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In the environment surrounding a court case, the United States Justice System requires that DNA evidence presented in a court of law be reliable, robust, and reproducible. For DNA laboratories to uphold these strict guidelines, all methods and instrumentation used in the lab must undergo validation studies. A validation study will measure the ability of the laboratory to perform a new method or use new instrumentation on a variety of samples before casework can be performed, ensuring that the method or instrumentation is indeed reliable, robust, and reproducible.

This study consists of the validation of Promega's PowerPlex® 16 Kit on the ABI 3100 Avant Genetic Analyzer instrumentation for Identigene, located in Houston Texas. The validation consists of sensitivity, routine, mixture, non-probative, allelic ladder size precision, and matrix evaluations. Completion of these studies allow Identigene to begin performing casework using the PowerPlex® 16 Kit. A validation study of the Takayama Reagent for Identigene was also performed. This procedure is used when a substance is presumed to be blood and the laboratory personnel want to confirm the presence of heme. The study consisted of a test determining the amount of reducing agent to add to the reagent, a dilution study, a chronological study testing the ability of the reagent to perform on older blood stains, and substrate studies, and after these tests were performed indicated the ineffectiveness of the Takayama procedure for the purposes of Identigene.

INTERNAL VALIDATION STUDY OF PROMEGA'S

POWERPLEX® 16 SYSTEM

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CHAPTER I

INTRODUCTION

In the field of forensic science, Short Tandem Repeats (STRs) are currently the major type of identification markers used to distinguish individuals. STRs have been an integral part of forensic science over the last fifteen years due to their ability to discriminate between biological evidence and individuals at a statistical power unheard of using previous methods (1). STRs are short sequences of DNA lined up adjacent to one another in a repeating sequence, and are seen throughout the human genome. The short size of STRs makes it possible to develop a DNA profile from degraded samples, further supporting their widespread use (1). Research has progressed based on the success of STRs leading to the development of Promega's PowerPlex® 16 System. The PowerPlex® 16 kit provides all the reagents necessary to amplify DNA and genetically analyze sixteen locations, or loci, in a single reaction, saving time, money, and DNA sample when compared to other kits requiring multiple reactions such as the Profiler Plus™ and COfiler™ Kit combination. In order for Promega's PowerPlex® 16 System to be utilized at the Forensics Laboratory at Identigene, a private corporation located in Houston, Texas, a validation study must be conducted. Validation studies are important in the field of forensic science because they directly evaluate a laboratory's ability to achieve accurate and consistent results using a new technique on a particular instrument.

This validation study will lead directly to the development of interpretation guidelines that will be used as a reference for forensic casework by analysis at Identigene.

Various kits or systems for STR study are available, and are recommended for use on a specific brand of instrument. These kits undergo developmental validation by the manufacturer, but all labs must still internally validate the kits on their own instruments before use. Occasionally a client will request a particular kit for which the laboratory does not have the recommended brand of instrument as selected by the manufacturer. It is in this case where the laboratory must validate the ability of its available instruments to perform tests using the requested kit. Applications of validated STR systems include forensic and paternity DNA testing, both of which Identigene performs.

Validation of the Promega PowerPlex® 16 System on an ABI 3100 Genetic Analyzer will allow for STR analysis on a particular instrument using a different kit than is normally used by the laboratory. Identigene normally uses the Profiler Plus™ and COfiler™ kits provided by Applied Biosystems (ABI) for use in forensic testing on an ABI 3100 genetic analyzer. A validation of the PowerPlex® 16 kit will extend the range of kits that Identigene can use to perform forensic analysis on its ABI 3100 instrumentation, extending its range of potential clients. The PowerPlex® 16 kit consists of 16 loci including Amelogenin (Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818) (4).

New or novel techniques in the field of Forensic Science must be validated for use in forensic casework according to TWGDAM, or the Technical Working Group for DNA

Analysis Methods that established the standards and guidelines in 1995, based on standards proposed by the DNA Advisory Board that are followed by forensic laboratories across the United States (1, 10). In 2003, SWGDAM, or the Scientific Working Group for DNA Analysis Methods, revised these standards and guidelines for validation purposes, and all laboratories performing DNA analysis are expected to follow these guidelines. In order for the PowerPlex® 16 System to be used in forensic casework in the Forensic Laboratory at the Identigene Corporation, a validation study must be performed so that the performance of the system can be evaluated in all aspects, and demonstrate the laboratory's ability to perform a technique in a robust, reliable, and reproducible manner. Reproducibility, mixture, and sensitivity studies are evaluated, as well as population studies, concordance, and environmental and substrate studies. Non-probative evidence, or previously analyzed samples coming from cases that have been adjudicated, must be used for validation studies.

The validation study for the PowerPlex® 16 System at Identigene consisted of four studies: sensitivity, mixture, a routine sample study, and a non-probative casework or concordance study. An additional validation of the Takayama technique for presumptive blood identification was also conducted. For the PowerPlex® 16 System, the sensitivity test is a serial dilution of known DNA concentrations that are tested to determine the optimal input amount of DNA. This was completed before the remainder of the experiments begun. The mixture study dealt with various ratios of male and female mixtures using two individuals per mixture. The mixtures represent a simulation of a sexual assault case or various types of mixture cases, where any particular ratio of

contributor's DNA may be encountered. The mixture test attempts to determine the sensitivity of the kit and instrument's detection ability when faced with a minor component in a mixture. The routine samples study was performed in order to determine the kit and instrument's ability to perform equally and reliably when faced with a variety of routine single source samples seen in casework. Calculations were performed including peak height ratio, stutter percentage, and standard deviation, helping to determine the bounds of both the kit and the instrument, and whether they perform within the bounds acceptable to the lab when performing routine casework. The non-probative casework study attempts concordance, or the ability of the kit to compare to the results already obtained using Profiler Plus™ and COfiler™ on the same non probative samples using the same instruments.

All samples used for this PowerPlex® 16 project contained DNA previously extracted by Identigene via the Chelex and Organic Methods (2). Quantification using Real-Time Polymerase Chain Reaction via the ABI Prism® 7000 Sequence Detection System using the Quantifiler™ Human DNA Quantification Kit was performed on all samples (3). STR Amplification was then performed on both the Perkin-Elmer GeneAmp® PCR System 9600 and 9700 Thermal Cyclers using the PowerPlex® 16 System. Finally, capillary electrophoresis was performed on the ABI Prism® 3100 Avant Genetic Analyzer, followed by analysis on GeneScan® Analysis Software and Genotyper® Program using the PowerTyper™ 16 Macro for the PowerPlex® 16 system (4, 5). Dye set "Z" was used for the PowerPlex® 16 kit, in addition to custom analysis

parameters (5). A matrix validation was also conducted for the PowerPlex® 16 system using the 3100-Custom matrix standards.

Four different studies were performed for the Takayama Test: DTT, dilution, environmental, and substrate. A DTT test was conducted in order to evaluate the performance of the oxygen scavenger DTT when used in the Takayama reagent. DTT supposedly helps to remove oxygen from the reaction, allowing pyridine to bind to the heme in blood easier, thereby causing hemachromogen crystals to bind quicker, giving an overall faster positive reaction for the presence of heme (6). The results of this study were then used to create a Takayama reagent for use in the remainder of the experiments. A dilution series was performed using liquid blood, to test the sensitivity of the test. An environmental study was performed to test the reagent's performance when attempting to identify blood stored for one week in various levels of heat, light, and moisture. A substrate study using blood stains on cotton swabs, stain cards, and cloth was performed to determine the ability of the Takayama reagent to detect blood found on these substrates. In addition, this substrate test helped to determine whether these substrates released enough heme into solution to allow the reagent to produce a positive result.

Each of the four PowerPlex® 16 studies conducted have a hypothesized result associated, as compared to previous validation studies performed by Promega and elsewhere (7). Promega recommends 1ng to 2ng as an optimal DNA template amount to be added to each sample with >2ng of male DNA template resulting in higher levels of stutter bands, PCR inhibition, and various artifacts (4). Too little DNA can result in allelic dropout and too much DNA can result in pull-up occurring if the peak height

exceeds 2,000RFU, or 2000 relative fluorescent units. RFUs help the analyst determine the amount of DNA added to the reaction, and the peak height, or RFU is usually directly proportional to the amount of DNA present in the sample. An exception occurs when too much DNA causes PCR inhibition, resulting in smaller peaks or allelic drop out at certain locations. For the routine samples, all samples are expected to have stutter percentages equal to or less than the maximum suggested stutter percentages reported on the Promega web site (7). Peak height ratios for heterozygotes are expected to be greater than 50 percent, and standard deviations are expected to be within margins reported on the Promega site (7). For the non-probative or concordance study, the profiles produced from the PowerPlex® 16 kit are expected to reveal the same alleles at the same locations when compared to results obtained for the same samples using the Profiler Plus™ and COfiler™ kits. Each of these four studies are vital to ensure the performance of the PowerPlex® 16 system is reliable, robust, and reproducible and can be used in forensic casework at Identigene.

CHAPTER II

BACKGROUND

Short Tandem Repeats

STR technology has been a reliable tool in forensic science ever since the middle to late 1990's (8). Short tandem repeat (STR) loci are segments of DNA consisting of identical repeating sequences approximately 3-7 base pairs in length (8). These repeats occur frequently throughout the human genome and are a source of highly polymorphic markers that can be detected through amplification using the polymerase chain reaction (PCR) (8). The flanking regions surrounding the STR sequences must be identified and the appropriate PCR primers designed before PCR amplification can proceed (8). Primers must be selected based upon the buffers, denaturing temperature, and denaturing times concordant with the other primers included in the reaction, as well as elongation and annealing time and temperature based upon the sequence of bases present in the primer. They must also reflect the actual length of sequence to be amplified, especially in multiplex reactions where the fragments are separated and characterized based on size. Once the primers have been designed, PCR can proceed to allow for the amplification of the STR markers in the sample. Following PCR, a technique called florescent detection, a common method of STR analysis, determines the numbers of repeat units present at the

loci, or locations on the DNA where the STRs are found, determining the alleles present at those loci (8).

Various STR multiplex kits have been developed to analyze the thirteen core loci selected in the U.S.A. for forensic testing simultaneously, and all thirteen loci are analyzed for every sample located in the CODIS (Combined DNA Index System) National DNA database. The thirteen core STR loci are all contain tetranucleotide repeat units and include CSF1PO, FGA, THO1, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11. (8) The Profiler Plus™ and COfiler™ kits (Applied Biosystems), when used together amplify the core loci, as do the PowerPlex 1.1™ and PowerPlex 2.1™ kits (Promega Corp) (8). The PowerPlex® 16 and Identifiler® kits also amplify the core 13 loci, but each includes two additional loci to further increase discrimination power. PowerPlex® 16 kits in particular add to the CODIS loci two low stutter pentanucleotide repeat loci, Penta D and Penta E (4).

The PowerPlex® 16 System by Promega offers a multiplex system that allows co-amplification and three-color detection for sixteen loci including the sex indicator Amelogenin (Figure 1). Each primer is labeled with a different fluorescent dye.

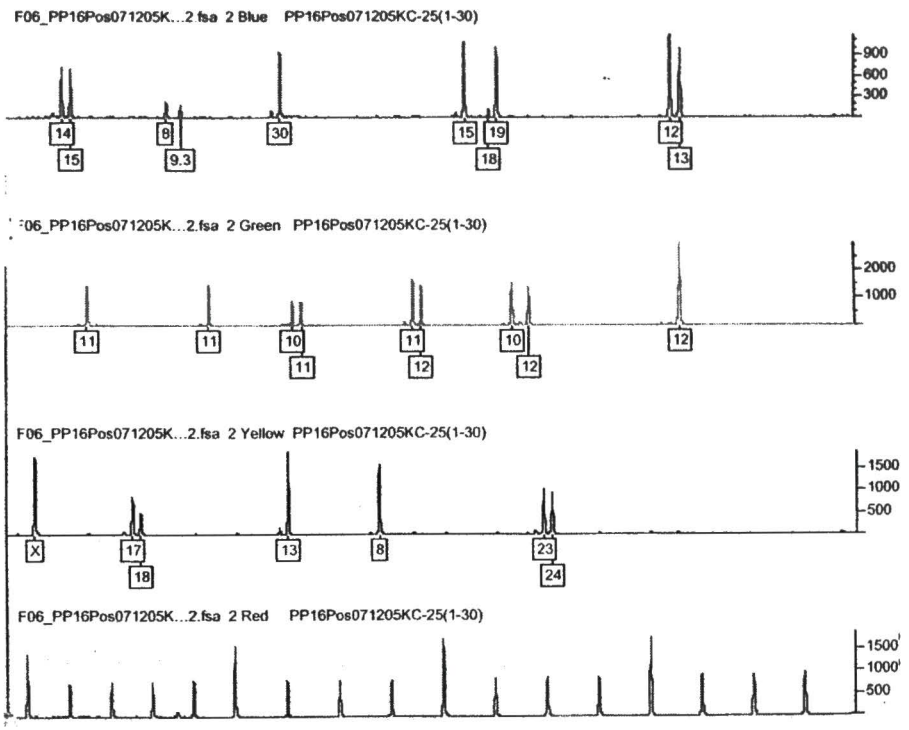

Figure 1: PowerPlex 16 STR Profile

The loci D3S1358, TH01, D21S11, D18S51 and Penta E are labeled with fluorescein, indicated by a blue dye (4). The loci D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D are labeled with JOE, or 6-carboyx-4', 5'-dicholor-2', 7'-dimethoxy-fluroescein, indicated by a green dye (4). The loci Amelogenin, vWA, D8S1179, TPOX and FGA are labeled with TMR, or carboxy-tetra-methylrhodamine, and are indicated by a yellow dye (4). The internal lane standard is indicated by a red dye, and is abbreviated ILS 600 (4). The use of the dye labels allows alleles present at loci with similar base pair sizes to be distinguished from one another. For example, the loci D3S1358, vWA, and D5S818 all share alleles with similar base pair sizes. Electrophoresis separates the DNA fragments by size, meaning that electrophoresis alone will not separate these loci. Dyes

must be added so that a software program can distinguish loci with fragments of similar length.

All sixteen loci including Amelogenin can be simultaneously amplified in a single tube and analyzed in single injection. An allelic ladder has been created in order to designate alleles through the software GeneScan® using the PowerTyper™ 16 Macro (Figure 2). In order for this new kit to be used at Identigene on the ABI 3100 Genetic Analyzer the PowerPlex® 16 System must be validated.

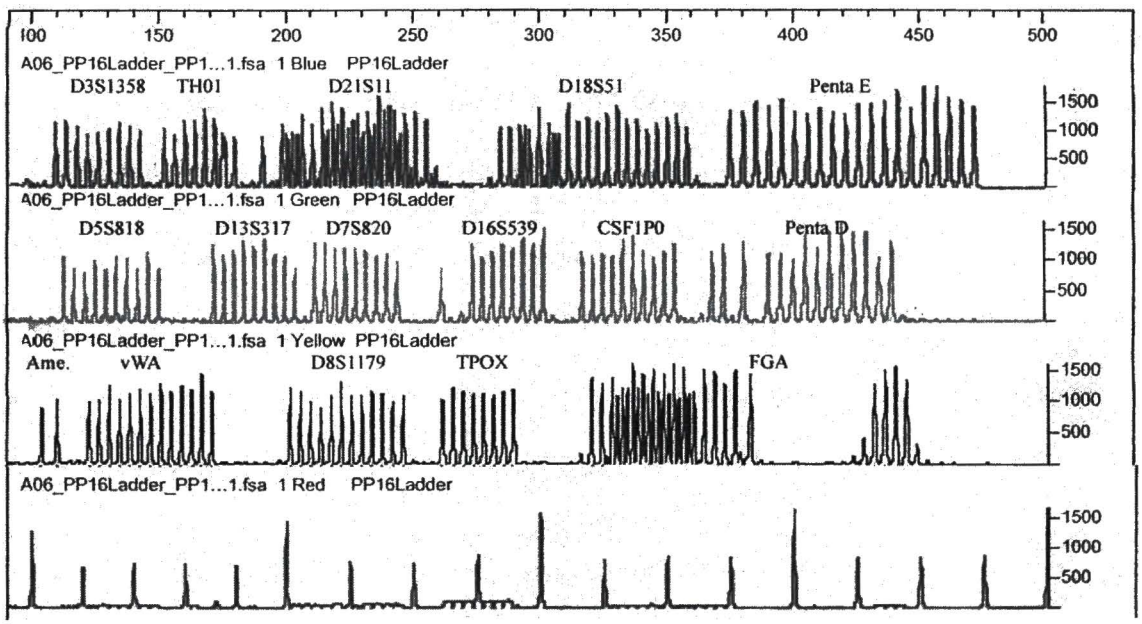

Figure 2: PowerPlex® 16 System Allelic Ladder

Validation Studies

Validation studies are important in the forensic field because they evaluate and document a laboratory's ability to perform a technique using the methods and equipment available in the lab. Currently, the forensic community is rarely challenged on the science behind DNA typing, but rather the public and the court system challenge the procedures used in a laboratory, and the talent of the technicians and analysts performing the casework. Validation studies allow a new technique or the use of an old technique on a new instrument to be evaluated in a variety of studies to ensure their performances are acceptable and reliable.

There are two types of validation studies, developmental and internal. A developmental validation study consists of the validation of a new technique, such as new primer sets for multiplex reactions, PCR cycle conditions, and new loci. Commercial manufacturers and large laboratories usually conduct this type of validation study prior to a product being released. The second type of validation study is an internal validation study, usually conducted by smaller local, independent, and state laboratories. An internal validation study addresses the performance of the technique in each lab on its equipment and is based on the developmental validation usually carried out by the manufacturers (8).

In order to make validation a standard in the forensic field, TWGDAM developed guidelines to be followed for validations in 1995 (10). These guidelines were revised in 2003 under SWGDAM. The guidelines state that reproducibility, mixture, population, environmental, and matrix studies should be conducted (10). Non-probative evidence

must be used. Non-probative evidence consists of old proficiency samples and samples from forensic cases that have been adjudicated. This demonstrates that the techniques can handle actual casework evidence situations (10). If applicable, non-human studies may be performed in order to show the technique is human specific. Mixture studies are performed to evaluate the ability of the technique to detect a mixture. Finally stutter percentages and peak height ratios are determined so that interpretation guidelines can be developed for laboratories to follow (10).

The validation study conducted for the PowerPlex® 16 System is an internal validation study. The Promega Corporation, the developers of the PowerPlex® 16 System, has already performed the developmental study for which this study is based (4, 7). In this internal validation study sensitivity, mixture, routine, and non-probative casework studies are performed. In addition, stutter percentages, standard deviation of base pair sizes and peak height ratios will be analyzed and recorded to ensure the system is working properly on the ABI 3100 Avant in the Forensics Laboratory at Identigene.

DNA Quantification

DNA Quantification is an important aspect of forensic DNA methodology due to its ability to establish the amount of DNA present in a sample in order to make appropriate dilutions for further analysis. It is important to establish the amount of human DNA present in a sample in order to obtain optimal results and minimize artifacts that can interfere with the interpretation of the DNA test. In regards to quantification, the DNA Advisory Board (DAB) established standard 9.3 that states, "The laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample

where possible" (10). The technique commonly used to quantify DNA in the field of forensic science is Quantiblot™, but this method is lengthy, qualitative, and not completely reliable. A new more reliable technique has been developed and is currently in use by Identigene for quantification purposes, and is called Real-Time Polymerase Chain Reaction using the Quantifiler™ kit on an ABI PRISM® 7000 Sequence Detection System, and is an automated quantification process performed in real time (1, 11, 12, 13). The Quantifiler™ kit was used to quantify all the samples used in this study.

Amplification

Amplification will be performed with the Polymerase Chain Reaction (PCR) on the Perkin-Elmer GeneAmp® PCR System 9600 and 9700 Thermal Cyclers. PCR is an enzymatic process that replicates specific regions of the DNA yielding multiple copies of particular DNA sequences (8). The PowerPlex® 16 System contains Gold Star 10X buffer, PowerPlex 16 10X Primer Pair Mix, and cell line 9947A control DNA. (4) AmpliTaq Gold DNA polymerase must be supplied by the user. The primer mix contains primers that amplify target regions on the DNA to help produce multiple copies of specific DNA regions so the sample can be analyzed. (Table 1)

Table 1: The PowerPlex® 16 System Locus-Specific Information

STR Locus	Label	Chromosomal Location	GenBank® Locus and Locus Definition	Repeat Sequence[1] 5′→3′
Penta E	FL	15q	NA	AAAGA
D18S51	FL	18q21.3	HUMUT574	AGAA (21)
D21S11	FL	21q11–21q21	HUMD21LOC	TCTA Complex (21)
TH01	FL	11p15.5	HUMTH01, Human tyrosine hydroxylase gene	AATG (21)
D3S1358	FL	3p	NA	TCTA Complex
FGA	TMR	4q28	HUMFIBRA, Human fibrinogen alpha chain gene	TTTC Complex (21)
TPOX	TMR	2p23–2pter	HUMTPOX, Human thyroid peroxidase gene	AATG
D8S1179	TMR	8q	NA	TCTA Complex (21)
vWA	TMR	12p12–pter	HUMVWFA31, Human von Willebrand factor gene	TCTA Complex (21)
Amelogenin	TMR	Xp22.1–22.3 and Y	HUMAMEL, Human Y chromosomal gene for Amelogenin-like protein	NA
Penta D	JOE	21q	NA	AAAGA
CSF1PO	JOE	5q33.3–34	HUMCSF1PO, Human c-fms proto-oncogene for CSF-1 receptor gene	AGAT
D16S539	JOE	16q24–qter	NA	GATA
D7S820	JOE	7q11.21–22	NA	GATA
D13S317	JOE	13q22–q31	NA	TATC
D5S818	JOE	5q23.3–32	NA	AGAT

[1]The August 1997 report (22,23) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5′ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used."

TMR = carboxy-tetramethylrhodamine

FL = fluorescein

JOE = 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein

NA = not applicable

In a reaction tube the following reagents were added: Gold Star 10X buffer, Primer Pair Mix, AmpliTaq Gold® DNA polymerase, and template DNA. The tubes were then placed on the Perkin-Elmer GeneAmp® PCR System 9600 or 9700 Thermal Cycler and the recommended cycling protocol used. For Study 1, the 9700 Thermal Cycler was used, and for Studies 2-4, the 9600 was used.

ABI Prism® 3100 Avant Genetic Analyzer System

The ABI Prism® 3100 Avant Genetic Analyzer System is a multi-capillary electrophoresis system with the ability to analyze 4 samples simultaneously on a 96 well plate. Using capillary electrophoresis to analyze DNA offers many advantages. For

example, the use of a narrow capillary gives more efficient heat dissipation versus a slab gel, capillaries allow the use of a higher voltage during loading, and this all means faster run times (8). The ABI Prism® 3100 Avant Genetic Analyzer System uses an electrokinetic injection loading the samples into a capillary, at which time 4 samples are injected simultaneously in 10 seconds. The 3100 Avant system differs from the regular 3100 in than it contains fewer capillaries in its array. The 3100 Avant array has four capillaries while the regular 3100 has sixteen. The 3100 Avant is cost efficient, but the 16 capillary 3100 gives higher throughput at a higher cost. Despite the type of instrument used, one of the greatest advantages of the 3100 in general is the near elimination of manual operation over its predecessor, the ABI 310, which of course leads to increased run-to-run consistency and reliability. The ABI 3100 Avant requires less cleanup, has an easier set up, has a more efficient polymer delivery system, and has more automated computer collection software set up than the ABI 310.

The ABI Prism® 3100 Avant Genetic Analyzer System is based on capillary electrophoresis (CE) consisting of an array of four narrow capillaries, two buffer vials, and two electrodes connected to a high-voltage power supply (8). The CE system includes a laser excitation source, a fluorescent detector, and a computer to track the location of each sample, control the sample injection, and record the results (8). As voltage is applied and the sample is injected, the negative charge present on the DNA causes the voltage to pull the sample through the capillary at a speed based upon size. A polymer, or a performance optimized gel medium is present in the capillary allowing the DNA to separate based on size. Bigger fragments of DNA move slower through the

polymer, while smaller fragments move quicker. A window is present at some point in the capillary, exposing the material flowing through to a laser excitation source (8). This excites the dyes attached to the DNA fragments during PCR causing the electrons in the dyes to jump an energy level. As the electrons fall back to their normal levels, they emit a fluorescence that is read by a fluorescent detector (8). The amount of DNA present in the sample containing a particular dye has a relative fluorescent intensity, and produces a peak of relative size on the electropherogram, or the readout generated by the software during the run. This readout produces a peak of the relative wavelength of the dye attached at the precise location at which it crossed the window in the capillary. The dye color combined with the peak location allows the software to determine the exact size or ultimately the number of short tandem repeats that the particular fragment of DNA possessed (8). The internal lane standard 600 (ILS 600) contains 22 DNA fragments with different base lengths, allowing for a standard to accurately size base pairs. Each of the ILS fragments are labeled with a fluorescent dye carboxy-X-rhodamine (CXR) which is detected separately as a fourth color in the amplified material, increasing the precision of the analysis when using the PowerPlex® 16 System (4).

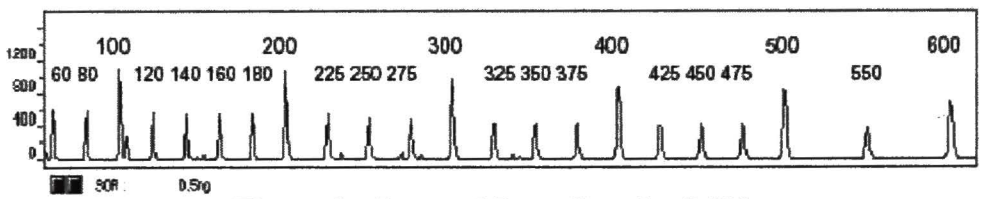

Figure 3: Internal Lane Standard 600

Analysis Software

The Genescan® Analysis Software is a program that automatically identifies and sizes each peak relative to an internal lane standard such as ILS 600 found in the PowerPlex® 16 System. The software also provides data involving peak area and peak height information. The results from GeneScan® can then be saved and imported into the Genotyper® software, for the second phase of analysis (14). Genotyper® compares a sample's genotype to a standard allelic ladder (8). The allelic ladder contains a peak for every common and uncommon allele in the population, as well as a great majority of the rare alleles. The difference between rare alleles and uncommon alleles are simply their occurrence in the population. If an allele is seen at very low frequency or close to 0% in a population, that allele may not be present in the allelic ladder depending on the manufacturer's decision. The Genotyper® software compares the base pair size of the peak read by Genescan® to the closest base pair size observed in the allelic ladder for that particular dye and assigns an allele call to that peak in the sample. This happens at all sixteen loci plus amelogenin, and in turn a DNA profile is developed based upon 32 allele calls, two per loci, and a sex call determination via the amelogenin sex indicator. For the PowerPlex® 16 System the samples have to be imported into Genotyper® and analyzed using the PowerTyper™ 16 Macro provided by Promega in order to designate alleles by comparison to the allelic ladder (4) (Figure 2). An analyst can then examine the peaks, create an allele table, and export it into a spreadsheet program for further analysis and comparison to other samples.

Spectral Evaluation

A minor yet important portion of this study involves evaluating the matrix, or the spectral set to be used on the ABI 3100 for PowerPlex® 16 validation. Identigene will use the 3100-Custom kit for matrix evaluation. A matrix is important for helping the software remove the emission overlap created by the dyes (5). The dyes have spectra that overlap one another, and if a matrix is not properly set up, raised baseline or an artifact known as "pull up" can occur (5). For example, the detection of a blue peak may cause pull up in the green and possibly the yellow dye if the matrix is not properly set up, artificially forming peaks in those yellow and green dyes that may be called by the software but are actually false alleles. A matrix is simply a computer algorithm that corrects this overlap, ensuring that "pull up" remains as minimal as possible and baseline remains stable (5). On the ABI 3100, multiple capillaries exist, and the dyes for the matrix must be tested on each capillary (5). For the ABI 3100 Avant 4-capillary instrument currently used by Identigene, at least 3 of the 4 capillaries must pass spectral calibration. If one fails, it will be automatically assigned the spectral data from the nearest passing capillary. (5). In order to pass, the Q value must be above 0.95 for each capillary and the C value, or condition bounds range, must fall in the range specified in the run protocol. These values are determined empirically and provide the software with information relating to the amount of spectral overlap and tolerance for pull-up and pull-down between the dyes in the set. Once this is complete, and all capillaries have spectral data assigned, the matrix algorithm is created which can then be applied to all further runs involving the PowerPlex® 16 kit.

Takayama Test

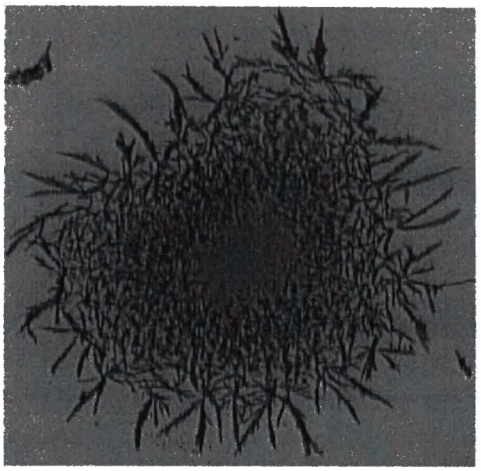

Figure 4: Takayama Crystal

The Takayama blood test is an older test developed to confirm the presence of the heme found in blood. Newer tests are available that are better indicators for the presence of blood. Still, clients may request only a certain type of test, and it is in these cases where the test must be validated in order to perform the test in the laboratory on casework and satisfy the client. The test relies on hemochromagens, or the ferroprotoporphyrin in where the valances of the hexacoordinate heme complex are occupied by nitrogenous bases like pyridine (9). Hemochromagen crystals are prepared at acidic or alkaline pH by various procedures (9). When performing this test, a portion of the stain suspected to contain blood is placed on a microscope slide. Two drops of Takayama reagent are then added. When placed under a microscope for analysis, two types of pink crystals will form after a few minutes if blood is presumptively present (9). One type of crystal appears to be a salmon pink shard, and the other crystal is similar to a dark red branching tree. An advantage of this kit over other crystalline detection methods includes the ability to detect the presence of blood on leather or wood surfaces (9). It is important to note that older blood samples may not completely form these crystals, or different crystals may form or take longer to form, so a negative result does not necessarily mean that blood is not present.

CHAPTER III

PROJECT DESIGN

The purpose of this project is to determine the performance of the PowerPlex® 16 System by running the following studies: sensitivity, routine, mixture, and non-probative sample studies. For the Takayama test, the purpose of the research is to determine the performance of the heme detection test by running the following studies: DTT, sensitivity, substrate, and environmental.

PowerPlex® 16 System

STUDY 1: Sensitivity

The objective of this study is to determine the optimal amount of DNA to be added to the Polymerase Chain Reaction, and determines the PowerPlex® 16 System's ability to generate results from blood and buccal swabs extracted both organically and using the chelex method. This study consists of four serial dilutions of known concentrations of DNA (4ng, 2ng, 1ng, 250pg, and 35pg. If too much DNA is added to a sample, artifacts could appear such as split peaks, stutter, pull up, and allelic drop in, or the sample could be inhibited during PCR and produce smaller peaks and allelic dropout. If too little DNA is added allelic drop out could occur and a complete profile will not be

obtained. Therefore the optimal input amount of DNA must be determined to enable the greatest opportunity for a complete profile to be obtained for each sample tested.

STUDY 2: Mixture

A mixture study was performed consisting of two unrelated sources, one male and one female. The purpose of this study is to test the sensitivity and overall ability of the PowerPlex® 16 System to detect mixtures at different ratios. To achieve a full view of the system's ability, the ratios generated involve both male and female DNA altering as the major and minor profile. The total amount of DNA added to each sample is 1ng. The dilutions were as follows: 1:30, 1:10, 1:3, 1:1, 3:1, 10:1, 30:1. In the 1:30 and 30:1 dilutions, 2ng/20ul of the major profile and 0.067ng/0.67ul of the minor profile was added. For the 1:10 and 10:1 dilutions, 2ng/20ul of the major profile and 0.2ng/2ul of the minor profile was added. For the 3:1 and 1:3, 1.5ng/15ul was added for the major, and 0.5ng/5ul was added for the minor. The 1:1 was simply 1ng/10ul for each contributor. Twenty microliters containing 2ng of DNA was prepared so the experiment could be run in duplicate. This study will gauge the sensitivity of PowerPlex® 16 in identifying mixtures.

STUDY 3: Routine Samples Study

The objective of the substrate study focuses on the ability of the PowerPlex® 16 system to produce accurate and reliable results on a variety of single source buccal and blood samples extracted with both organic and Chelex methods. The results from study 1

directly affect the input DNA of the samples that were used in this study. Originally, 15 Chelex and 15 organically extracted single source samples were intended, but if for a reason either type of extraction method fails to perform appropriately during the sensitivity study, the method will be replaced by additional samples extracted using the alternate method. From this study peak height ratios and stutter percentages will be reported individually and across an average of all samples in order to determine the ability of the PowerPlex® 16 system to obtain accurate and precise results across a routine amount of casework.

STUDY 4: Non-Probative Casework

The objective of the non-probative casework study is to compare the profiles generated from the samples used in studies 1 and 3 to the profiles originally generated from those cases using kits already validated in the lab. Additional artifacts and any differences in general between the kits will be noted. This study will help reinforce the ability of the PowerPlex® 16 system to compete with kits previously validated by the laboratory. Eight samples over four cases were used, two from each case. The results were then compared to Profiler Plus™ and COfiler™ data previously generated from those samples.

Additional Study: Promega Custom 3100 Matrix Evaluation

Two different spectrals were ran and used separately to ensure that pull up is minimal and baselines are low. The first spectral was ran with the first study of

sensitivity, while the second matrix was used on the re-injection of the first study as well as all subsequent studies. Both matrices passed on all four capillaries, and had to have passed on at least 3 of 4 capillaries in order to proceed with testing.

Additional Study: Size Precision Study on Allelic Ladder

Twelve ladder injections were also made in order to determine base pair size precision of the ladders. From this study standard deviations of base pair size from the repeated ladder injections will all be reported in order to determine the ability of the PowerPlex® 16 system's allelic ladder to call alleles effectively to the nearest base pair.

Takayama Test

STUDY 1: DTT

The objective of the DTT study is to compare the effectiveness of DTT in accelerating the Takayama reaction process. Four different reagents were mixed, each containing a different level of DTT, or dithiothreitol. DTT is a reducing agent, and should serve to occupy the available oxygen in the reaction, speeding up the process by allowing pyridine to bind heme freely (6). Different concentrations of DTT (200ul, 800ul, 1.4ml) as well as a reagent containing no DTT were tested on liquid blood in triplicate and the reagent that performed most effectively was subsequently used in the further experiments.

STUDY 2: Environmental

The objective of this study is to test the effectiveness of the Takayama reagent in achieving a positive reaction for heme when used on blood stored for one week in a variety of conditions. This study tested the ability of the procedure to identify blood left in various wet and dry, hot and cold, and dark and light environments. Blood was stained on sample collection cards and left for one week in the following environments: dark/wet/56°C, dark/wet/-20°C, dark/wet/RoomTemp, light/wet/RoomTemp, dark/dry/56°C, dark/dry/-20°C, dark/dry/RoomTemp, and light/dry/RoomTemp. The hot and cold temperatures do not have internal lighting to simulate light environment, so temperature cannot be varied in light conditions.

STUDY 3: Dilution

The objective of this study is to test the effectiveness of the Takayama reagent in identifying the heme found in liquid blood diluted to various concentrations. Liquid blood was diluted in distilled water to the following concentrations and the Takayama Test performed on each in triplicate: 1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000. The time to crystallize was examined, as well as the ability to crystallize the heme found in the sample.

STUDY 4: Substrate

The objective of the substrate test is to evaluate the effectiveness of the Takayama Test to detect heme recovered from blood stains on several different types of substrate.

Blood was dried on cotton tipped applicators, cloth, and blood stain cards and then each was placed in a microcentrifuge tube containing distilled water for fifteen to thirty minutes. After this time the liquid present in the tube was placed on a microscope slide and tested using the Takayama reagent. Time and ability to crystallize were both examined.

CHAPTER IV

MATERIALS AND METHODS

All reagents and instrumentation including the PowerPlex® 16 kit were provided by Identigene located at Houston, Texas. All procedures were performed in the Forensic Laboratory or Forensic Administration area (Extraction, Quantification, Polymerase Chain Reaction, and Analysis) of Identigene. Extraction was performed by qualified analysts employed at Identigene previous to my employment. Quantification was performed by technician Miranda Bussey of Identigene at my request. Technicians Miranda Bussey and Alex Berrios of Identigene assisted me in the loading step of the ABI 3100 Avant.

DNA SAMPLES

Buccal swabs were taken from unrelated male and female volunteers to be used for the sensitivity, routine sample study, non-probative casework, and mixture studies. In addition blood samples taken from proficiency tests were used for the sensitivity, non-probative, mixture, and routine studies as well as all four Takayama studies.

EXTRACTION

Biological samples like blood, tissue, saliva, or semen contain DNA but also contain cellular material and proteins that can affect DNA results in various ways, such as inhibiting the PCR process. Therefore an extraction must be performed in order to separate the cellular material and protein from the DNA molecules, isolating the DNA in a buffer so amplification can proceed unchallenged. Two types of extraction methods were used in this study. Organic Extraction, sometimes referred to as Phenol-Chloroform extraction was performed as well as the Chelex® 100 method of extraction, both of which are commonly used in the forensic field as well as at Identigene. For reference extractions, or pristine known samples received from individuals thought to be associated with the case, Identigene performs the Chelex extraction. This reaction is faster than the Organic reaction, yet offers a greater risk of PCR inhibition, especially with blood stains, since the reaction doesn't fully filter out heme, a PCR inhibitor. The substrate is also left in the final tube containing DNA. However, this technique has been validated at Identigene for the Profiler Plus™ and COfiler™ kits to produce full DNA profiles with little inhibition when used on pristine samples. For evidence samples, or biological evidence that usually is not in pristine condition, Identigene performs Organic Extraction, choosing at the end to micro-concentrate the sample instead of precipitation by ethanol, both of which can be performed to isolate DNA effectively. What follows are procedures based on the Identigene standard operating protocols.

Organic Extraction

Note: Based on Identigene's Organic Blood and Tissue standard operating protocol, April 2004. The following steps exclude the quality control, labeling, and paperwork procedures and focus only on the extraction process.

1) Add a fixed portion of the stain or liquid to the appropriately labeled tube.

2) Add 500µl Digest Buffer and 15.0µl of Proteinase K. Vortex to mix.

3) Incubate samples at 56°C for 2-24 hours, at least two hours for reference samples, and four hours to overnight for evidence samples.

4) After digestion, centrifuge tubes at 3000RPM for 30 seconds.

5) If substrate is present, remove it and place it in a Spin-X™ basket over the original extraction tube, spin for 1 minute at 11,000RPM.

6) Remove Spin-X™ basket and discard both substrate and basket.

7) Add 500µl of Phenol-Chloroform-Isoamyl Alcohol.

8) Vortex samples for 15 seconds, centrifuge at 11,000RPM at Room Temperature for five minutes

9) Transfer aqueous layer to a fresh Microcon YM-100 unit.

10) Centrifuge the Microcon YM-100 units for 30 minutes at 2,000 rpm. Discard the flow-through solution by removing the filter unit with a clean kimwipe, pouring out the flow-through, and then replacing the filter unit.

11) Add 500 uL TE Buffer to each Microcon YM-100 unit.

12) Centrifuge 20 minutes at 2,000 rpm. Discard the flow-through solution as described in step 10.

13) Repeat steps 11-12 TWO more times. On the final spin, centrifuge 15-20 minutes or until the retentate volume is less than 100 uL.

14) Turn the filter unit upside down onto a freshly labeled Microcon tube. Spin for 1 minute at 3,000 rpm to collect the retentate (purified DNA).

15) Most retentate volumes are 10 – 75 uL. The optimal amount is 40ul. If the retentate volume is less than 30 uL, add enough TE Buffer to the filter unit to

make a final volume of 30 uL, turn filter unit upside down in the Microcon tube, and spin 1 min at 3,000. The total liquid collected from both spins must be at least 30 uL.

16) Label a new microtube with a preprinted label and transfer the retentate to this tube.

17) Store refrigerated (if sample will be used the next day) or frozen (-20 degrees C) for long term storage.

Chelex Extraction

Note: Based on Identigene's Chelex Blood and Tissue standard operating protocol, March 2004. The following steps exclude the quality control, labeling, and paperwork procedures and focus only on the extraction process.

1) Add a fixed portion of the stain or liquid to the appropriately labeled tube.

2) Add 1ml of TE Buffer to each tube.

3) Incubate at room temperature for 15-30 minutes. Mix by inversion or gentle vortex.

4) Centrifuge samples for 3 minutes at 14,000RPM.

5) Remove and discard all but 20 uL of the supernatant. Leave the fabric substrate in the tube with the pellet if present.

6) Add enough 5% Chelex to each sample to get a final volume of 200uL. Vortex at high speed for 5 seconds.

7) Incubate samples at 56°C for at least 15 minutes or overnight.

8) Vortex at high speed for 5-10 seconds.

9) Centrifuge at 14,000 rpm for 30 seconds.

10) Incubate at 100°C for 8 minutes (+/- 30 seconds)

11) Vortex at high speed for 5-10 seconds.

12) Add 300 uL TE Buffer to a freshly labeled Microcon YM-100 unit.

13) Transfer Chelex Solution supernatant (approximately 180 uL) to the fresh Microcon YM-100 unit for each sample.

14) Centrifuge the Microcon YM-100 units for 30 minutes at 2,000 rpm. Discard flow-through solution. Note: align the cap strap toward the center of the rotor.

15) Add TE Buffer to each Microcon YM-100 unit, to a final volume of 500 uL.

16) Centrifuge for 20 minutes at 2,000 rpm. Discard flow-through solution.

17) Add TE Buffer to each Microcon YM-100 unit, to a final volume of 500 uL.

18) Centrifuge for 20 minutes at 2,000 rpm, or until the retentate volume is less than approximately 100 uL.

19) Turn the filter unit upside down in the fresh labeled 1.5 mL Microcon tube. Spin for 1 minute at 3,000 rpm to collect retentate (purified DNA).

20) Typical retentate volumes are 10 – 75 uL. If the retentate volume is less than 30 uL, then add TE Buffer to a final volume of 30 uL. Store refrigerated (if sample will be used the next day) or frozen (-20 degrees C) for long term storage.

QUANTIFICATION

Quantification is an important aspect of scientific methodology due to its ability to establish the amount of DNA present in a sample, in order to make the appropriate dilutions and achieve the best results with little to no unwanted artifacts. The Real-time Polymerase Chain Reaction technique was used to quantify the DNA in this validation study through the use of the Quantifiler™ Human DNA Quantification Kit by Applied Biosystems.

Note: Based on Identigene's Quantifiler standard operating protocol, May 2005. The following steps exclude the quality control, worksheet set up, labeling, and paperwork procedures and focus only on the quantification process and software setup.

Materials

- AB Quantifiler Human DNA Quantification Kit
- AB 96-Well Optical Reaction Plates, AB Optical Adhesive Covers, and AB MicroAmp Splash Free Support Base **or** AB Optical Tubes (8 tubes/strip, 125 strips), AB Optical Caps (8 caps/strip), and AB MicroAmp 96-Well Tray/Retainer Set
- AB Compression pad from Optical Adhesive Covers
- Pipettors and pipette tips
- Tabletop centrifuge with 96-well plate rotor
- AB Prism 7000 Sequence Detection System

Reagents

- AB Quantifiler Human DNA Quantification Kit
- Quantifiler Human DNA Standard dilution series (dilutions must be made within one week of use)
- TE Buffer

DNA Quantification Standard

Table 2: DNA Quantification Standards dilution series

Standards	Concentration (ng/µl)
Standard 1	50.00
Standard 2	16.70
Standard 3	5.560
Standard 4	1.850
Standard 5	0.620
Standard 6	0.210
Standard 7	0.068
Standard 8	0.023

Preparation of Dilution Standards

1) Label eight 1.5 mL polypropylene tubes 1 through 8.

2) Vortex DNA Standard to mix thoroughly.

3) Add 30 uL TE Buffer to the tube labeled 1 and 20 uL TE Buffer to tubes 2 thru 8.

4) Add 10 ul of DNA Standard to tube 1.

5) Add 10 ul of 1 to tube 2 , vortex and pulse spin tube 2; add 10 ul of tube 2 to tube 3, vortex and pulse spin.

6) Continue the serial dilution through tube 8.

Sample Preparation

1) To prepare the reaction mix, use the following calculation. Add 5.5uL Primer Mix and 6.5uL PCR Reaction Mix to each sample to give a total mix volume of 12uL per sample. Include approximately 5% additional reactions to provide excess volume for the loss that occurs during reagent transfer.

2) Thaw the primer mix completely, vortex 3 to 5 seconds and centrifuge briefly before opening the tube.

3) Pipette the required volumes of components into an appropriately sized polypropylene tube.

4) Vortex the PCR mix 3 to 5 seconds, the centrifuge briefly. Dispense 12μL into each well.

5) Add 1μL of sample, standard, or control to the appropriate wells of reaction plate. The 8 standard curve samples are run in duplicate.

6) Seal reaction plate with the Optical Adhesive Cover, or the MicroAmp 8caps/strips.

7) Centrifuge the plate at 3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.

8) Place the compression pad over the Optical Adhesive Cover with the gray side down and the brown side up and with the holes positioned directly over the reaction wells.

9) Place in the ABI Prism® 7000 Sequence Detection System and close the door.

Software Setup

1) Power on the ABI 7000 machine, computer, and software.

2) Select start >Programs>ABI Prism 7000>ABI Prism 7000 SDS software. In the SDS software, select File>New to open the New Document dialog box.

3) Select the following settings for Quantifiler Kit.

Assay:	Absolute Quantitation
Container:	96-Well Clear
Template:	Blank Document

4) Click Okay.

5) Open the pre-prepared Quantifiler worksheet.

6) Set thermal cycler conditions by selecting the plate document and next selecting the Instrument tab.

7) Press the Shift key and click within stage 1 hold step (50°C for 2 minutes) to select it. After the hold step is selected, press the Delete Key.

8) Change the Sample Volume to 13 (µL) and make sure that the 9600 Emulation box is selected.

9) Before running the reaction plate, save the plate document. Select File>Save>Enter a file name>select SDS Document (*.sds). Click Save.

10) Click Start to rotate the instrument tray to then IN position and start the run.

STR Amplification

Amplification will be performed by Polymerase Chain Reaction (PCR) on both the Perkin-Elmer GeneAmp® PCR System 9600 and 9700 Thermal Cyclers using the PowerPlex® 16 System. PCR is an enzymatic process that replicates specific regions on the DNA yielding multiple copies of particular DNA sequences.

Note: Identigene does not have a protocol for amplification using the PowerPlex® 16 System, but a draft has been created to be used in the future. The following is based upon this draft.

1) Determine the number of samples to be amplified

2) Determine the appropriate values as follows by entering the total number of PCR amplifications plus 2

   a. Nuclease Free water: 9.2µl X _____ = _____µl

   b. Gold STAR 10X buffer: 2.5µl X _____ = _____µl

   c. 10X Primer Pair Mix: 2.5µl X _____ = _____µl

   d. AmpliTaq Gold: 0.8µl X _____ = _____µl

3) Prepare the master mix and then vortex at medium speed for 5 seconds. Centrifuge at 3000 RPM for 30 seconds.

4) Add 15 µl of master mix to each sample tube and then add 10µl of DNA sample or diluted positive control to the appropriate tube. For the negative control, replace the DNA sample with TE buffer.

5) Thermal Cycler Parameters

For PowerPlex 16 on the TC9700, select the following program:

a.	HOLD	95 degrees C / 11 minutes
b.	HOLD	96 degrees C / 1 minute
c.	CYCLE	10 cycles of:
		- 100% to 94 degrees C / 30 seconds
		- 29% to 60 degrees C / 30 seconds
		- 23% to 70 degrees C / 45 seconds
d.	CYCLE	20 cycles of:
		- 100% to 90 degrees C / 30 seconds
		- 29% to 60 degrees C / 30 seconds
		- 23% to 70 degrees C / 45 seconds
e.	HOLD	60 degrees C / 30 minutes
f.	HOLD	4 degrees C / (forever)

For PowerPlex 16 on the TC9600, select the following program:

a.	HOLD	95 degrees C / 11 minutes

b.	HOLD	96 degrees C / 1 minute
c.	CYCLE	10 cycles of:
		- 94 degrees C / 30 seconds
		- 68 seconds to 60 degrees C / hold 30 seconds
		- 50 seconds to 70 degrees C / hold 45 seconds
d.	CYCLE	20 cycles of:
		- 90 degrees C / 30 seconds
		- 60 seconds to 60 degrees C / hold 30 seconds
		- 50 seconds to 70 degrees C / hold 45 seconds
e.	HOLD	60 degrees C / 30 minutes
f.	HOLD	4 degrees C / (forever)

6) Specify a 25uL reaction volume and after the run is over store the samples at -20°C in the post-PCR room

Analysis

The ABI Prism® 3100 Avant Genetic analyzer is based on capillary electrophoresis using a four capillary system, and is needed to determine the size of DNA fragments by fluorescent emission detection.

Note: Identigene does not have a protocol for capillary electrophoresis using the PowerPlex® 16 System, but a draft has been created to be used in the future. The following is based upon this draft.

Sample Preparation

1) Prepare a loading mixture by combining and mixing the internal line standard and deionized formamide [(1µl ILS600) X (# of injections) + (9µl deionized formamide) X (# injections)]

2) Vortex briefly

3) Pipette 10µl of formamide/internal lane standard mix into each well to be used

4) Add 1µl of amplified sample or allelic ladder

5) Cover wells with septa

6) Centrifuge briefly to ensure that the sample is at the bottom of tube

7) Denature samples just prior to load as follows at 95°C for 3 minutes and then immediately chill on crushed ice for 3 minutes

Instrument Preparation

1) Refer to ABI Prism® Genetic Analyzer User's Manual for instructions on set up of machine

2) In the plate view tab, click Import.

3) Import plate previously saved on share drive.

4) Highlight plate record name under Pending Plate Records and select the Edit option on the Plate View tab.

5) Complete the plate record as follows:
   a. Color Comment: "PP16" and "Ladder" as appropriate.
   b. Run Module: Genescan36_POP4DefaultModule
   c. Analysis Module: select the appropriate analysis parameters
   d. Dye Set "Z"

6) Fill down all columns, click OK.

7) Highlight the plate name to be used under Pending Plate Records.

8) Click the tray in position B on the autosampler. (Tray will change colors to show it is linked.) The plate name will appear under Linked Plate Records.

9) Click the green arrow at top to start run.

Sample Detection

1) Analyze the date through the GeneScan® Analysis software

2) Review the raw data for one or more samples. Highlight a sample file name then under the sample menu select raw data. By using the cursor move to the right of the primer peaks to the first internal lane standard peak (red) in order to determine the start position in the analysis parameters

3) Recommended analysis parameters

   a) Analysis Range Start: defined in step 2, Stop: 10,000

b) Data Processing: Baseline-checked, Multicomponent: checked, and Smooth options: Light[1]
c) Peak Detection: Peak Amplitude Threshold[2] at 75 for all dyes and Min. Peak half width: 2pt
d) Size call range: Min: 60 – Max: 600
e) Size calling method: Local southern method
f) Split Peak Correction: None

4) The above analysis parameters can and should be saved in the "Params" folder.

5) Create a new size standard according to the peaks on the next page and store in the "Size Standard" folder

6) Apply the analysis parameters and size standard to samples, then proceed to analyze the samples, saving after analysis.

Data Analysis

1) Load the PowerTyper™ 16 Macro to a designated location on the computer hard drive

2) Open the Genotyper® software and the PowerTyper™ 16 Macro template

3) Under "file" select "import" to import samples from the GeneScan® Project.

4) For Casework: double-click "Power" macro, and let the program proceed, identifying the alleles in the ladder sample and calculate offsets for all of the loci without filtering.

5) For data basing or paternity: double-click the "Power 20% filter" macro. Note: This can not be used if mixtures could be present.

6) Double-click on the "Display Fluorescein data" macro to display the blue dye and observe all the samples

7) Repeat for TMR data (yellow) and JOE (green)

8) Create the appropriate tables by selecting "PowerTable", "Make allele table" or "Make Vertical Table" macro. Print and save data.

Takayama Protocol

Four protocols for DTT addition were implemented, and four different Takayama reagents formed. A portion of the same fresh blood sample was individually tested three times with each the four reagents. Each Takayama reagent had a different concentration of DTT added. The speed that the crystals formed on the samples was closely measured and the time recorded. The protocols are as follows:

Protocol 1 – No DTT

Saturated Dextrose solution	--- 3ml
10% NaOH	--- 3ml
Pyridine	--- 3ml
Distilled Water	--- 7ml

Protocol 2

Saturated Dextrose solution	--- 3ml
DTT	--- 200uL
10% NaOH	--- 3ml
Pyridine	--- 3ml
Distilled Water	--- 7ml

Protocol 3

Saturated Dextrose solution	--- 3ml
DTT	--- 800uL
10% NaOH	--- 3ml
Pyridine	--- 3ml
Distilled Water	--- 7ml

Protocol 4

Saturated Dextrose solution	--- 3ml
DTT	--- 1.4ml
10% NaOH	--- 3ml
Pyridine	--- 3ml
Distilled Water	--- 7ml

Takayama Procedure

1) For liquid blood, add 5uL sample to a microscope slide.

2) If the sample is contained on a substrate, soak the substrate for 15 to 30 minutes in 300uL distilled water. After the required time, add 5uL of the liquid to a microscope slide.

3) Add 1 to 2 drops Takayama Reagent to the slide, and cover with a cover slip.

4) Place slide under microscope and wait to observe forming crystals. Two types of crystals will appear in a positive reaction. One type appears as a pink single shard, and the other type appears as a dark red branching crystal.

5) If no crystals are observed after 10 minutes, leave the slide and check again after 60 minutes. If no crystals are observed, the reaction is negative for heme.

For the following studies, use one of the four Takayama Reagents above that was found to exhibit the quickest positive reaction.

Dilution Study

Prepare liquid blood dilutions in distilled water in the following concentrations: 1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000. Follow the protocol steps 1-5 listed above in triplicate for each sample and determine the ability of the reagent to crystallize the heme in the sample.

Environmental Study

Prepare blood stains on blood stain cards and place the samples in the following environments for one week: dark/wet/56°C, dark/wet/-20°C, dark/wet/RoomTemp, light/wet/RoomTemp, dark/dry/56°C, dark/dry/-20°C, dark/dry/RoomTemp, and light/dry/RoomTemp. Follow the protocol steps 1-5 above in order to determine the ability of the reagent to crystallize the heme in the samples.

Substrate Study

Prepare blood stains on blood stain cards, sterile cotton swabs, and loose knit cloth, and place the samples in a dark dry room temperature environment overnight to dry. Use protocol steps 1-5 above to determine the ability of the reagent to crystallize the heme in the samples.

CHAPTER V

RESULTS AND DISCUSSION

Sensitivity Study

Purpose—The purpose of the study is to determine the optimal amount of DNA to add to the remaining studies, and also to observe the effects of too little and to much DNA and the observation of allelic dropout. In addition, blood and buccal swabs extracted using both Chelex and organic methods will be compared and evaluated using the PowerPlex 16 System.

Experiment—Using DNA extracted using the Chelex method for both buccal and blood swabs, and DNA extracted using the organic method for both buccal and blood swabs, dilutions of 4ng, 2ng, 1ng, 250pg, and 35pg were diluted, amplified, and ran through electrophoresis to develop a DNA profile.

Table 3: Dilution Series for Study 1

Method	**Source**	**Dilution**
Organic	Blood	4ng, 2ng, 1ng, 250pg, 35pg
Organic	Buccal	4ng, 2ng, 1ng, 250pg, 35pg
Chelex	Blood	4ng, 2ng, 1ng, 250pg, 35pg
Chelex	Buccal	4ng, 2ng, 1ng, 250pg, 35pg

Calculations

1) **Dilutions**--The amount of DNA present in each sample was established through Quantifiler to allow for the known amount to be determined and added to each of the six samples. The appropriate amount of DNA was added to each of the samples, prepared through normal dilution protocol.
2) **Peak height ratio**—determined by the minor peak height divided by the major peak height.
3) **Stutter %**-- determined by the stutter peak height divided by the adjacent peak height one repeat unit to the right and then multiplied by 100 to give a percent.
4) **Standard Deviation of the Mean**—determined by averaging the base pair sizes for all peaks in the dilution series, and then taking the absolute value of the base pair size for the peak in question subtracted from the average or the mean of the base pair sizes.

Allele and Base Pair precision

For the 62037:S147220 sample

(Organically extracted blood)

At D3S1358, alleles 16 and 18 were called for all dilutions except 35pg and base pairs were consistent between the samples. At TH01, alleles 6 and 9.3 were called for all dilutions except for 35pg and the base pairs were consistent between the samples. At D21S11, allele 28 was called for all dilutions except for 35pg and 250pg, where allelic dropout was observed. Allele 32.2 was called for all dilutions except for 35pg and the

base pairs were consistent between the samples. At D18S51, alleles 19 and 20 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At Penta E, allele 10 was called for all dilutions except for 4ng and 35pg and the base pairs were relatively not consistent between all the samples, which can be expected at higher base pair sizes. Only one allele was called and although another peak was present indicating a heterozygote, it fell below the 75RFU threshold in all samples. Stutter was not observed at this locus. At D5S818, alleles 10 and 11 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At D13S317, alleles 8 and 11 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. D7S820 was a homozygote so allele 10 was called for all dilutions except for 35pg and the base pairs were consistent between the samples. At D16S539, alleles 9 and 12 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At CSF1PO, alleles 10 and 12 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At Penta D, allele 9 was called for all dilutions except 35pg, and allele 11 was called for all dilutions except for 4ng and 35pg. Only one allele was called at 4ng, and although another peak was present indicating a heterozygote, it fell below the 75RFU threshold at 4ng. Stutter was not observed at this locus. Base pairs were relatively consistent between all the samples, but almost reached a 0.1 standard deviation at 4ng and 1ng. At amelogenin, X and Y were called for each of the dilutions except for 35pg and the base pairs were consistent between all the samples. For vWA, alleles 16 and 17 were called for all dilutions except for 35pg and the base pairs were exceptionally

consistent between all the samples. Stutter % did not exceed recommended value of 15% for any dilution. Peak height ratios suggest heterozygote for all available dilutions. At D8S1179, alleles 13 and 15 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At TPOX, alleles 8 and 11 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At FGA, alleles 21 and 24 were called for all dilutions except for 35pg and the base pairs were consistent between all the samples. The optimal concentration for organically extracted blood is 1ng of input template DNA. Organically extracted blood performed well under PowerPlex® 16, although the locus Penta E dropped out at even optimal concentrations (1ng).

Table 4: Peak height Ratio (Organically extracted Blood)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	89%	95%	85%	49%	Allelic Dropout
TH01	97%	84%	94%	43%	Allelic Dropout
D21S11	77%	73%	92%	Allelic Dropout	Allelic Dropout
D18S51	68%	95%	87%	72%	Allelic Dropout
Penta E	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
D5S818	75%	83%	74%	95%	Allelic Dropout
D13S317	95%	69%	96%	45%	Allelic Dropout
D7S820	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
D16S539	92%	81%	97%	68%	Allelic Dropout
CSF1PO	83%	78%	85%	42%	Allelic Dropout
Penta D	Allelic Dropout	90%	81%	77%	Allelic Dropout
Amelogenin	78%	92%	83%	62%	Allelic Dropout
vWA	92%	90%	100%	68%	Allelic Dropout
D8S1179	83%	97%	96%	56%	Allelic Dropout
TPOX	89%	77%	71%	62%	Allelic Dropout
FGA	85%	80%	92%	63%	Allelic Dropout

Table 5: Mean Stutter percentage (Organically Extracted Blood)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	25.5%	11.5%	11.5%	16.5%	Allelic Dropout
TH01	1.5%	1.5%	2.5%	0%	Allelic Dropout
D21S11	10%	9.5%	11%	11%	Allelic Dropout
D18S51	14%	16%	13%	12%	Allelic Dropout
Penta E	0%	0%	0%	0%	Allelic Dropout
D5S818	5%	6%	5%	8%	Allelic Dropout
D13S317	2.5%	4.5%	3.5%	7.5%	Allelic Dropout
D7S820	6%	6%	7%	7%	Allelic Dropout
D16S539	6.5%	6%	7%	7.5%	Allelic Dropout
CSF1PO	9.5%	7.5%	8.5%	12.5%	Allelic Dropout
Penta D	0%	0%	0%	0%	Allelic Dropout
Amelogenin	0%	0%	0%	0%	Allelic Dropout
vWA	9%	11%	8%	9%	Allelic Dropout
D8S1179	6.5%	7%	6.5%	11%	Allelic Dropout
TPOX	0%	0%	0%	0%	Allelic Dropout
FGA	11%	10%	10%	10%	Allelic Dropout

Allelic dropout—Allelic dropout occurred in all the 35pg samples. In the 4ng samples, allelic dropout was observed at Penta E and Penta D. In the 2ng and 1ng samples, allelic dropout occurred at Penta E. At 250pg, allelic dropout occurred at D21 and Penta E.

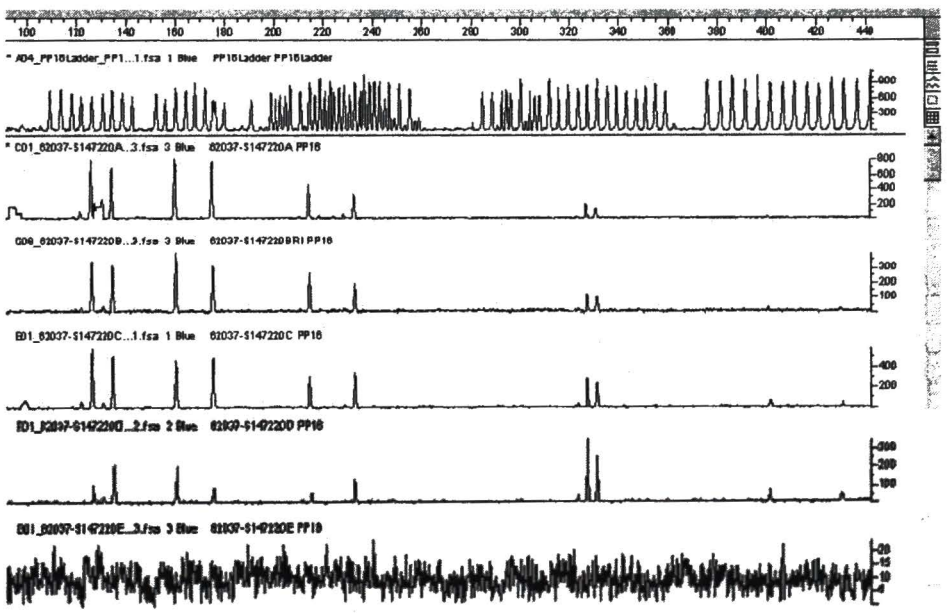

Figure 5: Blue Dye (Organically Extracted Blood)

From top to Bottom: (Ladder, 4ng, 2ng, 1ng, 250pg, 35pg)

Notice the peak height imbalances in the figure on the previous page as the dilution reaches 250pg. Also notice how the peaks at the larger loci seem to disappear as the concentration increases past the 1ng optimal amount to 4ng, and also as the concentration reaches 35pg. At 4ng, a dye blob appears between the two alleles at the first locus, D3. This artifact is not reproducible.

For the 62037:S147223 sample

(Chelex extracted blood)

At the locus D3S1358, homozygotic allele 16 was called for all dilutions except 35pg and base pairs were consistent between the samples. At TH01, allele 7 was called for all dilutions except 250pg and 35pg, and allele 8 was called for all dilutions except for 35pg. At D21S11, alleles 27 and 31 were called for all dilutions except for 250pg and 35pg and the base pairs were consistent between all the samples. For D18S51, allele 16 was called once at 2ng, and dropout was observed at all other dilutions. No other alleles were amplified, and even though two peaks are present, they are not above the 75RFU threshold. At Penta E, allelic dropout occurred at all dilutions across the locus. D5S818 exhibited a homozygotic allele 12 that was called for all dilutions except 35pg. Base pairs were consistent between the samples. At D13S317, allele 11 was called for all dilutions except 35pg, and allele 14 was called for all dilutions except for 250pg and 35pg. For D7S820, alleles 11 and 12 were called for all dilutions except for 4ng, 250pg and 35pg and the base pairs were consistent between all the samples. At D16S539, alleles 10 and 11 were called for all dilutions except for 4ng, 250pg and 35pg and the

base pairs were consistent between all the samples. At CSF1PO, allele 10 was called for all dilutions except for 4ng, 250pg and 35pg, while the 11 allele was only called at 1ng. The base pairs were consistent between all the samples. Penta D showed allelic dropout occurring at all dilutions across the locus. Amelogenin displayed a homozygotic X, and was called for each of the dilutions except for 35pg and the base pairs were consistent between all the samples. Locus vWA exhibited a homozygotic allele 16 and was called for all dilutions except for 35pg and the base pairs were consistent between all the samples. At D8S1179, allele 13 was called for all dilutions except 4ng, 250pg, and 35pg. Allele 15 was called for all dilutions except for 4ng and 35pg and the base pairs were consistent between all the samples. At TPOX and FGA, allelic dropout occurred at all dilutions across the locus. Allelic dropout occurred in abundance for Chelex extracted blood. This could potentially be due to the methodology by which the reaction is performed, allowing additional heme, and the substrate to affect the pure isolation of the DNA.

Table 6: Peak height Ratios (Chelex Extracted Blood)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
TH01	98%	96%	90%	Allelic Dropout	Allelic Dropout
D21S11	59%	81%	92%	Allelic Dropout	Allelic Dropout
D18S51	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
Penta E	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
D5S818	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
D13S317	64%	75%	94%	Allelic Dropout	Allelic Dropout
D7S820	Allelic Dropout	89%	93%	Allelic Dropout	Allelic Dropout
D16S539	Allelic Dropout	66%	51%	Allelic Dropout	Allelic Dropout
CSF1PO	Allelic Dropout	Allelic Dropout	96%	Allelic Dropout	Allelic Dropout
Penta D	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
Amelogenin	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
vWA	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
D8S1179	Allelic Dropout	81%	62%	Allelic Dropout	Allelic Dropout
TPOX	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
FGA	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout

Table 7: Mean Stutter percentages (Chelex Extracted Blood)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	9%	10%	10%	14%	Allelic Dropout
TH01	2%	3%	0%	Allelic Dropout	Allelic Dropout
D21S11	4.5%	8.5%	4.5%	Allelic Dropout	Allelic Dropout
D18S51	Allelic Dropout	0%	Allelic Dropout	Allelic Dropout	Allelic Dropout
Penta E	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
D5S818	8%	10%	9%	11%	Allelic Dropout
D13S317	9.5%	8.5%	9%	0%	Allelic Dropout
D7S820	Allelic Dropout	0%	0%	Allelic Dropout	Allelic Dropout
D16S539	Allelic Dropout	6%	7%	Allelic Dropout	Allelic Dropout
CSF1PO	Allelic Dropout	0%	0%	Allelic Dropout	Allelic Dropout
Penta D	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
Amelogenin	0%	0%	0%	0%	Allelic Dropout
vWA	7%	7%	8%	9%	Allelic Dropout
D8S1179	Allelic Dropout	9%	8%	Allelic Dropout	Allelic Dropout
TPOX	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
FGA	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout

Allelic dropout—Allelic dropout occurred in all the 35pg samples. In the 4ng samples, allelic dropout was observed at Penta E and Penta D, CSF, D18, D7, D16, D8, TPOX, and FGA due to PCR inhibition because too much DNA was added, the chelex reaction itself, and/or heme inhibition. In the 2ng and 1ng samples, allelic dropout occurred at Penta E, Penta D, TPOX, D18, and FGA. At 2ng CSF had an allele drop out. At 250pg, allelic dropout occurred at all loci but D3, D5, Amelogenin, and vWA.

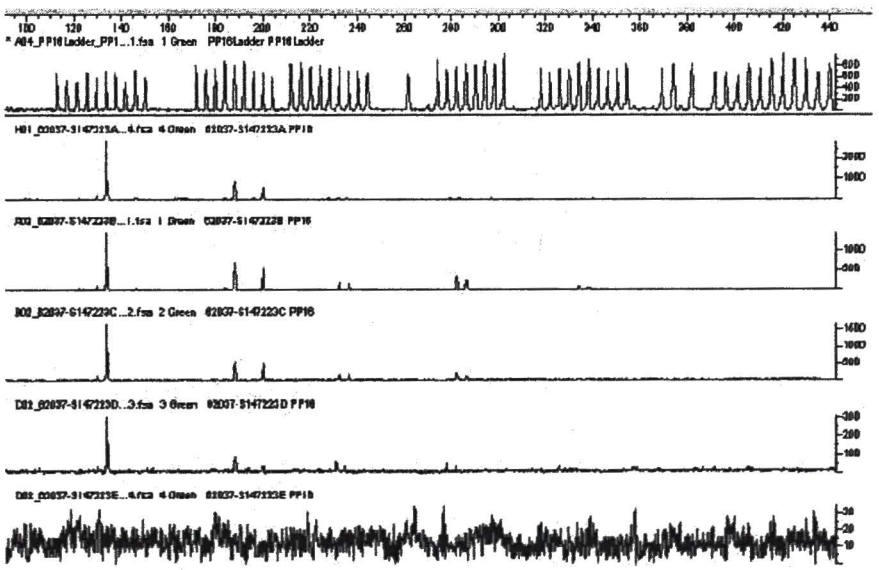

Figure 6: Green Dye (Chelex Extracted Blood)

From top to Bottom: (Ladder, 4ng, 2ng, 1ng, 250pg, 35pg)

Notice the allelic dropout displayed on figure 6 in the previous page. The remaining heme seems to be greatly inhibiting the reaction, in combination with the substrate contained in the final extraction tube. Dropout is widespread across all loci.

For the KC sample

(Chelex extracted buccal swab)

For locus D3S1358, alleles 16 and 17 were called for all dilutions and base pairs were consistent between the samples. At TH01, alleles 9 and 9.3 were called for all dilutions and the base pairs were consistent between the samples. At D21S11, alleles 29 and 30 were called for all dilutions and the base pairs were relatively consistent between the samples. For the locus D18S51, alleles 13 and 17 were called for all dilutions and the base pairs were relatively consistent between all the samples, although the 2ng and 35pg samples had standard deviations nearing 0.1. Penta E did not amplify, because only one allele (11) was called at 250pg. All other alleles dropped out. Stutter was not observed at this locus. At D5S818, alleles 9 and 11 were called for all dilutions and the base pairs were consistent between all the samples. For D13S317, alleles 11 and 12 were called for all dilutions and the base pairs were consistent between all the samples. At D7S820, alleles 8 and 12 were called for all dilutions and the base pairs were consistent between all the samples. At locus D16S539, alleles 11 and 12 were called for all dilutions except the 35pg sample where only the 11 was called. The base pairs were consistent between all the samples. At CSF1PO, alleles 11 and 12 were called for all dilutions and the base

pairs were relatively consistent between all the samples. At Penta D, allele 12 was called for all dilutions except 4ng, and allele 13 was called for all dilutions except for 4ng and 2ng. Stutter was not observed at this locus. Base pairs were inconsistent between all the samples, exceeding a standard deviation of 0.1 in the 2ng and 250pg samples (0.19 and 0.21 respectively). At amelogenin, X and Y were called for each of the dilutions and the base pairs were consistent between all the samples. For locus vWA, homozygotic allele 17 was called for all dilutions and the base pairs were consistent between all the samples. Peak heights were very high across all dilutions (>2000) except for the 35pg. At D8S1179, homozygotic allele 12 was called for all dilutions and the base pairs were consistent between all the samples. At TPOX, allelic dropout occurred at all dilutions across the locus, similar to that of the Chelex extracted blood. At FGA, alleles 21 and 25 were called for all dilutions except for 35pg where the 21 allele dropped out, and at 4ng where the 25 dropped out. The base pairs were consistent between all the samples.

Table 8: Peak height Ratios (Chelex extracted Buccal Swab)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	85%	86%	82%	93%	69%
TH01	93%	97%	98%	88%	100%
D21S11	88%	90%	90%	97%	79%
D18S51	75%	84%	71%	62%	79%
Penta E	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
D5S818	89%	93%	92%	85%	85%
D13S317	86%	83%	89%	90%	69%
D7S820	82%	74%	88%	96%	68%
D16S539	93%	86%	93%	88%	Allelic Dropout
CSF1PO	90%	98%	84%	88%	82%
Penta D	Allelic Dropout	Allelic Dropout	99%	90%	83%
Amelogenin	87%	92%	97%	98%	58%
vWA	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
D8S1179	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
TPOX	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
FGA	Allelic Dropout	87%	77%	72%	Allelic Dropout

Table 9: Mean Stutter percentages (Chelex Extracted Buccal Swabs)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	9%	10%	10%	8%	0%
TH01	4%	5%	5%	0%	0%
D21S11	9%	10%	9%	10%	16%
D18S51	5%	8%	8%	11%	14.5%
Penta E	Allelic Dropout	Allelic Dropout	Allelic Dropout	0%	Allelic Dropout
D5S818	2.5%	3%	3%	5%	7.5%
D13S317	3%	3%	3%	4%	16%
D7S820	0%	7.5%	6%	9.5%	6%
D16S539	8%	7%	7%	6%	29%
CSF1PO	0%	0%	9%	6%	10%
Penta D	Allelic Dropout	0%	0%	0%	0%
Amelogenin	0%	0%	0%	0%	0%
vWA	8%	8%	9%	9%	14%
D8S1179	4%	5%	7%	8%	9%
TPOX	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout	Allelic Dropout
FGA	0%	0%	0%	9.5%	0%

Allelic dropout—Allelic dropout occurred at TPOX and Penta E across all dilutions. In addition to TPOX and Penta E, the following dropouts were observed. In the 4ng samples, allelic dropout was observed at FGA and Penta D. In the 2ng samples, allelic dropout occurred at Penta D. At 35pg, allelic dropout occurred at D16.

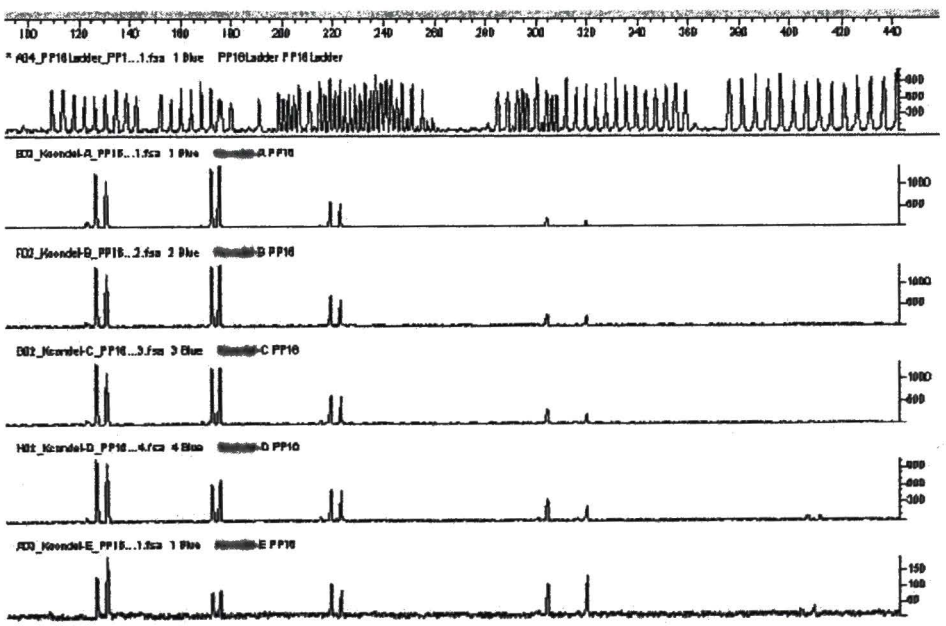

Figure 7: Blue Dye (Chelex Extracted Buccal Swab)

From top to Bottom: (Ladder, 4ng, 2ng, 1ng, 250pg, 35pg)

On figure 7, notice how allelic dropout spans the entire locus at Penta E, and peaks are only observable at the 250pg and 35 pg levels, although they do not exceed threshold. In comparison, look at the second locus vWA and how the peaks are incredibly high. This is a sign of competition between loci for reagents during PCR, causing inhibition, especially at high input template amounts.

For the SD sample

(Organically extracted buccal swab)

At D3S1358, alleles 15 and 16 were called for all dilutions except 35pg where the 15 dropped out. Base pairs were consistent between the samples. For TH01, alleles 7 and 9 were called for all dilutions but 35pg and the base pairs were consistent between the samples. At locus D21S11, alleles 28 and 31.2 were called for all dilutions except 35pg, where the 28 dropped out. Base pairs were consistent between the samples. At D18S51, alleles 12 and 21 were called for all dilutions except 35pg, where the 12 dropped out. The base pairs were exceptionally consistent between all the samples. For Penta E, alleles 5 and 13 were called for all dilutions except 35pg where both alleles dropped out and 4ng, where the 13 dropped out. The base pair sizes were inconsistent approaching standard deviations of 0.1 at 250pg (5 allele). Stutter % was barely seen at this locus. For D5S818, alleles 11 and 13 were called for all dilutions except 35pg where the 8 dropped out. Base pairs were consistent between all the samples. For locus D13S317, alleles 8 and 12 were called for all dilutions but 35pg where the 8 dropped out. Base pairs were consistent between all the samples. At D7S820, homozygotic allele 10 was called for all dilutions and the base pairs were consistent between all the samples.

D16S539 had a homozygotic allele 11 called for all dilutions. The base pairs were consistent between all the samples. At CSF1PO, alleles 10 and 11 were called for all dilutions except 35pg where the 11 allele dropped out. The base pairs were consistent between all the samples. At Penta D, alleles 12 and 13 were called for all dilutions except 35pg where the 13 allele dropped out. The base pairs were relatively consistent between all the samples, with the 1ng dilution standard deviation for the 12 allele at 0.094. Stutter was barely observed at this locus. At amelogenin, homozygotic X was called for each of the dilutions and the base pairs were consistent between all the samples. At vWA, alleles 17 and 18 were called for all dilutions except for 35pg. The base pairs were very consistent between all the samples. For D8S1178, alleles 8 and 13 were called for all dilutions except for 35pg. The base pairs were consistent between all the samples. At TPOX, alleles 8 and 11 were called for all dilutions except for 35pg, where the 11 dropped out. The base pairs were consistent between all the samples. At FGA, alleles 21 and 26 were called for all dilutions except for 35pg. The base pairs were relatively consistent between all the samples.

Table 10: Peak height Ratios (Organically Extracted Buccal Swab)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	84%	96%	92%	82%	Allelic Dropout
TH01	91%	78%	96%	86%	Allelic Dropout
D21S11	87%	79%	87%	52%	Allelic Dropout
D18S51	65%	88%	83%	73%	Allelic Dropout
Penta E	Allelic Dropout	60%	88%	81%	Allelic Dropout
D5S818	90%	99%	75%	79%	Allelic Dropout
D13S317	73%	70%	73%	61%	Allelic Dropout
D7S820	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
D16S539	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
CSF1PO	95%	89%	89%	99%	Allelic Dropout
Penta D	87%	99%	83%	67%	Allelic Dropout
Amelogenin	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
vWA	81%	79%	99%	92%	Allelic Dropout
D8S1179	76%	93%	50.2%	91%	Allelic Dropout
TPOX	87%	97%	84%	54%	Allelic Dropout
FGA	96%	81%	87%	64%	Allelic Dropout

Table 11: Mean Stutter percentage (Organically Extracted Buccal Swab)

Locus	4ng	2ng	1ng	250pg	35pg
D3S1358	10%	9%	11%	15%	Allelic Dropout
TH01	4%	7.5%	9%	5.5%	Allelic Dropout
D21S11	10%	9.5%	10.5%	10%	Allelic Dropout
D18S51	10.5%	7.5%	10.5%	11%	Allelic Dropout
Penta E	0%	2.5%	2%	0%	Allelic Dropout
D5S818	6%	9.5%	9%	9%	Allelic Dropout
D13S317	5.5%	5%	6.5%	5%	Allelic Dropout
D7S820	4%	5%	6%	8%	0%
D16S539	7%	8%	10%	10%	0%
CSF1PO	5%	8%	5%	7%	Allelic Dropout
Penta D	0%	0%	5%	0%	0%
Amelogenin	0%	0%	0%	0%	0%
vWA	8%	10%	10%	14%	Allelic Dropout
D8S1179	6.5%	5.5%	7%	10.5%	Allelic Dropout
TPOX	3.5%	3%	3.5%	6.5%	Allelic Dropout
FGA	9%	9%	14%	13%	Allelic Dropout

Allelic dropout—Allelic dropout occurred at 35pg for all loci but D7, D16, and Amelogenin. Allelic dropout occurred at Penta E at 4ng, due to overloading of DNA inhibiting PCR.

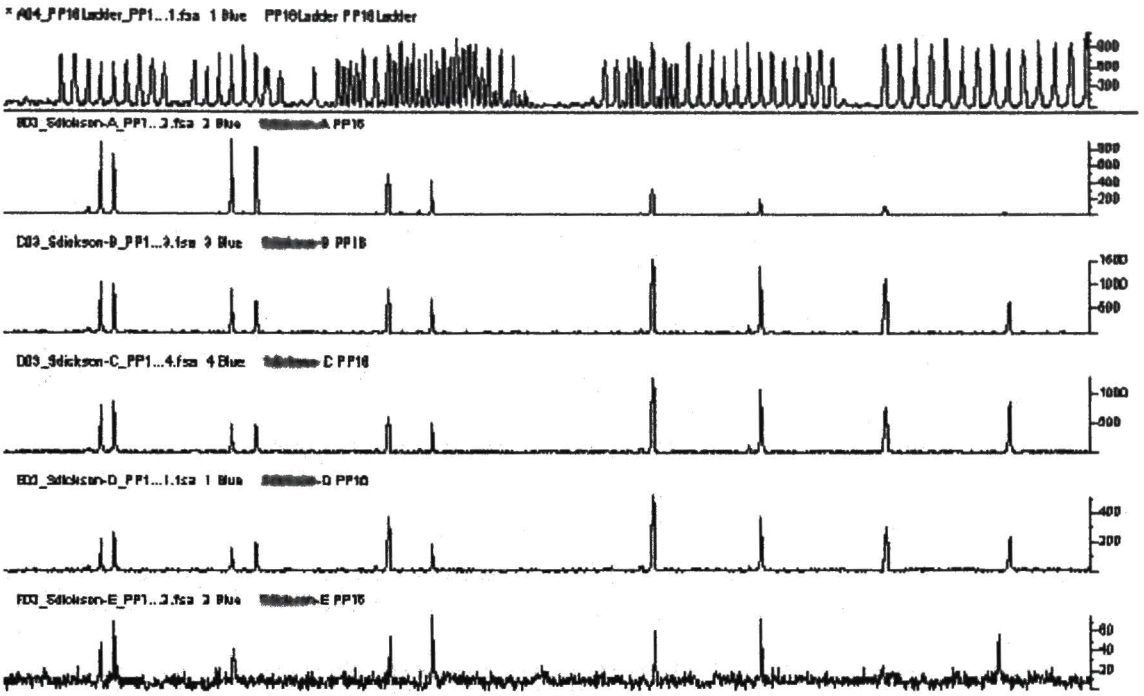

Figure 8: Blue Dye (Organically Extracted Buccal Swab)

From top to Bottom: (Ladder, 4ng, 2ng, 1ng, 250pg, 35pg)

Notice in figure 8 above how very little dropout is observed. DNA is recognized at 35pg. However, inhibition at Penta E due to D3 and vWA consuming all the reagents at higher concentrations is prevalent.

When determining the optimal amount of DNA to be put into a PCR reaction one must take into consideration stutter, allelic drop-out and allelic drop-in, and other artifacts. The appropriate RFU value must be used so the peaks are called accordingly. In this study, an RFU of 75 was analyzed and at that level stutter products were called occasionally, especially at the vWA locus and in loci with peaks exceeding 1500 RFU. The Chelex extracted blood sample was greatly inhibited by either heme inhibition or the Chelex process itself, and the Chelex buccal swab was also shown to be inhibited. Proper

storage was maintained since full profiles were obtained with other samples stored in the same container. With this experiment it was decided by Laura Gahn and I that the optimal amount of DNA to be added to a PCR reaction using PowerPlex® 16 is 1ng. At this amount there is very little if any allelic dropout or drop in, no pull up, the appropriate amount of stutter, no peak splitting or PCR inhibition, and the peak heights suggest a heterozygote when it occurs. Also the base pair sizes are consistent at this DNA input amount. In addition, Chelex extracted samples should not be ran using the PowerPlex® 16 kit, due to excessive allelic dropout. For the routine study, the 15 chelex samples were removed and replaced with 12 organically extracted samples. The mixture samples were also altered to contain different concentrations of male and female DNA from the two organically extracted blood and buccal swabs used in this study.

Controls

1) Reagent blank—produced the expected results (Previously analyzed with extractions)
2) Positive Control—produced the expected results. Peak heights are >6000RFU and pull up was observed, so in a reinjection, a 1:3 dilution was made to attempt to correct the RFU level. The pull up diminished in the reinjection, and the RFU values were near 2000 for most loci, but several remained high at 6000. A 1:30 dilution of the supplied positive control was made, and 1ul of that was added to 9ul of TE Buffer and used in Experiments 2-4.

3) Negative Control—produced the expected results. Pull-up observed in the yellow dye due to pull up for the Internal Lane Standard. This problem lies in the matrix. A spectral was re-ran and corrected the problem somewhat. Pull up due to the ILS 600 is still present in the yellow dye, but at no greater than 20-30RFU.

Mixture Study

Purpose— To determine the limits at which a mixture can be detected and interpret the sensitivity of the PowerPlex® 16 kit when dealing with mixtures.

Experiment—with the use of a male and female organically extracted sources of DNA, dilutions of 30:1, 10:1, 3:1, 1:1, 1:3, 1:10, and 1:30 were prepared at 1ng total concentration per sample. 30:1, 10:1, and 3:1 ratios represent female:male DNA, and 1:30, 1:10, and 1:3 ratios represent male:female DNA. Two samples for each dilution were prepared, and were run in tandem. The source of the female DNA came from organically extracted buccal swabs volunteered by an employee at Identigene. The source of the male DNA originated from an organically extracted blood source involved in a proficiency test with the sample name 62037:S147220. The samples were analyzed at RFU values of 75.

Calculations

1) *Dilutions*- Amount of DNA present in each sample was established through Quantifiler to allow for the optimal and known amount of 1ng of DNA to be added to each sample. The dilutions were prepared through normal dilution procedures and calculation.

2) *Peak height ratio*- Calculated by dividing the minor peak height by the major peak height to determine major/minor ratio to insure the peak height falls in the range of 7% to 50% of the highest peak to concluded the sample is a mixture and a peak above 50% being heterozygous region and below 7% being stutter, and the range of 7-15% being possible stutter or mixture depending on the locus. These peak height ratios will determine both range of mixture and range of heterozygosity, as well as averages.

3) *% Stutter*- Calculated by dividing the stutter peak by the larger adjacent peak to the immediate right and multiplying the number received by 100 to get a percent.

Table 12 – Called Alleles for Mixture

	Profiles		Number of Called Alleles						
Locus	Male	Female	30F:1M	10F:1M	3F:1M	1:1	1F:3M	1F:10M	1F:30M
D3	16, 18	15, 16	2	2	3	3	3	3	2
TH01	6, 9.3	7, 9	2	2	3	4	4	2	2
D21	28,32.2	28, 31.2	2	2	2	3	3	3	2
D18	19, 20	12, 21	2	2	2	4	4	2	2
Penta E	10, 16	5, 13	2	2	2	4	4	4	2
D5	10, 11	11, 13	2	2	3	3	3	3	2
D13	8, 11	8, 12	2	2	3	3	3	2	2
D7	10, 10	10, 10	1	1	1	1	1	1	1
D16	9, 12	11, 11	1	1	1	3	3	3	2
CSF	10, 12	10, 11	2	2	2	3	3	3	2
Penta D	9, 11	12, 13	2	2	3	4	4	2	2
Amel.	X, Y	X, X	1	1	1	2	2	2	2
vWA	16, 17	17, 18	2	2	3	3	3	2	2
D8	13, 15	8, 13	2	2	2	3	3	2	2
TPOX	8, 11	8, 11	2	2	2	2	2	2	2
FGA	21, 24	21, 26	2	2	2	3	3	2	2

How to recognize a mixture

First attempt to determine the sample origin and if the type of sample is more likely to be a sample mixture. Next, check for loci with two or more alleles. If there is more than one locus with two or more alleles, the sample is likely to be a mixture. Check for a peaks in the stutter position with peak height ratios greater than 7- 15% and between 15% and 50%. This could indicate the presence of a mixture in a single source sample. Peak height ratios greater than 50% in single source samples indicate that the peaks are likely heterozygotes.

Results of Mixture Study

An RFU of 75 is sufficient to call smaller peaks present in minor component mixtures of a 1:5 to 1:10 ratio. Any ratio with a greater difference than 1:10 (1:30 for instance) is not likely to develop a full mixture profile with all minor component peaks called using this RFU level. The RFU value of 75 will most likely call minor component peaks at the given 1:5 to 1:10 ratio with little stutter peak calling. As more samples are ran, further modification of the analysis parameters should proceed to determine the optimal RFU value for calling as many minor component peaks present in small concentrations (1:10 to 1:30) without calling stutter peaks. Considering that the sensitivity study showed that 250pg and less would not give full profiles, it is not surprising that the minor components of both the 10:1 / 1:10 (200pg) and the 30:1 / 1:30 (67pg) dilutions would not display a full profile, if any at all.

Routine Study

Purpose-- To determine the effectiveness of organic extraction methods using the PowerPlex® 16 kit on the ABI 3100 Avant, and determine percent and max stutter, as well as peak height ratio. The twenty eight samples should provide a wide enough distribution of alleles so stutter and peak height ratio for at least most of the alleles can be determined.

Experiment—28 organically extracted single source samples were amplified and ran on the ABI 3100 Avant Genetic Data Analyzer and profiles obtained. Calculations then proceeded in order to determine peak height ratio, and stutter percentages.

Calculations

1) *Dilutions*-- amount of DNA present in each sample was established through Quantifiler to allow for the optimal and known amount to be determined and added to each dilution. 1ng of DNA was added to each single source reaction, prepared through normal dilution protocol.
2) *Peak height ratio*—determined by the minor peak height divided by the major peak height
3) *% Stutter*- calculated by dividing the stutter peak by the larger adjacent peak to the immediate right and multiplying the number received by 100 to get a percent.

Results of Routine Study

Average Peak Height Ratio across all loci = **0.8638077**

Table 13: Peak Height Ratio Per Loci

D3	0.8991411
TH01	0.9002125
D21	0.8936971
D18	0.8478379
Penta E	0.7915993
D5	0.8368208
D13	0.8848919
D7	0.8659936
D16	0.8591207
CSF	0.8456088
Penta D	0.8111408
Amelogenin	0.8860125
VWA	0.8688544
D8	0.8824593
TPOX	0.8807805
FGA	0.8667514

Loci Penta E and Penta D show the lowest average peak height ratios, at 79% and 81%, respectively, but are still well within the recommended values for calling heterozygote peaks. Locus TH01 on average exhibits the most even heterozygote peaks at 90%.

Table 14: Mean % Stutter

Average Stutter %			
Locus	Allele 1	Allele 2	Total
D3	8%	6%	7%
TH01	3%	1%	2%
D21	8%	6%	7%
D18	6%	5%	6%
Penta E	1%	3%	2%
D5	7%	2%	4%
D13	6%	4%	5%
D7	6%	3%	4%
D16	6%	3%	4%
CSF	6%	2%	4%
Penta D	1%	1%	1%

Amelogenin	N/A	N/A	N/A
vWA	7%	7%	7%
D8	7%	7%	7%
TPOX	3%	3%	3%
FGA	7%	7%	7%

Stutter percentages are below 8% for all loci, and all fall within the recommended values. Penta E and Penta D exhibit the lowest stutter excluding Amelogenin at 2% and 1% respectively. Each sample exhibited a full profile at all 16 loci. Stutter percentages were within acceptable ranges, as were peak height ratios. At 1ng, some samples exhibited peaks in excess of 2000 RFU, perhaps indicating that the quantification process did not give completely accurate values. However, even in excess of 6000 RFU, few instances of pull-up were visible, and in cases where present, they were easily identifiable. If observed, much of the pull-up was observed and concentrated in the smallest base pair loci for each dye, such as D3, D5, and vWA. The positive control run in this study was in excess of 6000RFU at several loci, yet produced a full profile. The original positive control sample provided in the PowerPlex® 16 kit was diluted 1:30 for this study, and 1ul of that dilution was then diluted in 9ul TE buffer. This dilution still produced peaks in excess of 6000 RFUs at some loci. Negative controls showed the appropriate results.

Non-Probative Casework Study

Purpose— To determine the ability of the PowerPlex® 16 kit to perform to satisfaction when compared to the Profiler Plus™ / COfiler™ kits on the 3100 using proficiency casework. Non-probative casework is technically adjudicated casework, but for the purposes of this experiment, proficiency samples were appropriate.

Experiment—Compare profiles generated by existing Profiler Plus™ and COfiler™ data. Compare these profiles to those generated by PowerPlex® 16 and determine the percent accuracy for determining the correct allele calls.

Calculations

Percent Accuracy: Add the number of correct PowerPlex® 16 allele calls as compared to Profiler Plus and COfiler data, then divide by the total number of alleles and multiply by 100 to get a percent. Since Penta E and Penta D are not present in Profiler Plus™ and COfiler™, they will not be counted towards this percentage.

Table 15a: Allele calls for Non-Probative Samples

		Locus						
Sample	**Kit**	D3	vWA	FGA	D8	D21	D18	D5
62037-S147219	Pro/Co	16, 18	16, 17	21, 24	13, 15	28, 32.2	19, 20	10, 11
62037-S147219	PP16	16, 18	16, 17	21, 24	13, 15	28, 32.2	19, 20	10, 11
62037-S147220	Pro/Co	16, 18	16, 17	21, 24	13, 15	28, 32.2	19, 20	10, 11
62037-S147220	PP16	16, 18	16, 17	21, 24	13, 15	28, 32.2	19, 20	10, 11
66383-S152199	Pro/Co	15, 16	16, 20	21, 22	13, 15	31.2	14, 15	12, 13
66383-S152199	PP16	15, 16	16, 20	21, 22	13, 15	31.2	14, 15	12, 13
66383-S157297	Pro/Co	15, 16	16, 20	21, 22	13, 15	31.2	14, 15	12, 13
66383-S157297	PP16	15, 16	16, 20	21, 22	13, 15	31.2	14, 15	12, 13
56426-S133918	Pro/Co	13, 17	15, 17	20, 25	13, 16	29, 32.2	12, 15	11, 13
56426-S133918	PP16	13, 17	15, 17	20, 25	13, 16	29, 32.2	12, 15	11, 13
56426-S133919	Pro/Co	13, 17	15, 17	20, 25	13, 16	29, 32.2	12, 15	11, 13
56426-S133919	PP16	13, 17	15, 17	20, 25	13, 16	29, 32.2	12, 15	11, 13
57673-S136795	Pro/Co	15, 18	14, 18	20, 23	10, 13	30	13, 16	11, 12
57673-S136795	PP16	15, 18	14, 18	20, 23	10, 13	30	13, 16	11, 12
57673-S137136	Pro/Co	15, 18	14, 18	20, 23	10, 13	30	13, 16	11, 12
57673-S137136	PP16	15, 18	14, 18	20, 23	10, 13	30	13, 16	11, 12

Table 15b: Allele calls for Non-Probative Samples

		Locus								
Sample	**Kit**	D13	D7	D16	TH01	TPOX	CSF	Am.	Penta E	Penta D
62037-S147219	Pro/Co	8, 11	10	9, 12	6, 9.3	8, 11	10, 12	X,Y	N/A	N/A
62037-S147219	PP16	8, 11	10	9, 12	6, 9.3	8, 11	10, 12	X,Y	10, 16	9, 11
62037-S147220	Pro/Co	8, 11	10	9, 12	6, 9.3	8, 11	10, 12	X,Y	N/A	N/A
62037-S147220	PP16	8, 11	10	9, 12	6, 9.3	8, 11	10, 12	X,Y	10, Inc.	9, 11
66383-S152199	Pro/Co	8, 12	10, 11	9, 13	6, 7	9, 10	11	X,Y	N/A	N/A
66383-S152199	PP16	8, 12	10, 11	9, 13	6, 7	9, 10	11	X,Y	7, 12	9, 12
66383-S157297	Pro/Co	8, 12	10, 11	9, 13	6, 7	9, 10	11	X,Y	N/A	N/A
66383-S157297	PP16	8, 12	10, 11	9, 13	6, 7	9, 10	11	X,Y	7, 12	9, 12
56426-S133918	Pro/Co	12, 13	10, 11	9, 14	8, 9.3	8	11, 13	X,Y	N/A	N/A
56426-S133918	PP16	12, 13	10, 11	9, 14	8, 9.3	8	11, 13	X,Y	11, 15	12, 13
56426-S133919	Pro/Co	12, 13	10, 11	9, 14	8, 9.3	8	11, 13	X,Y	N/A	N/A
56426-S133919	PP16	12, 13	10, 11	9, 14	8, 9.3	8	11, 13	X,Y	11, 15	12, 13
57673-S136795	Pro/Co	11, 12	10, 11	9, 13	8, 9.3	8, 10	11, 12	X,Y	N/A	N/A
57673-S136795	PP16	11, 12	10, 11	9, 13	8, 9.3	8, 10	11, 12	X,Y	13, 18	9, 10
57673-S137136	Pro/Co	11, 12	10, 11	9, 13	8, 9.3	8, 10	11, 12	X,Y	N/A	N/A
57673-S137136	PP16	11, 12	10, 11	9, 13	8, 9.3	8, 10	11, 12	X,Y	13, 18	9, 10

With the exception of Locus Penta E for sample 62037-S147220, all alleles were called for all samples. Since Penta E and Penta D are not available in Profiler Plus™ or COfiler™ kits, it is still possible to receive a 100% for comparisons between the kits at the CODIS loci.

Calculations are as follows:

26 correctly called CODIS Alleles / 26 total CODIS alleles * 100 = 100% accurate

Conclusion

For this study, the PowerPlex® 16 kit has been validated to compare 100% to the Profiler Plus™ and COfiler™ kits on the ABI 3100 at Identigene. All alleles reported correctly on samples from actual proficiency testing. One allele dropped out at the Penta E locus in one sample, however all other alleles including all 26 CODIS alleles reported flawlessly.

Allelic Ladder Size Precision Study

Purpose- To determine the ability of Powerplex® 16 to size alleles accurately based upon repeated ladder injections.

Experiment—12 ladder injections were run and standard deviations calculated for each allele at each locus. Averages across the loci were calculated and are shown in the table on the next page.

Table 16: Standard Deviations from 12 Ladder Injections

D3	0.045386
TH01	0.032667
D21	0.031779
D18	0.065455
Penta E	0.042931
D5	0.044819
D13	0.033704
D7	0.036775
D16	0.045062
CSF	0.057292
Penta D	0.062004
Amelogenin	0.030556
VWA	0.030524
D8	0.031296
TPOX	0.050312
FGA	0.047887

Standard deviations for allelic ladders are less than 0.66 for all loci. Penta D and D18 exhibited the highest standard deviations at 0.062 and 0.065, which can be expected for loci with larger base pair sizes. Amelogenin and vWA exhibited the smallest standard deviations both at 0.030, a value that can be expected for loci of smaller base pair sizes. Ladder injections were within the suggested standard deviation values for all loci. However, for the locus D21, allele 36.2 was only called in five of the twelve ladder injections, and allele 39 was only called in ten of the twelve injections. In addition, the locus FGA exhibited an off ladder allele around 427.47 base pairs in every ladder injection, one repeat unit less than the allele 43.2.

Takayama Test

Purpose—The purpose of this experiment is to determine the effectiveness of heme identification using the Takayama Test, and to determine the ability of the reagent to detect heme in both liquid blood and substrate stained samples. Five studies were planned for this study, two involving liquid whole blood and three involving blood stained on stain cards.

Experiment 1—DTT

The pyridine in the Takayama reagent binds to the heme in blood, but so does oxygen (6, 9). Oxygen and pyridine are competing to bind to heme, slowing the reaction (6). DTT serves as an oxygen scavenger, binding the oxygen leaving the heme open for pyridine to bind so the crystals can form faster (6). Therefore, a test was conducted to determine what ratio of DTT works best. Four protocols for DTT addition were implemented, and four different Takayama reagents formed. Four samples of liquid blood from the same fresh blood sample were individually tested with the four reagents. Each Takayama reagent will have a different concentration of DTT added. The speed that the crystals form on the samples will be closely measured and the time recorded. The protocols are listed on the next page.

Protocol 1 – No DTT		**Protocol 2**	
Saturated Dextrose solution	--- 3ml	Saturated Dextrose solution	--- 3ml
10% NaOH	--- 3ml	DTT	--- 200ul
Pyridine	--- 3ml	10% NaOH	--- 3ml
Distilled Water	--- 7ml	Pyridine	--- 3ml
		Distilled Water	--- 7ml

Protocol 3		**Protocol 4**	
Saturated Dextrose solution	--- 3ml	Saturated Dextrose solution	--- 3ml
DTT	--- 800ul	DTT	--- 1.4ml
10% NaOH	--- 3ml	10% NaOH	--- 3ml
Pyridine	--- 3ml	Pyridine	--- 3ml
Distilled Water	--- 7ml	Distilled Water	--- 7ml

From these findings, one of these protocols was accepted for use in the creation of a Takayama reagent containing the proper amount of DTT to be used in experiments 2-4.

Results

***Experiment** 1 - DTT*

(1ul fresh liquid blood used / sample)

Reagent

1 = 0ml DTT 2 = 200ul DTT

3 = 800ul DTT 4 = 1.4ml DTT

X- indicates no crystal formation

Check mark – indicates Takayama crystal formation

Table 17: Takayama DTT Experiment

	Time required for crystal formation										
Reagent	30 sec	1 min	5 min	10 min	15 min	20 min	25 min	30 min	45 min	60 min	90 min
1	X	X	√	-	-	-	-	-	-	-	-
2	X	X	X	√	-	-	-	-	-	-	-
3	X	X	X	X	X	X	X	√	-	-	-
4	X	X	X	X	X	X	X	X	√	-	-

Conclusion – Takayama Experiment 1

In contrast to my hypothesis, it was found that DTT inhibited crystal formation on liquid blood in three ways. First, it was found that more time was needed to crystallize the heme present, smaller crystals were formed, and the crystals disintegrated over a quicker period of time as increased amounts of DTT were present. However, the only DTT available for use was expired, but the expiration of DTT would not explain why inhibition was occurring. It seems likely that if the DTT expired, all reactions would

proceed at the same pace. However this was not the case. Since reagent 1 with 0ml DTT added produced the quickest and best results on liquid blood, it was decided that this reagent be used for the remainder of the experiments.

Experiment 2

Dilution Study

A 1ul sample from each of the following dilutions of blood from the same source will be tested using the Takayama procedure in order to test the sensitivity of the method.

Blood: dH2O

1:1

1:10

1:100

1:1000

1:10000

1:100000

Results

Experiment 2 - Dilution

(Used DTT amount and optimal time required from Experiment 1)

1ul sample used

divided into 0.5ul aliquots

X- indicates no crystal formation

Check mark – indicates Takayama crystal formation

Table 18: Takayama Dilution Experiment

Dilution	
Blood:dH20	Crystal Formation
1:1	√
1:10	√
1:100	√
1:1000	√
1:10000	X
1:100000	X

Conclusion

I determined that this test gives positive results on blood diluted somewhere between 1:1000 and 1:10000 dilutions. All dilutions still gave the same result after a one hour period, and after testing in triplicate. 1 ul of liquid blood sample was used from the same source for each dilution, and was divided into two 0.5ul aliquots to keep the surface area of the stain as small as possible. One drop of Takayama reagent was added. The results shown here are concurrent with those reported in the Forensic Science Handbook (9).

Experiment 3

Environmental Test

Blood stains on blood stain cards were prepared and placed in the following environments for one week: dark/wet/56°C, dark/wet/-20°C, dark/wet/RoomTemp, light/wet/RoomTemp, dark/dry/56°C, dark/dry/-20°C, dark/dry/RoomTemp, and light/dry/RoomTemp. The stains were then tested in triplicate using the Takayama Method.

Results and Conclusion

Upon testing the first sample in this study, it was discovered that the blood stain cards, when cut and placed in 250ul of distilled water for 30 minutes, would not release enough heme into the liquid to give a positive result when 1ul liquid was taken for a sample. Attempts were made to vortex the liquid and substrate, centrifuge the liquid and take the sample from the bottom of the tube, use 2ul, 5ul, and even 50ul liquid sample, and an additional drop of Takayama Reagent, but still no results. The substrate was just too thick and tightly woven in my opinion to release any heme into solution. Although the liquid surrounding the substrate was yellowish/pink, not enough heme was present in even 50ul of the sample to give a result. Short of wasting the liquid sample by adding two drops of reagent directly to the tube, then taking 5ul of the combination after five minutes and placing it on a slide, I was almost out of options. Finally after placing a cutting of the substrate directly on the slide, adding 2 drops of Takayama reagent physically onto the substrate, placing a coverslip over the substrate, and placing it under a

microscope did I see results in under five minutes, but only on the substrate itself. The coverslip was at an angle, and the technique was very messy, eve though positive results were obtained. After discussion with my lab supervisor, it was decided that although this test works for liquid and dry blood or blood scrapings, blood on stain cards or thick substrate cannot be validated using the techniques used at Identigene. For example, it may be necessary to store slides for clients who request a positive sample to be kept for long periods of time. These slides do not store well, especially if the substrate has to be placed on the slide under a coverslip for the test to work correctly. Too much stain has to be used to obtain a positive result when compared to other methods. Old reagent still within the expiration date of one year may still give false negatives. The reagent has a very unpleasant odor. A cutting of the substrate must be brought into liquid, and then a portion of that liquid should be used to make the stain on the slide. This is not possible using stain cards because the substrate holds so much of the heme that the Takayama Reagent cannot detect any heme in the small amount of liquid used to make the slide. It was discussed and proposed that the stain cards quite possible were the only substrates causing the problem. Perhaps if the stains were on cotton swabs or lightly woven cloth, enough heme would release into solution to provide a positive result from a substrate without resorting to putting the actual substrate on the slide. In this respect, two studies including this one and a proposed chronological test were canceled because the stains were made using blood stain cards and there was simply not enough time left to re-stain swabs and cloth for one week's time. A new study was proposed, a substrate study.

Experiment 4

Substrate Test

Stains on stain cards, cotton swabs, and lightly woven cloth were made using blood recovered from non-probative proficiency testing and tested for blood using the Takayama Reagent. A $1cm^2$ blood stain was cut from the stain card and lightly woven cloth and each placed into an individual tube containing 250ul distilled water for 30 minutes. For the cotton swab 1/5 of the swab was taken and placed into a tube containing 250ul distilled water for 30 minutes. Each tube was vortexed twice during this time on medium speed, and centrifuging at 14000RPM for 2 minutes at the end to gather the heme to the bottom of the tube. After the time period, 1ul stains from the bottom of the tubes were made on microscope slides divided into two 0.5ul aliquots, one drop of Takayama reagent added to each, and a coverslip added. The slides were then closely observed under microscope for crystal formation.

Results and Conclusion

All samples produced negative results, even after one hour. Even the cuttings of light woven cloth and the cotton swabs would not release enough heme into 1ul of liquid to give a positive result. Attempts were made to vortex the liquid and substrate two extra times, centrifuge the liquid longer and take the sample from the bottom of the tube, use 2ul, 5ul, and 50ul liquid sample, and an additional drop of Takayama Reagent, but still no results. In addition, when using 50ul the coverslip was very uncooperative, making for a very messy process. Once again, although the liquid surrounding all the substrates was

yellowish/pink to translucent red in the tubes containing the swab and cloth, not enough heme was present in even 50ul of the sample to give any crystalline result. Wasting the liquid sample by adding two drops of reagent directly to the tube, then taking 5ul of the combination after five minutes and placing it on a slide, was against protocol and not feasible for a presumptive test. There are other tests currently available that use much less sample and can detect blood flawlessly in a far less dilute solution. Once again, after placing a cutting of the substrate directly on the slide, dropping 2 drops of Takayama reagent physically onto the substrate, placing a coverslip over the substrate, and placing it under a microscope did I see results in under five minutes, but only on the substrate itself. The coverslip was at an angle, and the technique was very messy, especially with the cotton swab. However, positive results were obtained, but only under non-workable conditions.

CHAPTER VI

CONCLUSIONS

Original expectations of the PowerPlex 16 validation study and results obtained are similar. For the sensitivity study, Promega documents the optimal amount of DNA to be added to the PCR reaction is 500pg to 1ng, (Promega) which is consistent with the results of this validation study (4, 7). For the mixture study of male and female DNA, Promega claims that the 3100 Custom Matrix kit would minimize the baseline enough so that minimal component peaks could be detected (5, 7). This was the case for this study, even though these peaks were not always called, especially at the 10:1 and 30:1 concentrations, but the peaks of the minimal component could be easily detected from the baseline. Peaks were seen for minimal components under 75RFU for most of the mixture samples, and outside of the 30:1 and 1:30 concentrations few instances of complete allelic dropout were observed. The routine sample study and non-probative casework study proves that the PowerPlex® 16 system can compare to results obtained from Profiler Plus™ and COfiler™ kits, exhibiting not only the correct allele calls, but also acceptable peak height ratios and stutter percentages. In conclusion, the Promega PowerPlex® 16 kit is validated for regular casework at Identigene on the ABI 3100 Avant Genetic Analyzer, with several limitations that must be discussed with every client

requesting Powerplex® 16. A component of Identigene's Chelex-extracted samples has been shown to inhibit the Powerplex® 16 amplification reaction. This can be due to Identigene's failure to remove their substrate from the final extraction tube. In addition, heme does not get filtered out in blood extractions. Using Identigene's methodology, it is difficult to add a sufficient amount of Chelex-extracted DNA to amplification without resulting inhibition. There is a lack of sensitivity in the Powerplex® 16 system when reading mixtures where the minor component is less than 200pg. Pull-up exists in the ladder over the red dye due to pull-up from TPOX in the yellow dye. This does not affect allele calling by Genotyper™. The software also labels peaks for samples where the max peak in the sample does not exceed 30 RFU. This was observed in the yellow dye of some negative control samples, and in a few of the 35pg samples in the dilution series. This does not happen every time the symptoms are present and the reason has yet to be determined. The problem may lay either in the macro itself, Genescan, or Genotyper. Until the problem is solved with both Promega and ABI, this issue will remain a limitation.

After discussion with my lab supervisor, it was decided that although the Takayama test works for liquid and dry blood or blood scrapings, blood on stain cards or thick substrate cannot be validated using the techniques used at Identigene for several reasons mentioned in the previous pages. Tests like phenolphthalein and leukomalachite green (LMG) are much better indicators for the presence of blood, give results faster, do not require a microscope, give positive results at smaller dilutions, and work well on both liquid blood and substrate. Storing slides of substrates using the only method I was able

to get to work is out of the question. The substrate has to be placed on the slide under a coverslip elevated from the slide itself for the test to work correctly, and that is not only messy, but violates good lab protocol, can lead to contamination and possible injury. In addition, dark dyes in the substrate could shroud a positive result completely using this method. Too much stain has to be used to obtain a positive result when compared to other methods. Old reagent still within the expiration date of one year may still give false negatives (9). The reagent has a very unpleasant odor. A cutting of the substrate must be brought into liquid, and then a portion of that liquid should be used to make the stain on the slide in order for this test to be validated. Since this is not possible with substrates, this test cannot be validated as an equal or better identification method for the presence of blood or heme in blood than the present tests already validated at Identigene.

There are several things I would have done differently, given more time, money, less competition with regular casework, and the opportunity to follow alternate policies and methodologies. I would have followed the UNTHSC Chelex protocol in contrast to Identigene's Chelex protocol, or run extracts of UNTHSC Chelex vs. Identigene Chelex to determine if there was a significant difference in inhibition. I would have run the study in duplicate, once on the ABI 9600 then again on the ABI 9700 to determine if a difference existed across either thermal cycler. I would have added more sensitivity dilutions, for example 1.5ng and 500pg. More samples in the mixture study would have been appropriate. Fore example, more female to male combinations and a greater number of people per mixture would have been informative. It would have been informative to look closer at the internal positive control in Quantifiler to determine

whether heme was inhibiting PCR in the Chelex reactions. If the IPC crossed the threshold, but wasn't as high in comparison to the organic extracted reactions, inhibition may have been present. I could have used more samples in the non-probative concordance study. Eight samples were not enough to get a complete view of concordance. It would have been informative to run Hematrace parallel to Takayama for comparison purposes. Quantifiler should have been run on the positive control instead of the dilution series, and should have been run after every dilution to ensure that these were indeed accurate. The addition of hair, bone, and tissue samples would have extended the variety of samples validated on the PowerPlex® 16 kit.

Validation studies are needed any time a new method or a new instrument is being used to run a procedure in a forensic laboratory contributing to results that may be submitted into a court of law. Forensic cases are frequently under scrutiny by the judicial system. The questions lie mostly in the processes by which the laboratory performs the DNA test. In order to reduce the amount of doubt as to the ability of the laboratory personnel's ability to perform the experiments properly, validation studies are performed and interpretation guidelines are developed from those validation studies.

Interpretation guidelines are written in order to direct and assist an analyst in making final decisions and interpretation with each individual sample. The guidelines consist of a control that must be run beside each sample along with accepted results. The guidelines include the types of identification for samples. For example, the identifications of no result, inconclusive, exclusive, and not excluded are common. DNA quantification information must be included along with internal lane standard and allelic

ladder guidelines as based upon the laboratory's equipment. Mixture interpretations are included, explaining peak height ratios and the proper way to determine a mixed sample using the equipment in the laboratory for which the guidelines were written. A calculation section included addressing the appropriate calculations needed to analyze the results. Interpretation guidelines will be produced based on the PowerPlex® 16 validation study for the forensic laboratory at Identigene.

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II. Figures

Figures 1, 2, 5, 6, 7, and 8 are original figures taken from electropherograms generated in this validation study.

Figure 3 Promega. "PowerPlex® 16 System Technical Manual No. D012" pdf manual. http://www.promega.com/tbs/tmd012/tmd012.pdf

Figure 4 State University of New York Canton. Public Safety Technology: Criminal Investigation. http://www.canton.edu/ci/previous_lessons.html

VIX. Tables

Table 1 Promega. "PowerPlex® 16 System Technical Manual No. D012" pdf manual. http://www.promega.com/tbs/tmd012/tmd012.pdf

Tables 2-18 are original tables created in Microsoft word or Excel using original data.