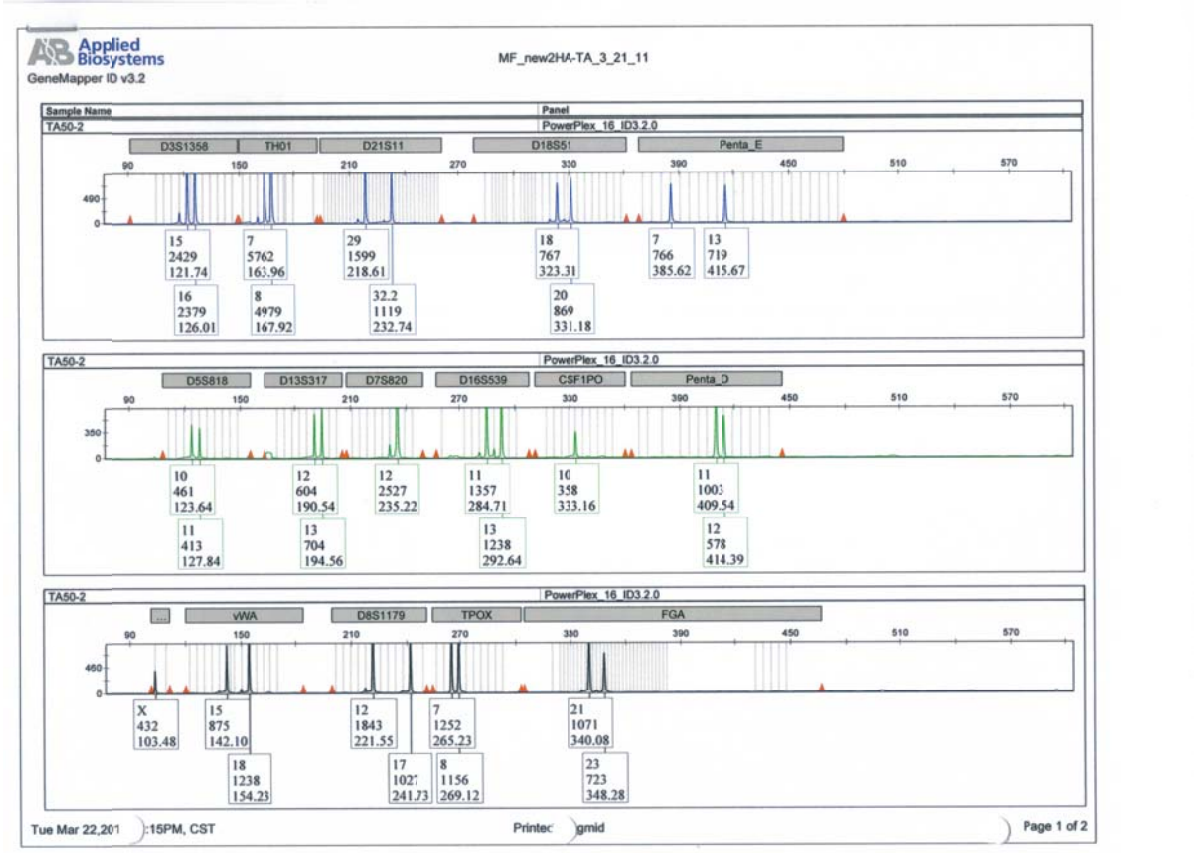

Figure G shows an electropherogram of a DNA sample following 50 ng/µl of tannic acid added directly to the PCR tube. All 29 true alleles are present.

Figure H. Tannic Acid 100 ng/µl electropherogram

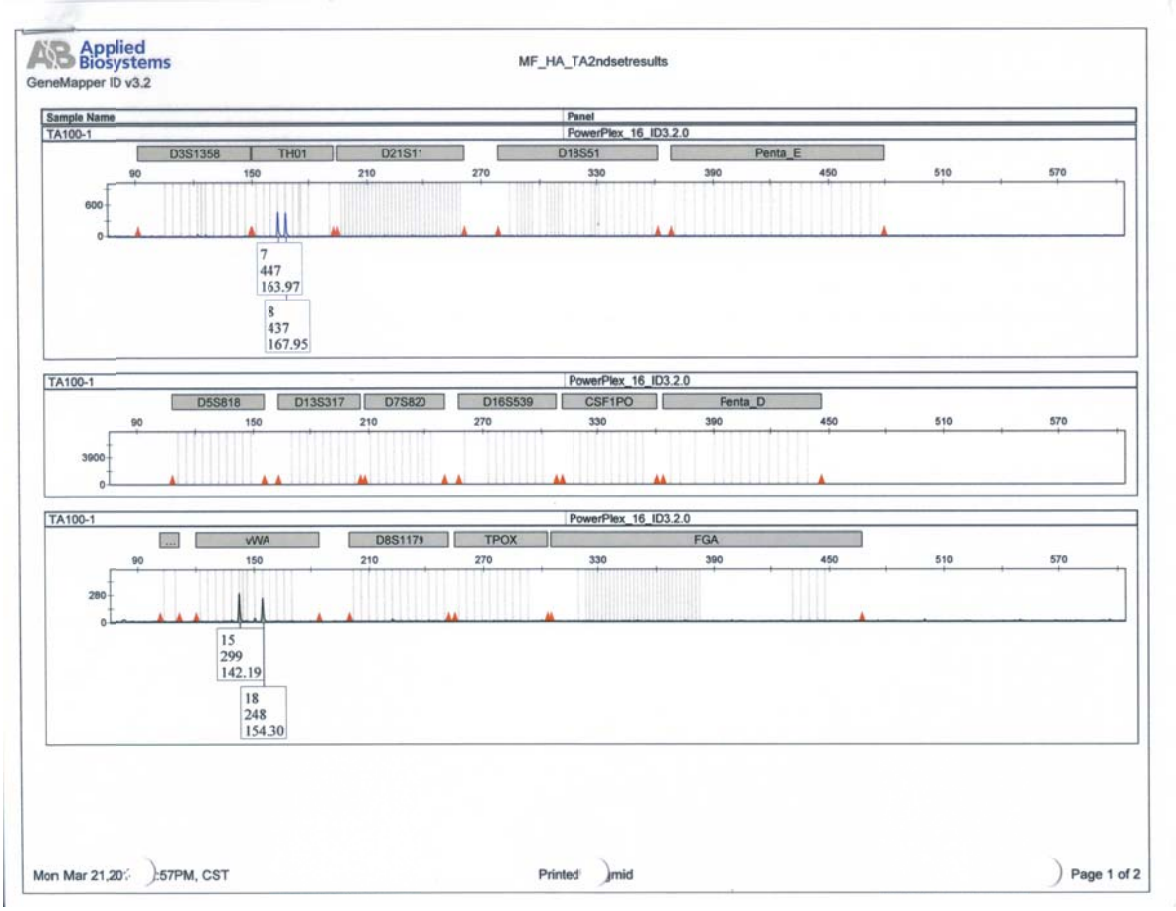

Figure H shows an electropherogram of a DNA sample following 100 ng/µl of tannic acid added directly to the PCR tube. Four alleles were present out of the 29 true alleles in the genetic profile.

Figure I. Tannic Acid 200 ng/µl electropherogram

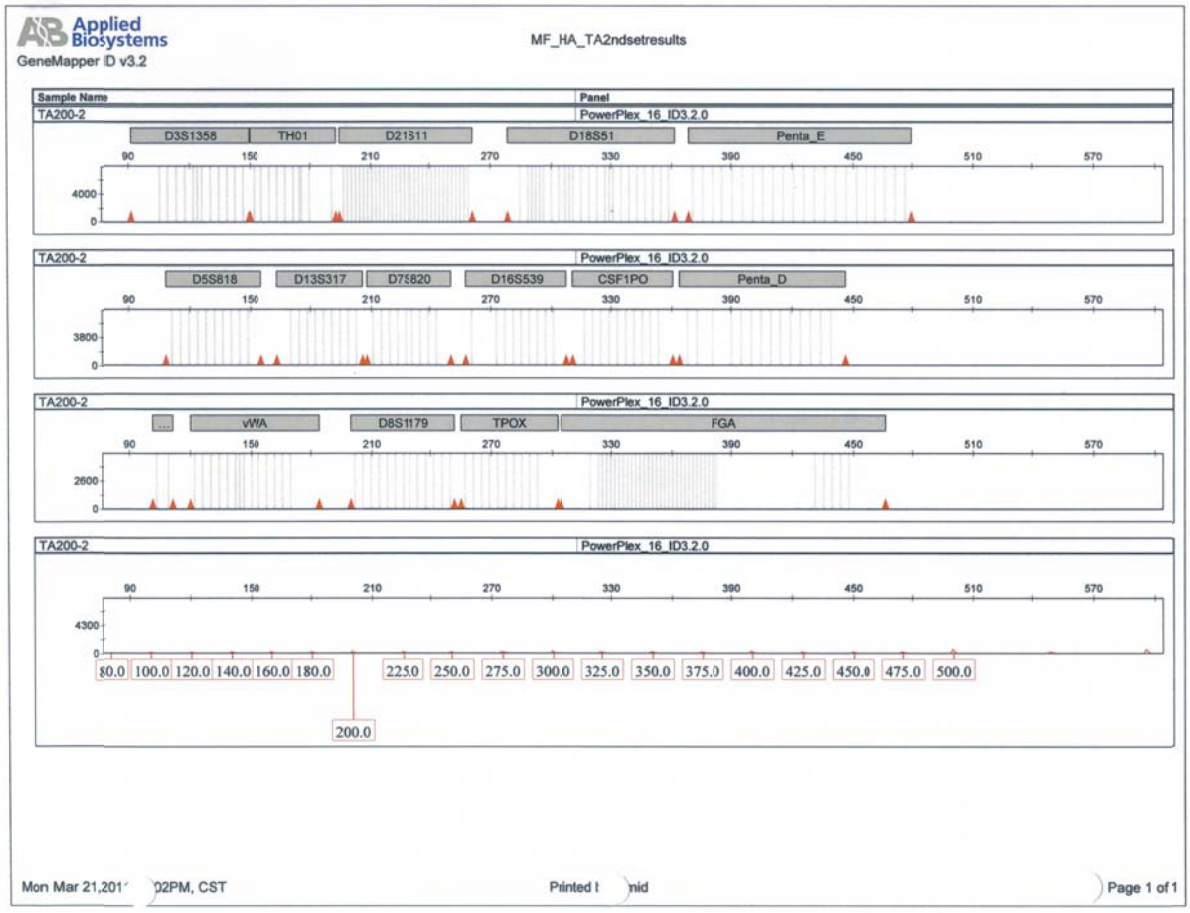

Figure I shows an electropherogram of a DNA sample following 200 ng/µl of tannic acid added directly to the PCR tube. No alleles were present out of the 29 true alleles in the genetic profile.

Figure J. Tannic Acid 400 ng/µl electropherogram

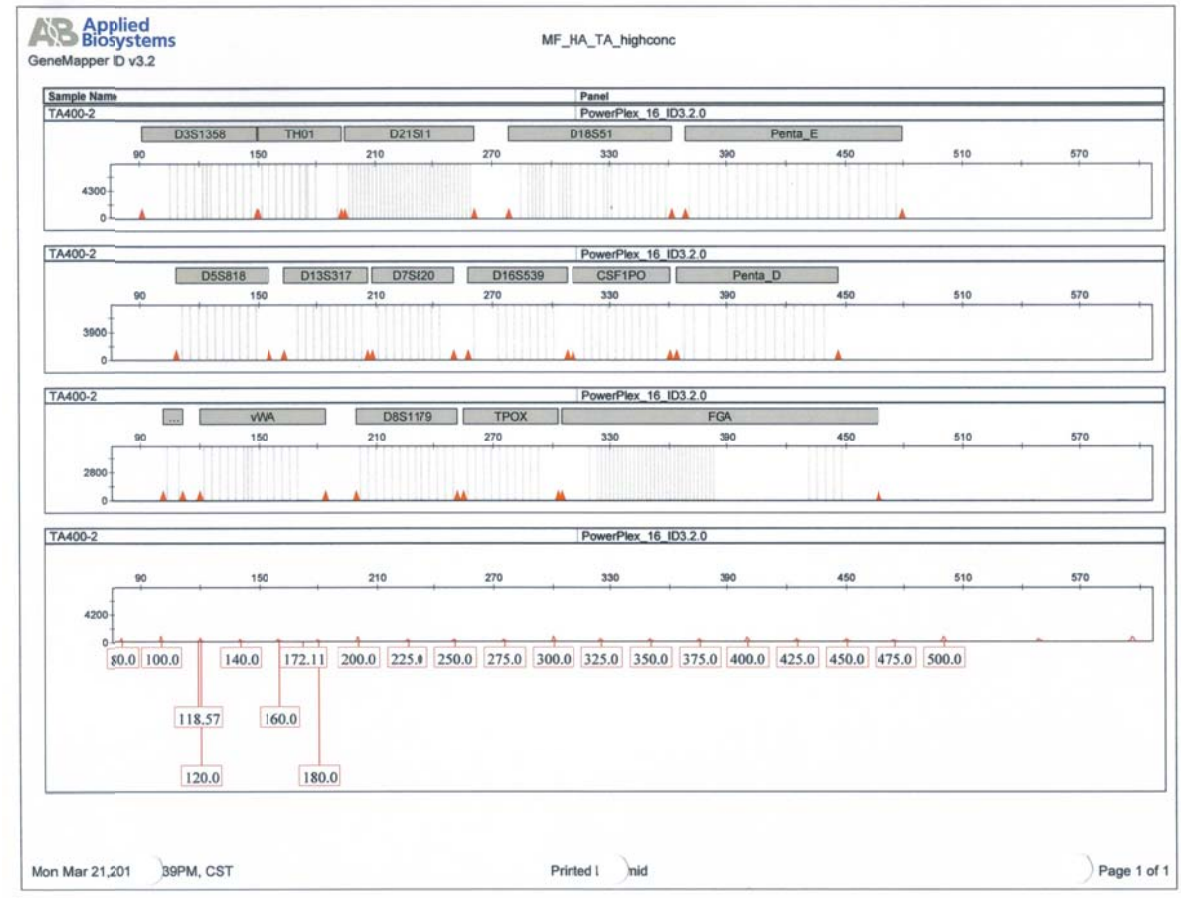

Figure J shows an electropherogram of a DNA sample following 400 ng/µl of tannic acid added directly to the PCR tube. No alleles were present out of the 29 true alleles in the genetic profile.

Figure K. Humic Acid 50 ng/µl electropherogram

Figure K shows an electropherogram of a DNA sample following 50 ng/µl of humic acid added directly to the PCR tube. All 29 true alleles are present.

Figure L. Humic Acid 100 ng/µl electropherogram

Figure L shows an electropherogram of a DNA sample following 100 ng/µl of humic acid added directly to the PCR tube. Eighteen alleles were present out of the 29 true alleles in the genetic profile.

Figure M. Humic Acid 200 ng/µl electropherogram

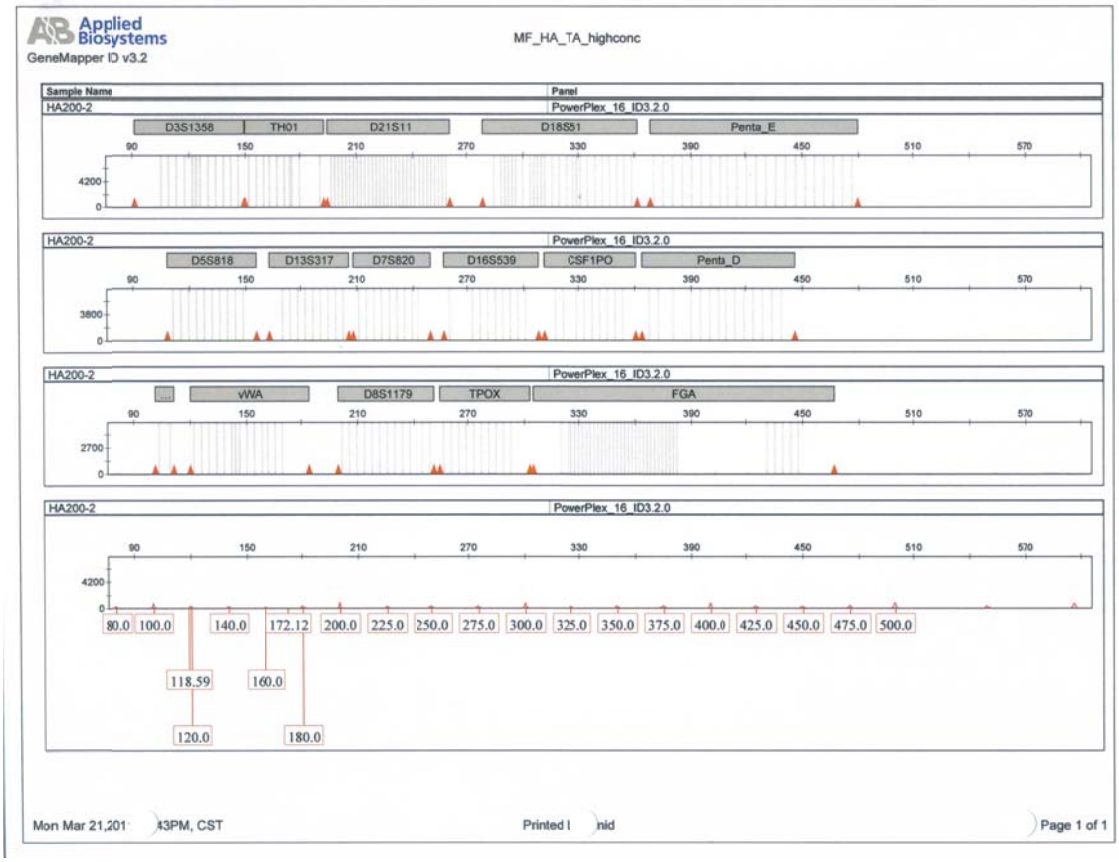

Figure M shows an electropherogram of a DNA sample following 200 ng/µl of humic acid added directly to the PCR tube. No alleles were present in the genetic profile.

Figure N. Humic Acid 400 ng/µl electropherogram

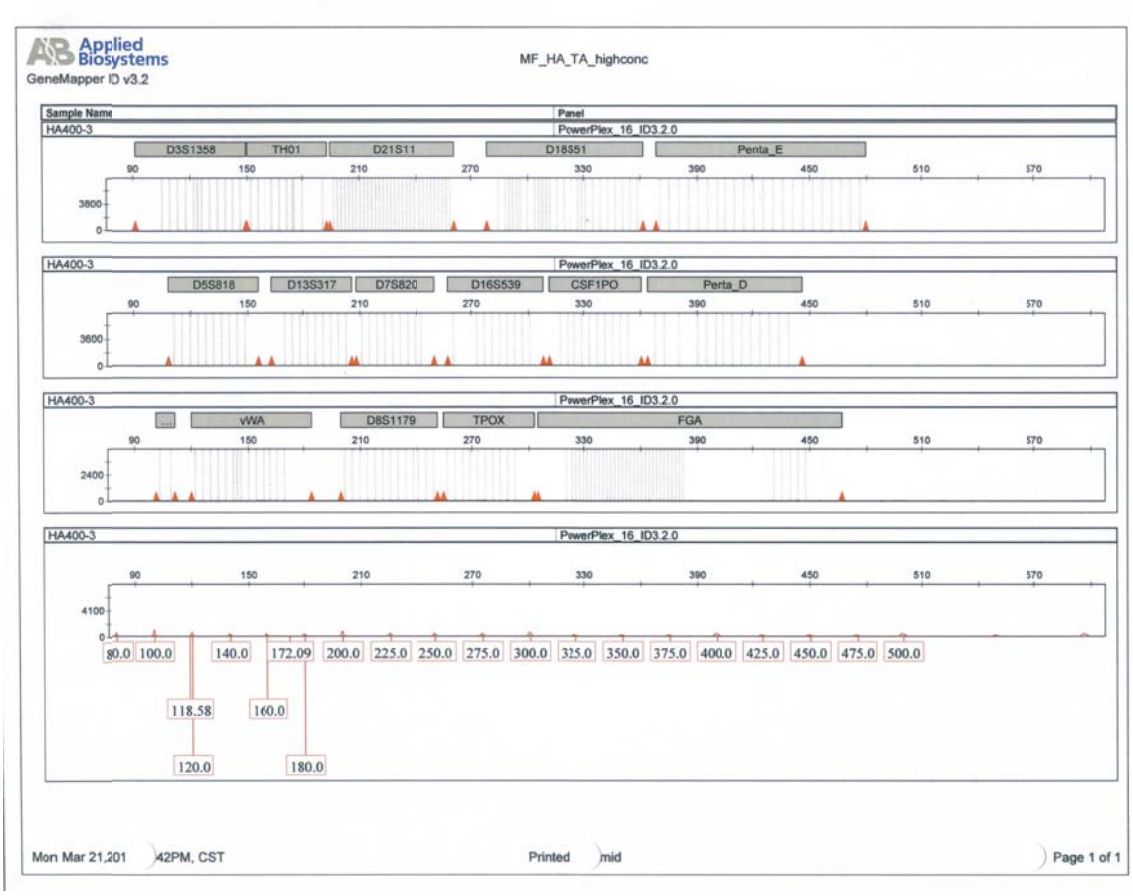

Figure N shows an electropherogram of a DNA sample following 400 ng/µl of humic acid added directly to the PCR tube. No alleles were present in the genetic profile.

Figure O. Hematin 125 µM electropherogram

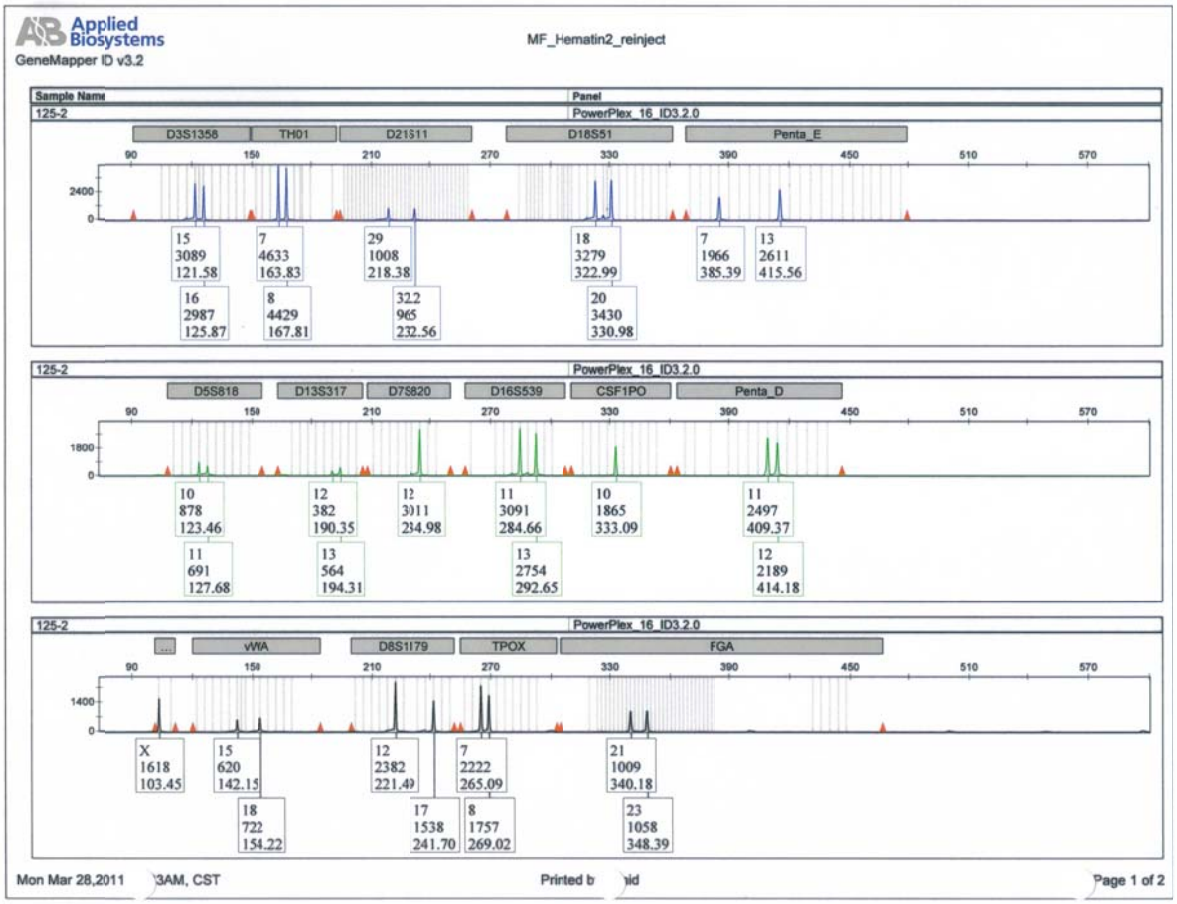

Figure O depicts an electropherogram of a DNA sample following 125 µM hematin added directly to the PCR tube. Twenty-nine alleles were present within the profile.

Figure P. Hematin 250 µM electropherogram

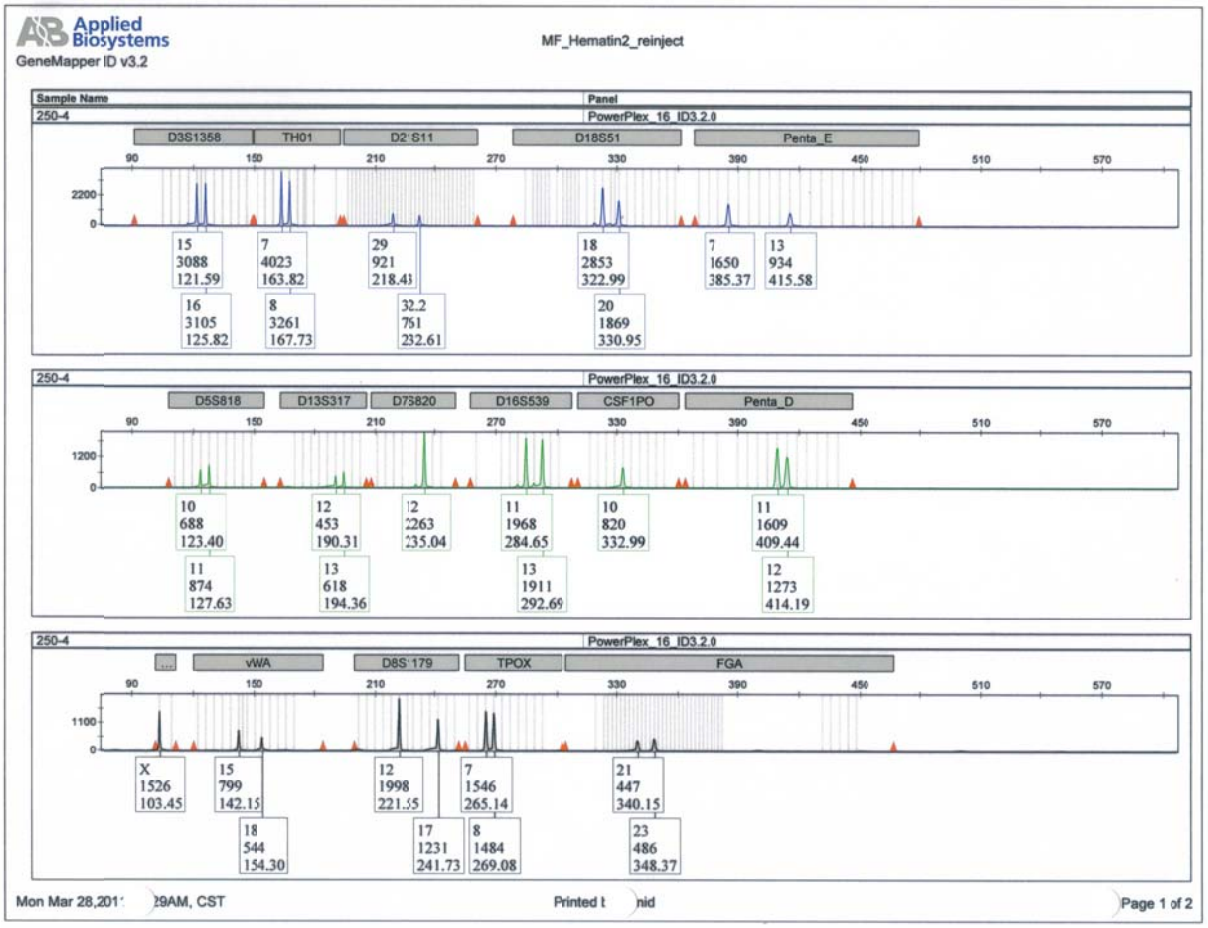

Figure P depicts an electropherogram of a DNA sample following 250 µM hematin added directly to the PCR tube. Twenty-nine alleles were present within the profile.

Figure Q. Hematin 500 µM electropherogram

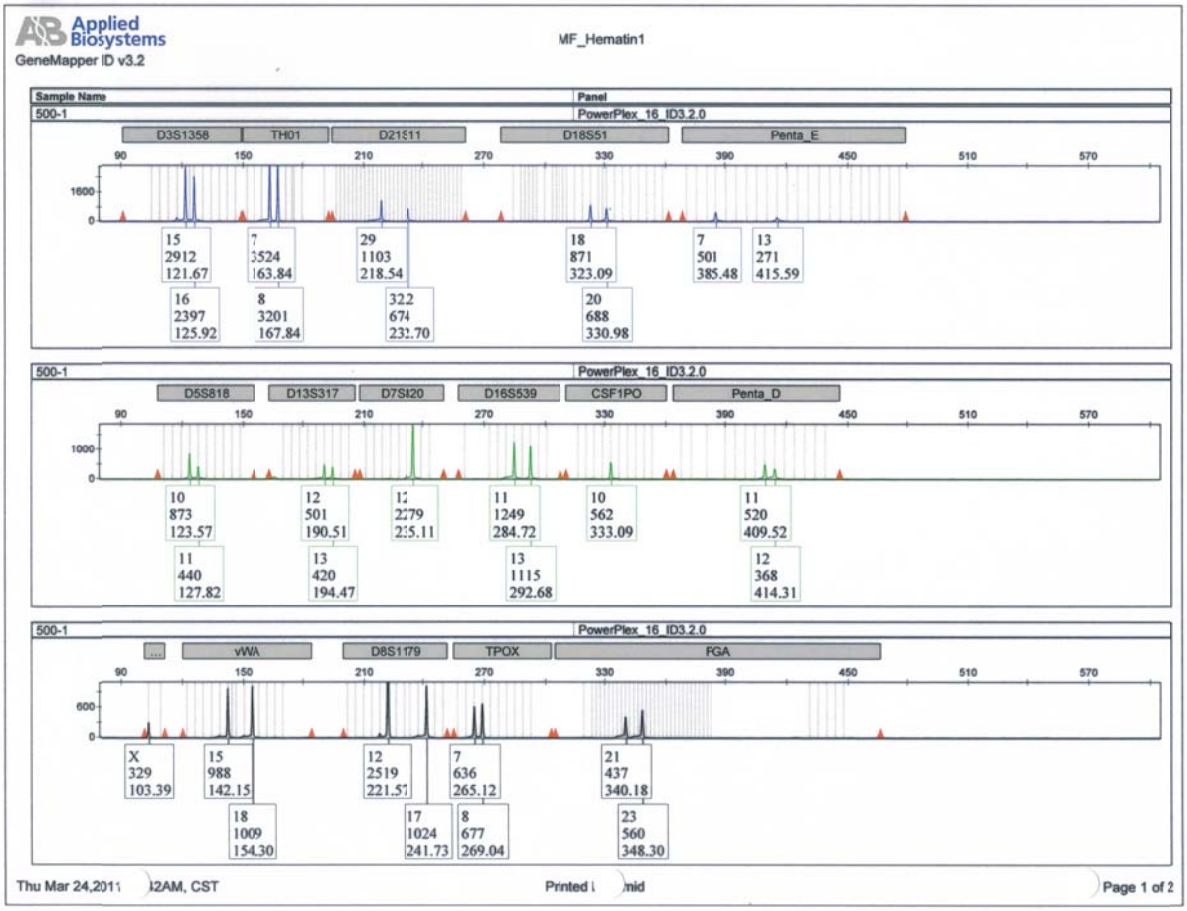

Figure Q depicts an electropherogram of a DNA sample following 500 µM hematin added directly to the PCR tube. Twenty-nine alleles were present within the profile.

Figure R. Hematin 1000 µM electropherogram

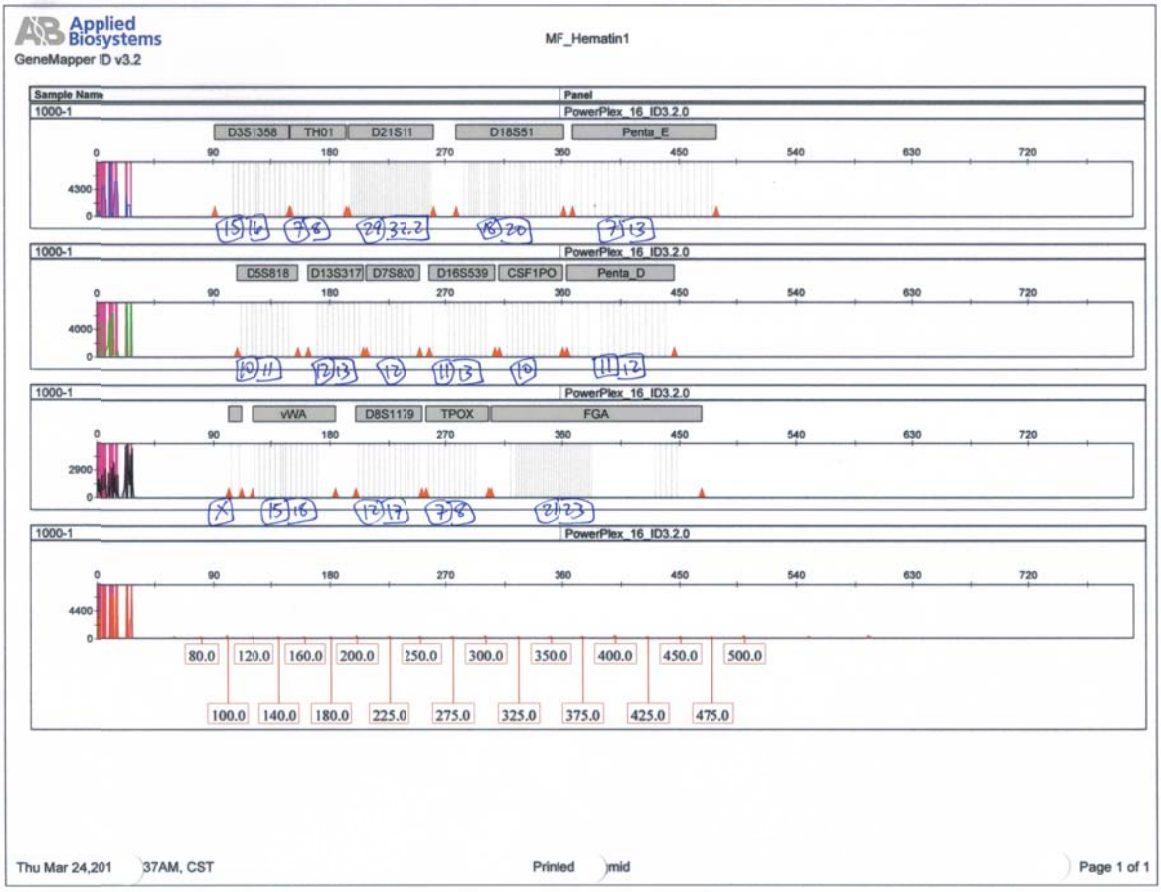

Figure R depicts an electropherogram of a DNA sample following 1000 µM hematin added directly to the PCR tube. No alleles were present within the profile.

Figure S. Photograph of Tannic Acid inhibition samples

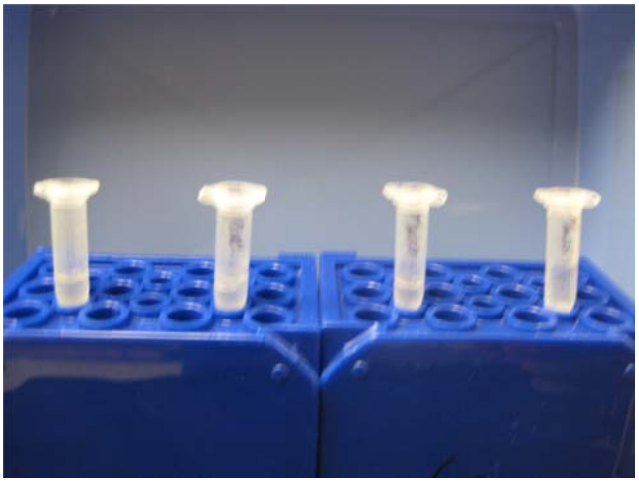

Figure S depicts the tannic acid inhibition tubes used. Starting on the left the first tube is 400 ng/µl, the second from the left is 200 ng/µl, the third from the left is 100 ng/µl, and the tube on the far right is 50 ng/µl.

Figure T. Photograph of Humic Acid inhibition samples

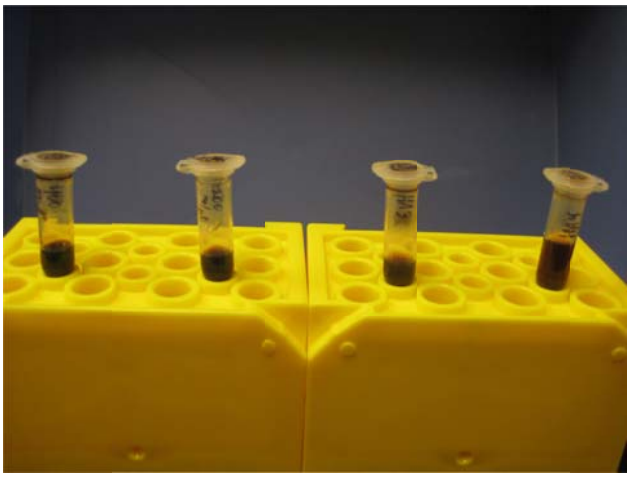

Figure T shows the inhibition tubes used for the humic acid study. The tube on the far left is 400 ng/µl, second from the left is 200 ng/µl, third from the left is 100 ng/µl, and the tube on the far right is 50 ng/µl.

Figure U. Photograph of Hematin inhibition samples

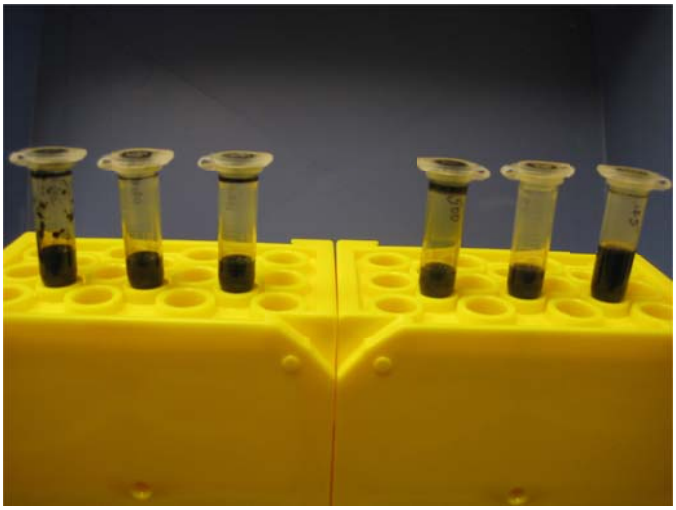

Figure U shows the tubes used for the hematin inhibition study. The tubes in order starting from the left are the following concentrations: 4000 µM, 2000 µM, 1000 µM, 500 µM, 250 µM, 125 µM. The 4000 and 2000 µM concentrations were not extensively tested during this research; however, the stock solution that serial dilutions were made from originated at a 4000 µM concentration.

Table V. Equipment used to conduct this research

Instrumentation used to conduct this research:			
Manufacturer	**Instrument**	**Serial No.**	
Applied Biosystems (Carlsbad, CA, USA)	3130 Genetic Analyzer	21364-025	
Applied Biosystems	GeneAmp PCR System 9700	805S8201803	
Applied Biosystems	7500 Real-Time PCR System	275001373	
USA Scientific (Ocala, FL, USA)	AirClean 600 PCR Workstation	AC648LFUVC-43352	
Thermolyne	Type 16500 Dri-Bath	229920807548	
Sartorius	Analytical Balance		
Consumables used to conduct this research:			
Manufacturer	**Item Name**	**Description**	**Catalog No./Part No.**
Promega Corporation (Madison, WI, USA)	DNA IQ™ System	400 Reactions	DC6700
Applied Biosystems (Carlsbad, CA, USA)	Quantifiler™ Human DNA Quantification Kit		4343895
Promega Corporation	PowerPlex® 16 HS System	400 Reactions	DC2100
Promega Corporation	PowerPlex Matrix Standards, 3100/3130		DG4650
Applied Biosystems	3130 POP4 (Performance Optimized Polymer 4)		4352755

Applied Biosystems	Buffer (10X) with EDTA		402824
Promega Corporation	DTT (Dithiothreitol)	Molecular Biology Grade	V3151
Applied Biosystems	3130 & 3100 – *Avant* Capillary Array	36cm	4333464
MP Biomedicals, LLC (Solon, OH, USA)	Hematin Porcine	Powder	198969
Fisher Science Education (Hanover Park, IL)	Tannic Acid	Powder	1401-55-4
Alfa Aesar, A Johnson Matthey Company (Ward Hill, MA, USA)	Humic Acid	Powder	1415-93-6
Sigma-Aldrich (St. Louis, MO, USA)	Sodium Hydroxide, pellets	97+%, A.C.S. reagent	221465-500G
Whatman	Human ID Bloodstain Card BFC 180		WB100014
Applied Biosystems	Plate Septa 96-well		4315933
Applied Biosystems	MicroAmp Optical 96-well Reaction Plate		N801-0560
Diversified Biotech (Boston, MA, USA)	Teeny Tough Tags		TT-TNY
USA Scientific (Ocala, FL, USA)	TipOne 0.5-20 µl Filter Tips		1121-4810
USA Scientific	TipOne 1-100 µl Beveled Filter Tips		1120-1840
USA Scientific	TipOne 101-1000 µl Filter Tips		1126-7810
USA Scientific	Seal Rite 0.2 ml Indiv. Thin Wall PCR Tubes with attached Dome Cap		1602-4300
USA Scientific	Seal Rite 0.5 ml Natural Microcentrifuge Tubes		1605-0000
USA Scientific	Seal Rite 2.0 ml Natural Microcentrifuge Tubes		1620-2700
Fisher Scientific	1 L BP561-1 Water	Sterile	1609-47-8

Table V. List the instrumentation and equipment used to conduct this research. The manufacturer is listed, along with the title of the item or instrument, and catalog number or serial number associated with the item.