
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

W 4.5 D687i 2004
Donovan, Erin Wynter.
Internal validation study
of

LEWIS LIBRARY
UNT Health Science Center
3500 Camp Bowie Blvd.
Ft. Worth, Texas 76107-2699

INTERNAL VALIDATION STUDY

OF PROMEGA'S POWERPLEX® Y SYSTEM

Erin W. Donovan, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Cell Biology and Genetics

Dean, Graduate School of Biomedical Science

INTERNAL VALIDATION STUDY

OF PROMEGA'S POWERPLEX® Y SYSTEM

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Science

University of North Texas

Health Science Center at Fort Worth

For the Degree of

MASTER OF SCIENCE

By

Erin Wynter Donovan, B.S.

Fort Worth, TX

August 2004

ACHKNOWLEDGEMENTS

I would like to acknowledge a few individuals, which assisted in the completion of this project. Shelley Crispin helping with the laboratory work. Hector Saenz, Linda Larose, Melody Moore, Christy Capt, Xavier Aranda, and Dixie Peters HybKi allowing and assisting me working in the DNA Identity Laboratories. Finally the members on my committee John Planz, Arthur Eisenberg, Stephan Grant, and especially Joseph Warren.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF APPRENDIXES

CHAPTER I

INTRODUCTION

Currently in the field of Forensic Science, Short Tandem Repeats (STR) are used as identification markers to distinguish individuals. STRs are repetitive sequences seen throughout the human genome. Due to the success of STRs in the field, research on Y-STRs has evolved leading to the development of Promega's Powerplex® Y System. Y-STRs are useful in the field of forensic science due to the Y-chromosome being mostly nonhomologous to the X-chromosome and changing only through mutations and not by recombination. In addition Y-STRs are especially useful in paternity and sexual assault cases. In order for Promega's Powerplex® Y System to be utilized in the DNA Identity Laboratory at University of North Texas Health Science Center a validation study must be conducted to ensure the laboratory is capable of achieving consistent and accurate results when using the new PowerPlex® Y System.

In order for a novel technique to be used in the forensic field, the technique must be validated according to the DNA Advisory Board (DAB), which is a governing body that established the National standards and guidelines to be followed by forensic laboratories throughout the United States. For Validations the DAB established Standard 8.1, which states, "The laboratory shall use validated methods and procedures for forensic casework analyses"(1). In order for the PowerPlex® Y System to be utilized in the DNA Identity Laboratory for casework a validation study must be performed to examine the performance of the system, in which it demonstrates a laboratory's ability to perform a technique in a robust, reliable, and reproducible manner.

The validation study for the PowerPlex® Y System for the DNA Identity Laboratory consisted of four studies: sensitivity, mixtures, substrate, and environmental. The sensitivity test is a serial dilution of known DNA concentrations, in order to determine the optimal amount input of DNA. The mixture study is a two-part study, with one study dealing with male: male mixtures and the other with female: male mixtures. The male: male mixture study is needed in order to determine how to recognize a mixture of multiple male contributors by examining different proportions of DNA in a mixture. The female: male mixture is used to simulate a sexual assault case, in which the female DNA in the study is much greater in proportion to the male DNA present. This mixture examines the kit's ability to detect male DNA at low levels with presence of high amounts of female DNA. The substrate study addresses the influence of potential substrates and contaminants to the performance of the PowerPlex® Y System. The final study, environmental, deals with the effects of temperature and humidity on blood samples. This study is useful because frequently forensic samples are subjected to harsh environments, which could alter the ability to receive reliable DNA results when using the PowerPlex® Y System.

The procedure of the validation study consists of DNA extraction via Chelex® 100 and Organic Extractions (8) and then quantification using Real-Time Polymerase Chain Reaction via the ABI Prism® 7000 Sequence Detection System using Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit (4). Next Amplification is performed on the Perkin-Elmer GeneAmp® PCR System 9700 Thermal Cycler using the PowerPlex® Y System. Finally, capillary electrophoresis is then performed on the ABI Prism® 3100 Genetic

Analyzer, followed by analysis on GeneScan® Analysis Software and Genotyper® Program using the PowerTyper™ Y Macro.

Currently in the forensic field STR kits have been developed to analyze the thirteen core loci, which are the basis for the CODIS (Combined DNA Index System) National DNA database. The STR multiplex kits are available to cover all thirteen loci, which are the Profiler Plus™ and Cofiler™ Kits and also the PowerPlex 1.1™ and PowerPlex 2.1™ (9). STRs have become a reliable tool in forensic science; due to their success research on Y-STRs have developed. Y-STRs are located on the Y-chromosome and found only in male individuals. Research has been conducted on the Y-chromosome that has lead to the discovery of over 250 Y-STR markers (12). Due to this discovery, the development of Y-STR multiplex kits is in high demand in order to use this beneficial tool in the field of forensic science leading to Promega's development of the PowerPlex® Y System.

The PowerPlex® Y system is significant because it utilizes the Y chromosome and can be especially useful in forensic casework, paternity testing, genealogical studies, and evolutionary studies. Advantages of the Y-chromosome include the ability to detect low levels of male DNA in a mixed sample especially in sexual assault cases. The chromosome can be used in paternity testing due to the non-mendelian inheritance pattern from father to son occurring with no recombination (9). Since the loci are all located on one chromosome, genetically linked, the product rule cannot be used to generate a profile frequency as in the case of autosomal short tandem repeats. To obtain a high level of discrimination a large number of Y-STRs need to be analyzed so that a complex haplotype is developed. The PowerPlex® Y System allows co-amplification of twelve loci to ensure a high level of discrimination between samples. (11)

The significance of the Y-chromosome shows the importance in validating the PowerPlex® Y System for the DNA Identity Laboratory at University of North Texas Health Science Center to be used in casework. Validation studies are a standard in the field. The science behind DNA testing is rarely questioned, but the process by which the laboratory performs the DNA analysis is challenged frequently. Therefore a validation study will enforce the accuracy of a laboratory due to the lab following the interpretation guidelines resulting from the validation study, which will guarantee that each sample is handled and processed appropriately (9).

The expected results of each of the four studies to be conducted in this validation study consist of the following expectations. For the sensitivity test the recommended amount of DNA to be added to each sample is 500pg to 1ng of male DNA template with >2ng of male DNA template added could result in higher levels of stutter bands (11). Too little DNA could result in allelic dropout and too much DNA could result in pull-up occurring if the peak height exceeds 2,000RFU. The mixture studies are being performed to determine how to recognize a mixture. For the female: male mixture female DNA at a concentration >100X that of the male component can decrease the relative yield of some loci, but for the majority of the mixtures the female DNA should have no effect on the male DNA and the ability to obtain a profile (11). For the male: male in any sample only one peak shows at each loci except for loci DYS385a/b two peaks will be displayed because the Y-chromosome does not have heterozygosity since the chromosome is only inherited by the father not both parents as in the case of STRs. Therefore, in the male: male mixtures peak height ratio will be examined to see the effect of mixed samples on the performance of the PowerPlex® Y System. Finally the substrate and environmental

tests will examine the ability for samples under certain circumstance to perform when analyzed with the PowerPlex® Y System. Some of the substrates and environmental conditions could inhibit PCR and degrade the DNA sample. Each of the four studies is vital to ensure the performance of the PowerPlex® Y system is reliable, robust, and reproducible and can be used in forensic casework at the University of North Texas Health Science Center DNA Identity Laboratory.

CHAPTER II

BACKGROUND

Short Tandem Repeats

Short Tandem repeats (STRs) provide useful and reliable genetic information for the forensic field. These genetic markers are used exclusively in forensic DNA typing because they are PCR-based and can produce results with low-quantity DNA or degraded DNA. In addition the method is automatable and is capable of testing a high number of samples over a short period of time. With STRs, multiple chromosomes can be examined, allowing a high amount of discrimination between individuals. (9)

Currently in the forensic field STR kits have been developed to analyze the thirteen core loci, which are the basis for the CODIS (Combined DNA Index System) National DNA database. The thirteen core STR loci include CSF1PO, FGA, THO1, VWA, D3S1358, D5S818, D7S820, D13S317, D16S539, D18S51, and D21S11. The STR multiplex kits are available to cover all thirteen loci, which are the Profiler Plus™ and Cofiler™ Kits by Applied Biosystems and also the PowerPlex 1.1™ and PowerPlex 2.1™ by Promega. For the Profiler Plus and Cofiler Kits three sets of fluorescent dyes are used to detect the loci and for PowerPlex 1.1™ and PowerPlex 2.1™ two fluorescent dyes are used (9). These fluorescent dyes label loci during PCR, the labeled STRs then undergo capillary electrophoresis, which detects the fluorescent dyes and distinguishes the allele(s) at each locus. The alleles are then analyzed using computer software and compared to allelic ladders for genotyping. The development of these kits to analyze the thirteen core loci has allowed DNA analysis to become more efficient and accurate. Due

to the use of STRs in identity testing, research has been conducted to evaluate on the usefulness of Y-STRs.

Y-STRs differ from autosomal STRs because they lie only on the Y-chromosome and they are male specific. The Y-chromosome is one of the smallest human chromosomes, consisting of an average of 60 million base pairs. (Figure 1) For most of the length of the Y-chromosome, except at the tips of each arm, the chromosome is nonhomologous changing only through mutation and not by recombination (16). In addition this means the Y-chromosome since it is male-specific is transmitted from father to son uncharged except for rare mutations.

Due to the properties of the Y-chromosome, evolutionary studies have been conducted using the Y-chromosome especially migration studies. In addition exploration of the Y-chromosome has been conducted for use in the forensic field. In 1994, the European Y Halplotype Reference Database (YHRD) was established in order to "identify polymorphisms capable of discriminating between majority of unrelated lineages, establish a database representative of the geographical and ethnical structure, and to aim at a database size that would allow accurate frequency estimations for rare haplotypes (17)". Thirty-one different forensic and anthropological institutions constructed the database. As of September 2000, the database contained 4688 haplotypes and has continued to grow to be the largest Y-STR database in the world (17). In Europe, nine loci are required for forensic comparison and these loci are included in Promega's PowerPlex® Y System, with the addition of three loci, two of which were recommended by SWGDAM for forensic work in the United States.

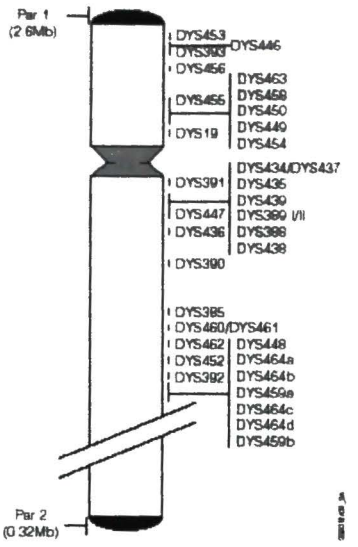

Figure 1: The Y-Chromosome (16)

As databases and multiplex kits become available for Y-STRs, the technique could greatly benefit forensic analysis especially in paternity disputes and sexual assault cases. For paternity testing any paternal relative could be used as a reference to identify the male child due to paternal inheritance of the Y-chromosome. The advantages of using Y-STRs in sexual assault investigations are where the female DNA present is in a greater volume than that of the male DNA. For example, cases with semen present in a high presence of female DNA, the female DNA will not interfere with or mask the results. With the Y-STRs only male DNA is detected allowing small amounts of male DNA to be detected with large amounts of female DNA present.

Promega's PowerPlex® Y System

The PowerPlex® Y System by Promega offers a multiplex system which, allows coamplification and three-color detection for twelve loci. The loci included are DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, and DYS439. (Figure 2)

STR Locus	Label	Chromosomal Location	GenBank® Accession Number	Repeat Sequence[1] 5'→3'
DYS391	FL	Yq	G09613	TCTA (14)
DYS389I/II	FL	Yq	AF140635	[TCTG][TCTA] Complex (14)
DYS439	FL	Yq	AC002992	GATA (29)
DYS393	TMR	Yp	G09601	AGAT (14)
DYS390	TMR	Yq	AC011289	[TCTG][TCTA] Complex (14)
DYS385a/b	TMR	Yq	Z93950	GAAA (14)
DYS438	JOE	Yq	AC002531	TTTTC (29)
DYS437	JOE	Yq	AC002992	[TCTA][TCTG] Complex (29)
DYS19	JOE	Yp	X77751	TAGA Complex (14)
DYS392	JOE	Yq	G09867	TAT (14)

Figure 2: PowerPlex® Y System Locus-Specific Information (11)

Three dyes are used in the kit in order to label each of the twelve loci. Each locus is amplified with a separate primer sets but only three dyes are needed due to the base length difference in the loci. For DYS389I/II, DYS391, and DYS439 the loci are labeled with fluorescein (FL) which is indicated by a blue dye; for DYS385a/b, DYS390, and DYS393 the loci are labeled with carboxy-tetra-methylrhodamine (TMR) which is indicated by a yellow dye; and the final dye, 6-carboxy-4', 5'-dicholor-2', 7'-dimethoxy-fluroescein (JOE), which is indicated by a green color identifies loci DYS19, DYS393, DYS437, and DYS438. (11) All twelve loci can be simultaneously amplified in a single tube and analyzed via capillary electrophoresis in a single injection. Allelic ladders

consisting of 102 alleles over the twelve loci have been created in order to make allele calls at each locus. (Figure 2) This process is performed using the software GeneScan® through the PowerTyper™ Y Macro.

STR Locus	Label	Size Range of Allelic Ladder Components[1] (bases)	Repeat Numbers of Allelic Ladder Components	Alleles Observed in 9948 Male DNA Standard
DYS391	FL	90–118	6, 8–13	10
DYS389I	FL	148–168	10–15	13
DYS439	FL	203–231	8–15[2]	12
DYS389II	FL	256–296	24–34	31
DYS393	TMR	104–136	8–16	13
DYS390	TMR	191–227	18–27	24
DYS385	TMR	243–315	7–25	11, 14
DYS438	JOE	101–121	8–12	11
DYS437	JOE	183–199	13–17	15
DYS19	JOE	232–268	10–19	14
DYS392	JOE	294–327	7–18	13

Figure 3: The PowerPlex® Y System Allelic Ladder Information (11)

Validation Studies

Validation studies are important in the forensic field, because the study documents and evaluates new techniques to be used in the field. The forensic community is rarely challenged on the science of DNA typing, but rather colleagues, the public, and the court system frequently challenge the procedures used in a laboratory. Therefore, validation studies allow the new technique to be challenged in a variety of studies to ensure the performance of the technique is acceptable and reliable.

Validation studies consist of two parts: developmental and internal. A developmental validation study involves the construction of the new technique, such as primer sets, PCR conditions, and new loci. Commercial manufactures and large laboratories, such as the FBI laboratory, typically conduct this part of the validation study. The second stage of validation studies is internal validation study, which is

conducted by smaller local and state laboratories. The internal validation study addresses the performance of the technique in each lab and is based on the developmental validation (9).

In order to make validation a standard in the forensic field, in 1995 TWGDAM developed guidelines to be followed for validations. The guidelines address sample source, which must be consistent with samples commonly seen in forensic cases. Reproducibility, population, mixture, environmental, and matrix studies are conducted. Non-probative evidence needs to be used, which is samples from forensic cases that have been closed in order to demstrate the technique can handle real casework situations. Non-human studies can be performed in order to show the technique is human specific. Finally stutter percentages and peak height ratios are determined in order to be used to develop interpretation guidelines for laboratories to follow (1).

The validation study conducted for the PowerPlex® Y System will be an internal validation study. The developmental study has already been performed by the Promega corporation the producers of the PowerPlex® Y System. In the internal validation study sensitivity, mixture, environmental, and substrate studies will be performed. In addition stutter percentages and peak height ratios will be analyzed and recorded to ensure the system is working properly in the DNA Identity Laboratory.

DNA Quantification

DNA quantification is an important aspect of forensic DNA testing. It is important to establish the amount of human DNA, which is present in a sample in order to obtain optimal results and minimal artifacts that can interfere with the interpretation of the DNA

test. In regards to quantification, DAB established standard 9.3 that states, "The laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample where possible" (1). The technique, which is commonly used in the field of forensic science, is the Quantiblot. This method is a reverse slot blot hybrization, which is very time consuming. The results produced by this technique are very subjective. A new technique is emerging in the field of Forensic science for quantification purposes, which is Real-Time Polymerase Chain Reaction. The advantage of real-time-PCR is the sensitivity of the system compared to other quantification methods. The reaction process entails three phases: exponential, linear, and plateau. The first phase exponential the sample is doubling with each cycle due to the availability of reagents and kinetics advancing the reaction. Next the linear phase results due to the reagents being consumed leading to the reaction decreasing along with the sample no longer doubling after each cycle. Finally the plateau phase is reached and the reaction stops with no more products being made. (Figure 4)

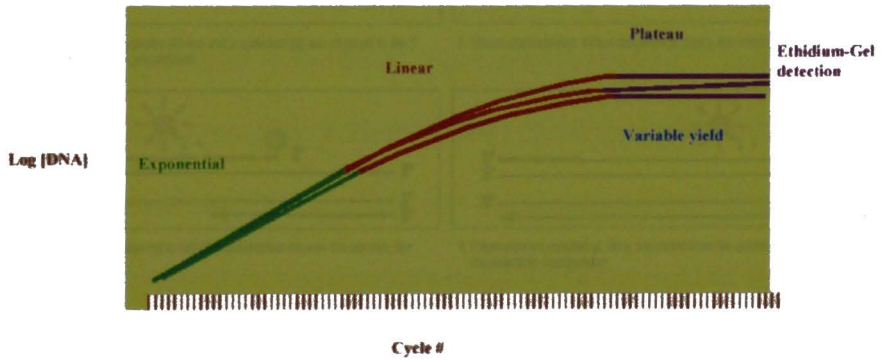

Figure 4: Polymerase Chain Reaction Phases (6)

Real-Time PCR is designed to collect data during the exponential phase, when the reaction is proceeding in order to provide fast and accurate results, and also to eliminate the need for post PCR methods, such as agarose gels. (6)

Real-Time PCR detection is based on the TaqMan® chemistry. This detection system uses a fluorogenic-labeled probe with a 5' nuclease activity. The first step involves the construction of an oligonucleotide probe consisting of a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. When both the quencher and reporter dye are attached to the probe, fluorescent emission is quenched. When the target sequence is present the probe anneals downstream from a primer and is then cleaved by the 5' nuclease activity of Taq DNA polymerase as the primer is extended. At this point the reporter dye is separated from the quencher dye increasing the reporter dye signal. In return, the probe is removed from the target strand allowing primer extension to the end of the template strand (figure 5). With each cycle additional reporter dye molecules are displaced from the probe resulting in an increase in the intensity of the fluorescent proportionality relates to the amount of DNA present. (7)

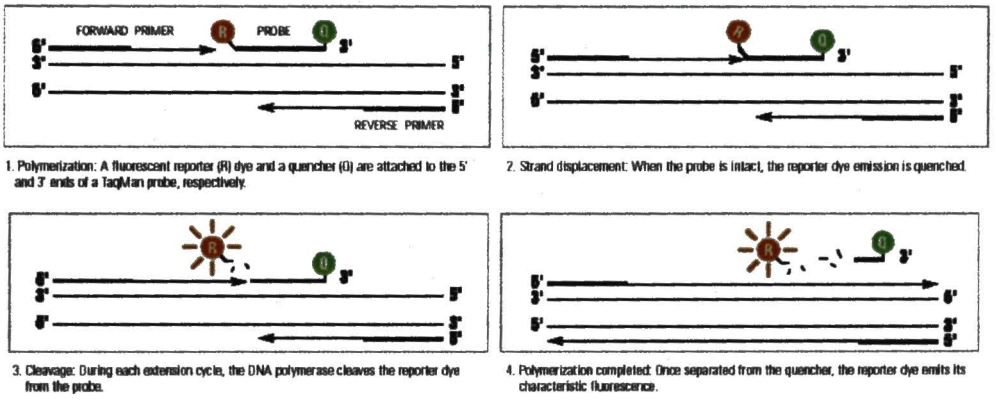

Figure 5: Real-Time Polymerase Chain Reaction (7)

As the fluorescent signal increases due to the reporter dye the fluorescent emission can be detected and displayed as an amplification plot. There is a direct relationship with the amount of product being produced for a given sample to the amount of fluorescent emission. The amplification plot gives the quantitative measurement of the DNA (Figure

6). The point at which the reaction has reached the detection level is at the threshold, which is set in exponential phase of the amplification in order to get the most accurate reading. Once this level has been reached the cycle is called the Cycle Threshold, Ct. Both of these measurements are needed to obtain accurate data analysis using the 5' nuclease assay. (6)

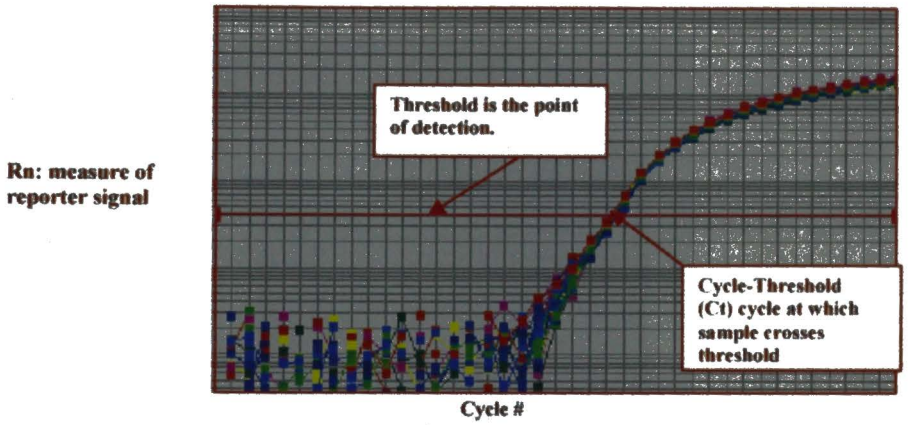

Figure 6: Amplification Curve (6)

For the forensic field of science real-time PCR will improve the method of quantification through its reliability, accuracy, and reproducibility. Applied Biosystems has developed two kits, which are beneficial to the field: which are Quantifiler™ Human DNA Quantification Kit and Quantifiler Y Human Male DNA Quantification Kit. Both kits use the 5' nuclease assay and TaqMan® probe-based technology. The Quantifiler kits offer a solution to the stringent requirements assigned to the field of forensic science. The Quantifiler Y Human Male DNA Quantification Kit adds the feature to analyze samples, which are a mixture of female and male DNA commonly seen in sexual assaults cases enabling the ability to only quantify the male DNA through the Y-chromosome. (4) Both kits will be used in this validation study to quantify the amount of DNA present in each of the samples.

ABI Prism® 7000 Sequence Detection System

All samples were quantified on the ABI Prism® 7000 Sequence Detection System, a 96-well Real-Time Polymerase Chain Reaction unit processing data at the expontial phase. (Figure 7)

Figure 7: ABI Prism® 7000 Sequence Detection System (14)

During excitation all 96 samples are illuminated with a tungsten-halogen lamp, in which the light excites the fluorescent dyes leading to the fluorescent emission between 500 and 660nm. The fluorescent emission is then directed to a charge-coupled device (CCD) camera through optical filters, which are based on wavelengths. The system has the ability to detect a variety of fluorescent dyes including FAM™/SYBR® Green 1, VIC™/JOE™, TAMRA™, and ROX™ dyes. The detection of multiple dyes allows flexiblity in the use of multiplexed quantifications, along with assays with an internal positive control (IPC). The final step is the analysis through software, which is based on

monitoring cycle number, the fewer cycles it takes to detect fluorescence, the greater the quantity of starting material. (5)

ABI Prism® 3100 Genetic Analyzer System

The ABI Prism® 3100 Genetic Analyzer System is a fully automatic multi-capillary electrophoresis system with the ability to analyze 16 samples simultaneously. Capillary electrophoresis for DNA analysis offers many advantages; more efficient heat dissipation versus slab gels, capillaries allow high run voltage, and allows fast run times. The system is based on electrokinetic injection to load samples into the capillary, at which time 16 samples are injected simultaneously in less then 30 seconds. In addition one of the greatest advantages of a CE is the elimination of manual operation, which lead to increase run-to-run consistency and reliability.

ABI Prism® 3100 Genetic Analyzer System is based on Capillary Electrophoresis consisting of a narrow capillary, two buffer vials, and two electrodes connected to a high-voltage power supply. In addition the CE system includes a laser excitation source, a fluorescent detector, and a computer to control the sample injection and to record the results. The sample passes through the capillary at which time the relative fluorescent intensity is observed as the sample passes the detector and by the fluorescent emission of dyes the DNA molecules can be detected and analyzed. (9) Sample preparation will include 1µl of the internal lane standard 600 (ISL 600), 9µl of deionized formamide and 1µl of amplified sample. The internal lane standard 600 (ISL 600) contains 22 DNA fragments with different base lengths, which allows for base pair sizing. Each of the fragments are labeled with a fluorescent dye carboxy-X-rhodamine (CXR) which is detected separately as a fourth color in PowerPlex® Y System-amplified

material, to increase the precision in the analysis when using the PowerPlex® Y System. (11)

Analysis Software

Genescan® Analysis Software is the first step in analyzing DNA after the genetic analyzer instrument such as ABI Prism® 3100 Genetic Analyzer System. The software automatically identifies and sizes each peak relative to an internal lane standard such as ILS 600 found in the PowerPlex® Y System. The software provides peak area and peak height information. The results from GeneScan® can then be imported into the Genotyper® software which is the second phase of analysis (15). Genotyper® compares a samples genotype to a standard allelic ladder (9). For the PowerPlex® Y System the samples can be imported into Genotyper and analyzed using the PowerTyper™ Y Macro to designate alleles by comparison to the allelic ladder, which consists of 102 alleles over the twelve loci. (11) (Figure 8) Finally an analyst can then examine the peaks and furthermore an allele table can be created and exported to a spreadsheet program for further analysis.

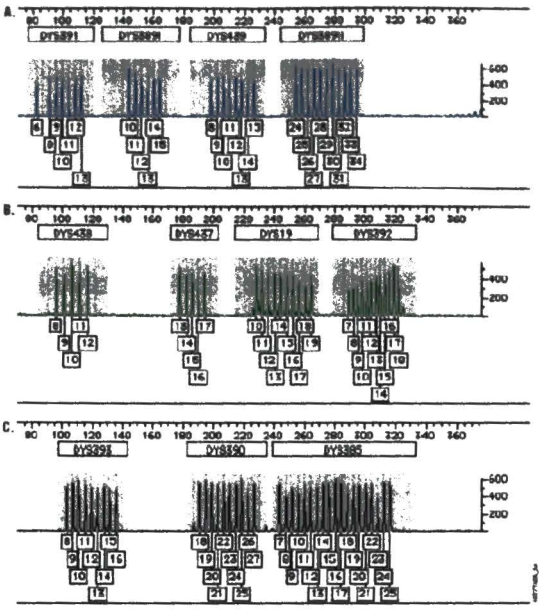

Figure 8: PowerPlex® Y System Allelic Ladder (13)

CHAPTER III

RESEARCH DESIGN

The purpose of this research is to determine the performance of the PowerPlex® Y System by running the following studies: sensitivity, mixtures, substrate, and environmental.

STUDY 1: Sensitivity

The objective of this study is to determine the optimal amount of DNA to be added to a Polymerase Chain Reaction. The study consists of a serial dilutions of know concentrations of DNA (10ng, 5ng, 2.5ng, 1ng, 750pg, 500pg, 250pg, 125pg, 75pg, and 50pg). If too much DNA is added to a sample artifacts could appear such as split peaks, stutter, and allelic drop in, but if too little DNA is added allelic drop out could occur and a complete profile will not be obtained. Therefore the optimal amount of DNA must be determined to enable a complete profile to be obtained for each sample tested.

STUDY 2: MIXTURES

Two mixture studies were performed, one consisting of two unrelated male sources, and the other consisted of male DNA and female DNA. For the male: male mixture the purpose is to determine the ability to intreprerate a mixture of two male individuals at a variety of proportions. The dilutions were as follows: 1:50, 1:25, 1:10, 2:8, 3:7, 1:5, 1:2, 1:1. This part of the study gives peak height ratios of mixtures in order to be included in the interpretation guidelines on how to determine mixtures. The female:

male mixture can determine the effect of female DNA in the PowerPlex® Y System. The system should only detect male DNA due to males only carrying a Y-chromosome. Therefore samples were overloaded with female DNA approximately 10ng and the male DNA was added in the following concentrations 3ng, 2ng, 1ng, 750pg, 500pg, 250pg, and 100pg. This proportion of the mixture study is representative of sexual assault cases, which usually have an overload of female DNA and a small amount of male DNA present. This part of the study ensures the system will not be inhibited by high concentration of female DNA.

STUDY 3: SUBSTRATE

The objective of the substrate study focuses on the influence of different substrates and substances on the performance of the PowerPlex® Y system and the ability to get interpretable results. In the forensic field samples are commonly found on a variety of material, which could alter the DNA results of a sample. Common material can be tested with the PowerPlex® Y System to identify if any of the substrates could inhibit the performance of the kit in producing interpretable results. The substrates used in this study were: jean material, black cotton, white cotton, nylon, leather, and towel material each was prepared by placing drops of blood on the material and allowing to air dry. Blood was mixed with different substance to see the effects of a variety of liquids either commonly used to clean up a crime scene or could be mistaken for blood. The substances used were bleach, water, hydrogen peroxide, ethanol, red nail polish, and soap with each substance mixed with blood and then place on a white cotton fabric to air dry.

STUDY 4: ENVIRONMENTAL

The objective of the environmental study is to determine the effects of temperature and humidity on bloodstains. Many forensic samples are subjected to harsh environments such as high or low temperature and humidity, which can lead to sample degradation. Samples were placed at temperatures of 37°C and 57°C along with humidity at room temperature and 57°C. The samples will be taken out of the environmental conditions at 24 hours, 72 hours, 7 days, 14 days, 21 days, and 1 month, in order to determine the effects of humidity and temperature on the PowerPlex® Y System.

CHAPTER IV

MATERIAL AND METHODS

All reagents including the PowerPlex® Y System were provided by the University of North Texas Health Science Center DNA Identity Laboratory in Fort Worth, TX. Also all procedures were performed in the Forensic Laboratory (Extraction, Polymerase Chain Reaction, and Analysis) or Research and Development Laboratory (Quantification) at the University of North Texas Health Science Center.

DNA SAMPLES

Buccal swabs were taken from four unrelated male volunteers to be used for the sensitivity and mixture studies, along with two unrelated female volunteers to be used for the mixture study. In addition blood samples taken from a male volunteer were used for the environmental and substrate studies.

EXTRACTION

A biological sample such as blood, salvia, or semen contains DNA but also contains cellular material and proteins, which can affect DNA results. Therefore an extraction must be performed in order to separate the cellular material and protein from the DNA molecules. Two types of extraction methods were used in this study, which are an Organic Extraction sometimes referred to as Phenol-Chloroform extraction along with the Chelex® 100 method of extraction, both commonly used in the forensic field.

Organic Extraction

Note: Base on Budowle, Bruce, DNA Typing Protocols: Molecular Biology and Forensic Analysis, Eaton Publishing. 2000.

1) Cut a piece of cotton swab from buccal swab and place into a microcentrifuge tube.

2) Add 300µl of Stain Extraction Buffer and 5.0µl of Proteinase K.

3) Incubate samples at 56°C for 6-24 hours

4) Remove the swab and place it in the cup of a Spin-EASE™ extraction tube, spin for 4 minutes at 13,000RPM

5) Remove Spin-EASE™ basket and discard

6) Add 300µl of Phenol-Chloroform

7) Vortex samples for 15 seconds, centrifuge at 13,000RPM at Room Temperature for five minutes

8) Transfer aqueous layer to a new tube

9) Add 1ml of cold absolute alcohol to the aqueous layer and let sit at -20°C for 30 minutes

10) Centrifuge for 20 minutes at 13,000RPM

11) Decant alcohol off

12) Add 1ml 70% ethanol, centrifuge for 10 minutes at 10,000

13) Pipette off ethanol, evaporate remaining by placing on a heat block

14) Add 56µl TE-4 and place in storage at 4°C for short term or for long term place at -20°C

Chelex Extraction

Note: Based on Budowle, Bruce, DNA Typing Protocols: Molecular Biology and Forensic Analysis, Eaton Publishing. 2000.

1) Take a cutting and place in a microcentrifuge tube

2) Add 1ml of sterile water to each tube containing a cutting

3) Incubate at room temperature for at least 30 minutes

4) Centrifuge samples for 3 minutes at 13,000RPM

5) Make a 5% Chelex® 100 mixture, and place on stirrer

6) Remove supernant

7) Add 200µl Chelex to each sample

8) Incubate samples at 56°C for 30 minutes

9) Vortex for 15 seconds

10) Incubate at 100°C for 8 minutes

11) Vortex for 15 seconds

12) Centrifuge for 3 minutes at 13,000

13) Transfer supernant to a new tube do not remove and they resin beads

14) Store at -20°C

QUANTIFICATION

Quantification is an important aspect of science methodology in order to establish the amount of DNA, which is present in a sample in order to make appropriate dilutions for further analysis. Real-time Polymerase Chain Reaction was used to quantify the DNA in this research study through the use of Applied Biosystems kits Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit

NOTE: This method is currently not used in the University of North Texas Health Science Center DNA Identity Laboratory so the protocol used is based on Applied Biosystems protocol for the Quantifiler kits (http://docs.appliedbiosystems.com/pebiodocs/04344790.pdf)

Material

1) Quantifiler™ Human Primer Mix or Quantifiler™ Y Human Male Primer Mix (Forward and reverse primers to amplify human DNA or human male DNA, probe to detect human DNA or human male DNA target, and IPC (internal positive control) primers, template, and probe)
2) Quantifiler Human DNA standard (200ng/µl) purified DNA standard)

3) Quantifiler PCR Reaction Mix (AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components

DNA Quantification Standard

Table 1: DNA Quantification Standards dilution series

Standard	Concentration (ng/µl)	Amount	Dilution Factor
Standard 1	50.00	10µl [200 ng/µl stock] + 30µl TE-4 buffer	4X
Standard 2	16.70	10µl [Std. 1] + 20µl TE-4	3X
Standard 3	5.560	10µl [Std. 2] + 20µl TE	3X
Standard 4	1.850	10µl [Std. 3] + 20µl TE	3X
Standard 5	0.620	10µl [Std. 4] + 20µl TE	3X
Standard 6	0.210	10µl [Std. 5] + 20µl TE	3X
Standard 7	0.068	10µl [Std. 6] + 20µl TE	3X
Standard 8	0.023	10µl [Std. 7] + 20µl TE	3X

1) Label eight micorcentrifuge tubes Std. 1 through Std. 8

2) Dispense the required amount of TE-4 to each of the tubes

3) Vortex the Quantifiler Human DNA standard for 5 seconds

4) Pipette 10µl [200 ng/µl stock] Quantifiler Human DNA standard into the tube marked standard 1

5) Vortex the sample

6) Add 10µl of standard 1 to the tube marked standard 2

7) Vortex

8) Repeats steps 6 and 7 until the dilution is completed

Sample Preparation

1) Determine the number of samples to be quantified

2) Calculate the volume of each component needed for the reaction

Quantifiler Human Primer Mix or Quantifiler Y Human Male Primer Mix

10.5µl (per reaction) X number of samples = total volume

Quantifiler PCR Reaction Mix

12.5 µl (per reaction) X number of samples = total volume

3) Thaw the primer mix and reaction mix then vortex the primer mix but only swirl the reaction mix

4) Pipette the appropriate volumes into a microcentrifuge tube

5) Vortex the mixture

6) Dispense 23µl of PCR mix into each reaction well

7) Add 2µl of sample, standard, or control to the appropriate well

8) Seal the reaction plate with the Optical Adhesive cover and centrifuge

9) Place the compression pad over the Optical adhesive cover with the gray side down and the brown side up and be sure that the holes are positioned directly over the reaction wells.

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

Software Setup

1) Open the ABI Prism 7000 SDS software

2) Select file, new to open the New document select Assay as absolute quantification, container 96-well clear, and template blank document and press OK

3) Select the wells which will include samples

4) Select view well inspector and select the dyes to be used

5) Label each of the wells with the appropriate name

6) For the standards this must be indicated in the Task box in the well inspector for each standard along with the sample amount in the quantity box

7) Set the thermal conditions by selecting the instrument tab, press the shift key and click within the stage 1 and then press the delete key

8) Select file and save

9) After plate has been loaded press the start bottom and the run take approximately 1 hour and 45 minutes

Y-STR Amplification

Amplification will be performed by Polymerase Chain Reaction (PCR) on the Perkin-Elmer GeneAmp® PCR System 9700 Thermal Cycler using the PowerPlex® Y System. PCR is an enzymatic process to replicate specific regions on the DNA in order to yield multiple copies of a particular DNA sequence.

Note: The University of North Texas Health Science Center DNA Identity Laboratory does not have protocol for amplification with the PowerPlex® Y System, but one has been created to be used in the future (appendix 1). Based on the Promega's PowerPlex® Y System Technical Manual. http://www.promega.com/tbs/tmd018/tmd018.pdf

1) Determine the number of samples to be amplified

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

   a. Distilled water: 9.45µl X ____ = _____µl

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

   c. Primers: 2.5µl X _____ = _____µl

   d. AmpliTaq Gold: .55µl X _____ = _____µl

3) Prepare the master mix and then vortex

4) Add 15 µl of master mix to each sample tube and then add 10µl of sample to the appropriate tube

5) Thermal Cycler Parameters

95°C for 11 minutes
96°C for 1 minute

10 cycles of: 94°C for 30 seconds
60°C for 30 seconds
70°C for 40 seconds

18 cycles of: 90°C for 30 seconds
57°C for 30 seconds
70°C for 45 seconds

60°C for 30 minutes
4°C hold

6) Store samples in the -20°C in the post-PCR room

Analysis

The ABI Prism® 3100 Genetic analyzer is base on capillary electrophoresis to analysis determine the size of DNA fragments by fluorescent emission detection.

Note: The University of North Texas Health Science Center does not currently have a protocol for analysis with the PowerPlex® Y System. The following is based on Promega's PowerPlex® Y System Technical Manual. http://www.promega.com/tbs/tmd018/tmd018.pdf

Sample Preparation

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

2) Vortex briefly

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

4) Add 1µl of amplified sample or allelic ladder

5) Cover wells with septa

6) Centrifuge briefly to insure all the sample is at the bottom of tube

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

Instrument Preparation

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

2) Open ABI Prism® 3100 collection software

3) Open a new plate record

4) Name the plate and select "GeneScan" and select the size of the plate either 96-well or 384-well

5) Fill the sample name in for all wells to be loaded, for ladders be sure to include the work ladder

6) Select Color Info as Blue, Red, Yellow, and Green

7) Copy and paste sample information to the color information column

8) In the "BioLIMS Project" column, select "3100_Project 1" from the pull down menu and fill down

9) In the "Dye Set" column select "Z" from the pull down menu and fill down

10) In the "Run module 1" column select the one for the PowerPlex® Y System and fill down

11) In the "Analysis Module 1" column select the one for the PowerPlex® Y System and fill down

12) Click OK, the new plate then will appear in the pending plate records table on the plate setup page of the collection software

13) Place samples into the instrument and close the door

14) In the pending plate records table, click on the name of the plate record you just created

15) Once the plate is highlighted, click the plate graphic which corresponds to your plate on the autosampler

16) The plate is then linked and the plate graphic should change from yellow to green

17) Click the "run instrument" button on the toolbar to start the sample run

Sample Detection

1) Analyze the date through the GeneScan® Analysis software

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

3) Recommended analysis parameters

   a) Analysis Range Start: defined in step 2, Stop: 10,000
   b) Data Processing: Baseline-checked, Multicomponent: checked, and Smooth options: Light[1]
   c) Peak Detection: Peak Amplitude Threshold[2] for 100 for all dyes and Min. Peak half width: 2pt
   d) Size call range: Min: 60 – Max: 600
   e) Size calling method: Local southern method
   f) Split Peak Correction: None

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

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

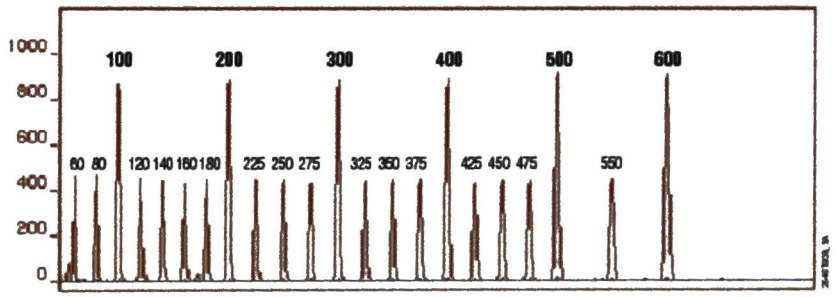

Figure 9: Internal Lane Standard 600

6) Apply the analysis parameters and size standard to samples, then analyze the samples

Data Analysis

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

2) Open the Genotyper® software and then the PowerTyper™ Y Macro

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

4) For Casework: double-click "Power" macro which will identify the alleles in the ladder sample and calculate offsets for all of the loci

5) For data basing or paternity: double-click on the "Power 20% filter" macro, should not be used if mixtures could be present

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

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

8) Create the appropriate tables bye selecting "PowerTable", "Make allele table" or "Make Vertical Table" macro

CHAPTER V

RESULTS AND DISCUSSION

Sensitivity Study

The sensitivity study was performed by a serial dilutions of known DNA concentration in order to examine the performance of the PowerPlex® Y System. The dilutions consisted of the following 10ng, 5ng, 2.5ng, 1ng, 750pg, 500pg, 250pg, 125pg, 75pg, and 50pg. All twelve loci were examined for each of the DNA concentrations, concluding the optimal amount of DNA is between 1ng-500pg.

Sample	DYS391	DYS389I	DYS439	DYS389II	DYS438	DYS437
10ng	7665	7695	4449	7553	7444	7844
5ng	7586	6250	2200	5359	7832	7832
2.5ng	3856	4523	687	5072	7716	7716
1ng	1281	2367	150	1840	1776	1776
750pg	2940	2629	819	2740	6421	6421
500pg	2211	1171	588	1243	1903	2719
250pg	329	373	145	557	358	1208
125pg	1146	233	232	368	857	857
75pg	436	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out	567	567
50pg	389	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out	290	290
	DYS19	DYS392	DYS393	DYS390	DYS385a	DYS385b
10ng	4161	4504	7600	7302	2500	2405
5ng	2476	2518	5676	4071	1784	1488
2.5ng	2356	2589	3741	1095	1121	307
1ng	1812	2664	1307	620	1307	879
750pg	1398	1196	4488	2033	552	933
500pg	823	1085	1343	1231	381	196
250pg	201	333	927	259	Allelic Drop-out	Allelic Drop-out
125pg	599	176	819	379	Allelic Drop-out	Allelic Drop-out
75pg	196	Allelic Drop-out	500	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out
50pg	124	Allelic Drop-Out	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out

Table 2: RFU values of the twelve PowerPlex® Y loci for the sensitivity study

This amount was determined by reviewing the peak heights through the Relative Flurescent Unit (RFU) value of each of the samples at each locus (Table 2), with optimal RFU value at less than 2,000 RFU. Also split peaks were recognized at most loci for the 10ng, 5ng, and 2.5ng samples which indicates too much DNA is present this is due to incomplete nucleotide addition. (Figure 10)

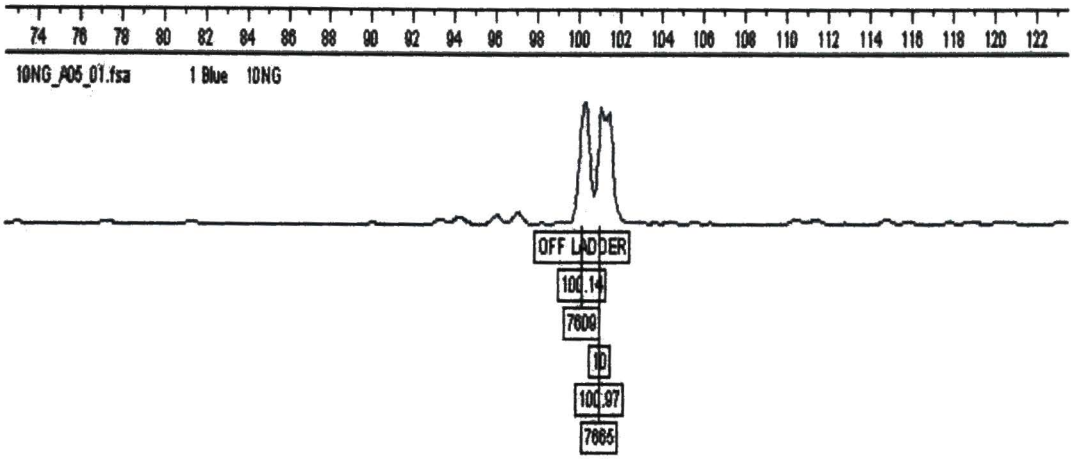

Figure 10a: 10ng sample with split peaks

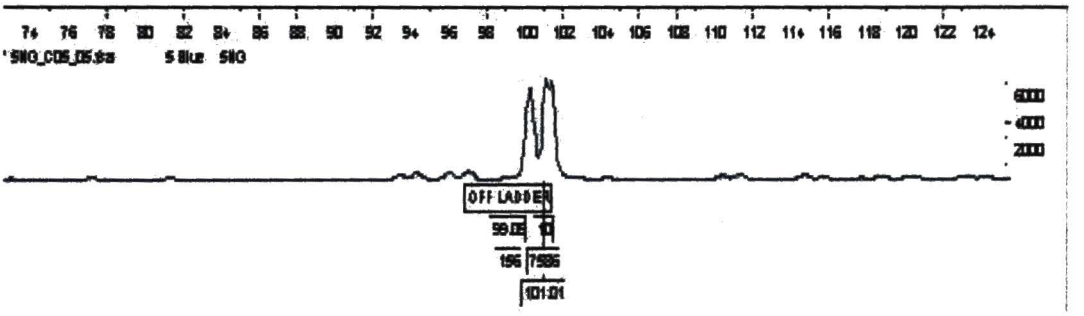

Figure 10b: 5ng sample with split peaks

Allelic dropout was observed at 250pg, 125pg, 75pg, and 50pg indicating that too little DNA is present in the sample in order to obtain a full profile. For the 250pg and 125pg sample's allele drop out occurred at loci DYS385a/b. (Figure 11) The profile obtained

using 75pg of DNA showed allele drop out at loci DYS392, DYS390, DYS385a/b, DYS389I, DYS389II, and DYS439. For the 50pg sample only four of the twelve loci had an allele call and these loci were the one's with the fewest bases within the PowerPlex® Y System.

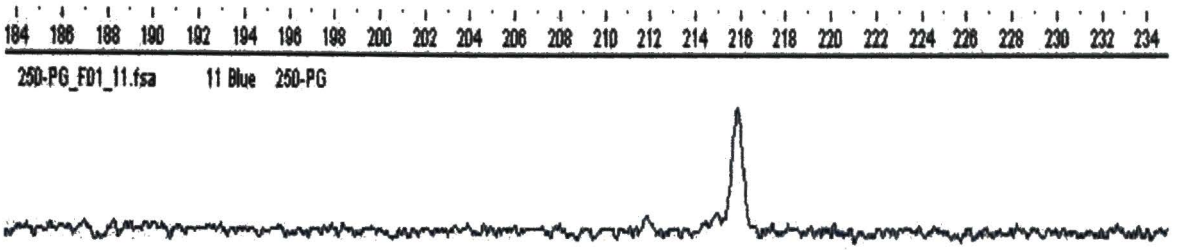

Figure 11a: 250pg sample with allelic drop out

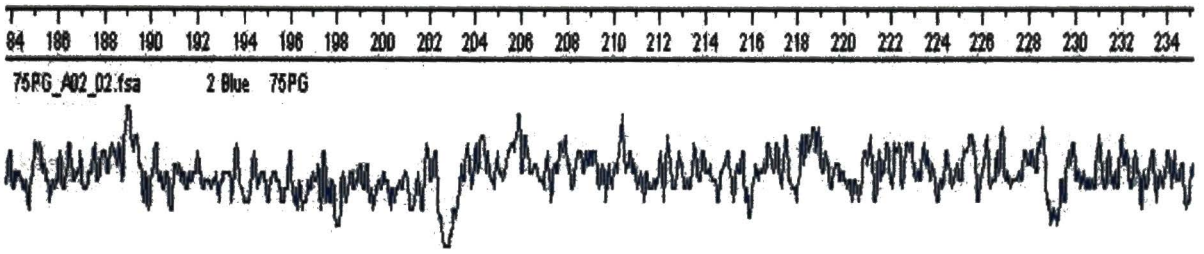

Figure 11b: 75pg sample with allelic drop out

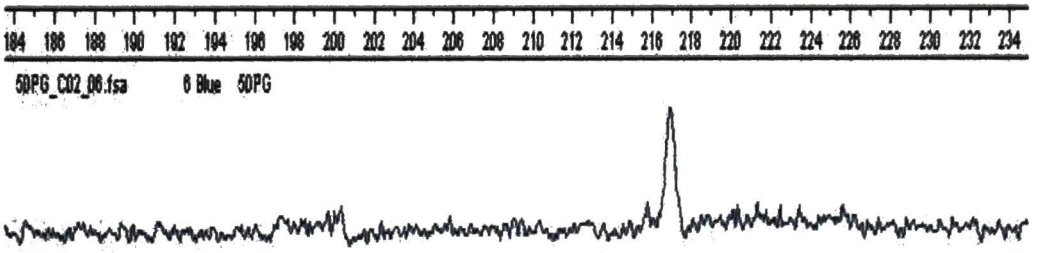

Figure 11c: 50pg sample with allelic drop out

Therefore the optimal amount of DNA to be added to PCR reaction for the PowerPlex® Y System is 1ng-500pg.

Two Male Mixture Study

The mixture study was performed in order to determine how to recognize a mixture and the sensitivity of the mixture with different proportions of DNA. Two unrelated male samples, one designated as a minor contributor and the other as a major contributor were used in the study. Seven of the twelve loci were similar in alleles; therefore these loci cannot be used to compare peak height ratios between a major and minor component. The peaks can be evaluated to determine the average peak height for common alleles of a male-male mixture at different dilutions. (Table 3)

Sample	DYS437	DYS439	DYS393	DYS438	DYS19	DYS392	DYS385b	Average
1:50	7836	961	5774	1066	1381	835	628	2640
1:25	7474	1193	2329	1181	1718	860	835	2227
1:10	7726	868	6807	1469	1166	724	700	2780
2:8	7867	1641	5099	817	613	411	431	2411
3:7	3713	714	3332	547	368	391	174	1319
1:5	4040	651	2491	603	268	458	379	1270
1:1	7355	448	4074	1522	1560	875	790	2365

Table 3: Peak heights and average of common alleles of male-male mixture

The study examined five of the twelve loci, DYS391, DYS389I, DYS389II, DYS385b, and DYS390 with the minor male the allelic profile obtain was 10, 13, 29, 11, and 23 respectively and for the major male the allelic profile was 12, 12, 28, 13, and 24. The male-male were prepared by combining DNA from the minor and major male volunteers in the following proportions: 1:50, 1:25, 1:10, 1:5, 2:8, 3:7, and 1:1. The RFU values were compared to determine if accurate results could be detected with the male-male mixture proportions. (Table 4)

Mixture Ratio	Major Component			Minor Component		
	DYS391	DYS389I	DYS389II	DYS391	DYS389I	DYS389II
1:50	4910	3003	3486	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out
1:25	1284	1183	764	Allelic Drop-out	Allelic Drop-out	Allelic Drop-out
1:10	5773	3477	2570	1334	Allelic Drop-out	Allelic Drop-out
2:8	7414	2879	1886	3359	712	541
3:7	1367	1206	1397	1002	471	456
1:5	3718	3057	863	1187	623	Allelic Drop-out
1:1	1621	3664	739	2658	2533	705
	Major Component			Minor Component		
		DYS390	DYS385b		DYS390	DYS385b
1:50		1676	515		Allelic Drop-out	Allelic Drop-out
1:25		2195	808		315	Allelic Drop-out
1:10		1024	482		243	Allelic Drop-out
2:8		639	302		294	101
3:7		349	339		236	200
1:5		885	138		344	Allelic Drop-out
1:1		1040	140		1236	148

Table 4: Male: Male Mixture Peak Heights

The peak height average was determined across the five loci for each of the samples both minor and major components, which can be compared to the average of the loci which had the same loci for both individual. (Table 5)

Mixture Ratio	Major Component Average	Minor Component Average
1:50	2718	Allelic Drop out
1:25	1247	Allelic Drop out
1:10	2665	315
2:8	2624	1001
3:7	932	473
1:5	1732	430
1:1	1441	1456

Table 5: Peak Height Average across five loci

For the 1:50 ratio allelic drop out occurred for the minor allele in all the five loci (figure 12) and for the 1:25 allelic drop occurred at all the loci but DYS390. Allelic dropout occurred for the minor allele at proportions 1:10 and 1:5.

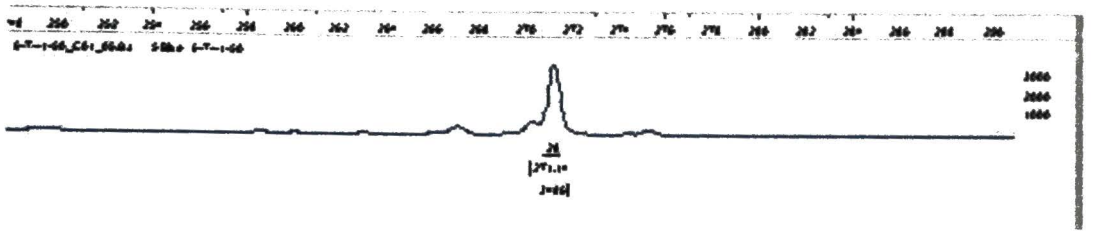

Figure 12: Male-Male Mixture 1:50

For the 1:1 samples two peaks can be seen of similar heights showing equal amounts of both contributors are present within that sample. (Figure 13)

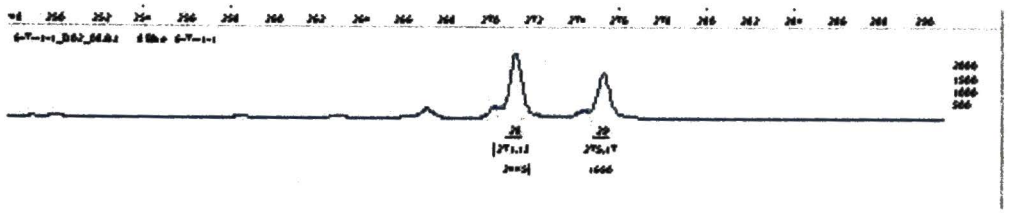

Figure 13: Male-Male Mixture 1:1

Peak height ratios were calculated for all loci producing a profile of both the minor and major male contributor by dividing the minor peak height by the major peak height to determine major/minor ratio. (Table 6)

Mixture Ratio	DYS391	DYS389I	DYS389II	DYS390	DYS385b
1:10	.231076	Allelic Drop-out	Allelic Drop-out	.237305	Allelic Drop-out
2:8	.453062	.247308	.28685	.460094	.334437
3:7	.732992	.390547	.326414	.676218	.589971
1:5	.319258	.203795	Allelic Drop-out	.388701	Allelic Drop-out
1:1	1.639729	.691321	.953992	1.188462	1.057143

Table 6: Peak height Ratios for male: male mixtures

Male-Female Mixture Study

The male-female mixture samples were prepared by adding 10ng of female DNA to each sample and the following proportions of male DNA 3ng, 2ng, 1ng, 750pg, 500pg, 250pg, and 100pg. A complete male profile was obtained at each of the proportions showing the female DNA at 10ng did not effect the performance of the PowerPlex® Y System. (Figure 14)

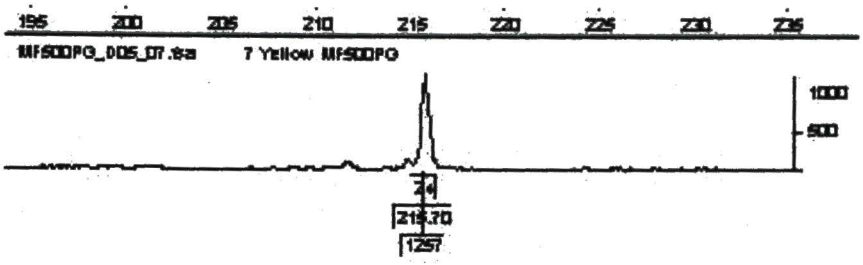

Figure 14: Male-Female Mixture 500pg

Therefore the system would perform accurately when used in a sexual assault case to obtain a male profile. The importance of this finding is especially useful for vaginal swab samples containing high levels of female DNA compared to male DNA. The PowerPlex® Y System would eliminate the need to perform a differential extraction which allows for the separation of female and male DNA. Eliminating this extraction and using the organic extraction method for vaginal swab samples time would be saved for these samples.

Substrate Study

The effect of different substrates was examined by adding 50µl of male blood samples onto a variety of substrates frequently seen at crime scenes. The substrates used in this study were; blue jean material, black cotton, white cotton, nylon, terry cloth

material, leather, and pink cotton. The samples were extracted using both Chelex® 100 and organic extraction. A complete profile (Table 7) was obtained from all the substrates except for the leather substrate.

Substrate	Complete Profile	Partial Profile	No results
Blue Jean Material	Positive	N/A	N/A
Black Cotton	Positive	N/A	N/A
White Cotton	Positive	N/A	N/A
Nylon	Positive	N/A	N/A
Terry Cloth	Positive	N/A	N/A
Leather	Negative	Negative	Positive
Pink Cotton	Positive	N/A	N/A

Table 7: Chelex and Organic Substrate Results

This could be due to inhibitors such as tannic acid found in the leather, which could affect amplification via PCR. Also when cutting the samples to be added to the extraction tube the leather was difficult to cut which could have lead to the DNA sample never being added to the tube. (Figure 15)

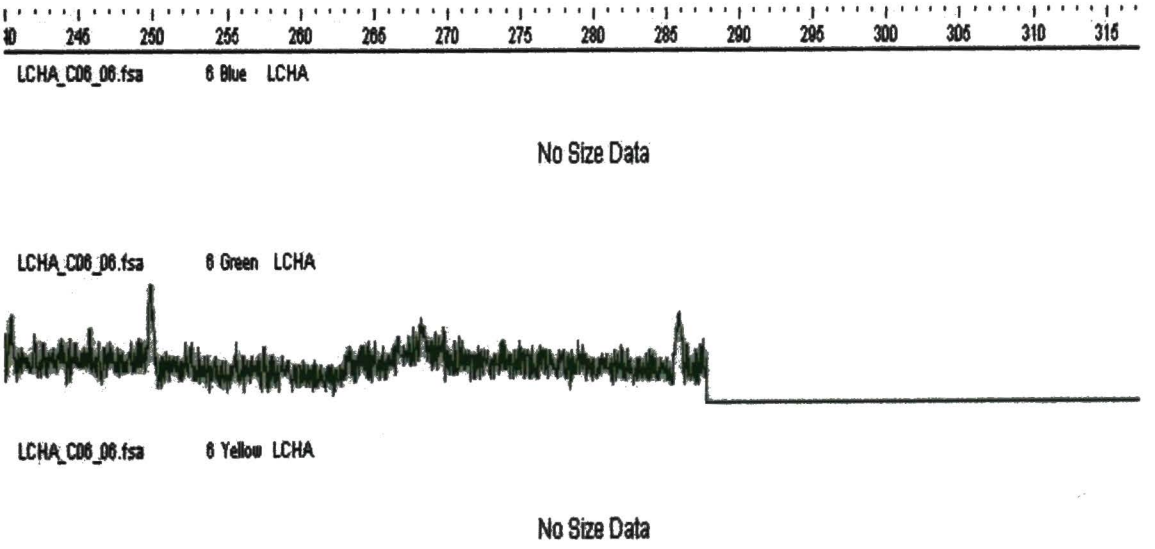

Figure 15: Leather Sample

The issue of leather not working with the PowerPlex® Y System is not too significant because leather is not one of the most common substrates found in sexual assault cases.

Chemical Contamination

The effect of different substances was examined by adding 50µl of the male blood sample and 50µl of the substance being examined. The substrates used in this study are frequently used to clean crime scenes or can be mistaken for blood which are hydrogen peroxide, 10% and 100% bleach, water, alcohol ethanol, red nail polish, cleanser and water, soup and water, and spic & span.

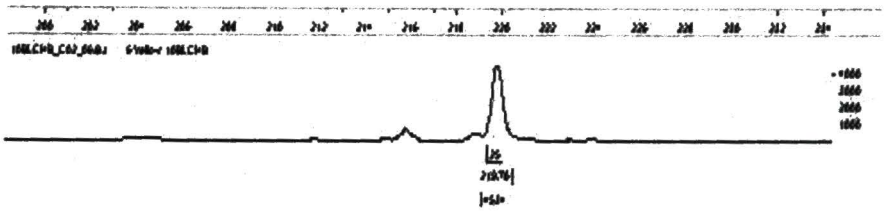

Figure 16: 100% Bleach sample

The samples were extracted using both chelex and organic extraction. A complete profile resulted in all the samples mixed with the different substances, even the 100% bleach, (figure 16) with no allelic drop out at any of the twelve loci (Table 8).

Chemical Contaminate	Complete Profile	Partial Profile	No Results
Hydrogen Peroxide	Positive	N/A	N/A
10% bleach	Positive	N/A	N/A
100% bleach	Positive	N/A	N/A
Water	Positive	N/A	N/A
Alcohol ethanol	Positive	N/A	N/A
Red nail polish	Positive	N/A	N/A
Cleanser and water	Positive	N/A	N/A
Soup and water	Positive	N/A	N/A
Spic and span	Positive	N/A	N/A

Table 8: Organic and Chelex Chemical Contaminant Results

Environmental Study

In order to determine the effects of humidity and temperature on the PowerPlex® Y System samples were placed at temperatures of 37°C and 57°C along with subjected to humidity at room temperature and 57°C. The samples were taken out of the environmental conditions at 24 hours, 72 hours, 7 days, 14 days, 21 days, and 1 month and placed in the freezer until extractions via chelex (Table 9) or organic (Table 10) were performed.

	24 hours	72 hours	7 days	14 days	21 days	1 month
37°C	+	+	+	+	+	+
57°C	+	+	+	+	+	+
Room Temperature with humidity	PP, DYS391 DYS389II DYS389I DYS437 DYS19 DYS392	+	+	PP, DYS391 DYS389I DYS389II DYS438 DYS439	PP, DYS389II DYS19 DYS393 DYS390	PP, DYS389I DYS389II DYS438 DYS19 DYS393 DYS392 DYS390 DYS385a/b
57°C with humidity	+	PP, DYS391 DYS389I DYS389II DYS438 DYS19 DYS392 DYS393 DYS390 DYS385a/b	+	+	+	PP, DYS389II

Table 9: Chelex Environmental Results: complete profile (+), partial profile (PP), and the drop out alleles are indicated

Allelic drop out occurred in some of the samples limiting the results of complete profile. The samples, which allelic drop out occurred were those submitted to humidity both at room temperature and 57°C. Comparing samples at 57°C without and with humidity differences can be seen with the non-humidity sample having a complete profile, which the sample submitted to humidity had allelic dropout. (Figure 17)

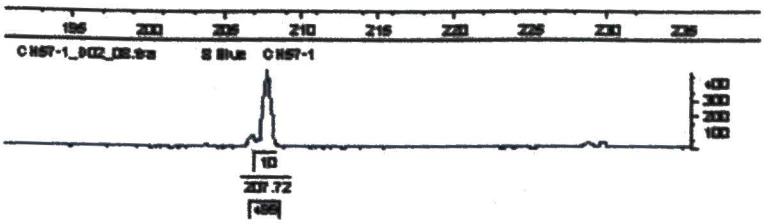

Figure 17a: Environmental Study: 57°C without humidity

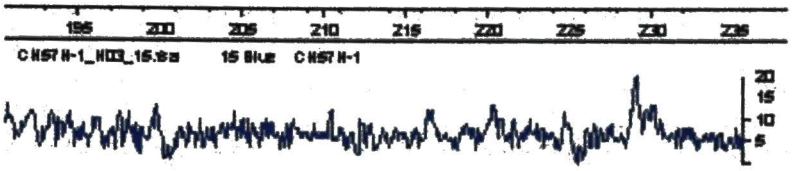

Figure 17b: Environmental Study: 57°C with humidity

This concludes that humidity has an effect on the PowerPlex® Y System and precautions should be made when samples are analyzed under those conditions.

	24 hours	72 hours	7 days	14 days	21 days	1 month
37°C	+	+	PP, DYS385a/b	+	+	+
57°C	+	+	+	+	+	+
Room Temperature with humidity	+	+	+	PP, DYS391 DYS389I DYS439 DYS389II DYS390 DYS438 DYS392	PP, DYS391 DYS389I DYS439 DYS389II DYS393 DYS390 DYS385a/b DYS438 DYS437 DYS19	PP, DYS389I DYS439 DYS390 DYS385a/b DYS438 DYS437 DYS19 DYS392
57°C with humidity	+	PP, DYS391 DYS389I DYS439 DYS389II DYS393 DYS390 DYS385a/b DYS438 DYS437 DYS19 DYS392	+	+	+	PP, DYS389I DYS439 DYS389II DYS393 DYS390 DYS385a/b DYS437 DYS19 DYS392

Table 10: Organic Environmental Results (complete profile (+), partial profile (PP), and the drop out alleles are indicated

Controls

Controls are important aspect of the validations study along with any scientific experiment, in which they verify that all areas of the experiment are working appropriately and no contamination has occurred. Reagent blanks are started at the beginning during extractions to ensure no contamination has occurred doing the extraction procedure along with the reagent being contaminated. For this validation study, reagent blanks showed no contamation in any of the extractions performed. DNA controls are run starting at the PCR point of the procedure, which include a negative female DNA, positive male DNA, and negative male DNA. For the negative female DNA, 9947A DNA was used which is included in the PowerPlex® Y System Kit. The function of this control is to ensure no female DNA is being called by the PowerPlex® Y System since it is male specific. In the case of this validation study no female DNA was seen in the electrophegrams on the negative female DNA ensuring the system is working properly. The positive male DNA used in the kit is 9948 Male DNA. This control will give a complete know male profile to ensure that the PowerPlex® Y System is interpreting the results accurately. (Figure 3) Negative male DNA, distilled water used during sample preparation, is ran with the samples to ensure no contamination occurred during amplification along with capillary electrophoresis, and the results in this validation study were accurate with no contamination.

Furthermore the Internal Lane Standard 600 is used to determine base pair size and the allelic ladder is used to call alleles. The Internal Lane Standard 600 (ILS 600) contains a total of 22 DNA fragments of the following base length: 60, 80, 100, 120, 140,

160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, and 600. (Figure 9) The ILS 600 is added to each capillary electrophoresis injection in order to increase precision in analyzes when using the PowerPlex® Y System. The fragments are labeled with carboxy-X-rhodamine (CXR) and are detected as a fourth color besides the three colors designating the loci. The ILS 600 was setup using the protocol stated in the methods and material and functioned correctly. The allelic ladder assigns alleles to each of the samples by comparing the sample to the allelic ladder, which consists of 102 alleles over the twelve loci. (Figure 8) For each of the studies performed for this validation study the allelic ladder functioned correctly.

CHAPTER VI

CONCLUSIONS

The expectations of this validation study and results obtained are similar. For the sensitivity study, Promega documents the optimal amount of DNA to be added to the PCR reaction is 500pg to 1ng, (11) which is consistent with the results of this validation study. For the mixture study of male and female DNA Promega claims up to a concentration >100X female DNA compared to male DNA is acceptable with no effects. This was the case in the validation study, which had a proportion of 1:50 with no effect. The substrate study showed leather to inhibit the PowerPlex® Y System which has been see to happen in other DNA testing (11). The environmental study test showed humidity effects the results of the PowerPlex® Y System. Also this frequently has been seen in other DNA testing due to the humidity causing DNA degradation, which results in a partial profile or no results for that sample.

The results obtained from this internal validation study can be compared to other validation studies for Y chromosome specific STR multiplex systems. For example the paper "Validation and casework application of a Y Chromosome specific STR multiple" written by Mechthild Prinz is comparable to that of this validation study performed (3). The Y-STR multiplex tested only four loci versus the PowerPlex® Y System which tests twelve. The studies addressed in each of the validation studies consist of mixture, sensitivity, environmental, and substrate. For the male-female mixture study, the female DNA had no effect on the results up to 1:4000 ratios. These results are similar to the

PowerPlex® Y System because no effect on the ability to obtain a full profile with a mixed sample of female and male DNA was observed. The sensitivity for the multiplex system showed allelic drop out at 125pg of DNA and optimal amount of DNA at 1-2ng. The PowerPlex® Y System showed allelic drop out at 250pg and optimal amount of DNA to be 500pg-1ng. The environmental studies were similar because both showed allelic drop out for the humidity conditions. The multiplex with four loci did not use leather as a substrate, the other substrates were similar in both validation studies and reported similar results.

Validation studies are frequently needed in order to validate a technique to be used in a forensic laboratory. Forensic cases are frequently under scrutiny by the judicial system. The questions due no lie in the science behind DNA testing but rather the process by which a laboratory performs the DNA test. In order to reduce the amount of questions validation studies are performed and interpretation guidelines are developed from those validation studies.

Interpretation guidelines are written to direct and assist an analyst in making a final interpretation of each individual sample. The guidelines consist of the control, which must be run along side each sample along with the excepted results of each of the controls. The guidelines include types of identification for a sample such as no result, inconclusive, exclusive, and not excluded. Also DNA quantification information is included along with internal lane standard and allelic ladder guidelines. Interpretations of mixtures are included, which explains peak height ratios and how to determine a mixed sample. A calculation section is included to address the appropriate calculations to be used with the system to analyze the results. Interpretation guidelines will be produced

based on this validation study for the University of North Texas Health Science DNA Identity laboratory.

REFERENCES

1. DNA Advisory Board 1998. "Quality Assurance Standards for Forensic DNA Testing Laboratories." Forensic Science Communications, July 2000: 1-15. http://www.fbi.gov/programs/lab/fsc/backissu/july2000/codis2a.htm.

2. Butler JM, Schoske R, Vallone PM, Kline MC, Redd AJ, Hammer MF. A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers. *Forensic Science International*, 129 (2002) 10-24.

3. Prinz M, Ishii A, Coleman A, Baum HJ, Shaler RC. Validation and casework application of a Y chromosome specific STR multiplex. *Forensic Science International*, 120 (2001) 177-188.

4. Applied Biosystems. "Quantifiler™ Human DNA Quantification Kit Quantifiler Y Human Male DNA Quantification Kit" pdf product bulletin. http://www.docs.appliedbiosystems.com/pebiodocs/00112974.pdf

5. Applied Biosystems. "ABI Prism 7000 Sequence Detection System" pdf manual. http://www.docs.appliedbiosystems.com/pebiodocs/00104039.pdf

6. Applied Biosystems. "Real-Time PCR Vs. Traditional PCR" pdf manual. http://www.appliedbiosystems.com/support/tutorial/pdf/rtpcr_vs_tradpcr.pdf

7. Applied Biosystems. "Essentials of Real Time PCR" pdf manual. http://www.docs.appliedbiosystems.com/pebiodocs/00105622.pdf

8. Budowle, Bruce, *DNA Typing Protocols: Molecular Biology and Forensic Analysis*, Eaton Publishing. 2000.

9. Butler, John. *Forensic DNA Typing*. Academic Press. 2003.

10. "YSTR Postitions along Y Chromosome" Internet. 7 May. 2004. Available. http://www.cstl.nist.gov/div831/strbase/ystrpos1.htm

11. Promega. "PowerPlex® Y System Technical Manual No. D018" pdf manual. http://www.promega.com/tbs/tmd018/tmd018.pdf

12. Butler, John. "NIST Developments in Y-Chromosome Analysis" 20 August 2003. pdf. http://www.cstl.nist.gov/biotech/strbase/pub_pres/ButlerAug2003.PDF

13. Krenke BE, Fulmer PM, Miller KD, Spreacher CJ. "PowerPlex® Y System" pdf. http://www.promega.com/profiles/602/ProfilesInDNA_602_06.pdf

14. Applied Biosystems. "Quantifiler™ Kits User's Manual" pdf manual. http://docs.appliedbiosystems.com/pebiodocs/04344790.pdf

15. Applied Biosystems. "GeneScan® Analysis Software Product Bulletin" pdf. http://docs.appliedbiosystems.com/pebiodocs/00103532.pdf

16. Promega. "Y-Chromosome Specific STRs" pdf. http://www.promega.com/profiles/601/ProfilesInDNA_601_03.pdf

17. Willuweit S, Roewer. Y-STR Haplotype Reference Database. Internet. 2004. Available: http://ystr.charite.de/index_gr.html

UNT Health Science Center
DNA Identity Laboratory

Case Number _____ **AGENCY NUMBER(S)** _____

Y-STR Amplification Setup - 25μl reactions

Principle:

PCR Set-Up and thermal cycling: Promega's PowerPlex® Y System. This protocol is for a 25 uL PCR final reaction volume and is used as the standard reaction for most samples. Some samples, i.e. those that may contain inhibitors, etc may be amplified using an alternate procedure for 50 µl reaction volumes.

Date Started

SAMPLE IDENTIFICATION AND LABELING. — ORGANIZE THE SAMPLES TO BE AMPLIFIED WITH THIS PROTOCOL AND FILL IN THE CHART WITH THE REQUIRED INFORMATION. *DO NOT FORGET POSITIVE AND NEGATIVE CONTROLS.*

Tube	System	Cycles	Sample ID	Dilution	DNA	Water

NOTE: Evidentiary DNA extracts and extracts from known reference samples must be amplified seperately

NOTE: If several samples from individual cases are being amplified in a batch format, list all case numbers in header and include a copy of this worksheet in each case file

☐ **Check Here if Batch Processed**

Analyst Initials: _____
Date: _____

Case Number _____ **AGENCY NUMBER(S)** _____

Safety:

When handling any potentially biohazardous material, always wear personal protective equipment and follow universal safety precautions. All personnel working with biological specimens must wear a lab coat, powderless latex gloves, and eye protection.

Quality Assurance:

1. Verify identification numbers, as appropriate.
2. Amplification tubes containing DNA template must remain outside of the PCR set-up hood.
3. Only one reagent tube should be open at any point in time.
4. Clean benchtops and hood daily with 10% bleach solution.
5. PCR Set-Up must take place in a different room from thermal cycling and other activities that generate or involve handling of PCR products.
6. Use dedicated space and dead-air hood and dedicated pipettors for handling PCR reagents and master mix set-up.
7. Store PowerPlex® Y System kits at-20° C.
8. PCR Set-up and amplification of evidence (unknown) samples must take place prior to PCR Set-up and amplification of reference (known) samples.

Equipment & Supplies:

- Microcentrifuge
- Fisher Vortex Genie 2
- Micropipettes : P10, P 20, P100, P200, P1000
- ART pipet tips (barrier tips)
- 1.5 mL microcentrifuge tubes
- Thermal Cycler ABI 9700 Thermal Cycler ID: _____
- MicroAmp Tubes w/ Caps (sterile)

Reagents:

- PowerPlex® Y System Kit PowerPlex Y System lot# _____ Exp. Date: _____
  - Gold STAR 10X Buffer
  - AmpliTaq Gold Taq lot#: _____ Exp. Date: _____
  - PowerPlex® Y 10X Primer Pair mix
  - Male Positive Control
  - Female Negative Control

Analyst Initials: _____
Date: _____

PROCEDURE - STEPWISE:
A. Sample Set-Up and Preparation

Step Completed

1. Complete the chart on Page 1 with the appropriate sample information for the group of samples to be amplified with this Master Mix preparation. ☐

2. Label each 0.2 mL MicroAmp tube with specimen ID or tube number and place into a 96-well thermocycler retainer/rack. **Confirm concordance with the information listed on Chart on Page 1.** ☐

B. Set-Up and Prepare Master Mix

1. Vortex the PowerPlex® Y Gold Star 10X buffer, PowerPlex® Y Primer Set and AmpliTaq Gold DNA Polymerase for 5 seconds. Centrifuge the tubes briefly to remove any liquid from the caps. Place these items within the PCR setup dead-air hood.
2. When making up a Master Mix for multiple samples, always take the total number of samples (including controls) + 2 and multiply the single sample amount of each component.
3. Fill in the appropriate values on the chart below for Master mix preparation.

PowePlex® Y System Enter total # PCR Amplifications:_____(Add 2 reactions) Not performed ☐

a.	Gold Star 10X Buffer:	2.5	x	_____	=	_____ uL
b.	AmpliTaq Gold:	2.5	x	_____	=	_____ uL
c.	Primer Set:	2.5	x	_____	=	_____ uL
d.	Nuclease-Free Water:	9.45	x	_____	=	_____ uL
	Final volume PCR Master Mix				=	_____ uL

4. Mix thoroughly by vortexing for 5 seconds. Centrifuge briefly.
5. Dispense 15 uL of PCR Master Mix into each PCR tube.

Analyst Initials: _____
Date: _____

C. Add DNA to PCR:

1. Vortex DNA extracts for 5 seconds and centrifuge briefly.
2. Prepare any necessary dilutions of DNA extracts as per quantitation results.
3. Add appropriate diluted DNA extract volume (typically 2 µl) to each MicroAmp tube.

NOTE: Add 2 ng (or up to 2 ng if sample is limiting) to each PCR tube.

- PCR Male Positive Control: Add 1 – 2 ng of control DNA (9948)
- PCR Female Negative Control: Add 1 – 2ng of control DNA (9947A)
- PCR Negative Control: Add 10 µL of sterile water

NOTE: Sample DNA volume must total 10 ul, such that the final volume of all PCR amplification tubes is 25 uL.

4. Seal the tube/rack assembly making sure that each tube is properly capped to avoid evaporation.

D. Thermal Cycling:

1. Transport the tube/rack assembly to the Post-PCR laboratory within the dedicated lock box . Walk directly between the pre-PCR area and the Post-PCR laboratory – do not stop to talk with anyone. Do not bring the lockbox and cart into the Post-PCR laboratory.
2. Place the tube/rack assembly into the block of a ABI 9700 Thermal Cycler and close the lid Record the Thermal Cycler number in the designated space on page 2 of this protocol and start the appropriate cycling program.
3. Record run information in Thermal Cycler Run Log.

NOTE: Do not bring this document into the Post-PCR laboratory.

E. Thermal Cycler Parameters – Profiler Plus *ID* & CoFiler

HOLD	95 degrees C / 11 minutes
	96 degrees C/ 1minute
CYCLE	**10 cycles** of:
	- 94 degrees C / 30 seconds
	- 60 degrees C / 30seconds
	- 70 degrees C / 45 seconds
	18-22 Cycles of:
	- 90 degrees C / 30 seconds
	- 58 degrees C / 30 seconds
	- 70 degrees C / 45 seconds
HOLD	60 degrees C / 30 minutes
HOLD	4 degrees C / (forever)

Note any changes to standard protocol:

The preceding procedure was completed as described in this protocol:

Case Number _____ **AGENCY NUMBER(S)** _____

Analyst's signature _____ Date ____/____/____

Appendix 2

UNT Health Science Center
DNA Identity Laboratory
Proposed
W(A). Interpretation Guidelines for Y STR Analysis

Note: This guide is not meant to be a set of rigid rules for the analyst to follow. Rather, it is recognized that the responsibility of the analyst is to utilize the knowledge that he or she has developed in their own experience to make the final interpretive judgment. The reality of forensic biology is that each case, each sample, has it's own individual character. Therefore, it is not possible to develop a set of rules to be rigidly followed. These guidelines are just that, guidelines to be used by the analyst as an aid in making that final interpretation on an evidentiary sample.

1. A positive (+) and negative (-) control will be run with each and every amplification set of samples to be processed.
2. A reagent blank will be run with each and every extraction set of samples to be processed.
3. It is recommended that all DNA extractions and amplifications be performed by female analysts. In those instances where that is not possible, then the Y- STR profile of the male analyst must be known prior to him performing casework.
4. In certain instances, due to the quantity and/or quality of the DNA in a sample, it may not be possible to obtain conclusive or reportable results.
5. The three controls (+,- and reagent blank) should be fully analyzed and produce the expected results.
6. If the reagent blank or negative control produce amplified product, then refer to the *Contamination Control Policy*.
7. The positive control must always yield the expected results. Refer to manufacturers guidelines for the proper allele designations.
8. If the positive control fails to amplify or produce the expected results, the analyst will investigate the cause and take appropriate corrective action. Samples amplified along with the failed positive control must be re-amplified/re-analyzed.
9. The following definitions apply to casework samples involving identification either for unknown remains or stain analysis.

No result (NR)- no visible result is present.
Inconclusive (INC)- a result is present but not interpretable.
Exclusion/Excluded- a reference sample is excluded as a donor of the sample in question when an allele present in a reference sample is not found in an unknown or evidentiary sample. The reference is eliminated as a possible source of origin of the unknown or evidentiary sample.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

Not excluded/Included- when an allele or alleles found in a reference sample are also found in an unknown or evidentiary sample. It is not necessary that all loci produce profiles. Calculations to determine the haplotype frequency will be performed in all cases where a reference sample is not excluded.

10. The following definitions apply to cases involving criminal paternity/ missing person body identification.

    No result (NR)- no visible result is present.
    Inconclusive (INC)- a result is present but not interpretable for any reason.
    Exclusion/Excluded- a reference sample is excluded at a particular loci when the obligate allele found in the evidentiary sample is not present in the reference sample. When this occurs at two or more loci, then the reference sample is excluded as sharing the paternal lineage of the evidentiary sample.
    Not Excluded/Included- the reference sample is not excluded at a particular loci when the obligate allele in the reference is also found to be present in the evidentiary sample. If this situation holds for all the interpretable loci analyzed, then the reference sample is not excluded as sharing the paternal lineage of the evidentiary sample. Calculations are performed to determine the haplotype frequencies

11. Cases involving kinship, reconstruction or other situations not listed above will be dealt with on an individual, case by case basis.

W(A).1 STR Analysis with the /3100 Genetic Analyzer

Note: In order to obtain optimal results using the Promega Power Plex Y System it is recommended that the amount of DNA template added to the reaction mixture fall within the 1.0 – 0.50 ng range.

1. All of the 22 peaks in the internal lane standard must be checked to make sure they fall within the accepted range of 60-600 bp.
   Do not use the results of sample in which the internal lane standard gives unacceptable readings.
2. The allelic ladder must be checked to make sure that all of the alleles for that particular ladder have been called correctly.
3. Several ladders may be run with each set of samples processed. If one ladder does not produce usable data when analyzing with *Power Typer Y Macro*, an analyst may delete that ladder from the project and re-run the *Power Typer Y Macro* with one of the other ladders.

   A positive control will be analyzed with every allelic ladder used to analyze a set of samples to insure accurate typing of unknown samples.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

4. Base pair sizing in the *GeneScan* printout may be periodically spot-checked to make sure that alleles being called by *Power Typer Y Macro* are correct.

5. All peaks with an RFU (relative fluorescence unit) of at least 100 will be assigned an allele designation by the analytical software.

6. Samples with peak heights of greater than 6000 rfu will be interpreted with caution and may be re-injected with a dilution or smaller volume of amplified product to better resolve results.

7. All results or electropherograms must be checked and approved by two qualified DNA analysts.

8. The general shape and appearance of the peak should be sharp and well defined so as to discriminate between a true allelic peak and any artifacts caused by sample overload, poor electrophoretic run, degraded or to little DNA, old matrix etc.

9. The locus DYS385a and DYS 385b are the only loci that more then one allele (peak) will be observed for single source samples. Allele dropout for those loci has been observed at RFU levels below 400. If only a single peak (allele) is observed at those loci that falls below 400 RFU that loci will be called inconclusive.

10. In single source samples, the typical peak height ratio between two heterozygote alleles for loci DYS385 a and b is not less then 70%. This figure is a rule of thumb as some peak height ratios may be as low as 50% in low- level evidentiary samples. Contrary results may indicate a mixture. (see mixture interpretation below)

11. Reference samples must produce a maximum of one peak per locus for the loci DYS391,DYS439, DYS438, DYS437, DYS19, DYS392, DYS393,DYS389I,DYS389II and DYS390 with the exception of stutter peaks and chromosomal abnormalities. See #20

12. Stutter peaks are usually 4 base pairs (one repeat unit) shorter then the true allele though they can sometimes be 8 or 12 bases shorter or rarely 4 bases longer.

13. Stutter peaks can be recognized by this distinctive position, decreased intensity and do not typically exceed 18% of the true allele.

14. Off-ladder alleles may be called true alleles that fall outside the range of the ladder. Microvariants that fall outside the range of an allelic ladder will be given a greater than, or less than the largest or smallest marker designation.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

15. Possible microvariant samples must either be re-injected, or if possible, re-amplified to confirm the presence of the microvariant.

16. If the microvariant falls within the allelic ladder, within a given marker range that allele can be designated with a 0.X (ex. 21.1, 11.2 etc)

17. If many off-ladder peaks appear at any one locus it is an indication that too much DNA was amplified. That sample may be diluted and re-injected or amplified using less DNA.

18. Spurious peaks may result from fluctuations in current. Such spikes will be present in all colors. They are not reproducible, not associated with peak height or width and posses a thin, characteristic shape. Peaks demonstrating these characteristics may be disregarded.

19. Pull-up is a matrix effect caused by spectral overlap of the dyes. It is recognized a being in a different fluorescent color but corresponds almost exactly (+/-0.07bp) in size to a true allele of another fluorescent color. Pull-up data is generally reproducible and typically a result of off-scale peaks. If pull–up is observed then the following steps are to be followed:

    A. The matrix in use may be evaluated and if need be, replaced.

    B. The sample may be diluted which may decrease the appearance of pull-up.

    C. Loci demonstrating these pull-up characteristics may be considered inconclusive if pull-up is not resolved.

20. Chromosomal abnormalities may be observed. They may appear as an extra peak (two or three alleles at one locus), in cases of trisomy, translocations or duplications. They are
typically restricted to one locus and will be observed in both reference and evidentiary samples.

21. Mutations in the primer- binding region can lead to allelic drop-out.

22. If a peak or peaks appear between two loci due to non-specific amplification or other causes, they may be ignored at the analyst's discretion after investigation of the cause of the spurious peaks.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

23. If a locus is considered *Inconclusive*, efforts will be made to troubleshoot the cause. A reasonable effort to re-amplify, re-inject and/or re-analyze the sample will be made in order to better resolve the results. If the situation is still not resolved, the locus will be called inconclusive.

W.2 Interpretation of Mixtures

1. With regards to mixtures (DNA from two or more individuals in one sample), results must be interpreted for one marker in conjunction with the results from other markers. The analysts experience will be factored into the interpretation.

2. Several factors can indicate whether a mixture is present in a sample. They are: Any locus with two or more alleles. A peak in the stutter position with a peak height ratio greater than 18%. Peak heights in any locus that differ by more then 40%. Sample origin- is it the type of sample that a mixture of DNA might be found.

3. If the relative peak heights of two alleles are significantly different, one allele is considered the major component and the other is considered the minor component (an allele that is less than 60% peak height ratio of the major component).

4. Samples that produce results consistent with a mixture of two or more individuals are Reported as such. The report will include a statement with regards to who may or may not be included in the mixture.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

W.3 Calculations

Note: The Y- STR markers used by the UNTHSC are all located on the non-recombining region of the Y-chromosome and are inherited as a single unit or haplotype. Haplotype frequencies will be calculated using the counting method.

1. Frequencies for three racial groups (African-American, Caucasians and Hispanics) are calculated for single source profiles.

2. The UNTHSC DNA Identity Laboratory will only use databases that have been independently tested for linkage, homozygosity and adequate sample size. The database and search software used to calculate haplotype frequencies, ***Power Plex Y Haplotype Database*** is found on the Promega Corporation web site, http://www.promega.com/techserv/tools/pplexy/

3. All results and calculations are documented in the case file.

Reviewed:_____ Date:_____

Approved:_____ Revised:_____

ECKMAN
ERY, INC.
nd-To-Please"
OV 04
STER, INDIANA 46962