Validation and evaluation of the Identifiler® Plus system using environmentally compromised DNA samples

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Abstract

Internal validations are required for every laboratory for every kit chemistry used. This study validated the Identifiler® Plus STR Amplification kit for use at the University of Central Oklahoma. Validations examine the sensitivity, precision, reproducibility, peak height ratio, and stutter observed in testing. In addition to these components, the validation also sets mixture interpretations, evaluates contamination, and conducts a mock case study. At the conclusion of the validation, the Identifiler® Plus kit was also tested using common inhibitors such as UV light, humic acid, tannic acid, and hematin. Guidelines for interpretation and an analysis of the affect of inhibitors were drawn from the results obtained in the study.

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Introduction

In the field of forensic human identification, consistency and the use of quality controlled protocols are paramount. The use of commercial kits makes consistency, as well as the overall analysis process, convenient and faster for the analyst. Forensic analysts depend on these materials in order to obtain reliable results and expect on the kit to perform according to manufacturer claims. In order for the analyst to be confident in the use of a new commercial product within their laboratory, a validation of the product must be completed to know how well it performs. The Applied Biosystems AmpFℓSTR® Identifiler® Plus PCR Amplification Kit was released in January 2010. Applied Bioystems has made minor changes to this second generation amplification system to allow for more efficiency in providing a complete genetic profile. In order to utilize the Identifiler®Plus STR kit properly, a validation study must be done for the laboratory at the University of Central Oklahoma.

The DNA Advisory Board (DAB) requires that all kits must be validated to test for optimum and consistent results (SWGDAM, 2010). Environmental insults provides information on how well the kit performs if the deoxyribonucleic acid (DNA) is exposed to UV light, humic acid, tannic acid, and hematin that may appear at crime scenes. Performing an internal validation of the Identifiler® Plus kit demonstrates the reliability and limitations of this product both under normal conditions, and under conditions that may arise from case samples.

The goal of this research project was to conduct an internal validation of the Identifiler® Plus kit for the Forensic Science Institute at the University of Central Oklahoma. In order to thoroughly validate the system, the sensitivity, precision, and reproducibility of the chemistry were evaluated. The research also tested the efficiency and effectiveness of the kit using human

blood stains exposed to a variety of environmental insults. This study showed that the Identifiler® Plus STR kit can provide optimum results for casework and research.

The DNA Molecule

DNA provides the needed information to allow cells to develop and work together to sustain life. The content of its structure also makes each individual unique. In its simplest form, the DNA molecule is a double helix of two anti-parallel strands of repeated monomers called nucleotides. Each nucleotide consists of three functional groups: a nitrogenous base, a sugar, and a phosphate. The sugar present in each nucleotide is a 2-deoxy-D-ribose sugar. As shown in Figure 1, deoxyribose is a 5 carbon ring that has a hydroxyl group on the 3’ and 5’ carbons. The sugar differs from the ribose by one hydroxyl group, which is present on the 2’ carbon in the RNA sugar.

Figure 1– The structures of DNA and RNA are made of two separate sugars. Deoxribose is present in DNA whereas ribose is present in RNA (Wiebe, 2007).

Two different types of bases exist in the molecule. Purines are double-ringed structures and consist of the bases adenine and guanine. Pyrimidines are single-ringed structures and are made up of the bases thymine and cytosine (Figure 2). Bonding between nucleotides maintains the classic double helix shape of the DNA molecule and allows for flexibility. The nitrogenous bases bond to the 2’-deoxyribose sugar at the 1’ carbon site via a β-N-glycosidic bond. These bonds are covalent bonds between the sugar and the nitrogenous base group. For purines, the

glycosidic bond forms at the ninth nitrogen of the base, and for pyrimidines the glycosidic bond forms at the first nitrogen of the base.

Figure 2 - Four different bases are included in the nucleotides to add variation to the DNA molecule. In base pairings, there will always be one pyrimidine bound to one purine (Wiebe, 2007).

The phosphate group of the nucleotide forms a phosphoester bond between the 5' carbon of the 2'-deoxyribose sugar and a hydroxyl group of the phosphoric acid. A phosphodiester bond links two nucleotides together by creating a bond between the 3' carbon of one nucleotide to the 5' carbon of the next nucleotide, creating a bridge between the nucleotides. The phosphodiester bond creates the backbone of the molecule and helps give DNA its stability. On the inside of the molecule, the bases are linked together by hydrogen bonds. Adenine bonds to thymine via a double hydrogen bond, whereas guanine bonds to cytosine via a triple hydrogen bond (Figure 3).

Figure 3 – DNA is made up of two strands of nucleotides connected together by various bonds. Phosphodiester bonds hold the backbone together, whereas hydrogen hold the bases together. Glycosidic bonds are also present within the base holing the individual components together (MIT, 1998).

In 1953, James Watson and Francis Crick discovered the three dimensional double helix structure of the DNA molecule. The structure was based on Chargaff's rule of comparative base pairing, that the A/T and G/C bonds are present equally in the molecule. Under physiological conditions the bases possess a hydrophobic nature which in turn stabilizes the double helix.

β -DNA is the most common form of DNA. This form possesses inconsistent spacing between bases, which results in a major and minor groove in the turning of the structure (Bettelheim, Brown, Campbell, & Farrell, 2007). The pattern of nitrogenous bases within the strand is what codes for the information that is necessary to produce proteins. In turn, this pattern may also be unique between individuals and becomes useful in human identification.

Genome Architecture

The entire human genome is made up of approximately three billion base pairs organized into 22 pairs of autosomes and two sex chromosomes. The human genome can be broken down into two main groups; non-coding and coding regions. Intergenic, or non-coding, DNA is the larger of the two groups, with genes and gene related sequences being the smaller (Figure 4).

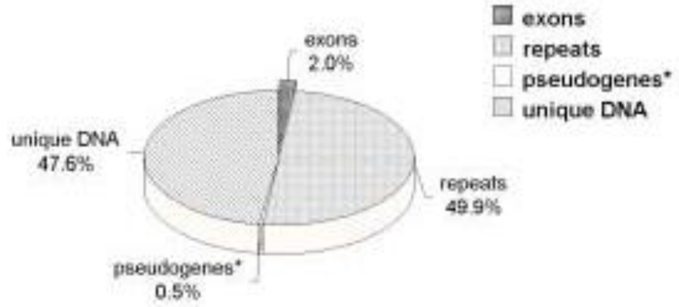


Figure 4 – A pie chart representing the different components of DNA and how much each component contributes to the overall structure of DNA (Makalowski, 2001)

Intergenic DNA contains few or no genes in its sequences. It can contain genome wide repeats or other regions such as unique DNA sequences, gene regulatory regions, or microsatellites. In a review article of the structure and organization of the human genome, Makalowski (2001) describes the unique DNA percentage shown in Figure 4 as the rest of DNA that does not make up exons or repetitive sequences. This accounts for the high percentage represented. In actuality, much of the genome is the same from individual to individual (Makalowski, 2001).

Genes and gene related sequences make up the other portion of the genome and include introns, gene fragments, and pseudogenes. Pseudogenes are remnants of genes that no longer have the ability to code for proteins. Each gene contains exons, or the coding region, and introns which are the non-coding region of DNA. Within introns of one gene, exons of another gene may be present. Therefore, even if a region of DNA in a gene sequence is not directly used for that gene product, it may in fact be used for another gene product. The genome also contains repetitive sequences dispersed throughout. These sequences known as satellite DNA can be tandem or isolated and can be classified by function, dispersal patterns, and sequence relatedness (Makalowski, 2001). Using these characteristics of satellite DNA, an analyst can pinpoint a region for use in genetic identification.

Microsatellite DNA

Satellite DNA comes in three different sizes; macrosatellites, minisatellites, and microsatellites. Macrosatellites are up to hundreds of kilobases long, while minisatellites are repeat units that consist of 10-100 base pairs repeated in tandem. Minisatellites, also known as variable number of tandem repeats (VNTRs), were previously used for human identification (Tautz, 1993). These larger stretches of satellites usually occur at the ends and the center of the chromosomes (Bettelheim, Brown, Campbell, & Farrell, 2007). Microsatellites, on the other hand, can be found throughout the human genome and are tandemly repeated units of 2-6 base pairs long. Repeat units of 4-5 base pairs are the predominant genetic marker used in forensic science (International Human Genome Sequencing Consortium, 2001). They are simple repeat sequences with no known function. A key reason that microsatellites have become predominant in forensic science is the method used to analyze them. Due to the characteristics of microsatellites, polymerase chain reaction (PCR) can be used to multiply a target sequence of DNA millions of times. PCR reduces the time, labor, and amount of DNA used, and can also have successful results with partially degraded DNA.

Polymerase Chain Reaction

In 1985, PCR was first described by Kary Mullis. PCR creates millions to billions of copies of DNA in just hours, and is a key step in forensic DNA analysis. Many components work together to complete the PCR process. The boundaries of the targeted sequence are designated by forward and reverse primers specific to the DNA sequence of interest, acting as a guide for where the synthesizing begins and ends. The excess concentration of primers drives the reaction to occur. However, if too much primer is present, mispriming or primer dimers occur resulting in a lower yield. Likewise, if not enough primer is present then they may run out

before the cycles are completed. A DNA template of the target sequence must be present in just the right concentration. Too much can lead to non-specific primer binding or even change the pH of the system, while too little can lead to a poor yield. Deoxyribonucleotide triphosphates (dNTPs) must also be present in equal concentrations to use as the building blocks in the extension process. Taq polymerase is used in extension to incorporate the bases because it is a thermostable enzyme that can withstand the temperatures used during the process. Newer STR systems incorporate Ampli*Taq* Gold®, which is chemically modified to be present in an inactive state until the initiation step in the amplification process. Magnesium chloride is a cofactor for the DNA polymerase, while a buffer maintains the overall pH of the reaction. Some manufacturers include bovine serum albumin (BSA) in their STR kits to overcome any inhibitors that may be present (President's DNA Initiative: DNA Analyst). Water is added last to bring the entire mixture up to the correct volume and concentration.

Three main steps are vital for the DNA amplification process and can be seen in Figure 5. During denaturation, samples are heated to 95°C to break apart the DNA into two single strands. Having the DNA as a single strand allows the target region to be exposed. Samples are cooled to approximately 50°C to allow the primers to anneal to the flanking regions. In order for the polymerase to bind to the DNA and begin extension, the samples are heated to 72°C. In newer STR kits, the annealing and extension steps are combined and the temperature set at 59°C. Taq polymerase moves along the target sequence creating a complimentary strand using free dNTPs.

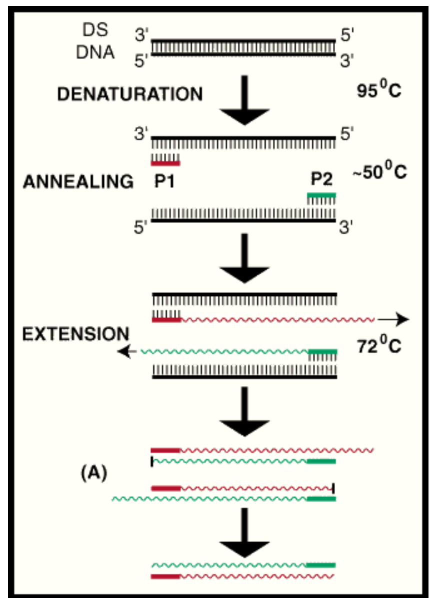


Figure 5 – A step by step representation of the polymerase chain reaction. The polymerase chain reaction consists of denaturation, annealing, and extension steps. With the introduction of hot start STR kits, an initiation step has been added to the beginning. (University of Florida)

PCR is a cyclic process that increases the amount of DNA exponentially, using a thermal cycler to accurately heat and cool the samples during amplification, simultaneously making it an automated process (Rapley & Whitehouse, 2007). "PCR is the mainstay in molecular biology because of its simplicity and ease of manipulation of the steps. It provides a rapid means for DNA identification and analysis, and has also opened up for the investigation of cellular and molecular processes to those outside of the field of molecular biology" (Altshuler, 2006).

Hot Start PCR. In the past, low temperature mispriming was observed in some results of amplification. This resulted in the amplification of primer dimers instead of the desired target sequences. The development of hot start PCR protocols avoids this, and uses the thermally stable Ampli*Taq* Gold® polymerase. This polymerase has been modified with "a derivitization of the epsilon-amino groups of the lysine residues" (Innis & Gelfand, 1999). In a solution with a pH below 7.0, the modification separates and activates the polymerase. It was found that the pH of a system decreases by 0.02 pH units for every 1°C increase in temperature, meaning the hot

start systems must start with a pH of 8.3 in order for the polymerase to become activated and denature the DNA at 95°C. At this temperature, the DNA is denatured at the same time that the polymerase becomes ready for amplification. Hot start PCR allows the polymerase to remain stable for a longer period of time, resulting in the target sequences being properly amplified throughout the entire process. Overall, the introduction of hot start PCR into the forensic community has eliminated a step during sample set up, and has eliminated a problem observed in PCR products (Innis & Gelfand, 1990a).

Primer Design. Primer design is crucial to the overall amplification process. Forward and reverse primers must be specific to the target region they are flanking. It is also important that the primers have similar annealing temperatures, do not interact with one another, and be structurally compatible. The sequence of the DNA that the primers are binding to should not change significantly between individuals. Primers are fluorescently dye labeled at the 5’ end in an attempt to not allow interference with extension. A series of guanine bases are added at the end of the primers to make sure they stay annealed to the DNA. All these factors, along with those present in Table 1, are used to make a primer for amplification (Dieffenbach, Lowe, Dveksler, 1993).

Primer Design	
1. primer length	18-30 bases
2. Primer Melting Temperature (T_m)	55-72°C
3. Primer Annealing Temperature (T_a)	~5 ^{o}C < the lowest T_m of the primers
primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;	
4. T_m difference between forward and reverse primers	≤5 ^{o}C
5. Max 3' Stability	ΔG value for five bases from 3' end
6. Percentage GC content	40-60%
7. No secondary Structures	Identify primer pairs which do not assume secondary structure
8. primer self-complementarity (ability to form 2° structures such as hairpins) should be avoided;	<4 contiguous bases
9. No complementarity to other primers	<4 contiguous bases
10. No long runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.	<4 contiguous bases
11. Distance between two primers on target sequence	<2000 bases apart
12. Plateau Effect	Accumulation of product ≤0.3 to 1 pmol

Table 1 - Guidelines to follow for primer design for amplification of DNA. Primers must follow these guidelines if they are to work properly for amplification. (Innis & Gelfand, 1990b and Butler, 2005).

PCR inhibitors

A variety of natural and chemical materials have been shown to interfere with the PCR process. This interference prevents the amplification of the DNA template, therefore providing profiles that resemble degradation (Hudlow, Chong, Swango, Timken, & Buoncristiani, 2008). Inhibition can occur by binding directly to the polymerase leaving it unavailable to bind to the DNA template. An inhibitor can also interact with the DNA to prevent the primers from binding to the template, or can interact with the polymerase during extension affecting the size of the amplicon. Some examples of common PCR inhibitors are tannic acid, humic acid, and hematin. Tannic acid is a Taq polymerase inhibitor and affects the availability of the DNA, whereas humic

acid only affects the amount of DNA available for amplification. Hematin is also known as an inhibitor of Taq polymerase (Opel, Chung, McCord, 2010).

Overcoming Inhibitors. To overcome inhibition, the samples can be diluted and reamplified, in turn diluting the inhibitor. In situations where the inhibitor affects the function of Taq polymerase, additional Taq polymerase can be added so that it is available to bind to the DNA. Some inhibitors bind to magnesium, preventing it from binding to the Taq polymerase, therefore preventing amplification. Addition of more magnesium allows it to be present to bind to the polymerase and leads to amplification (Opel, Chung, McCord, 2010). Bovine serum albumin (BSA) can be added in samples that are contaminated with some chemicals, such as humic and tannic acid, leaving the polymerase free for amplification. Phenols present in plant matter bind to proteins via hydrogen bonds. The addition of BSA allows the phenols to bind to it instead of the polymerase allowing the polymerase to bind to the target sequence of DNA for amplification (Kreader, 1996). Other ways to remove inhibitors are by re-extracting using kits that are designed to help remove inhibitors, or even the use of a Microcon® to clean up the sample (President's DNA Initiative: DNA Analyst).

Characteristics of STRs

STRs are easy to use, but have many traits associated with them that need to be considered for the data analysis process. One characteristic is an artifact known as stutter. Stutter peaks charted on an electropherogram include one more or one less repeat than the true allele, most often occurring with one less repeat (Mulero, Chang, & Hennessy, 2006). This characteristic takes place less frequently as the size of the repeat unit increases. However, as the total length of the fragment increases, the chance for stutter also increases (Edwards, Civitello, Hammond, & Caskey, 1991). It is theorized that stutter occurs when the Taq polymerase slips

during the PCR process. Primer slippage is when the primer-template complex unpairs during extension and slips creating a loop. When stutter is present it becomes harder to determine if the peak present is stutter or in fact a true allele, making the profile interpretation more difficult, especially in the case of a mixture, (Walsh, Fildes, & Reynolds, 1996). Analytical thresholds are set to try and categorize any stutter that may occur in a profile and to determine if a peak is a true allele. If the peak passes the threshold, the analyst must determine the peak height ratio (PHR) to determine if it is a true allele or stutter. In the field, it has been agreed that the peak height ratio for stutter should not exceed 15% (Budowle et al., 2009).

Other artifacts that occur include dye blobs, pull-up, spikes, and noise. Dye blobs are disassociated primer dyes, and may occur because the fluorescent dye tags breakdown over time. An analyst can distinguish peaks associated with dye blobs, because they are usually broader than an allele peak. Some complications can still arise when analyzing a profile. If it becomes difficult for the analyst to tell if it is a dye blob or an allele, the sample can be reinjected. The peak may still reappear, but may have moved, indicating it is not reproducible and not a true allele (Applied Biosystems, 1988).

Pull-up is a result of the detection laser and spectral spacing failing to differentiate between the different fluorescent dyes, and can also occur if the data is oversaturated. These peaks will most likely be smaller than the other alleles in the dye channel, and will also directly correspond to an allele in an adjacent dye channel of the same base pair length. Peaks that are caused by fluctuation in voltage or the presence of air bubbles in the capillary are known as spikes. When present, spikes appear as sharp peaks with no distinguishable space between the sides of the peak. Crystals in the polymer or fluorescent material in the polymer or formamide can also cause spikes. Non-reproducible peaks along the baseline are known as noise, and are

caused by current fluctuation, air bubbles, or urea crystals. If close enough to the analytical threshold, noise can be confused as a true allele (President's DNA Initiative: DNA Analyst).

Non-template nucleotide addition can also appear in an STR profile. Taq polymerase naturally adds an adenine to the 3' carbon end of a PCR product. The addition of an adenine is known as adenylation. This phenomenon can create double peaks of different lengths at a single allele site known as split peaks. To prevent the addition of an extra nucleotide to the product, a guanine can be added to the 5' carbon end of the reverse primer (Brownstein, Carpten, Smith, 1996). Another way to overcome incomplete adenylation is to add an extra adenine to every fragment, so that all the fragments are uniform. Adding an adenine to the PCR product is the most common method, and is done by letting the PCR products sit at 72ºC for an extra 15-45 minutes at the end of the cycles. Taq polymerase is allowed time to add the extra nucleotide to every product. If not enough Taq polymerase is present to add an adenine to every product, incomplete adenylation can occur. Not having enough of the polymerase most often occurs when the DNA input is too large for the reaction (Clark, 1988). Overall, non-template nucleotide addition can be easy to distinguish if the peak heights are low enough in the profile. They can also be removed by diluting a sample that may be oversaturated.

Microvariants are another characteristic of STR loci. It manifests itself as an incomplete repeat that differs from a true allele by one or more base pairs, but never a full repeat (Crouse, Rogers, Amiott, Gibson, & Masibay, 1999). New microvariant alleles are constantly being discovered and are detected as off-ladder alleles. It has been shown that not only do partial repeat sequences occur, but insertion/deletions also occur in the flanking region. In the D7S820 locus of Hungarian and Albanian individuals, an insertion of multiple thymine nucleotides has

been observed. This causes many microvariants to occur at this locus, and predictably with one or three extra bases (Egyed et al., 2000).

Another characteristic of STRs that affect the number of alleles detected are as null alleles, which occur when an allele that is actually present in the DNA fails to amplify (Clayton, Hill, Denton, Watson, & Urquhart, 2004). A null allele can happen if there is a single nucleotide change in the primer binding site, not allowing the primer to bind and therefore preventing amplification of the region of DNA. This can make data interpretation difficult due to the given locus appearing homozygous when in fact it is heterozygous (Callen et al., 1993). For loci that frequently show null alleles, the manufacturer may produce a new kit with extra primers. This was the case with the STR kit Identifiler ® Direct. Some mutations were observed in the primer binding sites, so Applied Biosystems added new primers to the already existing primers of the Identifiler® kit. Doing this allowed the previous null alleles to be amplified (Applied Biosystems, 2009a).

Mutations such as those that occur in null alleles can happen anywhere in the DNA and are repaired or remain within the genome. Mutations can cause a primer not to bind resulting in a failure to amplify the region, or they can result in the number of repeats to be incorrectly expressed, showing a different allele than is truly present. Primer binding site mutations are not often seen because extensive studies are done with the primers being used to make sure that they work properly during the reaction (Clayton, Hill, Denton, Watson, & Urquhart, 2004).

Each mutation event causes genomic variation between generations, and the accumulation of variation results in individualization. Mutations within an individual, over time, integrate into a population which results in new alleles. As new alleles increase, so does the

genetic variability within a given population (Johnson, 1999). Variability of the genetic sequence occurs in the sections of repeated sequences.

Processing DNA for Forensic Use

Extraction. Extracting DNA from an evidentiary or known sample is the first step in the analysis process. DNA extraction is vital to the success of downstream analysis because it releases the DNA from the nucleus and separates it from other cellular material. Solid phase extraction is a frequently used form of DNA extraction, and uses a solid stationary phase to extract molecules from the liquid phase based on the physical properties of the DNA molecule. The DNA IQ™ system by Promega Corporation utilizes this technology. The DNA IQ™ system uses a paramagnetic beads solid phase to separate the DNA from other cellular material and inhibitors that may be present. This process is not perfect, and may leave some of the inhibitor present. If left in the sample, the paramagnetic beads are also known to inhibit the PCR reaction. Due to the negatively charged phosphate backbone of DNA, binding to the positively charged silica beads will occur. The DNA is separated from the beads and left in a purified form that is carried on to the quantification process. Though many extraction methods exist, the utilization of the DNA IQ™ system provides an easy and efficient way of extraction.

Quantification. Quantification is the step in the DNA analysis process that determines the amount of amplifiable DNA present in the sample. The DAB guidelines state that in order to comply with quality assurance for analytical procedures, an analyst must always perform a quantification step when it is appropriate (SWGDAM, 2010). In forensic science, real-time PCR is used to quantify the amount of amplifiable DNA. Quantification is performed by amplifying the human telomerase reverse transcriptase gene (hTERT) and measuring how much fluorescence occurs at the end of amplification, which directly corresponds to how much of the

gene is present. An accurate measurement is obtained by detecting a fluorescent probe attached to forward primer. Fluorescence occurs when the polymerase cleaves the quencher dye from the probe, allowing the reporter dye to fluoresce and be detected. The amount of DNA present is based on the cycle number at which the product crosses the threshold. This cycle number is then compared to a standard curve to determine the amount of amplifiable DNA present in the sample. After quantification, the samples go through the amplification process using PCR technology present in commercially available STR kits.

Separation. The PCR process produces several different DNA fragments that need to be separated for analysis. STR fragment sizes can be similar, and the DNA Advisory Board states that the precision of sizing should be established for allele designation (SWGDAM, 2010). Separation of amplified DNA occurs by a form of chromatography known as capillary electrophoresis. Capillary electrophoresis technology allows for single base pair resolution to be used to discriminate between the fragments. A stationary and a mobile phase interact together during the physical separation of molecules. Separation is based on the size and charge of the amplified DNA fragments. Since all the molecules have a negative charge, size is the key indicator for separation. The stationary phase in electrophoresis is the polymer that fills the capillaries, while the mobile phase is a medium that allows the samples to move with it. Throughout the entire separation process, the sample will be present in both phases, and is known as retention time (Poole, 2003).

An electrical current is added to the system, moving DNA away from the negative electrode and towards the positive electrode based on the natural repulsive force of the molecule (Jorgenson & Lukacs, 1981). As the molecules move along the capillary, they interact with the stationary phase (Figure 6). Polymer forms a series of pores that the DNA molecules must move

through. The larger DNA molecules have more interaction with the polymer and therefore move slower through the system. Based on this interaction, the smaller fragments of DNA will move through the capillary first, and each molecule thereafter will be bigger than the last. Separation resolving down to a single base pair resolution is accomplished by keeping the molecules single stranded with a formamide solution (Poole, 2003).

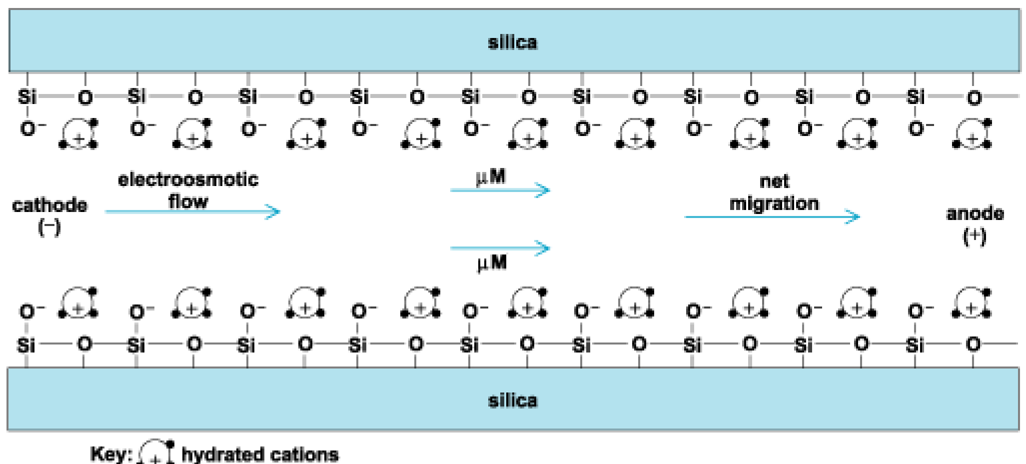


Figure 6 – Shows the direction of flow and the interaction of the molecule with each phase in the capillary during electrophoresis (McGraw-Hill, 2005)

The development of capillary electrophoresis has made the separation process in human identification more efficient and automated. Capillary electrophoresis uses an array of capillaries to run multiple samples simultaneously and consumes only a small amount of sample, allowing enough left over for the sample to be injected again if needed (Poole, 2003). A capillary has a high surface area to volume ratio, which allows heat to be dissipated more effectively; therefore, this process uses a higher voltage, making it faster than the previous slab gel methods of separation. A laser excitation source and a fluorescent detector are used to detect the samples as they move through the capillary. When the separation is complete, the results are available electronically, making it suitable to store and analyze results (Tagliaro, Manetto, Crivellente, & Smith, 1998).

Forensic use of STRs

STRs have many characteristics that make them suitable for forensic use of human identification. One of which is that partially degraded or limited amounts of DNA can be amplified to produce a profile (International Human Genome Sequencing Consortium, 2001). Forensic analysis of microsatellite DNA loci for the purpose of human identification is frequently utilized due to their small size, wide distribution, and high rate of polymorphism (Weber & Wong, 1993). Microsatellites are widely distributed throughout the genome, making it easier to find loci that are not genetically linked to one another. Microsatellites are also highly polymorphic, meaning that there are many different forms, or alleles, in a population. Being highly polymorphic helps create an abundance of possible allele combinations and also adds statistical value to the individualization of a profile (Rodrigues, LoVerde, Romanha, & Oliveira, 2002). There are 5-30 repeats at each STR site, meaning there could be up to 30 alleles for one locus. By using the product rule, increasing the number of loci increases the probability for individualization. Another characteristic of microsatellite alleles is that a heterozygous person will have relatively the same peak height for both alleles at the same locus. Since they are approximately the same size, PCR can be used to easily amplify STRs ensuring that all fragments will be completely replicated (Weber & Wong, 1993).

A full genetic profile can be created with one nanogram of DNA, even if the DNA is partially degraded, because only the regions of DNA that include the STRs need to be intact for amplification. Due to the nature of microsatellites, these markers typically are not affected by fragmentation. The technology of capillary electrophoresis allows for single base pair resolution, allowing fragments to be successfully be separated so that there is a clear distinction (Edwards, A., Civitello, Hammond, Caskey, 1991).

STRs have a low rate of mutation compared to previously used VNTRs, (Weber & Wong, 1993). Though the current alleles were created by mutation, it takes time for a new allele to integrate into the population (Johnson, 1999). In a study done in 1993, Weber and Wong determined that the mutation rate for chromosome 19 was "1.2 x 10^{-3} per locus per gamete per generation." They also stated that the mutations were more often full repeats. This would change alleles in the individual, but not in the population (Weber & Wong, 1993). In order for a new allele to be integrated into a population, a mutation must occur in an individual, and they must pass it on to their offspring. The mutation would have to keep passing from generation to generation to eventually become integrated.

STR analysis is ideal for human identification, because forensic analysis of DNA can be used to provide evidence for a variety of scenarios. Current circumstances in which human identification is utilized includes identifying genetic material from a crime scene, establishing paternity, identifying victims both from criminal activity and catastrophic disasters, and exonerating wrongly accused people. Using STR information has allowed research to be done in other life forms including identifying endangered species, detecting bacteria, matching organ donors with recipients, determining livestock pedigree, and authenticating consumables (Human Genome Project, 2009). Due to their genetic characteristics, STR use has significantly increased, and new uses will continue to be identified.

Use of Fluorescent dyes. Detection of amplified DNA during capillary electrophoresis is key to allowing analysts to view results. Fluorescent dyes can be attached to primers, allowing the fragments to be detected via a laser as they pass through the capillaries. In forensic science, it has become crucial for results to be obtained reliably, while at the same time quickly and efficiently. This can be done by multiplexing, which is the ability to amplify multiple fragments

at once. Using multiple dyes and STRs with different amplicon lengths, multiplexing has easily been incorporated into the field via commercial STR kits. The use of fluorescent dyes in these kits has placed the quality of the materials with the manufacturer, ultimately allowing results between laboratories to be consistent and reliable (Andersen et al., 1996). Combining fluorescent dyes and the information of the amplicon length of loci, manufacturers have increased the number of loci present to 16 and most recently 18 loci. Using this information, the match probability has been lowered without sacrificing the sensitivity of the system (Tamaki, 2007; Promega Corporation, 2011).

Identifiler® Plus

Applied Biosystems has provided the instruments most widely used in the field, complete with their own commercial STR kits. In the infancy of STR technology, it was standard to use multiple kits to obtain the core CODIS loci. In 2006, Applied Biosystems developed their first commercial kit which included the 13 core loci, amelogenin, and two additional loci. The Identifiler® STR kit was and still is widely used in the forensic science community. As with any product, improvements were made and in 2010, the next generation kit, Identifiler® Plus was released.

Since Identifiler® Plus is an improvement on the first generation, there are a lot of similarities between the two. Primer sequences, control DNA, dye set, reaction volume, and cycle number have all remained the same. The same 16 loci are included, which means the same allelic ladder is used. The amplicon sizes remain the same using the same non-nucleotide linkers for spacing.

Improvements were made to make results more robust and to increase sensitivity of the kit. An improved buffer allows for higher peaks, and the inclusion of BSA helps with inhibited

samples. A slight modification to Taq polymerase allowing for heat activation, allows the polymerase to be incorporated into the master mix component. Cycling conditions have changed to enhance sensitivity, by changing the ramping speeds to combine the annealing and extension steps. The manufacturing of the primers has improved, resulting in a cleaner baseline. All of these changes allow the Identifiler® Plus STR kit to produce results from samples that previously did not provide any, and to allow for a higher level of confidence (Applied Biosystems, 2010).

Materials and Methods

The purpose of this project was to validate and determine the limitations of the Identifiler® Plus STR kit. All tests were conducted in the biology laboratory in Howell Hall at the University of Central Oklahoma (UCO). Samples were obtained from whole blood that was provided by a contractor (Innovative Research, Novi, MI) and was tested for blood-borne pathogens before being received. For all validation and inhibitor studies, a single female blood source was used throughout. A second female source was used for the mixture study. After receiving the aforementioned blood samples, a representative sample was placed on a Whatman® FTA® card (Fisher Scientific, Fair Lawn, NJ, WB120205) for storage and extraction purposes. FTA cards were designed to preserve blood samples for long-term storage and transportation. Upon application of blood to the FTA card, the cells become lysed and the DNA is preserved in the matrix of the cards (Sigma-Aldrich, 2011). Extracted DNA was obtained from reference samples, quantified to determine the concentration, amplified, and separated based on fragment length for STR analysis. Data collected from STR analysis were analyzed using GeneMapper® version 3.2.1 software (Applied Biosystems, Foster City, CA, 4359225).

Extraction

Extraction of DNA from blood on FTA cards was performed using Promega's DNA IQ™ kit (Promega Corporation, Madison, WI, DC6700). For a given sample, a 4mm^2 cutting of the stain was added to a microcentrifuge tube, and 100µL of lysis buffer was added to cover the sample. Samples were heated to 70ºC for 30 minutes in a Thermolyne Type 16500 Dri-Bath (Thermo Scientific, Waltham, MA, D816525). Samples were removed from the heat source, the cutting was removed from the microcentrifuge tube, placed into a spin basket (Promega Corporation, Madison, WI, V1221), and placed back in the tube. The microcentrifuge tube

containing the spin basket was inserted in an Eppendorf Centrifuge 5430 (Eppendorf, Hauppauge, NY, 022620525) for two minutes at room temperature to remove the majority of lysis buffer from the sample. Seven microliters of well-mixed stock resin were added to each sample. Samples were incubated for five minutes at room temperature, vortexing once every minute. Following incubation, all tubes were placed on the Promega magnetic stand. Doing so allowed for separation to take place between the liquid and paramagnetic beads. Upon complete separation of the lysis buffer and paramagnetic beads, the lysis buffer was removed and 100µL of lysis buffer was added to the tube. The samples were removed from the magnetic stand, vortexed, placed back on to the magnetic stand. Following separation of the lysis buffer and paramagnetic beads, the buffer solution was once again removed. One hundred microliters of wash buffer was added to each sample, vortexed, placed back in the stand to allow separation, and the wash buffer was removed. The wash buffer step was repeated for a total of three times. Following the wash step, the paramagnetic beads were allowed to air-dry for five minutes. To the dried sample, 40-50µL of elution buffer was added to the tube and allowed to incubate for five minutes at 65ºC. The elution buffer containing the DNA was removed and placed in a clean tube (Promega Corporation, 2009).

Quantification

Quantification of the DNA was performed using Applied Biosystems' Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Warrington, UK, 4343985). A standard curve, using a DNA standard of known concentration, was made for comparison to the unknown samples to determine their concentration. The DNA standard, with a concentration of 200ng/µL, was diluted to 50ng/µL. A serial dilution was made from this concentration to range from 50ng/µL to 0.023ng/µL. A solution of reagents was created by adding 10.5µL of Quantifiler

Human Primer Mix and 12.5µL of Quantifiler PCR Reaction Mix per sample to a tube. Twenty-three microliters of this solution was added to each well of the MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA, N8010560) that contained a sample. Two microliters of standard or sample was added to the appropriate wells. The plate was sealed with a MicroAmp® Optical Adhesive Film (Applied Biosystems, Foster City, CA, 4311971) and placed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, 4345241). Results were viewed using the 7500 System SDS version 1.2.3f2 (Applied Biosystems, Foster City, CA,) (Applied Biosystems, 2006).

Amplification

Amplification of STRs was performed using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, Warrington, UK, 4427368). A solution of reagents was created by adding 10µL of AmpFℓSTR® Identifiler® Plus Master Mix and 5µL AmpFℓSTR® Identifiler® Plus Primer Set per sample to a tube. Fifteen microliters of this solution was added to each 0.2 mL PCR tube (USA Scientific, Ocala, FL, 1602-4300). The volume of DNA added to the PCR tubes depended on the concentrations obtained during quantification. This number could not exceed 10µL of DNA sample. For a positive control, 10µL of 9947A control DNA was added to the PCR tube, while 10µL of sterile water was added to a second tube for a negative control. Enough water was then added to each tube to bring the final volume up 25µL. The PCR tubes were capped, vortexed, and centrifuged before being placed in the GeneAmp® PCR System 9700 with the Silver 96-Well Block (Applied Biosystems, Foster City, CA, N8050200) for amplification. The Identifiler® Plus amplification profile is shown in Table 2.

Initial Incubation Step	Cycle (28 cycles)		Final Extension	Final Hold
	Denature	Anneal/Extend		
HOLD	CYCLE		HOLD	HOLD
96ºC 11 min	94ºC 20 sec	59ºC 3 min	50ºC 10 min	4ºC ∞

Table 2 - AmpFℓSTR® Identifiler® Plus PCR Amplification Profile. An initiation step has been added with the introduction of hot start technology. Identifiler® Plus has combined the annealing and extension steps in this new generation. A final extension is present so Taq polymerase can add an additional adenine to all amplicons.

After amplification, a solution was created by adding 0.2µL GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Warrington, UK, 4322697) and 8.8µL Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, 4311320) per sample to a tube. Nine microliters of the solution was added to each well that contained a sample. One microliter of PCR product or 1.2µL of allelic ladder was added to the appropriate wells. A snap cooling step was performed by placing the plate on the thermal cycler for three minutes at 95°C then immediately placing it on ice for three minutes. The plate was sealed with a 96-Well Plate Septa (Applied Biosystems, Foster City, CA, 4315933) and place on the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, 627-0040) (Applied Biosystems, 2009).

Internal Validation

According to the DNA Advisory Board, all laboratories must perform an internal validation on all new kit chemistries (SWGDAM, 2010). The following phase is in accordance with these standards.

Baseline Study. In order to determine the best injection condition and the detection threshold, six negative controls with an Internal Lane Standard (ILS) were injected according to the manufacturer's protocol. The plate map was set up to run continuously through each of the following injection conditions: 1 kilovolt 5 seconds, 1kV 11 sec, 1kV 22 sec, 2kV 5 sec, 2kV 11 sec, 2kV 22 sec, 3kV 5sec, 3kV 11 sec, and 3kV 22 sec.

After the samples were analyzed on the Applied Biosystems 3130 genetic analyzer, the highest peak for every dye channel for every negative control was recorded, along with the average peak height and the standard deviation. The threshold for each dye channel was calculated by doubling the height of the highest peak. To determine the detection threshold for an injection condition, the highest threshold of the four dyes was used.

Dynamic Range. Ten different masses of DNA were amplified: 5ng, 2.5ng, 1.25ng, 0.63ng, 0.31ng, 0.16ng, 0.08ng, 0.04ng, 0.02ng, and 0.01ng. This method was repeated five times. After analyzing the results, the profiles were evaluated for off scale, full, and usable profiles. The dynamic range was determined by recording the lowest amount of DNA that produced a full profile, and recording the highest amount of DNA that produced a full profile without going off-scale.

Precision and Reproducibility. The precision and reproducibility of the system was determined using data from the same five amplified sets of DNA previously analyzed. For precision of the system, the base pairs for each allele for each profile were recorded. Since the fragments should always be the same size, base pairs can be used to compare allele migration and therefore precision of the systems. The base pairs of the alleles were then compared to the allelic ladder in the same run. The mean and standard deviation of the alleles were calculated. For reproducibility, alleles were recorded and compared to each profile to make sure that the same alleles were called every time.

Stochastic Threshold. Stochastic threshold used the same five sets of amplified DNA. The peak height ratios for heterozygous loci were calculated by dividing the shorter peak's RFUs (reflective fluorescent units) by the taller one. No points above a 60% peak height ratio were analyzed, and a threshold was set at the point where a rapid drop-off in the peak height ratios

began. An equation (mean PHR – 3* std. dev = stochastic threshold percentage) was used to help determine the percentage at which this drop off would occur (Promega Corporation, 2006).

Stutter. The percent of stutter was calculated for every allele for every profile. To calculate stutter the analysis method was altered, setting the threshold and the stutter distance to zero. This was to allow every single peak to show up on the electropherogram so that all stutter could be calculated. The peak height for the allele in the stutter position was divided by the peak height of the true allele. After analyzing the data, a stutter percentage was determined for each locus.

Mixture Study. A mixture study was conducted using two blood sources at the following ratios: 0:1, 19:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:19, 1:0. Five samples from each source were extracted and quantified. They were then diluted to equal the sample with the lowest concentration. This allowed all samples to be equal so that a unit of microliters could be used to make the ratios. The resulting electropherograms were analyzed to set interpretation guidelines based on a previous study (Budowle et al, 2009).

Contamination Study. A contamination study was conducted using two plates set up in a checkerboard pattern. Plates contained wells with samples and blank wells; all wells contained ILS and Hi-Di formamide. Having an ILS in the blank wells allowed for any peaks that may have shown up to be sized and called as an allele. This study was to determine if contamination occurred from well to well from the capillaries.

Mock Case. A qualifying test was performed to make sure that the genotypes obtained were correct for the known references. A Collaborative Testing Services, Inc. (CTS) test was used as mock case evidence. There was one blood stain of unknown origin and two blood stains of known origin. The stain of unknown origin (questioned sample) was processed first and at a

separate time and place from the known references. This was accomplished by sterilizing the work area and tools for 15 minutes with UV light. After analysis, the questioned sample was compared to the known references.

At the conclusion of this validation study, parameters were set that were followed for the environmental insults study and by anyone in the UCO laboratory that used this STR kit in the future.

Environmental Insults

The goal of this study was to test the limitations and strengths of the Identifiler® Plus kit. According to the DAB standards, the limitations of a kit must be set, and samples must be tested that mimic those that may appear in casework (SWGDAM, 2010). This was done by using environmental insults that may encountered in casework and to determine how well the Identifiler ® Plus kit responded to these insults.

UV Light. The use of UV light at an intensity of 254nm was used to simulate exposure to sunlight at the times of 0, 30, 60, 100, 200, 300, and 400 seconds. An intensity of 254nm was used because it was found to efficiently degrade DNA (Pang & Cheung, 2007). A sample of DNA was diluted to a 1ng/µL concentration. Three microliters of DNA were placed in a PCR tube. Five tubes for each time were used, resulting in a total of 35 sampels. The samples that were to be exposed to UV light were placed in a tube tray and placed on the shelf of the Air Clean 600 PCR Workstation (USA Scientific, Ocala, FL, AC648LFUVC-4852). The UV light was turned on and the tubes were left exposed for the allotted time. One microliter of DNA from each tube was added to the PCR tubes for amplification.

Humic and Tannic Acid. Humic (The Right Chemicals – Alfa Aesar, Ward Hill, MA, 41747) and tannic (Fisher Scientific Education, Hanover Park, IL, 1401-55-4) acids were used

to replicate exposure to soil contaminants, both at concentrations of 0, 50, 100, 200, and 400ng/µL. Concentrations of humic and tannic acid were obtained by weighing out 0.01g of each acid and placing it into separate tubes. One milliliter of sterile water was added to each tube. The concentration of the initial sample was 400ng/µL. A dilution was made by adding 500µL of sterile water to a tube, and adding 500µL of the initial concentration to the tube. The serial dilution was continued until 50ng/µL was obtained. One microliter of the inhibitor was added to the PCR tubes for amplification. Five sets of tubes each containing different concentrations of the inhibitor were amplified, totaling 25 samples.

Hematin. Hematin (MP Biomedicals, LLC, Solon, OH, 198969) simulated contact to hemoglobin, a contaminant in blood, at the concentrations of 0, 125, 250, 500, and 1000µM (Applied Biosystems, 2009b). Hematin is not soluble in water, so a 0.1N NaOH solution was made. The NaOH solution was made by weighing out 0.01995g of 97%+ NaOH pellets (Sigma Aldrich, St. Louis, MO, 1310-73-2) and adding it to a tube. Five milliliters of water was then added to the tube to fully dissolve the NaOH pellet. To make the hematin solution, 0.06335g of hematin was added to a tube. One milliliter of the 0.1N NaOH solution was added to the tube. The concentration of the initial solution was 4,000µM. A dilution was made by adding 500µL of the solution to a tube and adding 500µL of the NaOH solution. Another dilution was done the same way, which then resulted in the 1000µM. This concentration was the greatest concentration needed for the hematin study. A serial dilution was performed exactly as stated above until a 125µM concentration was obtained. One microliter of the inhibitor was added to the PCR tubes for amplification. Five sets of tubes containing samples were amplified for each concentration of inhibitor, totaling 25 samples.

The results of these tests were analyzed and the concentration resulting in the first loss of an allele was recorded, as well as the concentration at which each insult provided a complete loss of a profile. This provided information on the limitations of the Identifiler® Plus STR kit.

Results

It is mandated by SWGDAM and the DAB that all kits used for forensic DNA analysis must be validated for every lab. Each validation process is composed of individual studies that compile the results together for an overall conclusion. Each study is conducted in a certain order to build off of the previous results. Below is the data obtained from this internal validation of the Identifiler® Plus STR kit for the UCO laboratory.

Validation Study

Baseline Study. The results of this study provided a range of peak heights from 18-214RFUs across every dye channel for each injection. Illustrated in Figure 7 were the highest peak heights observed for each injection condition. Each of these peak heights corresponded with the green dye channel. Also illustrated was the standard deviation for each injection condition.

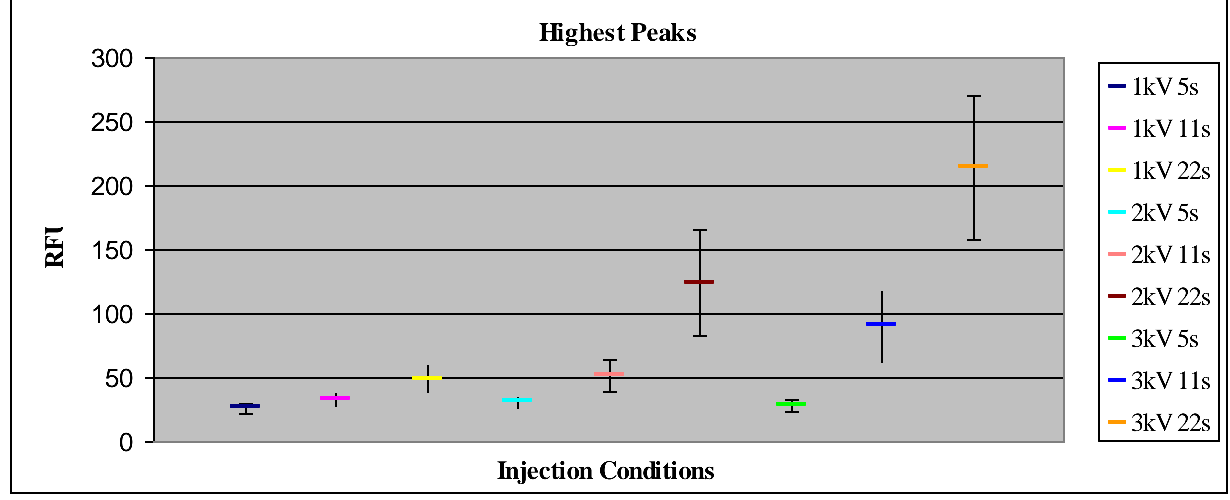


Figure 7 – Shown above are the highest peaks observed for each injection condition over all dye channels. Error bars represent the standard deviation calculated for each data set. Conditions using a five second injection time show the lowest standard deviation, and 3kV 5 seconds proved to be the best injection condition.

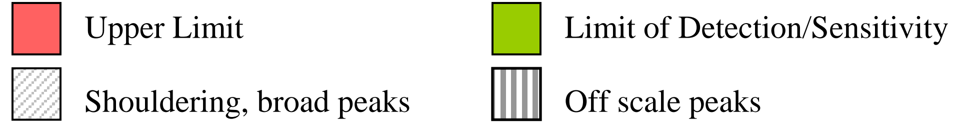
Within the data set, a pattern was seen. For each condition, the peak heights and standard deviations increased with the length of injection time. This resulted in the highest peak height

being recorded at 214 RFUs and the greatest standard deviation at 55.82 RFUs. After analyzing all the results, the injection condition of 3kV 5 seconds was chosen as the optimal condition for analysis. The highest peak observed in the 3kV 5 seconds condition was doubled and rounded up to set the analytical threshold. An analytical threshold of 60 RFUs was chosen to distinguish the true alleles from electrophoretic noise within the baseline.

Dynamic Range. Results of this study were recorded for the concentration range of 5.0 to 0.01ng of DNA. Table 3 exemplifies a summary of what was observed. At the upper end of the range, 5.0 and 2.5ng both produced off scale data. The greatest concentration also produced signs of peak broadening and shouldering. The middle concentrations of 1.25-0.08ng had full profiles with only the occasional off scale peak present at 1.25ng. The profiles within this off scale data were easy to distinguish. At the lower end of the concentration range, allele dropout occurred starting at 0.04ng. Sample FTA4b appeared to be an outlier in this study by not having off scale data for 2.5ng and starting allele drop out at 0.08ng. Though an outlier, the samples were still used to help determine the dynamic range. To alleviate any off scale data that may occur, the upper limit of the dynamic range was determined to be 1.0ng. Even though FTA4b possessed allele dropout at 0.08ng, this concentration was included in the dynamic range as the limit of detection.

	5.0ng	2.5ng	1.25ng	0.63ng	0.31ng	0.16ng	0.08ng	0.04ng	0.02ng	0.01ng
ERFTA1b	OS	OS	full profile	full profile	full profile	full profile	full profile	full profile	-9 alleles	-21 alleles
ERFTA2b	OS	OS	OS	full profile	full profile	full profile	full profile	-2 alleles	-14 alleles	-17 alleles
ERFTA3a	OS	OS	OS	full profile	full profile	full profile	full profile	full profile	no profile	no profile
ERFTA4b	OS	full profile	full profile	full profile	full profile	full profile	-7 alleles	-2 alleles	-4 alleles	-27 alleles
ERFTA5b	OS	OS	full profile	full profile	full profile	full profile	full profile	-2 alleles	-13 alleles	no profile

Table 3 – The results for the dynamic range study can be seen above. The samples with 5.0ng of DNA showed shouldering and broadened peaks along with off scale data. Samples with 2.5ng of DNA showed off scale data. FTA4b was an outlier at these two concentrations as well as at 0.08ng of DNA. Some samples at 1.25ng of DNA showed off scale data, but the profiles could easily be distinguished.



After the baseline and dynamic range studies were completed, the remaining results were obtained within these parameters. No data outside of the dynamic range was used for analysis. Since 1.0ng was considered the upper end of the dynamic range and was not directly tested, the 1.25ng samples were used in its place. These samples were expected to produce similar results as the 1.0ng for the remaining studies of the internal validation.

Precision and Reproducibility. Results were obtained by comparing the base pairs of the profiles to the base pairs of the allelic ladder run with the sample. Compared in Figure 8 were three standard deviations of the allele size for each locus. The standard deviation that was plotted was the highest produced per locus.

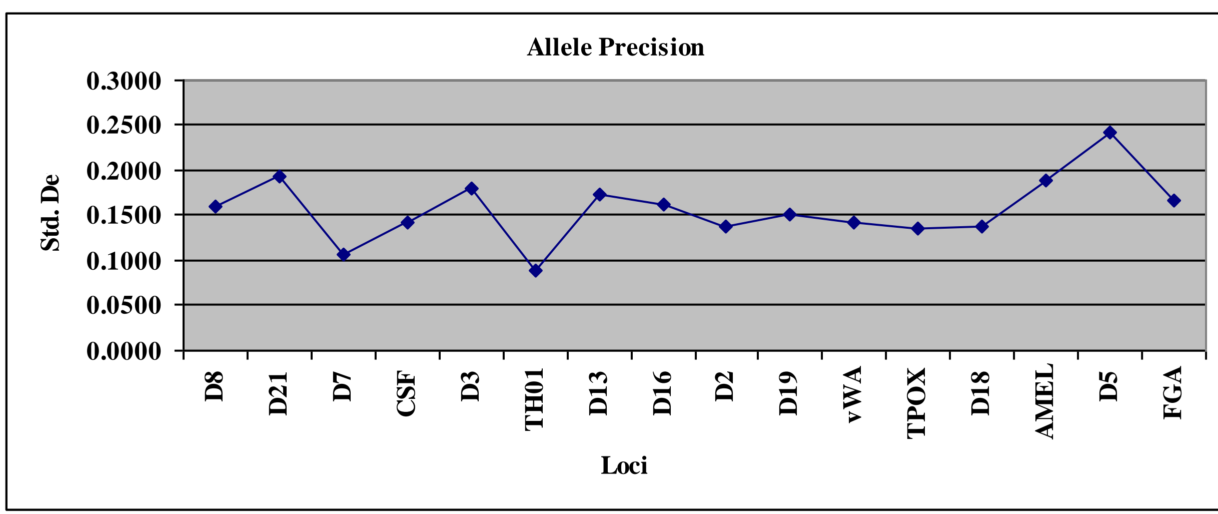


Figure 8 - The chart shows the standard deviation for each locus. For each locus, the standard deviation is less than 0.5 base pairs, which falls below the 0.5 base pair mark that all loci must fall below in order to be considered precise.

All the data fell well below 0.5 base pairs. The data must fall below the 0.5 base pair mark in order for the system to be precise. Alleles D21S11, AMEL, and D5S818 showed the most deviation with D5S818 producing a 0.24 base pair standard deviation, while TH01 produced the least deviation of 0.09 base pairs.

For reproducibility, it was demonstrated that all samples produced the expected profile. Based on the results, it was concluded that the system would provide reproducible results. A chart of the profile obtained from each sample was illustrated in Appendix A.

Stochastic Threshold. There were a total of 50 samples, five samples per ten concentrations. Included were 536 data points ranging from 61 to 9395 RFUs. The mean peak height ratio was 87.9% and the standard deviation was 10.9%. Figure 9 arranged all data points with the highest peak of the heterozygous pair plotted against the peak height ratio. Figure 10 illustrates only the data points up to 1000 RFUs. This allowed the data points to be spread out and the peak height ratio drop off point to be easier to see.

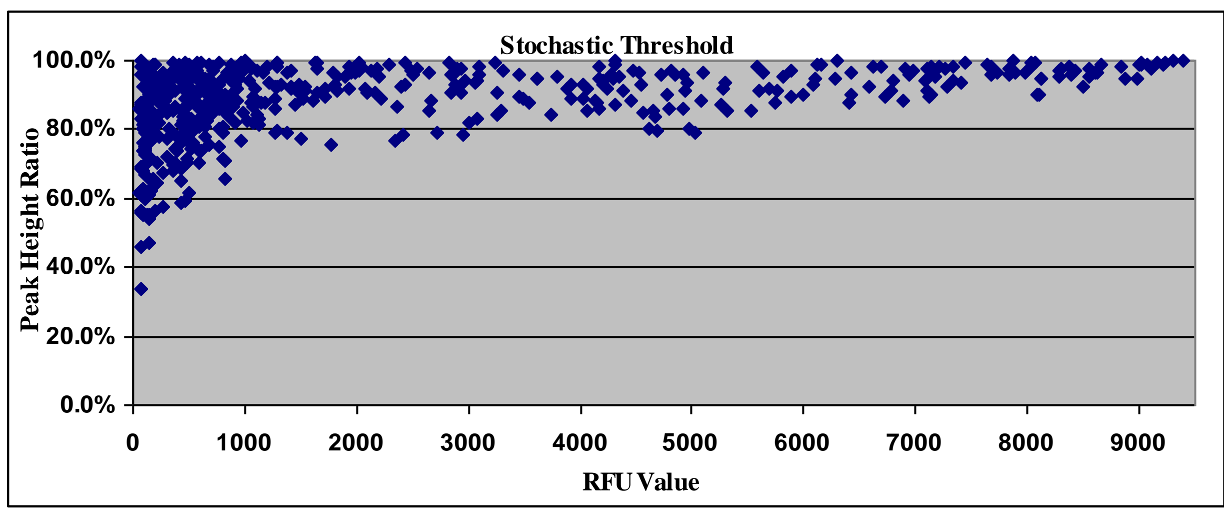


Figure 9 – All 536 data points can be seen above. Most of the points observed in the study are present below the 1000 RFU mark. Only analyzing the points below 60% provides just 14 points to determine the threshold.

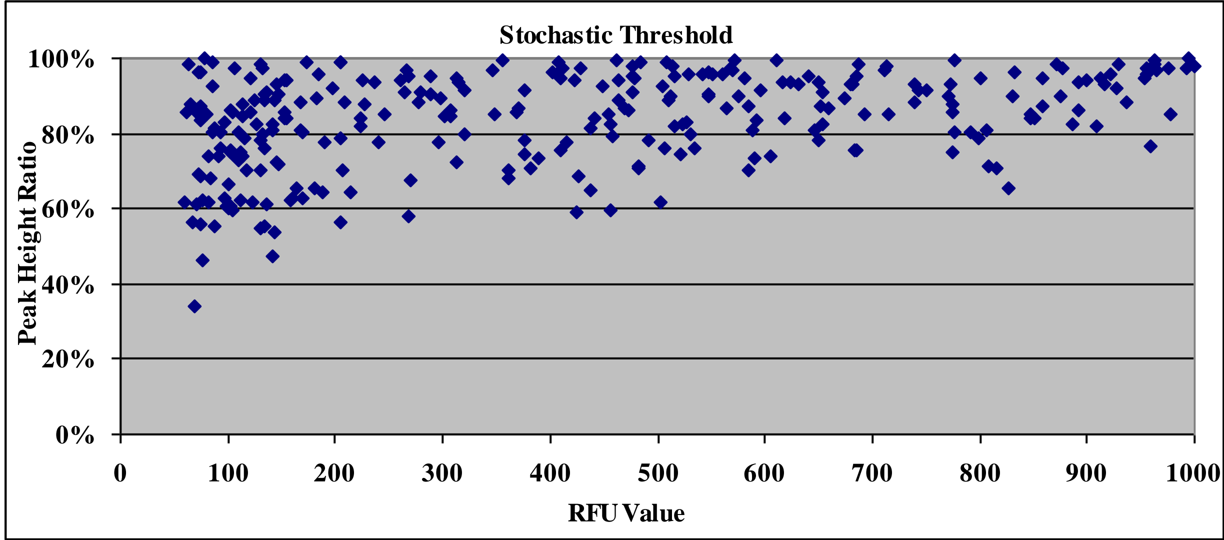


Figure 10 - Peak height ratios for all heterozygous loci results across all concentration for each FTA card in the range of 0-1000 RFUs. The smaller range was used so as better to see the point of dropoff. A cluster of points from 131-143 RFUs can be seen at about 55%. This is the point of dropout used to determine the threshold.

To determine the stochastic threshold, no points above 60% were analyzed. This leaves just 14 points used to determine where the drop off in peak height ratios occurred. There was a cluster of points from 131-143 RFUs indicating the point of the drop off. The highest value was taken and rounded up to result in a stochastic threshold of 150 RFUs.

Stutter. Figure 11 exhibited the data range for the stutter study. The chart mapped out stutter percentages observed at each locus. Stutter was mostly seen with concentrations above the dynamic range. Very little was present within the dynamic range itself. Likewise, +4 stutter was hardly seen over the course of the study.

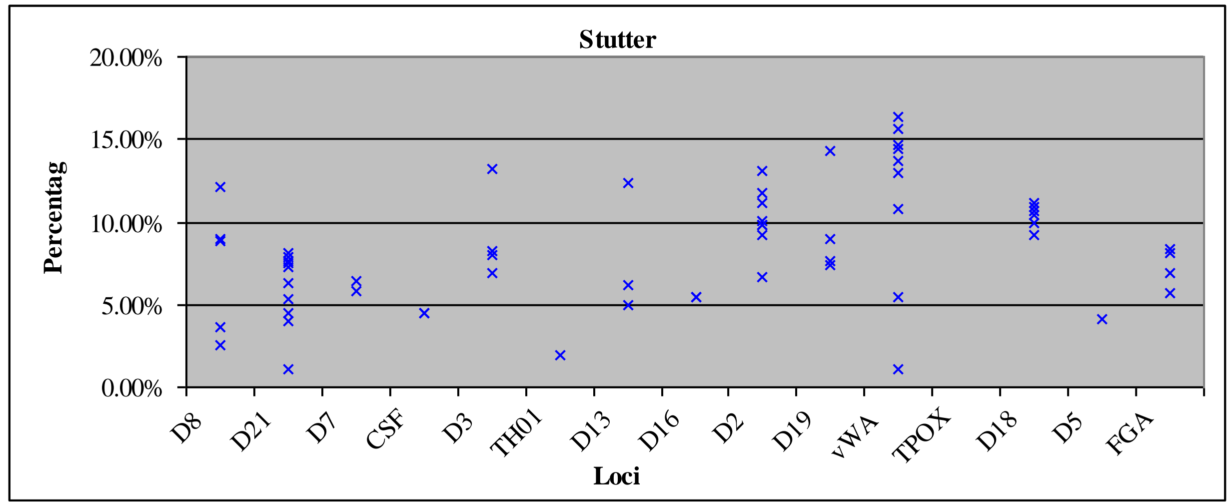


Figure 11 – A comparison of the stutter percentages observed throughout the internal validation. Percentages outside the dynamic range are also illustrated since this is where most of the stutter occurred.

The highest percentage observed for each locus can be seen in Table 4, along with the manufacturer's recommended percentages. Table 4 also provided the suggested stutter percentage at the completion of the study. Stutter percentage was calculated for each locus by comparing the percentages seen in the internal validation and the manufacturer's recommendations. Of the two, the highest percentage per locus was taken and recorded for the suggested stutter percentage of the study.

Stutter Percentages								
	D8	D21	D7	CSF	D3	TH01	D13	D16
Internal validation	12.15%	7.91%	6.41%	4.53%	13.16%	1.97%	12.39%	7.77%
Manufacturer	10.32%	10.67%	9.69%	9.20%	12.27%	4.08%	9.93%	10.39%
suggested	***12.15%***	***10.67***	***9.69***	***9.20***	***13.16%***	***4.08***	***12.39%***	***10.39***
	D2	D19	vWA	TPOX	D18	D5	FGA	
Internal validation	13.05%	14.27%	14.72%		11.10%	4.12%	8.37%	
Manufacturer	12.44%	11.21%	12.45%	6.38%	13.68%	10.06%	13.03%	
suggested	***13.05%***	***14.27%***	***14.72%***	***6.38***	***13.68***	***10.06***	***13.03***	

Table 4 – Comparison of the stutter ratios observed in this study and the manufacturer's suggested ratios. Comparing the two percentages, the higher one observed was chosen and can be seen as the final suggested stutter percentage for each locus.

Mixture Study. The results of the mixture study were placed in Table 5. The table shows what each allele call was for the major and minor profiles in the samples. Concentrations are listed in order from a single profile from one source to a single profile from the other source. Since the study was done in duplicate, the ratios are listed together so a comparison from each sample can also be compared. A legend was placed below to explain what various symbols present in the chart indicated.

Loci	1:0-1	1:0-2	1:4-1	1:4-2	1:9-1	1:9-2	1:19-1	1:19-2	1:1-1	1:1-2	19:1-1	19:1-2	9:1-1	9:1-2	4:1-1	4:1-2	0:1-1	0:1-2
D8	12,17	12,17	14,15 (12,17)	14,15 (12,17)	14,15 (12,17)	14,15 (12,N)*	14,15 (12,17)*	14,15 (12,N)*	12,14,15,17	12,14,15,17	12,17 (15,N)*	12,17 (N)	12,17 (14,15)*	12,17 (14,15)	12,17 (14,15)	12,17 (14,15)	14,15	14,15
D21	29,32.2	29,32.2	30,32.2 (29,N)	30,32.2 (29)*	30,32.2 (N)	30,32.2 (N)	30,32.2 (N)	30,32.2 (N)	30,32.2 (29,N)	29,30 (32.2)	29,32.2 (30,N)*	29,32.2 (N)	29,32.2 (30)*	29,32.2 (30,N)*	INDIST.	29,32.2 (30,N)	30,32.2	30,32.2
D7	12	12	10 (12)	10 (12)*	10 (12)*	10 (N)	10 (12)*	10 (N)	10 (12)	10 (12)	12 (10,N)*	12 (10)*	12 (10)*	12 (10)	12 (10)	12 (10)	10	10
CSF	10	10	10 (13)	10,13 (N)	10,13 (10)	10,13 (10)	10,13 (N)	10,13 (N)	10,13 (N)	10,13 (N)	10 (N)	10 (N)	10 (13,N)*	10 (13,N)*	10(13,N)*	10 (13,N)*	10,13	10,13
D3	15,16	15,16	16(15)	16 (15)	16 (N)	16 (N)	16 (N)	16 (N)	16 (15)	16 (15,N)	15,16 (N)	15,16 (N)	15,16 (N)	15,16 (N)	15,16	15,16 (N)	16	16
TH01	7,8	7,8	8,9.3 (7)	8,9.3 (7)	8,9.3 (7,N)	8,9.3 (7)	8,9.3 (7,N)	8,9.3 (7,N)	7,8,9.3	7,8 (9.3,N)	7,8 (9.3,N)	7,8 (9.3,N)	7,8 (9.3,N)	7,8 (9.3,N)	7,8 (9.3,N)	7,8 (9.3,N)	8,9.3	8,9.3
D13	12,13	12,13	8,9 (12,13)*	8,9 (13,N)*	8,9 (13,N)*	8,9 (N)	8,9 (12,N)*	8,9 (N)	8,9 (12,13)	8,9 (12,13)	12,13 (N)	12,13 (N)	12,13 (8,9)*	12,13 (9,N)*	8,9,12,13	12,13 (8,9)	8,9	8,9
D16	11,13	11,13	9,12 (11,13)	9,12 (11,13)	9,12 (11,13)	9,12 (11,13)*	9,12 (11,13)*	9,12 (11,13)*	9,11,12,13	9,11,12,13	11,13 (12,N)	11,13 (12,N)	11,13 (9,12)*	11,13 (12)	11,13 (9,12)	11,13 (9,12)	9,12	9,12
D2S	25,27	25,27	16,25 (N,27)	16,25 (25,27)*	16,25 (25,27)*	16,25 (25,27)*	16,25 (N)	16,25 (27,N)*	16,25 (27,N)	25 (16,27)	25,27 (16,N)*	25,27 (16,N)*	25,27 (16,N)*	25,27 (16,N)	25,27 (16,N)	25,27 (16,N)	16,25	16,25
D19	14.2,15.2	14.2,15.2	12,14 (14.2,15.2)	12,14 (14.2,15.2)	12,14 (14.2,15.2)	12,14 (14.2,15.2)*	12,14 (14.2,15.2)*	12,14 (15.2,N)*	12,14,14.2,15.2	12,14,14.2,15.2	14.2,15.2 (14,N)*	14.2,15.2 (12,N)*	14.2,15.2 (12,14)*	14.2,15.2 (12,14)	14.2,15.2 (12,14)	14.2,15.2 (12,14)	12,14	12,14
vWA	15,18	15,18	16,19 (15,18)*	16,19 (15,18)*	16,19 (N)	16,19 (18,N)*	16,19 (N)	16,19 (N)	15,16,18,19	15,16,18,19	15,18 (N)	15,18 (N)	15,18 (N)	15,18 (19,N)	15,18 (16,19)*	15,18 (16,19)	16,19	16,19
TPOX	7,8	7,8	8,9 (7,N)	8,9 (7)*	8,9 (7,N)*	8,9 (7,N)	8,9 (7,N)	8,9 (N)	7,8,9	7,9 (8,N)	7,8 (9,N)*	7,8 (9,N)*	7,8 (9,N)*	7,8 (9,N)	7,8 (9,N)	7,8 (9,N)	8,9	8,9
D18	18,20	18,20	14,18 (20,N)	14,18 (20,N)*	14,18 (20)	14,18 (20,N)	14,18 (N)	14,18 (N)	18,20 (14,N)	14,18,20	18,20 (N)	18,20 (14,N)*	18,20 (14,N)*	18,20 (14,N)	18,20 (14,N)	18,20 (14,N)*	14,18	14,18
AMEL	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)	X(X)
D5	10,11	10,11	11,12 (10,N)*	11,12 (10)*	11,12 (10)*	11,12 (N)	11,12 (N)	11,12 (10,N)*	10,11,12	10,12 (11)	10,11 (N)	10,11 (N)	10,11 (N)	10,11 (N)	10,11 (12,N)*	10,11 (12,N)*	11,12	11,12
FGA	21,23	21,23	22,24 (21,23)*	22,24 (21,23)*	22,24 (21,23)*	22,24 (21,N)	22,24 (N)	22,24 (21,23)*	23,24 (21,22)	21,22,23,24	21,23 (N)	21,23 (22,N)*	21,23 (22)*	21,23 (22,N)	21,23 (22,24)*	21,23 (22,24)*	22,24	22,24

Table 5 - Profiles resulting from mixture deconvolution for each ratio are summarized above. Ratio 4:1-1 had some indistinguishable loci, but the 1:1 ratios were the samples producing the most indistinguishable loci. Dropout started at the 1:9 and 9:1 ratios. This is also where the major/minor distinction became evident.

*minor not included in statistics ( ) minor N – possible allele included or not include

Some mixtures could easily be distinguished while others could not be deconvoluted. Sample 4:1-1 had a few loci that were indistinguishable, but most of the indistinguishable loci were in the 1:1 ratios. Dropout was seen starting at the 1:9 and 9:1 ratios. Also starting with these ratios and for ratios 1:19 and 19:1, a major and minor profile was easily distinguished. There was no clear ratio where the minor profile fell below the stochastic threshold. Minor profiles that could not be used for statistics were scattered across all ratios. After analyzing all the data, ratios of 1:4, 1:1, 4:1 or ratios close to these should not be attempted because too many factors arise that make deconvolution difficult, and can lead to a wrong allele call for a profile.

Contamination Study. The contamination study was done with two plates set up in a checkerboard pattern. Figure 12 demonstrated what one plate setup looked like. Wells were filled alternating between a sample or a blank well that contained Hi-Di formamide and an ILS. In this figure, the red cells represented a sample and the white cells represented the blanks. The second plate for the study had the samples in the opposite wells as the plate shown below.

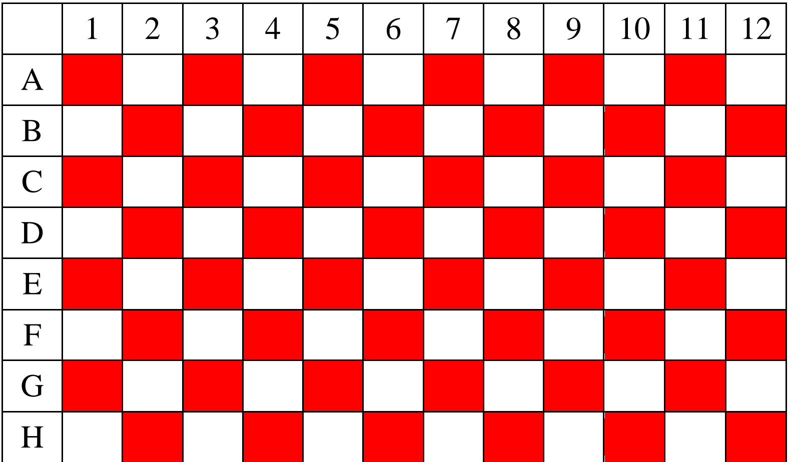


Figure 12 - Checkerboard pattern representing the plate set up for the contamination study. White cells represent the blank wells while the red cells represent the well with a sample. No contamination was seen suggesting the system of the genetic analyzer will not be a source of contamination if some occurs.

Hi-Di Formamide and ILS

Sample

No contamination was seen in the two plates, showing that the system of the 3130 genetic analyzer was capable of running samples without the analyst fearing contamination. If contamination did occur, the analyst could easily rule out the genetic analyzer and look for other instances that it may have occurred.

Mock Case. The final study of the validation was analyzing samples that represented casework. These samples demonstrated to the analyst that all parameters previously set and combined work correctly, and could be used for casework. For this validation, a CTS test was used for the mock case samples. Results were analyzed and the profiles obtained can be seen in Table 6. These profiles were compared to the information provided by CTS regarding what the true profile was; the data can be seen in Appendix B. After comparison, all profiles were identified correctly. The mock case showed that the Identifiler® Plus STR kit can produced the correct profiles from unknown casework samples. The study also showed that the parameters set in the previous studies were sufficient in producing reliable results.

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
Q1	13, 14	28, 32.2	9, 11	12, 12	17, 18	7, 8	11, 12	11, 12	18, 21	14.2, 15	18, 19	8, 8	15, 15	X, Y	8, 11	19, 22
K1	12, 15	29, 31.2	11, 11	11, 12	14, 17	9.3, 9.3	11, 12	9, 12	17, 20	14, 16.2	16, 18	8, 8	12, 12	X, X	10, 13	19, 23
K2	13, 14	28, 32.2	9, 11	12, 12	17, 18	7, 8	11, 12	11, 12	18, 21	14.2, 15	18, 19	8, 8	15, 15	X, Y	8, 11	19, 22

Table 6 – Profiles resulting from the CTS test used for the mock case study. All profiles matched the information from CTS. The study shows that the Identifiler® Plus STR kit obtained the correct profile from unknown samples simulating casework.

At the conclusion of the internal validation, all parameters were set for DNA analysis. An optimal injection condition of 3kV 5 seconds produced an analytical threshold of 60 RFUs. A dynamic range of 1.0-0.08ng of DNA was determined, along with an optimal DNA input of 0.63ng. A stochastic threshold of 150 RFUs was determined, giving support that all heterozygous alleles above this can be considered sister alleles. Locus by locus stutter percentages were calculated and entered into GeneMapper® for further analysis. These

parameters were used for analysis in the environmental insults study conducted after the conclusion of the internal validation.

Environmental Insults

UV Light. Displayed in Table 7 were the results for each sample for each time of exposure to UV light. This table provided the information on the state of the profile, including whether it was a full profile or if any alleles were missing. If allele dropout occurred, the number of alleles missing was indicated.

UV Light							
	0 seconds	30 seconds	60 seconds	100 seconds	200 seconds	300 seconds	400 seconds
1	full	-9 alleles	full	full	full	-18 alleles	-1 allele
2	full	full	-4 alleles	full	full	-18 alleles	-2 alleles
3	full	full	full	full	full	-24 alleles	-15 alleles
4	full	full	full	full	-6 alleles	-13 alleles	-23 alleles
5	full	full	-3 alleles	-9 alleles	-15 alleles	full	-27 alleles

Table 7 – A summary of results for the UV light study over time of exposure is shown above. The affect of exposure varied from 30-200 seconds. At 300 seconds allele dropout became consistent from sample to sample with a few outliers present.

Allele dropout was random across the time of exposure. One or two profiles were affected from 30-200 seconds, and at 300 seconds allele dropout started to be consistent, with every profile being affected. When alleles started to dropout with 30-200 seconds of exposure, the loci D18S51, D5S818, and FGA were consistently affected. At 100 seconds D7S820 started to dropout consistently. A few outliers were present with 300 and 400 seconds. At 300 seconds a full profile was obtained from one sample, and at 400 seconds two profiles lost one or two alleles. These results showed that a profile could still be obtained up to 200 seconds of direct exposure. After this time, results became more variable.

Humic and Tannic Acid. The results for humic acid (Table 8) and tannic acid (Table 9) are summarized below.

Humic Acid					
	0ng/µL	50ng/µL	100ng/µL	200ng/µL	400ng/µL
1	full	full	full	-11 alleles	-29 alleles
2	full	full	full	-6 alleles	-21 alleles
3	full	full	full	full	-29 alleles
4	full	full	full	full	-29 alleles
5	full	full	full	full	-29 alleles

Table 8 – This table summarizes how many alleles were present, and if there were any missing how many. An affect on the profiles was not seen until 200ng/µL of humic acid was present. Two samples showed dropout with the profiles sharing the same loci with dropout. At 400ng/µL the system is overwhelmed with the inhibitor and full profiles were inhibited.

Humic acid did not have an affect on the samples until a concentration of 200ng/µL. Two profiles saw dropout, with as many as 11 alleles not amplifying. Comparing these two profiles showed that loci D7S820, CSF1PO, D13S317, and D18S51 were affected in both. At 400ng/µL the Identifiler® Plus system became overwhelmed and could not compensate for the affects of the inhibitor. Four of the five samples showed complete loss of alleles, with the last only providing eight alleles in the profile. The one sample at 400ng/µL that produced a profile showed slight shouldering with some of the alleles. This sample only produced eight alleles in the profile which is only enough information for inclusion or exclusion purposes.

Tannic Acid					
	0ng/µL	50ng/µL	100ng/µL	200ng/µL	400ng/µL
1	full	full	full	full	-21 alleles
2	full	full	full	full	-19 alleles
3	full	full	full	-3 alleles	full
4	full	full	full	full	full
5	full	full	full	full	-1 allele

Table 9 – Full profiles were obtained for concentrations 0-100ng/µL. Sample 3 at 200ng/µL possessed a profile with three alleles dropping out. The concentration of 400ng/µL had results with full profiles and up to 21 alleles dropping out. Slight shouldering was present in the peaks at the greatest concentration due to the affect of the inhibitor.

Identifiler® Plus established that it withstands the affects of tannic acid better than humic acid. Full profiles were obtained for concentrations of 0-100ng/µL. At 200ng/µL, one profile had three alleles dropout at loci D18S51 and D5S818, and showed a slight shouldering of the

alleles present. At 400ng/µL, results ranged from a full profile to only eight alleles present. The profiles that were affected by tannic acid showed split peaks at 400ng/µL. Sometimes the split peaks were much shorter than the true peak, while other times the peaks were the same height. Results showed that the Identifiler® Plus system could produce an interpretable profile in the presence of a high concentration of tannic acid.

Hematin. Compiled in Table 10 were the complete results for the hematin study. Again, the results were for five samples per concentration. Also, the table indicated the state of the profiles obtained and how many alleles were missing, as with the previous studies.

Hematin					
	0µM	125µM	250µM	500µM	1000µM
1	full	full	full	full	-16 alleles
2	full	full	full	full	-4 alleles
3	full	full	full	full	full
4	full	full	full	full	-21 alleles
5	full	full	full	-1 allele	-26 alleles

Table 10 – A summary of results for each concentration of hematin is shown above. Full profiles were obtained up to 500µM with sample five as the outlier. At concentration 1000µM, the samples that resulted with at least 16 alleles dropping out possessed the loci with longer amplicons.

Full profiles were obtained for concentrations of 0-250µM. One sample at the 500µM concentration was an outlier, with one allele dropping out at the D13S317 locus. Results for 1000µM varied from a full profile to only three alleles present. The three samples with at least 16 alleles dropping out showed a pattern, with those alleles that were obtained corresonding to the shorter amplicons. Identifiler® Plus could endure the affects of hematin up to 500µM. Beyond this, the samples would not be of much statistical use other than inclusion and exclusion.

Discussion

Validation Study

Baseline Study. After comparing the negative controls, the height of the baseline noise, and the amount of artifacts in each sample, the injection condition of 3kV 5 seconds was considered optimal. The peak heights for this injection condition were consistent and low from dye channel to dye channel, and the baselines were clean. The standard deviation for this condition was also low, with the highest being recorded at 4.84 RFUs in the red dye channel. The highest peak seen across the dye channels was 28 RFUs occurring both in the green and red dye channels. To set the threshold, the 28 RFUs was doubled and rounded up to 60 RFUs. This threshold was acceptable and proved to be close to the 50 RFU threshold the manufacturer recommends. This was higher than what would have been obtained if the other method of adding three standard deviations to 28 RFUs was used.

Dynamic Range. Shouldering and broad peaks were seen at 5.0ng and 2.5ng. Off scale data was present at these concentrations along with samples at 1.25ng. These three concentrations were not included in the dynamic range. Samples included in the 0.63ng to 0.08ng range showed clean, full profiles. The samples at 0.04ng started to show loss of alleles and should not be used for the dynamic range for STR analysis along with the concentrations 0.02 and 0.01ng. The ideal mass to use was determined to be 0.63ng because it consistently gave full profiles with good sized peaks, and no off scale data. The peak height of these profiles made it easier for the analyst to distinguish true alleles from other artifacts. The peak heights were well above the stochastic threshold that was later determined, and illustrated that heterozygous peaks were sister alleles. The peak heights also were not too high to be off scale so that the whole peak could be seen. Though this is the ideal mass of DNA, other masses that are included

in the dynamic range can be used. After completing the analysis, the dynamic range was determined to be 1.0ng to 0.08ng of DNA. Though not directly tested, 1.0ng was suggested to alleviate the off scale data that can occur with 1.25ng. Applied Biosystems recommended 1.0ng for the input concentration of DNA for the kit, though they do not list a range of concentrations.

Even though sample FTA4b showed allele dropout at 0.08ng, the concentration was included in the dynamic range. This sample showed that you can get a full profile with the optimal DNA concentration, but on the lower end of the dynamic range there may be times when results would not produce a full profile based on the condition of the DNA. Quantification is done using the hTERT gene, not the STR locations. If the sample contains degradation at these primers sites, amplification would not occur, but the analyst would not know this until after the amplification results were obtained.

Precision and Reproducibility. After analyzing the data and recording the base pairs, all of the samples within the dynamic range had a 3 standard deviation calculation of 0.5 base pairs or less. The precision was calculated the same way the manufacturer did in their developmental validation. The original way was to calculate the standard deviation between all five samples in each concentration. When FTA4b was added to the samples, the three standard deviation calculations were above 0.5 base pairs. While looking at the data, the alleles for FTA4b were lower than the other four FTA samples. This is believed to have happened because the series of concentrations for FTA4b were amplified about a month after the other FTA samples and on a different set of capillaries. It should be noted that all of the alleles for samples in FTA4b were called correctly using the allelic ladder with which they were run.

Since the results of FTA4b were skewing the data of the study, the standard deviation between the samples and the allelic ladder was calculated for every sample. These differences

between the sample and the allelic ladder were calculated and averaged for each locus, and a standard deviation for each locus was also calculated. By doing this, the three standard deviations calculation was less than 0.5 base pairs for every locus and FTA4b provided acceptable results.

While recording the base pairs for every allele, the allele call was also recorded. The same profile was obtained and called correctly for every sample in the study, showing that the results of the Identifiler® Plus kit are reproducible. Overall, the results show that the kit was precise and could repeatedly produce the same profile from a given sample.

Stochastic Threshold. Illustrated in Figure 11 was the peak height ratio compared to RFUs for all the samples observed. Using all of the data points, the mean peak height ratio was determined to be 87.9%. To determine the stochastic threshold, only data points below 60% were analyzed. The point at which a rapid drop occurs is where the stochastic threshold was set. Since this can be subjective, an equation (Promega Corporation, 2006) was used to add weight to where the threshold was set. This equation used the mean peak height ratio and standard deviation to set the threshold at 55.1%, which is where the drop off occurred. At this percentage, a cluster of data points occurred. The highest RFU value of this cluster was rounded up to set a threshold of 150 RFUs. It should be noted that this was not an attempt to set a new peak height ratio for sister alleles. The 60% recommended by the field would still be used. The 55.1% was simply a percentage used on the chart generated from the results to correspond with the RFU value at which the stochastic threshold was set.

The peak height ratio for the point used to determine the threshold was 53.8%, which was close to the calculated percentage. Looking at the chart, one could see that four points dropped below the 60% line before 150 RFUs. Using a 98% confidence interval, 11 points were allowed

to drop below the set threshold and still be statistically valid. Based on this, the 10 points that fell after the 150 RFU mark are acceptable. This was the same threshold that the manufacturer recommended and other studies and agencies have recommended for Identifiler® Plus.

Stutter. The stutter percentages were calculated, and most of the stutter that occurred was in the samples above the upper limit of peak detection, though only the stutter in the dynamic range was used for the study. After those the percentages were recorded for every stutter peak shown, the highest stutter percentage for each locus was recorded, and was placed in Table 4 and corresponded to internal validation. The recommended stutter percentages from Applied Biosystems were also recorded in the table. These two percentages were compared for each locus, and in order to try and prevent stutter from showing up in the profiles the higher of the two percentages was chosen. Table 4 illustrated what the final stutter percentages were for the study. Ten of the 15 loci used percentages recommended by Applied Biosystems. vWA showed the most stutter of any locus, while TPOX had the least. Six loci had occurrences of stutter at least 25 times in the study. The remaining loci only had a maximum of 14 occurrences during the study. After obtaining these results, the stutter parameters in GeneMapper® were set to reflect the data.

There was not a major difference between the stutter percentages observed and those recommended by the manufacturer except for the TPOX, CFS1PO, D5S818, and FGA loci. There were no stutter peaks observed for the TOPX locus over the course of the study, so the manufacturer's recommendation was suggested by default. For the locus CSF1PO, a difference of 4.67% was observed. A 4.66% difference was observed for the FGA locus and a 5.94% difference was seen for the locus D5S818. Of the remaining loci, the greatest difference in percentage was 3.28 present in the D7S820 locus. All of the loci listed above had a lesser

percentage of stutter than recommended by the manufacturer. The only suggested percentages that were seen in the internal validation were the loci D8S1179, D3S1358, D13S317, D2S1338, D19S433 and vWA. The rest of the suggested percentages were the ones that he manufacturer recommended.

Mixture Study. This study was designed to determine when it would be possible to distinguish the profiles present in a two source mixture by using nine different concentrations. The 1:4 and 4:1 ratios were a good example of the possibilities that could arise in mixtures. With the 1:4 ratio, the profiles could be distinguished, while with the 4:1 ratio there were some loci that were determined indistinguishable. With these ratios, most of the loci of the minor profiles could be used for statistics, which could be very beneficial when another profile is compared to the mixture.

At the ratios 1:9 and 9:1, allele dropout started to occur and more loci of the minor profile dropped below the stochastic threshold. However, distinction of a major/minor profile became possible. Allele dropout also occurred for the 1:19 and 19:1 ratios, and a clear distinction of a major/minor mixture could be made. At the 1:1 ratios, analysis became more complicated. There were more instances of indistinguishable loci, but those loci that were present, could be used for statistics. As more loci became distinguishable, inclusion and exclusion purposes were the most probably route of analysis. It should be noted that for the ratios with a clear major and minor profile, the major profile could be easily distinguished in most cases. Therefore the major profiles could always be used for statistics.

After analyzing these results, a recommendation was made on how and when to interpret mixtures. Based on the results of this study, 1:4, 1:1, and 4:1 ratios or ratios close to this should not be attempted for deconvolution. Too many factors arose within these ratios that could create

difficulty for the analyst and even resulted in a wrong allele call for a profile as was shown for the 1:1 ratios that were distinguished. The 1:9, 1:19, 19:1 and 9:1 ratios showed clear major/minor profiles, and were acceptable to attempt a deconvolution. In some cases, the loci could be used for statistics and should be calculated to add weight to the analysis results.

It should be noted that there are calculations that could be done to analyze a profile that may have allele overlap, and to use this equation to make that distinction. However, these calculations were not done in this study. In the case of two or three alleles present, it was only calculated if the minor profile was homozygous or heterozygous. Overlap was not distinguished because a level of conservatism was wanted. Also, it was feared that since the analyst knew the profile of both sources to the mixture, a level of bias might inadvertently be incorporated into the analysis.

Contamination Study. Two successful checkerboard patterns were completed. There was no contamination in the plate due to the capillaries contaminating other wells. It was concluded that there was no contamination within the Genetic Analyzer 3130 system. If any contamination occurred, it would have taken place before the plate was placed on the genetic analyzer. This study also further tested the baseline study. After setting an analytical threshold, this study showed that 60 RFUs was high enough to filter out peaks that were not true alleles.

Mock Case. The questioned and known samples were analyzed to determine their profiles. Full and complete profiles from single sources were obtained for each source. After the samples were analyzed separately, the questioned sample was compared to the known samples. Table 6 illustrated that the questioned sample matched the second known sample. These results were compared to the information obtained from the manufacturer of the CTS test, and all samples matched the profiles provided by the manufacturer. These results show that the

Identifiler® Plus STR Kit successfully provided the correct profiles from unknown sources from case simulated samples. The study showed that the kit is sufficient for use on case samples and, therefore, also for research purposes.

Environmental Insults

UV Light. The affect of UV light on DNA is a random occurrence. Evidence of this was given in the results for the UV light study displayed in Table 7. In sample one, the profile had allele dropout at 30 seconds, whereas, the other samples showed full profiles for this exposure time. This same sample later produced a full profile at 100 and 200 seconds. Again at 300 seconds it resulted in significant allele dropout, only to lose one allele at 400 seconds.

After 300 seconds of exposure, most of the profiles showed significant allele dropout. In an attempt to eliminate the profile altogether, the samples were then exposed to UV light for 400 seconds. The results of this exposure did not differ much from 300 seconds of exposure. What was surprising was that two profiles had a loss of only one or two alleles.

These results further provided evidence to the randomness of UV light's affect. While these results could cause concern that DNA may still be present in the PCR workstation that was used, it should be noted that the study only used a maximum exposure time of 6.7 minutes. The protocol called for an exposure time of 15 minutes before and after PCR setup, which would result in 30 minutes of exposure before samples are placed in the workstation. This amount of time would be more than sufficient in decontaminating the work surface to prevent contamination from one set of samples to the next. Keeping all of this in mind, the results of this study showed that the Identifiler® Plus STR system could produce results that may be useful on case samples exposed to sun light at the crime scene.

Humic and Tannic Acid. The results of this study showed that the Identifiler® Plus STR kit could withstand the affect of humic and tannic acid on a DNA profile. Both studies used the same concentration ranges of the acids. For each set of samples, dropout did not occur until 200ng/µL was reached. Humic acid went from a few alleles missing at 200ng/µL to almost a complete loss of profile at 400ng/µL. While the kit seemed to withstand the affects of humic acid rather well, there seems to be a point between 200 and 400ng/µL that overwhelms the system having adverse affects on the profile.

As for tannic acid, the results showed that Identifiler® Plus could hold up to the affects better than humic acid. At 400ng/µL, the results ranged from a full profile to a loss of all but 3 alleles. The reason for this may be that the concentration of BSA in the kit was more suitable for the affects of tannic acid, but not for humic acid. No matter the reasoning, the results of this study illustrated that Identifiler® Plus could effectively produce results when exposed to these two inhibitors.

Hematin. Identifiler® Plus produced similar results with hematin as with tannic acid. At the highest concentration of 1000 µM, results ranged from a full profile to a loss of all but three alleles. The range demonstrated that the affects of hematin could vary from sample to sample. Major allele dropout occured only in the highest concentration, showing that the kit could withstand exposure to hematin. Also, split peaks with the concentration of 1000 µM affects the look of the profile but not the alleles that were called. The improvements made could counteract the presence of hematin and still provide acceptable profiles from case samples.

Results illustrated that the Identifiler® Plus STR kit was capable of producing sufficient data for use in the laboratory at UCO and for casework. A list of parameters was suggested for

use during future analysis. An injection condition of 3kV 5 seconds was used with an analytical threshold of 60 RFUs. The dynamic range of the system showed to be 1.0-0.08ng of DNA, with 0.63ng being the optimal input. Identifiler® Plus showed to be precise and provided reproducible results. A stochastic threshold of 150 RFUs allowed the analyst to be confident of the fact that two heterozygous peaks above this threshold were in fact sister alleles. Stutter was evaluated and guidelines were set on a locus by locus basis. A mixture study showed it was difficult to interpret results with similar contributions from two sources, but proved to give reliable results with major/minor profiles. The contamination study showed that no contamination would occur within the closed system of the genetic analyzer. Mock case samples were obtained from a CTS test and demonstrated that the system could produce the correct results from unknown samples. UV light impacted the DNA by breaking the strands to prevent successful amplification, whereas the other inhibitors affected the components of the amplification. For UV light, an exposure time of 300 seconds greatly impacted the profiles obtained. Humic and tannic acid were used in the same concentrations for the study. Humic acid inhibited amplification by binding to the target sequence of DNA and tannic acid inhibited by binding to the polymerase preventing it form binding to the DNA. While humic acid showed complete loss of profiles at 400ng/µl, tannic acid produced a range of results at this concentration from a full profile to only eight alleles remaining. Hematin proved to be an inhibitor by binding to the polymerase as well, and produced a range of results for its highest concentration of 1000µM. Results range from a full profile to just three alleles present.

These results allow an analyst within the laboratory to know what parameters must be set to analyze a DNA profile. They also provide information on how well the Identifiler® Plus kit will perform when in the presence of an known inhibitor of PCR. As with the laboratory at

UCO, other labs have also completed a validation on another STR amplification kits from other companies. This allows the analyst the option of which kit to use, and the results obtained during this research will give the analyst enough information on the performance of the kit to make their decision.

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Appendix A

5.0ng

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23

2.5ng

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23

1.25ng

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23

0.63ng

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23

0.31ng

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23

	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
0.16ng																
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X,	10, 11	21, 23
FTA4b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
0.08ng																
	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	12,N	N, 32.2	N	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	N,N	7, N	18, 20	X,X	10, 11	21, N
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
0.04ng																
	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA2b	12, 17	N, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, N	18, 20	X,X	10, 11	21, 23
FTA3a	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA4b	N, 17	N, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	18, 20	X,X	10, 11	21, 23
FTA5b	12, 17	29, 32.2	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, 18	7, 8	N, 20	X,X	10, 11	N, 23
0.02ng																
	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, 17	29, N	12	10	15, 16	N, 8	12, 13	11, 13	N, 27	14.2, 15.2	15, 18	7, N	18, 20	N,N	10, N	N, N
FTA2b	N, 17	N, 32.2	N	10	15, 16	N, 8	12, 13	11, 13	25, N	N,N	N, 18	7, N	N, 20	X,X	N, N	21, N
FTA3a	N, N	N, N	N	N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N
FTA4b	12, 17	29, N	12	10	15, 16	7, 8	12, 13	11, 13	25, 27	14.2, 15.2	15, N	7, 8	N, 20	X,X	10, 11	21, N
FTA5b	N,N	N,N	12	10	15, 16	7, 8	N, 13	11, 13	N,N	14.2, 15.2	15, N	7, 8	N, 20	X,X	N, N	N, N
0.01ng																
	D8	D21	D7	CSF	D3	TH01	D13	D16	D2	D19	vWA	TPOX	D18	AMEL	D5	FGA
FTA1b	12, N	N, N	N	N	N, 16	7, 8	N, N	11, N	N, N	N, N	15, N	N, N	N, N	X,X	10, N	N, N
FTA2b	N, 17	N, 32.2	N	10	15, 16	7, 8	12, N	N, 13	N, 27	N, N	N, N	7, 8	N, N	N, N	N, N	N, N
FTA3a	N, N	N, N	N	N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N
FTA4b	N, N	N, N	N	N	N, N	7, N	N, N	N, N	25, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N
FTA5b	N, N	N, N	N	N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N	N, N

Table 11 – The table compiles all of the profiles recorded for all of the samples used in the validation study. The sample name is given so that each profile corresponds with the sample that it was obtained from. An N present in the place of an allele call represents an allele that dropped out during analysis.

Appendix B

Collaborative Testing Services, Inc.

FORENSIC TESTING PROGRAM

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

Manufacturer's Information

Test No. 10-573: Forensic Biology

Each sample pack consisted of two known bloodstains on FTA Genecards (Items 1 & 2), and two questioned stains, one on clean red material (Item 3) and one on clean yellow material (Item 4). The stains in Items 1, 2 and 3 were prepared from human whole blood which was drawn into citric acid preservative bloodbank bags. Item 4 was a mixture prepared from human whole blood and semen. The semen, procured from a cryobank, was from a single donor, but stored frozen in multiple vials at -196° C in liquid nitrogen tanks. The semen was thawed and first mixed 1:1 with TAE buffer, then mixed 1:1 with the blood. This mixture was used to spot the Item 4 substrate. Item 1 was blood collected from a female donor and Items 2 and 3 were blood collected from one male donor. The Item 4 mixture contained the blood of the Item 1 female donor and semen from a donor whose known blood standard was not provided to participants. Stains from different sources were prepared at separate times and were packaged once they were thoroughly dried.

Sample Preparation Schedule

Item	Sample Collected	Prepared	Packaged	Volume
1	March 4, 2010	March 5, 2010	March 5, 2010	75 µl
2	March 4, 2010	March 8, 2010	March 8, 2010	75 µl
3	March 4, 2010	March 8, 2010	March 9, 2010	50 µl
4	Blood: March 4, 2010; Semen: Sept 2005-June 2006	March 5, 2010	March 8, 2010	50 µl

Completed sample sets were stored at -20°C until shipment on April 19, 2010 following receipt of predistribution results.

Amelogenin and STR Results

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

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

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

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

YSTR and Mitochondrial results on next page.

The information presented here is that received from the sample manufacturer. It presents details of the design specification for the test samples and/or details of how they were prepared. This information does not necessarily represent the answers that should or could be obtained from an examination of the sample. Final interpretation of the results should be deferred until the summary report is available.

Figure 13 - The mock case study used a CTS test for the samples. The results of the profiles for the sample present in the test are shown above. The samples used in the study are 1, 2, and 3. Only the blood samples were tested in the mock case study.

Appendix C

Item Description	Part Number	Manufacturer	Location
Whole Blood		Innovative Research	Novi, MI
Whatman® FTA® card	WB120205	Fisher Scientific	Fair Lawn, NJ
GeneMapper® version 3.2.1 software	4359225	Applied Biosystems	Foster City, CA
DNA IQ™ kit	DC6700	Promega Corporation	Madison, WI
Thermolyne Type 16500 Dri-Bath	D816525	Thermo Scientific	Waltham, MA
spin basket	V1221	Promega Corporation	Madison, WI
Eppendorf Centrifuge 5430	022620525	Eppendorf	Hauppauge, NY
Quantifiler™ Human DNA Quantification Kit	4343985	Applied Biosystems	Warrington, UK
MicroAmp® Optical 96-Well Reaction Plate	N8010560	Applied Biosystems	Foster City, CA
MicroAmp® Optical Adhesive Film	4311971	Applied Biosystems	Foster City, CA
Applied Biosystems 7500 Real-Time PCR System	4345241	Applied Biosystems	Foster City, CA
7500 System SDS version 1.2.3f2		Applied Biosystems	Foster City, CA
AmpFlSTR® Identifiler® Plus PCR Amplification Kit	4427368	Applied Biosystems	Warrington, UK
0.2 mL individual thin wall PCR tubes with attached dome cap	1602-4300	USA Scientific	Ocala, FL
GeneAmp® PCR System 9700 with the Silver 96-Well Block	N8050200	Applied Biosystems	Foster City, CA
GeneScan™ 500 LIZ® Size Standard	4322697	Applied Biosystems	Warrington, UK
Hi-Di™ Formamide	4311320	Applied Biosystems	Foster City, CA
96-Well Plate Septa	4315933	Applied Biosystems	Foster City, CA
3130 Genetic Analyzer	627-0040	Applied Biosystems	Foster City, CA
Air Clean 600 PCR Workstation	AC648LFUVC -4852	USA Scientific	Ocala, FL
humic acid	41747	The Right Chemicals – Alfa Aesar	Ward Hill, MA
tannic acid	1401-55-4	Fisher Scientific Education	Hanover Park, IL
hematin	198969	MP Biomedicals, LLC	Solon, OH
97%+ NaOH pellets	1310-73-2	Sigma Aldrich	St. Louis, MO
Small gloves	MF-300-S	Microflex Corporation	
Medium gloves	MF-300-M	Microflex Corporation	
1-100µL beveled filtered tips	1120-1840	USA Scientific	Ocala, FL

0.5-20 µL beveled filtered tips	1121-4810	USA Scientific	Ocala, FL
101-1000 µL beveled filtered tips	1126-7810	USA Scientific	Ocala, FL
Teeny tough tags®	TT-TNY	Diversified Biotech	Boston, MA
2mL SealRite natural microcentrifuge tubes	1620-2700	USA Scientific	Ocala, FL
0.5mL SealRite natural tubes	1605-0000	USA Scientific	Ocala, FL
Analytical Balance		Sartorius	Goettingen, Germany
Multicapillary DS-33 (Dye Set G5) Matrix Standard Kit	4345833	Applied Biosystems	Foster City, CA
1L BP561-1 water, steril	1609-47-8	Fisher Scientific	Fair Lawn, NJ
3130 POP-4™	4352755	Applied Biosystems	Foster City, CA
96 well plate base	4317237	Applied Biosystems	Foster City, CA
3130 and 3100 series plate retainer 96-well	4317241	Applied Biosystems	Foster City, CA
3130 and 3100 Avant capillary array 36cm	4333464	Applied Biosystems	Foster City, CA

Table 12 - The table summarizes all of the materials and equipment used throughout the research process. Some materials were previously stated in the materials and methods section, while other materials were not listed but included since they were used for research.