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The Development of Direct Ultra-Fast PCR for Forensic Genotyping Using Short Channel Microfluidic Systems With Enhanced Sieving Matrices

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THE DEVELOPMENT OF DIRECT ULTRA-FAST PCR FOR FORENSIC GENOTYPING USING SHORT CHANNEL MICROFLUIDIC SYSTEMS WITH ENHANCED SIEVING MATRICES

A dissertation submitted in partial fulfillment of

the requirements for the degree of

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in

CHEMISTRY

by

Maurice J. Aboud

2012

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Maurice J. Aboud, and entitled, The Development of Direct Ultra-Fast PCR For Forensic Genotyping Using Short Channel Microfluidic Systems With Enhanced Sieving Matrices, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Martin Tracey

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Watson Lees

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John Berry

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John Landrum

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Bruce McCord, Major Professor

Date of Defense: July 16, 2012

The dissertation of Maurice J. Aboud is approved.

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Dean Kenneth G. Furton
College of Arts and Sciences

_____
Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2012

DEDICATION

I would like to dedicate this dissertation to my Grandparents and Parents. Without their patience, family values, hard work, love and unending support, the completion of this work would not have been possible.

ACKNOWLEDGMENTS

In life we are sometimes so anxious to reach our destination that we forget the journey in arriving there, the years of studying and the many people whom we have encountered along the way. I would like to take this opportunity to remember that journey and thank everyone who motivated me along the way and imparted some little bit of knowledge or support to keep me going.

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My family, you have made this whole process worth it, as for Dad, thank you for lessons on sacrifice, commitment and your constant patience with me on this journey. Mom your constant questions have always driven me to make you proud and I will continue to strive to always be the best at what I do. You have always believed and encouraged me with your constant prayers. My sister Justine thanks for your constant support and help along the way. My Godson Zane, thanks for the happiness and joy that you have brought to the family and I am sure you will make us all proud.

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ABSTRACT OF THE DISSERTATION

THE DEVELOPMENT OF DIRECT ULTRA-FAST PCR FOR FORENSIC GENOTYPING USING SHORT CHANNEL MICROFLUIDIC SYSTEMS WITH ENHANCED SIEVING MATRICES

by

Maurice J. Aboud

Florida International University, 2012

Miami, Florida

Professor Bruce R. McCord, Major Professor

There are situations in which it is very important to quickly and positively identify an individual. Examples include suspects detained in the neighborhood of a bombing or terrorist incident, individuals detained attempting to enter or leave the country, and victims of mass disasters. Systems utilized for these purposes must be fast, portable, and easy to maintain. The goal of this project was to develop an ultra fast, direct PCR method for forensic genotyping of oral swabs.

The procedure developed eliminates the need for cellular digestion and extraction of the sample by performing those steps in the PCR tube itself. Then, special high-speed polymerases are added which are capable of amplifying a newly developed 7 loci multiplex in under 16 minutes. Following the amplification, a postage stamp sized microfluidic device equipped with specially designed entangled polymer separation matrix, yields a complete genotype in 80 seconds. The entire process is rapid and reliable, reducing the time from sample to genotype from 1-2 days to under 20 minutes. Operation requires minimal equipment and can be easily performed with a small high-speed

thermal-cycler, reagents, and a microfluidic device with a laptop. The system was optimized and validated using a number of test parameters and a small test population. The overall precision was better than 0.17 bp and provided a power of discrimination greater than 1 in 10^6.

The small footprint, and ease of use will permit this system to be an effective tool to quickly screen and identify individuals detained at ports of entry, police stations and remote locations. The system is robust, portable and demonstrates to the forensic community a simple solution to the problem of rapid determination of genetic identity.

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ACRONYMS AND ABBREVIATIONS

°C	Degree Celsius
A	Adenine
ABI	Applied Biosystem by Life Technologies
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
C	Cytosine
ccd	Charged Couple Device
CE	Capillary Electrophoresis
CGE	Capillary Gel Electrophoresis
CODIS	Combined DNA Index System
Ct	Critical Threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double Stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ENFSI	European Network of Forensic Science Institutes
EOF	Electroosmotic Flow
FBI	Federal Bureau of Investigations

G	Guanine
HEC	Hydroxyethyl Cellulose
I.D.	Internal Diameter
ILS	Internal Lane Standard
Iso-C	5-methylisocytosine
Iso-G	Isoguanine
LIF	Laser Induced Fluorescence
LPR	Low Profile Rapid
LTDNA	Low Template DNA
$MgCl_2$	Magnesium Chloride
MW	Molecular Weight
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NRC	National Resource Council
PCIA	Phenol- Chloroform Isoamyl
PCR	Polymerase Chain Reaction
PDMS	PolyDiMethylSiloxane
PHR	Peak Height Ratio
PMMA	PolyMethy-MethAcrylate
POP	Performance Optimized Polymer
PVP	Polyvinyl Pyrrolidinone
QC	Quality Check

qPCR	Quantative PCR
RD	Rapid Direct
RD-PCR	Rapid Direct PCR
RFLP	Restriction Fragment Length Polymorphism
rtPCR	Real-Time PCR
SDS	Sodium Dodecyl Sulfate
SINE	Short Interspersed Nuclear Elements
ssDNA	Single Stranded DNA
STRs	Short Tadem Repeats
T	Thymine
T_a	Primer Annealing Temperature
TAPS	3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid
Taq	Thermus aquaticus Polymerase
Tm	Primer Melting Temperature
UV	Ultra Violet Radiation
VNTR	Variable Number Tandem Repeats

CHAPTER I: FORENSIC DNA ANALYSIS

a) History & Technology

Forensic science, the application of science to law, has been used from as early as society developed a system of order. Quintilian, a Roman attorney in the year 1000 used a bloody palm print to frame a blind man for his mother's murder, while the Chinese book 'His Duan Yu' written 1248 shows the difference between drowning and strangulation.[1] While these early accounts were seen as mere logical correlations, many of these disciplines have advanced greatly and have become detailed scientific areas of study. It was not until the late 1800's that technological advances and application of science was truly seen as a useful tool to solve crimes, and forensics as we know it today was born. Mathiew Orfila, the scientist who developed testing for the presence of blood, who is considered the father of toxicology, Sir Francis Galton and Sir Edward Richard Henry who developed fingerprint classifications for identification of individuals are among the many scientists who have laid a solid foundation for the science of forensics.[1] It is important to note that in this manuscript the author places emphasis solely on the history of forensic DNA analysis and the technological advancements relevant to its development.

Deoxyribonucleic acid (DNA) is the genetic material that makes up human and living organisms. The purpose of DNA is to store genetic information that codes for biological processes. Work done by Erwin Chargaff on the composition of DNA and its nucleotides resulted in a discovery that helped James D. Watson and Francis Crick with the structure of DNA. Chargaff was able to show that the percentages of the four bases

connected in this structure, Adenine (A), Thymine (T), Cytosine (C) and Guanine (G) were present in a ratio of 30.9: 29.4: 19.8: 19.9 respectively.[2-5] The ratio meant that there was an almost even number of A and T and also G and C in their compositions. While Chargaff himself was unable to figure out exactly what this meant for the structure of DNA, a visit with James D. Watson and Francis Crick at Cambridge in 1952 provided valuable data to the two scientist, who at that time were trying to solve the structure of deoxyribonucleic acid. The following year, Watson and Crick discovered the structure of DNA with insight from Chargaff's results. They found that a DNA molecule existed as a double stranded helix made from a chain of nucleotides unlike the alpha helix that was previously thought to be the correct structure.[6]

The beginning of forensic DNA analysis can be ascribed to the work done by Sir Alec Jeffreys, a British geneticist from the University of Leicester, England. Sir Alec discovered a set of variable regions within the human genome that were able to differentiate between two individuals within a given population. These regions known as Variable Number of Tandem Repeats (VNTRs) are based on the number of times a specific sequence of DNA is repeated in tandem within the DNA.[7-10] Because of the nature of these polymorphisms and the number of length variations that can occur within individuals, a very high power of discrimination can be achieved. These repeats were analyzed using a method called restriction fragment length polymorphism (RFLP) developed in the 1970's for the detection of single base variations within humans.[11] A restriction enzyme is used to cut the DNA at a specific sequence into smaller fragments, which are then separated by their differences in size using electrophoresis in an agarose gel. The smaller fragments travel faster through the gel and hence appear further along

the gel strip. The fragments are then fixed to a nylon membrane which is labeled with a radioactive (P_{32}) probe. The probe binds to only specific regions on the fragments and when exposed to X-rays result is a barcode like image. The image was referred to as a "DNA fingerprint" by Sir Jeffreys, and then compared to a known sample for identity (Figure 1).[7]

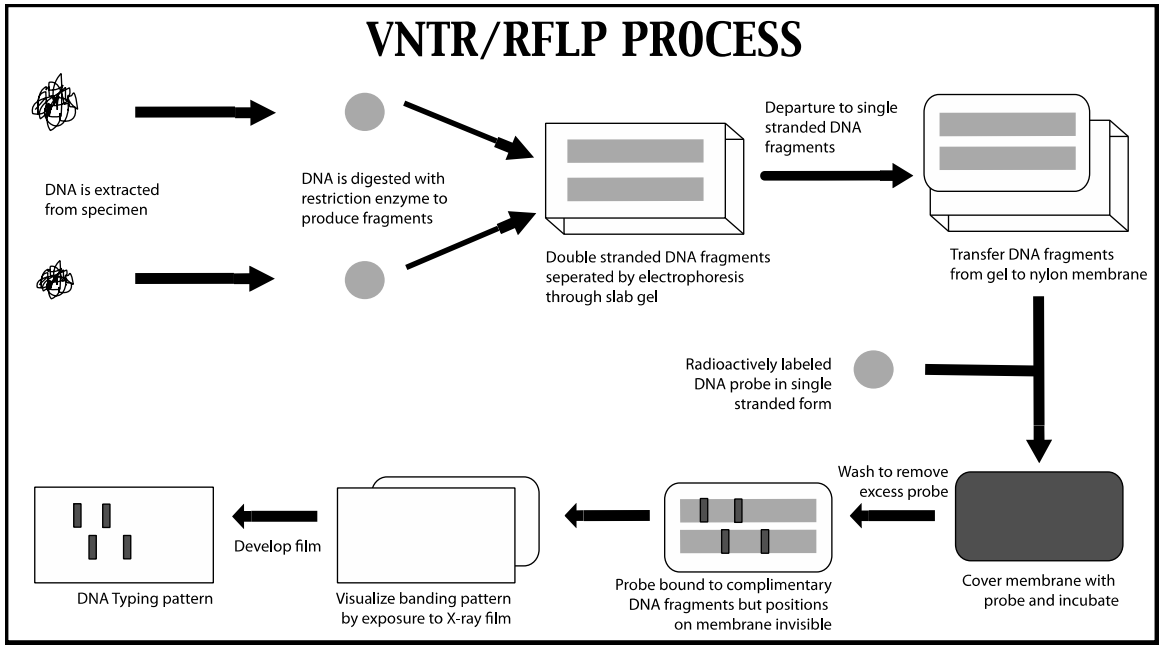


Figure 1: Schematic of the VNTR-RFPL process. DNA is extracted and digested with a restriction enzyme. After electrophoresis the fragments are transferred to a nylon membrane and a radioactive probe binds to its complimentary position. The membrane is then exposed to X-rays and visualized. If two bands align with each other then a match occurs at that marker. Adapted from How Stuff Works website.

In his original work Jeffreys' used Hinf I as a restriction enzyme to digest DNA using multi-locus VNTR probes. These multi-locus probes contain common sequences for several different VNTR loci that contain sites of different length that could be observed. The ending result made interpretation very difficult and complex which lead to the development of single locus probes. The first use of the RFLP-VNTR method was in

a civil immigration case in the year 1985 in England, where the paternity of a young boy from Ghana to relatives living in England was being questioned. The results showed that indeed the young boy was related and allowed entry into the country and reunion with his family.[10] The following year, 1986 marked the first criminal case tested using the muti-locus RFLP/VNTR method for identification. The case involved rape and homicide of two girls in which the only suspect at that time confessed to only one of the murders. Police believed that the suspect was also responsible for the death of the second victim and with no additional evidence all members of the small village were asked to submit a DNA sample for comparison to the DNA found at the scene.[12] After all the samples were tested including the original suspect with no match, the police were confused. It was not until a man from the village bragged about submitting a sample under a friend's name (Pitchfork) that the police were able to find out who the person was and his actual DNA tested. His DNA matched to the evidence found at the crime scene. The man in this case, Colin Pitchfork was convicted of his crime and sentence to life in prison.[12]

Around the same time of Sir Jeffreys and his "DNA fingerprinting" method, an extremely important biological technique was discovered. In 1983 Kary Mullis came up with an idea where he would use a pair of primers to encase a small region of DNA, add polymerase and nucleotides to make an infinite number of copies. This method, known as the Polymerase Chain Reaction (PCR), was capable of exponentially amplifying a nucleic acid sequence and making millions of copies of the same sequence.[13]

The early process of performing PCR using the Klenow region of the *E.coli* polymerase required that new polymerase be added after each denaturation stage because the enzyme was not thermally stable.[14, 15] After discovery of a new thermally stable

polymerase isolated from bacteria found in geothermal springs, *Thermus Aquaticus* (Taq), the need for adding additional polymerase was not required and the process performed without human intervention.[16] Later developments in polymerases led to a "hot start" polymerase, which required that the enzyme be heated before it was activated. This greatly improved the reaction specificity and efficiency by not allowing amplification to take place at undesired temperatures.[16]

Unlike the RFLP/VNTR method of "DNA fingerprinting" which required large amounts (0.5μg or greater) of intact DNA, PCR permitted the analysis of samples with low amounts (0.5ng or less) of DNA.[17] The first PCR based analysis used in forensics was the amplification of the HLA DQα1 marker. This region of the genome codes for the alpha subunit of the DQ protein of the major histocompatibility-complex on chromosome 6 and contains a polymorphic 242bp region that has eight or more alleles, which can be used in human identification.[18, 19] The method used to detect these variations is known as the reverse "dot blot" method. Using the dot blot method the DNA templates are bound to a substrate, then a complimentary, evidence or reference probe to the desired allele is hybridized to the DNA. Following successful binding of the complimentary probe to the DNA, reagents are added which produce a color change due to an enzyme attached to the probe by means of the biotinylated end.[20] The HLA DQα1 dot blot method was difficult to interpret when mixtures were present, and the method had a low power of discrimination.

To improve the power of discrimination, an additional dot blot kit was used, known as AmpliType PM which co-amplified five additional loci. The five markers were low density lipoprotein receptor (LDLR), glycophorin A (GYPA), hemoglobin gamma

globin (HBGG), D7S8 on chromosome 7 and a group-specific component (Gc). Each marker had a specific hybridization probe and contained 2-4 alleles.[21] The combination of these markers allowed for higher discrimination powers than the original dot blot kits.

About the same time another PCR method was established that became very popular using a single locus VNTR marker known as D1S80. The marker amplified a highly polymorphic 369bp to 801bp region that contained 27 alleles (each containing a variable number of 16 base repeat units).[21] The amplicons were then separated via electrophoresis on a polyacrylamide gel. The size of the amplicon determines how fast it moved through the polyacrylamide gel with small molecules migrating faster than larger ones. The gel was visualized using ethidium bromide, an intercalating agent that binds to double stranded DNA (dsDNA) under an ultra-violet light source (UV) or silver staining. The alleles are then determined by comparison to an allelic ladder, which contains all known alleles for a given locus. The large size of some of the D1S80 amplicons, made it difficult to amplify degraded DNA samples. The D1S80 method was more sensitive than some of the earlier RFLP based methods, but was not as statistically powerful even when combined with the DQA1 polymarker.

The next most important development in forensic DNA analysis was the identification of microsatellites or short tandem repeats (STRs). Short tandem repeats are simple sequence repeats, which contain a core-repeating unit of between two and six nucleotides in length.[22] There are abundant numbers of STR markers in the human genome, but only a small set of these markers were chosen as identification markers for forensic DNA analysis. While the most typical STRs markers currently being used contain a four base or tetrameric repeat unit, pentameric (5 base) nucleotide repeats have

also been used.[23*] STRs can be multiplexed permitting multiple loci to be simultaneously amplified. Current commercial kits may contain up to 21 different sets of alleles on as many chromosomes. The overall result is a very high power of discrimination (greater than 1 in 10^{16}) along with the added advantage of increased sensitivity and reduced analysis time.

By the year 1992, the practice of forensic DNA analysis had become routine and the interpretation of results were constantly being challenged in court. As a result of the confusion, the National Research Council (NRC) was asked to form a committee on forensic DNA to help resolve the outstanding issues in interpretation of results. As a result, the NRC I report was issued with guidelines to be followed by all forensic laboratories. In 1996, because of continuing issues with statistical interpretation and the development of new technologies a second report, NRC II, was issued. The report improved statistical methods used in data evaluation and assisted with application of PCR-based methods.[24] In order to further help standardize the markers being used and allow for the establishment of a DNA database that was compatible nationwide, the Federal Bureau of Investigations (FBI) coordinated a study in 1997 and developed a set of 13 core loci for use with a computer database known as the Combined DNA Index System (CODIS). The 13 loci were chosen based on discrimination power and quality of the results. The loci chosen were: CSF1PO, FGA, THO1, TPOX, vWA, D3S1358, D5S818, D7S820, D13S317, D16S539, D18S51, D21S11 and a sex determining marker Amelogenin.[17, 25] The sex-determining marker Amelogenin utilizes a 6bp difference

[*] Note: the use of pentameric STRs markers have additional benefits over tetrameric STRs due to fewer micro-variants and less stutter.

between the X and Y sex chromosomes allowing for the identification of the sample sex, making it a useful tool for investigators.[26 27] While these markers are still considered the core loci, many commercial kits use more loci than the 13 listed above for increased discrimination. Up to 21 different makers can be multiplexed today and more loci may be added to CODIS in the future.[28] At present there has been extensive validation of amplification procedures for the 13 core loci and large databases have been developed.[29]

STR alleles today are separated using a technique called capillary gel electrophoresis, which is much faster than agarose gel systems and achieves greater resolution. The markers are multiplex by size and by labeling the forward primer with a fluorescent tag at the 5' end. Different dyes may be used to allow for simultaneous detection depending on the instrument being used for the analysis. Most systems today are capable of exciting and detecting a wide range of dyes. Generally dyes are excited by a single laser and detected over a range of emission wavelengths. The most common instruments used today for forensic STR DNA typing are the Applied Biosystems (ABI) Genetic Analyzers. These capillary electrophoresis (CE) systems come with various numbers of capillaries starting with the single capillary ABI 310, which has been the standard instrument for most crime labs until recently, where the 16-capillary ABI 3130XL has become more popular for higher throughput.

b) DNA: Its Biology

Robert Hooke, an English physicist and prominent microscopist, first discovered the cell in 1665 under a compound microscope.[30] In the mid 18th century scientists, Theodor Schwann, Matthias Schleiden and Rudolf Virchow postulated a theory that cells

were the basic unit of life and present in all-living organisms.[30] Cells are responsible for the activity, and contain the hereditary information, which can be passed on through replication and cell division. Organism with cells containing a nucleus are called eukaryotes. The nucleus contains DNA and other proteins organized in chromosomes, which store the genetic information. The genetic information gives instructions for growth, development and reproduction. The entire ensemble of information contained on the chromosomes is known as the genome. The human species contains two copies of each of the twenty-three chromosomes, and is referred to as a diploid system. We inherit half our chromosomes from each one of our parents, generation to generation.[31]

i) DNA Structure

The structure of DNA is divided into three main nucleic acid components; a base, a sugar and a phosphate group. The nucleotides are connected by a phosphate and sugar backbone, which forms its basic structure. DNA contains four bases; adenine (A), cytosine (C), guanine (G) and thymine (T) and these account for the structural variation.[6] Adenine and guanine are referred to as purines a two ring structure, while cytosine and thymine are pyrimidines containing a single ring (Figure 2). The bases are attached to the sugar phosphate backbone such that adenine only bonds to thymine while cytosine only bonds to guanine as shown by Watson and Crick based on Chargaff's data. The differences and combinations of these bases result in different coding messages that make living organism different physically and genetically. The bases are linked together by a phosphodiester bond between the 5' hydroxyl of one pentose sugar to the 3' hydroxyl of the adjacent sugar.[32]

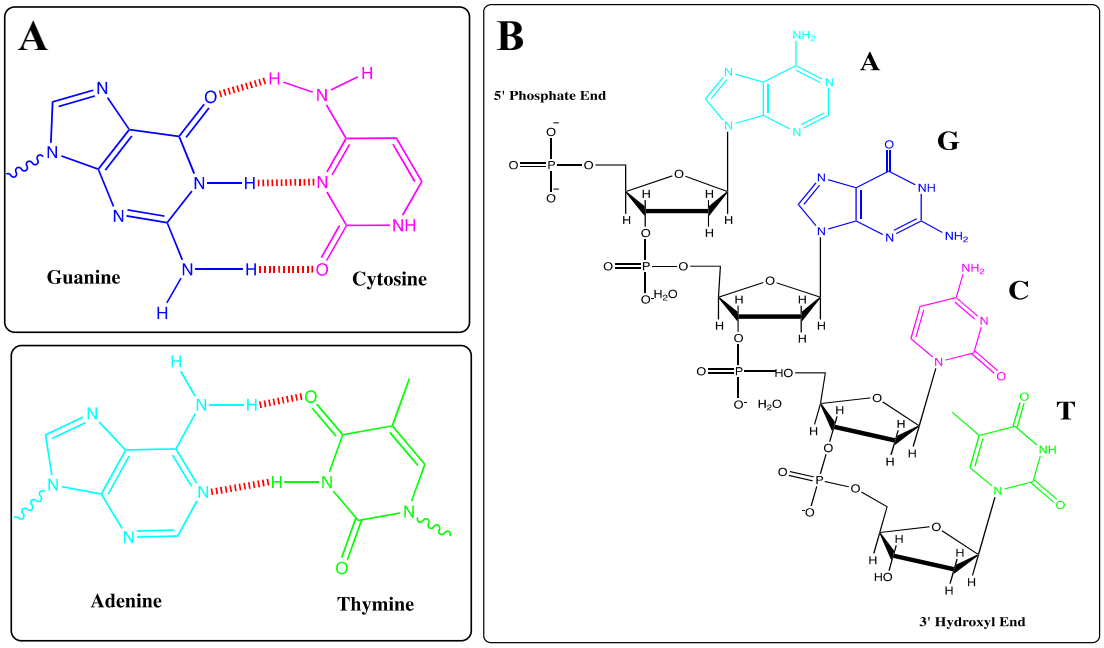


Figure 2: A: The chemical structures of the 4 DNA nucleotides, adenine (A), cytosine (C), guanine (G) and thymine (T) showing the Hydrogen bonding, A-T double H-bonds and C-G triple H-bonds. B: The DNA sugar phosphate backbone with attached nucleotides showing the 5' phosphate and 3' Hydroxyl ends.

The two strands of DNA are held together by hydrogen bonds. Even though hydrogen bonds are weak chemical bonds, they occur in such high numbers that the two-stranded helix is held together strongly via hybridization. The strands run in opposite direction one from 5' to 3' and the other 3' to 5' and bind at its complimentary base. (Figure 2) As a result of the structure and available H-bonding sites, G-C bonds have three H-bonds while A-T pairing only contains 2 H-bonds. The G-C bonds require more energy to break than A-T bonds and hence plays important roles when designing primers or target sequences to be amplified.[33]

ii) DNA Arrangement: Chromosomes, Genes and Loci

DNA is organized into tightly compact structures along with proteins known as chromatin, which are located in the chromosome. In the human cell there are 46 different chromosomes 23 coming from the maternal and 23 from the paternal parent. Two of these chromosomes are sex chromosomes that determine the gender of an offspring. Females contain two X-chromosomes and male contain one X-chromosome and one Y-chromosome. During fertilization of the egg (haploid) with the sperm (haploid) the zygote cell becomes diploid because of inheritance from each parent.[34]

When the compact DNA structure is unwound, it contains stretches of DNA sequences that code for polypeptides or proteins. These regions known as genes, consist of coding regions called exons and non-coding spacers called introns. Markers typically used for human identification are found in the non-coding region or introns and do not code for genetic variations (Figure 3).[35] [33]

The location of a gene or marker within the chromosome is known as the locus (or loci). This terminology is used to describe the exact location of the targeted region along the chromosome.

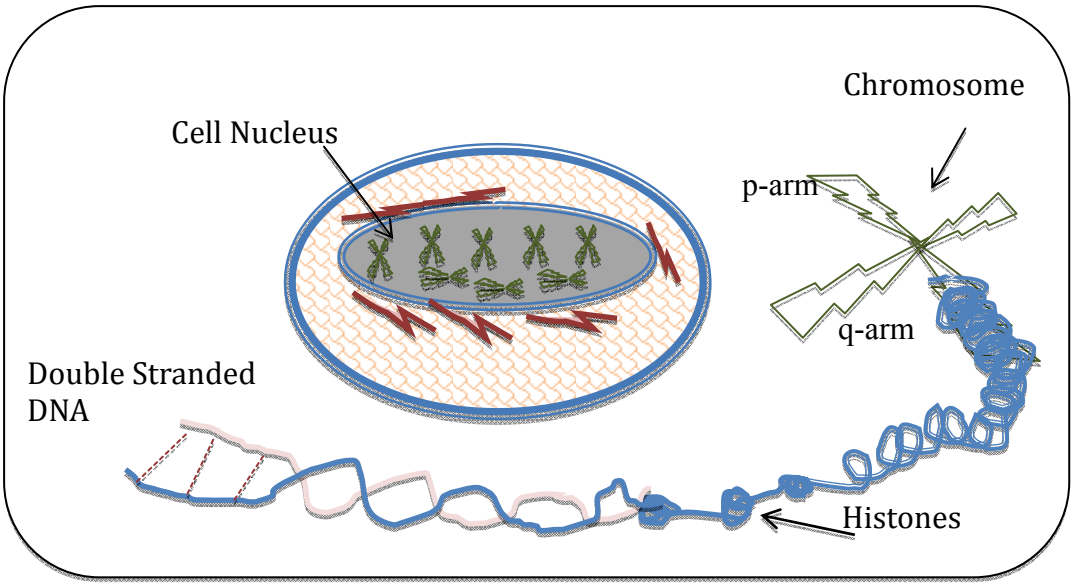


Figure 3: The structure of cell nucleus containing chromosomes. These chromosomes contain tightly bound DNA organized into X-like structures which when unwound code for genes and other proteins. Adapted from National Human Genome Research Institute website.

iii) DNA Marker nomenclature

The DNA markers are named depending on their position on the chromosome. For example the forensic STR locus D5S818 can be described as follows; The 'D' stands for DNA, the 5 refers to its location, on chromosome 5, 'S' indicates that it is a single copy sequence and the 818 represents the order in which it was discovered and characterized for that particular chromosome, hence it is the 818th marker described on chromosome 5. The same can be said for other markers like D13S317, D16S820, D7S820 and others. If the marker falls within the coding region of a gene, such as TPOX (thyroid peroxidase gene) or TH01 (tyrosine hydroxylase gene), it is named after that gene (Figure 4). [17]

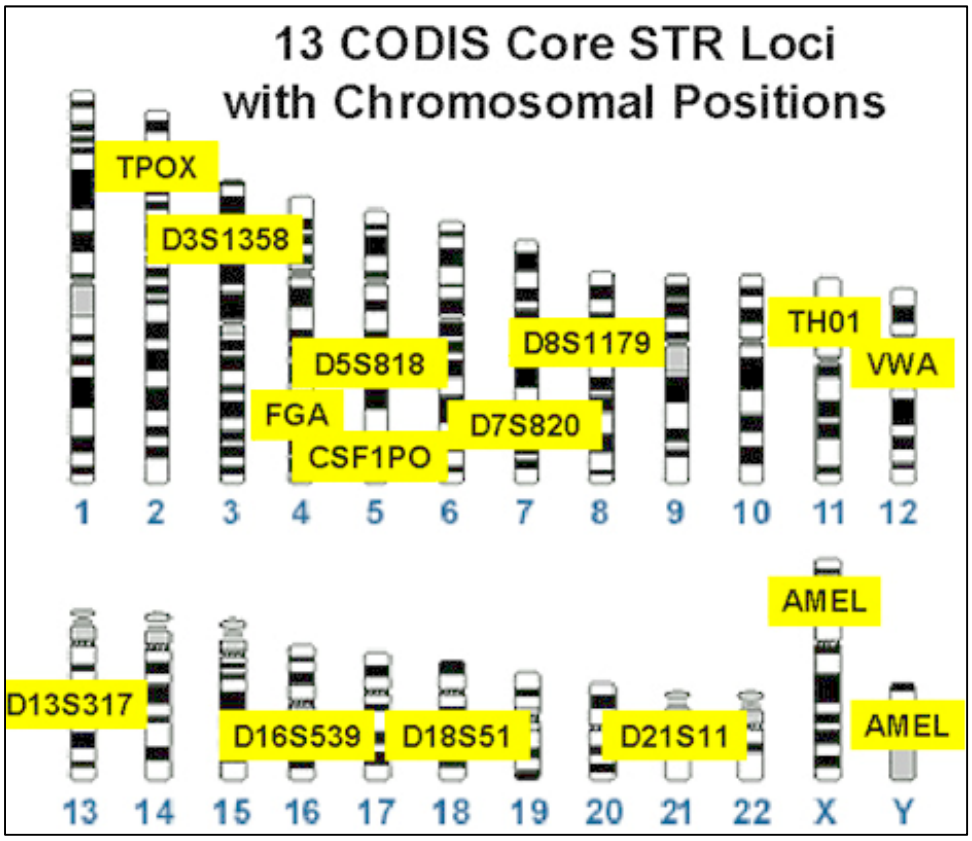


Figure 4:The chromosome location and nomenclature of the 13 core CODIS loci.[17]

iv) DNA Degradation

The biological evidence found at crime scenes is often not in pristine condition, exposed to extreme environmental conditions, and the DNA can be degraded causing problems in the quality of the amplified product. The degradation of DNA can take place by biochemical processes such as hydrolysis, oxidation and by bacterial activity or enzymatic digestion.[36] A number of environmental and external factors can also degrade DNA such as ultraviolet light, temperature change, time, enzymes such as nucleases, microbial bacteria and chemicals such as bleach.[37] Post mortem samples are greatly affected by enzymes liberated during autolysis. Endonucleases cut the DNA into smaller

fragments while exonucleases removes nucleotides from the terminal end one after another reducing the size of the overall length of the fragment.[36]

Hydrolysis can result in depurination and depyrimidination of nucleotides as the glycosidic base-sugar bond is cleaved. Once the glycosidic bond is broken, a β-elimination reaction can reduce the strand breaking at the 3' phosphodiester bond of the sugar. The rate of this process is greatly affected by the environmental and physiological conditions such as pH, temperature and ionic strength. For example, a typical 800bp DNA fragment can take about 5,000 to 10,000 years at 15°C to fully degrade.[38] Pyrimidines, cytosine and thymine, are not as affected as purines by full hydrolysis because they are more stable and less affected by pH.[39] An excision repair process quickly corrects the hydrolysis of DNA in living cells.[40] The damaged site is cleaved and DNA polymerase I and DNA ligase comes in and repairs the damage.[39]

Nucleotides containing secondary amino groups, such as adenine and cytosine, can also undergo hydrolytic loss of the amino group, and be converted into uracil and thymine which causes mis-binding and transition mutations. Cytosine and its 5-methylycytosine are especially prone to deamination even though this process occurs very slowing under natural physiological conditions (Figure 5). DNA strands can also be cross-linked to proteins as inter molecular bonds form between the open-ring of an abasic sugar to an amino group on the opposite strand.[38, 39]

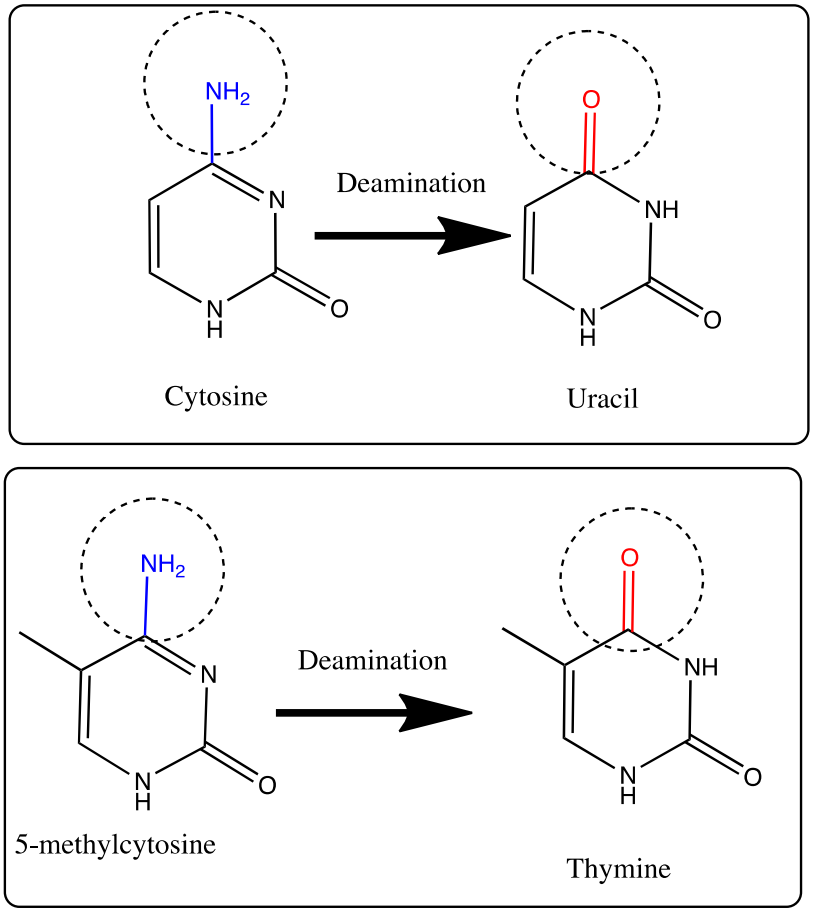


Figure 5: The hydrolytic loss of an amino group, which can result in translation mutations. The deamination of cytosine to uracil (above) and 5-methylctosine to thymine (below).

Another common DNA degradation process occurs through oxidation. During oxidation, reactive species such as oxygen radials ($O_2^{\cdot-}$), hydroxyl radials (OH^-) and hydrogen peroxide (H_2O_2) can cause endogenous damage to the DNA molecule. These radicals result from metabolic processes and attack the DNA sugar backbone. The radical, stabilized by its proximity to an oxygen atom, reacts with the phosphoester group at the C3 position and causes strand breakage by removal of the phosphate group. The reaction progresses in presence of water with the radical cation causing the elimination of another phosphate group at the C5 position (Figure 6). Oxidants can also cause base

mutations, for example, in the presence of an hydroxyl radical, adenine can bind with the mutated base 8-oxo-7-hydroxydeoguanosine causing a mis-binding and translation problem.

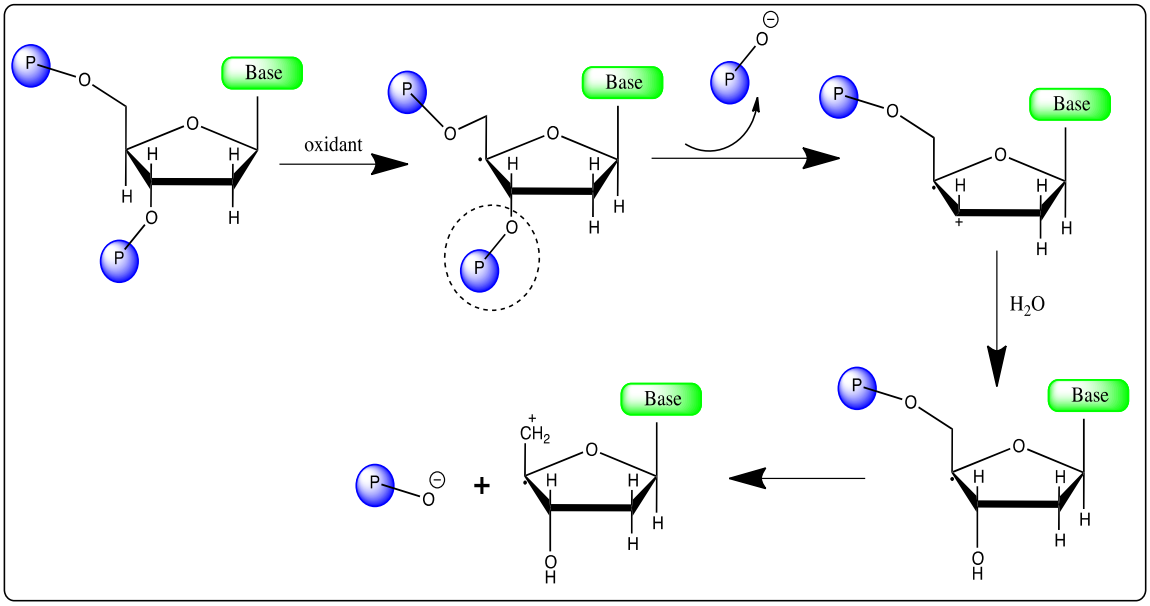


Figure 6: The attack of an oxidant on a DNA strand. The radial attacks the sugar residue first at the C4 position, where the nearby oxygen atom stabilizes it. The Phosphate group is ejected resulting in a positively charged radical. This radical can then react further with a different backbone DNA molecule, in the presence of water, removing yet another phosphate group. The end result is a DNA strand that is broken.

Ultra-Violet light causes DNA lesions that are can be mutagenic and toxic to the cells destroying the DNA. The most common and harmful are cyclobutane-pyrimidine dimers and 6-4 photoproducts which cause structural damage and distort the DNA (Figure 7).[40] Temperature change, freeze and thaw cycles also cause damage to the DNA by cleavage and random breakage. Forensic DNA samples found at crime scenes are commonly not pristine and affected by degradation. It is important to understand how the

degradation process affects the sample, quality of amplification and electropherogram data.

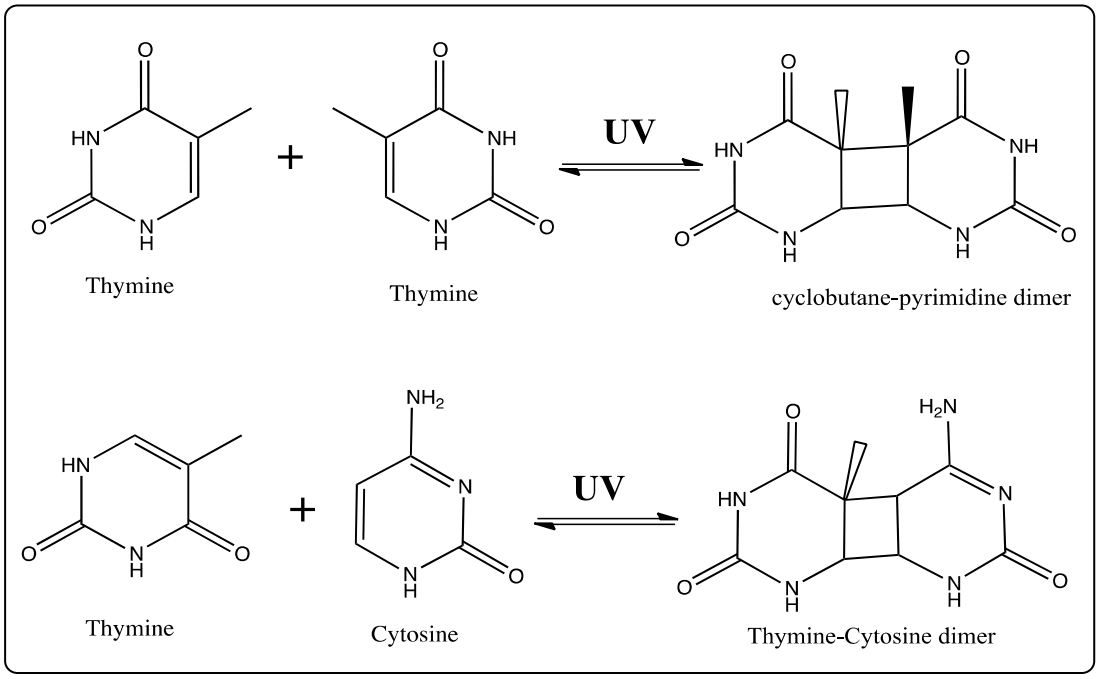


Figure 7:Formation of dimers from exposure of UV radiation. (A) cyclobutane-pyrimidine dimer (B) Thymine-cytosine dimer.

CHAPTER II: COLLECTION AND ISOLATION OF BIOLOGICAL EVIDENCE

a) Collection of Biological Evidence

The collection of evidence at a crime scene should be done in such a manner to avoid contamination and preserve its quality to ensure the best results when tested. However, one of the most common problems encountered by forensic scientists in the laboratory is poor collection, preservation and transport of biological evidence. With today's emphasis on DNA, the presence of biological evidence at a crime scene is often the most incriminating and valuable source. Within most of the 50 states in the United States of America, forensic scientists rarely leave the lab, and therefore, the collection of evidence is done by police personnel, investigators or special crime units. At a crime scene the most common types of biological evidence found are: blood, semen, saliva, hair, teeth, tissue, bone and urine.[41] These are not the only items found at a crime scene, but account for majority of the evidence collected. There are different collection standards that vary among departments, but there are some common protocols for the collection and preservation of evidence. There are numerous techniques that are used by forensic scientists, and where possible items should be left in their original state and sent to the laboratory for the DNA collection.[*42]

[*] Note: Before any item of physical evidence is collected it must be properly photographed and fully documented. You must also have the required search and seizure documents. After collection each item should be carefully labeled and sealed so as to establish a chain of custody.

i) The Collection of Blood Evidence

Blood is the most commonly biological sample found at crime scenes, and is a major contributor to successful recovery of DNA profiles. DNA is found within the nucleus of the white blood cells. The white blood cells are less abundant than red blood cells, which have no nucleus and no DNA. However, most blood samples once collected properly yield good results when DNA is extracted. A wet blood sample is collected using a sterile swab passed over the stained area. The swab is then allowed to dry completely before being packaged. Dry bloodstains are collected using a sterile swab slightly moistened with distilled water. Like the wet sample it is important that the swab be allowed to dry.

If the sample is packaged wet, it may promote the growth of mold and bacteria and degrade the DNA within the sample. All blood samples are packaged in paper or cardboard, but never in plastic. If the sample collected is not going to be analyzed within a short time frame it should be refrigerated at -20°C to help preserve the DNA, or stored on an FTA card for later analysis. A reference sample should also be collected where possible.[41, 43*]

ii) The Collection of Semen Evidence

Semen is another common source of evidence that is critical especially in rape cases. Semen is often detected as a dry crusty white stain. An alternate light source may also be used to aid in its location because semen fluoresces under UV radiation when

* Note: In some cases it may be more important to preserve the blood splatter pattern than to collect and interfere with the evidence.

viewed with the appropriate goggles.[41, 43] In the majority of cases, semen is collected from sexually assaulted victims and bed linens. A semen stain would be collected in the same manner as a blood sample mentioned before. When possible the entire item containing the semen sample should be collected and sent to the laboratory, as there may be trace evidence present.*

iii) The Collection of Salvia Evidence

Saliva samples and buccal swab samples may often be taken as control samples. Saliva can also be collected from items such as cigarette butts, chewing gum, soda cans, envelopes, stamps and bite marks. A saliva stain would be collected in the same manner as blood using a sterile swab. The sample must then be allowed to dry and packaged in a paper envelope or cardboard swab box. Buccal swabs are the most common method of reference samples used today (Figure 8).

* Note: A rape kit should be conducted by the hospital when possible, with dialogue from the evidence collector.

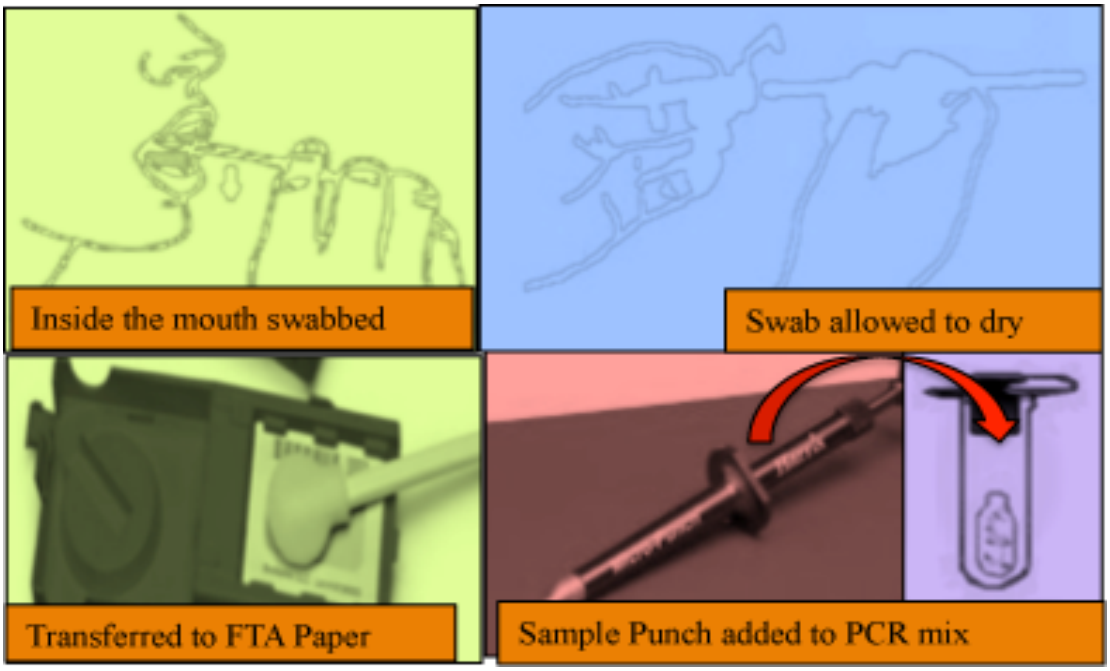


Figure 8: DNA swabbing and collection of control or reference samples. A sterile cotton swab is opened, the inside of the suspect's check is swabbed for 1 minute firmly, the swab is then allowed to dry and cut for the extraction process.

iv) The Collection of Hair, Tissue, Bone, Urine and Teeth Evidence

Hair samples are often considered trace evidence but a forensic biologist may be required to collect such a sample for DNA analysis. Hair can be found anywhere as it may easily be transferred throughout a crime scene. Special precautions must be taken as not to overlook and destroy hair samples. The samples should be collected with a tweezers and placed in a druggist fold or paper envelope. Each piece of hair should be separately packaged to avoid any cross contamination.

The use of tissue, bone, urine and teeth to provide DNA samples is more commonly associated with identification of a victim rather than obtaining evidence from a suspect. There are specialized fields in forensics that deal with these items of evidence, such as forensic odontology, forensic anthropology and forensic toxicology. These

samples are not as commonly used as blood and semen and will not be discussed in this manuscript. However there are special procedures for the collection and preservation of these types of samples, which may prove to be valuable evidence from case to case.

It is important that the evidence collected and submitted for DNA analysis is collected and persevered in such a manner to maintain the integrity of the sample to permit successful amplifications.

b) Isolation (Extraction) of Biological Evidence

Biological samples are often first detected using some type of presumptive test not discussed in this manuscript. However, once identified as a possible source of DNA, there are a few different methods used by forensic laboratories to break open the nucleus, release and purify the DNA for downstream STR analysis.

i) Phenol-Chloroform Isoamyl Alcohol (PCIA)

The PCIA extraction is also known as organic extraction, and is one of the most common methods used to isolate DNA from cells or samples. Many forensic laboratories, despite newer chemistries that have been developed, still utilize this method of extraction because of its high rate of recovery. While this method involves numerous steps and is difficult to automate, it yields a high concentration of DNA that is relatively clean of contaminants and inhibitors and can work on a variety of sample types. However, phenol can be toxic and must be carefully handled and used under a hood with proper ventilation.

The first step in a PCIA extraction is the addition of an enzyme, proteinase K, a detergent, sodium dodecylsulfate (SDS), and a disulfide bond-breaking reagent,

dithiothreitol (DTT). These chemicals are used to break open the cell membrane digest and remove proteins that bind and stabilize DNA in the nucleus.* This process is often aided by heating in a water bath at 56 °C for 2- 4 hours. After heating and lysing of the cells, phenol, chloroform, and isoamyl alcohol are added to separate the proteins from the DNA via a liquid/liquid extraction. Because the DNA is soluble in the aqueous phase, it can be separated from the organic layer by centrifugation at high speed, followed by pipetting of the aqueous layer into a new tube. The isoamyl alcohol is used to help stabilize the chloroform and reduces foaming, leading to a clearer interphase between the aqueous and organic phases (Figure 9).

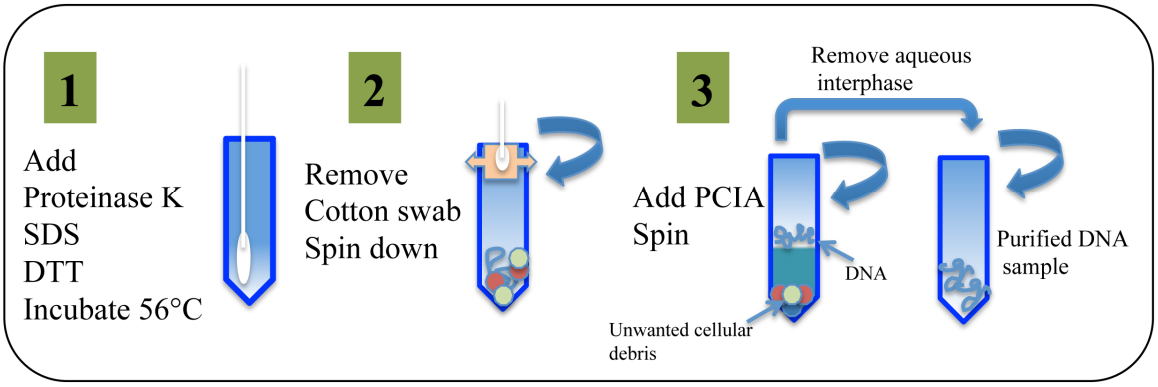


Figure 9: Overview of the phenol-chloroform isoamyl alcohol extraction process. Step 1; addition of proteinase K, SDS, DTT incubated at 56°C for 2-4hrs. Step 2; remove cotton swab and spin down. Step 3, add PCIA spin and remove aqueous layer into a new tube.

ii) Chelex Extraction

Another method used for extraction of DNA for PCR based approaches involves chelating resins. The method requires fewer pipetting steps and tube transfers, which makes it faster than PCIA extractions. Chelex® 100 (Bio-Rad Laboratories) is a highly

* Proteinase K is an enzyme capable of digesting proteins. SDS, a detergent is used to aid in the lysis of cells and in unraveling proteins.

selective cation exchange resin containing iminodiacetate ions which can chelate polyvalent metal ions, and inactivate PCR inhibitors and nucleases which damage DNA. The solution is heated for several minutes at 100°C which causes the cell membranes to be broken and the DNA exposed. However, this high temperature also denatures the DNA leaving it single stranded. Thus, procedures such as RFLP, which require intact DNA, cannot be used. Pre-wash steps and the ion exchange resin may also help remove inhibitors that may affect PCR.[44] The selectivity of the resin is 5000:1 for divalent versus monovalent ions, even in solutions with high salt concentrations.

iii) FTA® Paper

Fast Technology for Analysis of nucleic acids (FTA) was developed in the late 1980's at Flinders University in Australia. Burgoyne and Fowler were able to design a special paper capable of protecting nucleic acids from degradation such as nucleases, bacterial activity and preserving it for long-term storage.[45] They applied a weak base, chelating reagent, anionic surfactant and uric acid onto a cellulose based paper. As a result of contact with the mixture of chemicals embedded on the paper, the cells present in the biological fluid are lysed and their proteins are denatured. The DNA samples deposited on FTA® paper are stable and can be stored at room temperature for years with minimal DNA degradation. This is a great advantage for forensic samples, and the paper can provide long-term storage that requires less space than traditional tubes in freezers. However, some studies suggests that after 19 months, there is little to no improvement in STR profiles obtained from this paper when compared to traditional storage methods.[46]

One of the most valuable attributes of FTA paper is that you can add similar amounts of DNA to a PCR reaction by simply using the same size of paper punch each time. This eliminates the need for quantification of the DNA sample before genotyping and saves valuable time in the genotyping process. The DNA can be pre-washed and eluted using an elution buffer that solubilizes the DNA off the paper if higher purity is needed for analysis. Newer FTA paper products have included an indicating color spot showing where the DNA is on the paper to eliminate the possibility of punching a sample with no DNA. Recent elution methods permit the use of water with a small heat step, reducing the potential of contamination that occurs during pipetting and transferring of reagents.

iv) Solid Phase Extractions

It is possible to automate the DNA extraction process to permit high-throughput analysis with robots. A number of methods have been developed in which a solid phase substrate is used that binds DNA selectively, while proteins, cellular materials and other artifacts are washed away. The purified DNA is later released during an elution step. While the composition of the solid substrate varies depending on the solid-state chemistry, the two most common substrates used for forensic work are silica and magnetic beads.

An example of a silica based solid phase extraction is the the QIAamp® Kit (QIAGEN, Valencia, CA).[47] In this kit, the nucleic acids are selectivity bound to silica on a glass bead support. The cells are then lysed in the presence of a chaotropic salt

solution.* These salts help stabilize the denatured DNA and proteins. In acidic conditions below pH 7.5, DNA is absorbed to the surface of the silica beads with up to 95% occupancy. The remaining proteins and unwanted molecules are then washed away. The pH is then raised to generate basic conditions at low ionic strength, and the DNA is readily eluted. The beads can then be spun down via centrifugation, and the DNA collected in the aqueous portion. This process can be automated using a robotic process platform.[48]

A similar chemistry developed by Promega Corporation (Madison, WI) called the DNA Isolation Quantitation kit (DNA IQ) uses a magnetic bead solid phase instead of silica glass beads. The chemistry used is the same as that used with silica glass beads except a magnetic solid substrate is used to manipulate the DNA by holding it against the tube using a magnet. The results are a less tedious sample processing with fewer transfers and pipetting steps. The sample may also be washed several times for higher purity before being eluted. Other chemistries used in DNA extraction and purification includes ion exchange, charge-switch and sucrose based methods.† [49-53]

* A chaotropic salt is one that disrupts intra- molecular forces such as hydrogen bonding.
† Note that differential extraction is considered a form of the other types of extractions with an added step using dithiothreitol (DTT) a chemical that is responsible for breaking disulfide bonds that are in high concentrations in sperm membranes.

CHAPTER III: AMPLIFICATION: POLYMERASE CHAIN REACTION

Kary Mullis in 1985 was able to demonstrate a method to make millions of copies of a DNA fragments know as the polymerase chain reaction. The process revolutionized many of the biological sciences and forensic DNA testing. He won the Nobel Prize in 1993, very shortly after the discovery of PCR, which further illustrates the significance of the methodology. The value of PCR to forensic samples is critical as many of the samples found at crimes scene are in low quantities or degraded. Its ability to make millions of copies and amplify selected regions or STRs are paramount to forensic DNA typing.

a) PCR: Basics

The polymerase chain reaction occurs in three general stages; denaturation, annealing and extension. Denaturation occurs with an increase of temperature above 90 degrees Celsius (°C) where the weak hydrogen bonds are broken and the double strand DNA separates into two single strands. The temperature at which this step is done may vary according to the DNA polymerase enzyme. During the annealing stage, the primers bind to the separated complement strands when temperature is lowered (55-60°C) and the DNA is allowed to recombine. The primer concentrations are added in excess to ensure that the primer anneals and binds to the single strands minimizing the recombination of the original DNA. The final stage is the extension where the polymerase adds deoxyribonucleotide triphosphates (dNTPs) in the 5’ to 3’ direction. The primers are then extended by addition of complementary bases A, T, C & G at a temperature of 72°C. These steps are repeated such that, after one cycle, the amount of DNA is increased two

fold (After n cycles the amount of DNA will be approximately 2^n). For example after a typical PCR run of 30 cycles the amount of DNA would be copied 2^{30} or 1.073 x 10^9 copies (Figure 10).

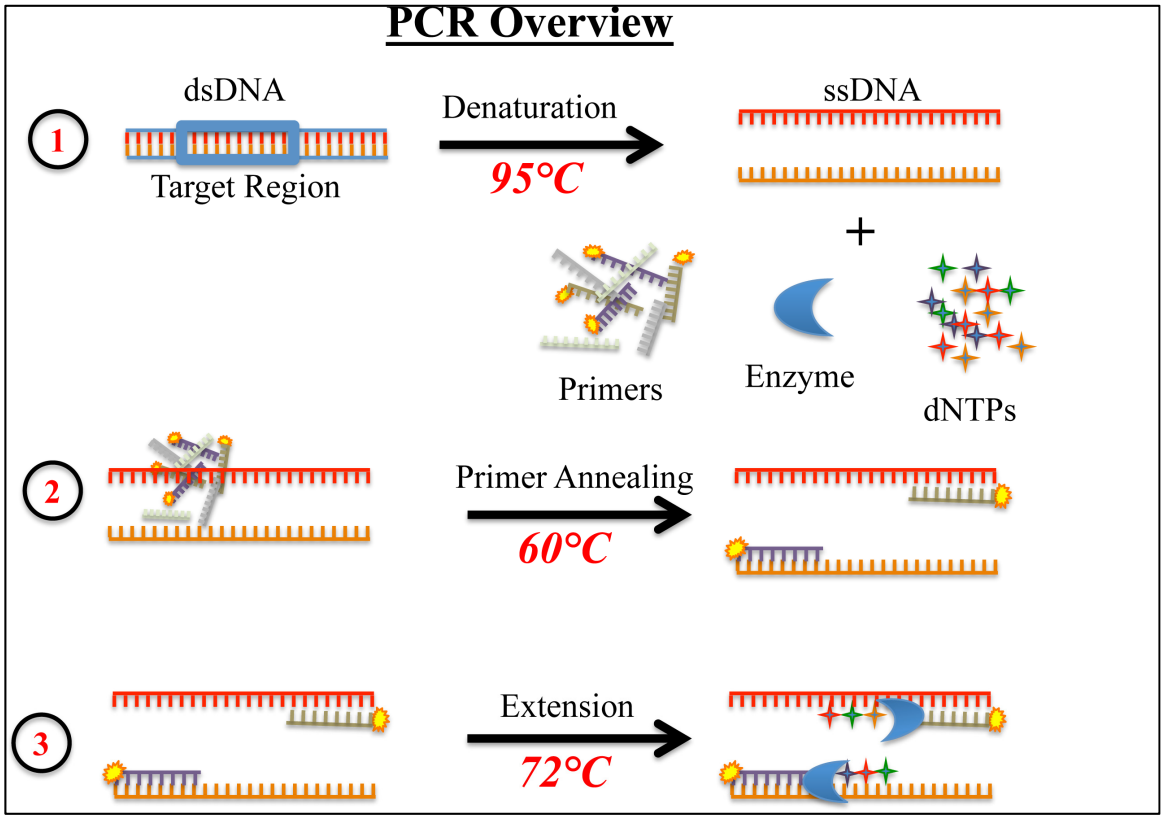


Figure 10: Overview of the PCR process. Denaturation takes place once the sample is heated 95°C. The addition of enzyme, dNTPs, and primers and buffer are all required for the PCR process. Primer annealing takes at a lower temperature. After the primers bind, the enzyme complex can come in and start to add new nucleotides. The overall result is a copy of that template. This process is repeated over and over until the desired amount of product is reached.

b) PCR Components

The polymerase chain reaction involves a mixture of reagents and amplification conditions that must be optimized in order to achieve accurate and well-balanced products. These products are known as amplicons. The main components used for PCR

include polymerase (Taq) with a co-factor, primers, dNTPs, buffers (that maintain proper ionic strength) and the template DNA.

The DNA polymerase Taq is the enzyme that binds and incorporates the new nucleotides. The speed at which it can add new nucleotides is known as its processivity, and the percentage likelihood of adding a correct nucleotide is known as its fidelity. The Taq polymerase requires the addition of a co-factor, magnesium chloride ($MgCl_2$) which activates the enzyme.[16]

The primers are responsible for specifying the region of DNA template targeted for amplification. The optimal primer lengths are 15-35 basepairs (bp) and should have a similar GC percentage.[54] Primers must also be designed to avoid primer-primer binding and hairpin structures that can cause the primers to fold, reducing their propensity to bind to the template region. The concentration of primers used for PCR may vary from a final concentration of 0.2 to 1.5μM depending on concentration of DNA and the number of simultaneously reactions. The dNTPs are utilized by the Taq enzyme for the addition of nucleotides during the PCR reaction. The template DNA should be present at an optimal concentration and the mixture should be as free as possible from inhibitor substances.

The buffer is specific to the type of Taq enzyme that is being used and it is important to keep the reaction within the correct pH range and ionic strength. It is not uncommon to find enhancing reagents added to the PCR to help increase the stability, reaction rates and selectivity. Common additives are non-acetylated bovine serum albumin (BSA) and dimethylsulfoxide (DMSO), both of which improve the reaction efficiency. BSA also binds to some substances which inhibit the PCR reaction.[55]

The template DNA, often obtained from one of the extraction methods described above should be as free from contimination as possible. Any inhibitors present greatly affect the efficiency and amplification during the PCR process. The amount of DNA added should also be sufficient. Too little DNA, or too much DNA can greatly affect the balance of the amplicons leading to allele drop out, allele drop in and other artifacts.* [17]

c) PCR Controls

As a result of the sensitivity of the PCR method a series of controls must be amplified along with the sample to ensure that reagents are working properly and that no contamination has occurred. A blank or negative control is run with the same PCR master mix without any DNA template. Running a blank would assure that no non-specific amplification or contamination has occurred within the lab. A positive control is also amplified using high quality DNA standards, with a known profile, to ensure that the amplification process and reagents are working correctly. It is important that these controls are run with every reaction to ensure quality control.[16]

d) Thermal Cycling

There are a number of thermal cyclers on the market that are capable of performing the heating and cooling cycles required for PCR. Currently most forensic labs use a system from Life Technologies formerly Applied Biosystems called the GeneAmp 9700. The system is capable of heating rates up to 4°C/s. While this thermal cycler is outdated by today's technology, it is still currently the number one choice used in the

* It is important to quantify the amount of template DNA as to ensure a consistent and balanced amplification process.

forensic community. Faster thermal cyclers such as the SpeedCycler^2 (Analytik Jena, Germany) and Philisa thermal cycler (Streck, USA) can perform amplification with faster cycling times (Figure 11). These thermal cyclers increase the cycling speed through the use special coated heating blocks, faster cooling fans and specialize tubes that permit efficient heat transfer.

Figure 11: Ultra fast thermal cyclers from Analytik Jena and Streck technologies. The cycler pictured on the left, is the SpeedCycler^2 capable of heating rates of 15°C/s and 10°C/s cooling. The Philisa cycler is shown on the right with heating rates of 15°C/s and 12°C/s cooling.

There are a number of thermal cycling parameters than can be changed and optimized during the PCR process. These include the length of time at each stage, the denaturing, annealing and extension temperatures, the ramp rate and the number of cycles. It is important to note that by increasing the amount of cycles to improve sensitivity, it must be accompanied by a detailed validation as artifacts may also be increased.

e) PCR inhibition

During PCR amplification because of co-extraction, exogenous molecules may be present and combined in the DNA samples. These compounds can have a great effect on the efficiency of the reaction leading to poor amplification or in some cases no amplification at all.[56] These types of compounds may be endogenous to the sample due to insufficient purification, and may be the result of contaminants from the environment. It is important to develop steps and mechanisms to prevent the inhibition of PCR, in order to obtain reliable results.

Some inhibitors that are commonly found in forensic samples include calcium, hematin, humic acid and indigo.[57] This list of inhibitors is not all-inclusive, but these compounds have been well studied and identified in a variety of biological samples found at crime scenes. Endogenous inhibitors on this list include hematin, a chelating component found in red blood cells, as well as calcium, which competes with magnesium as an enzyme co-factor disrupting the activity of the polymerase.[58, 59] Humic acid from soils, and indigo, a common dye found in denim, are examples of external inhibitors that also decrease the amplification efficiency.[60, 61]

There have been a number of methods used to describe the effects of inhibitors on forensic DNA samples. PCR inhibition results from binding to the polymerase or DNA template stopping the amplification. Real time PCR can be used to monitor these effects by monitoring the degree of amplification.[62] These types of analysis are important to determine whether inhibition is taking place, or, if in fact the sample is degraded since their electropherograms may look similar. However, different approaches are needed to address inhibitors versus degraded samples.

f) Real time PCR

Real time or quantitative PCR is a method that uses the same steps as traditional PCR with the addition of monitoring a fluorescent dye either directly attached to the primers/probe or via a double-stranded intercalating agent such as SYBR Green I. The fluorescent tag is incorporated as the PCR amplification takes place, the intensity of the signal increases as the DNA is amplified and more copies of DNA are produced. A typical analysis has three stages, the initial phase in which little to no product can be measured, an exponential phase and then a plateau (Figure 12). The DNA quantification is determined by its critical threshold (Ct) value which is proportional to the log of the initial copy number seen during the exponential phase.[63] The Ct can be modeled from the Equation 1:

Equation 1: Ct

$$C_t = m \log N_t + b$$

Where N_t is the amount of template DNA, m and b is calculated from the regression analysis of the Ct values of the standards. The unknown concentration is then calculated from the Equation 2:

Equation 2: Unknown concentration

$$N_u = 10^{\left((C_t - b)/m\right)}$$

Where N_u is the unknown concentration, Ct is the critical threshold, b and m are obtained from the line regression of the standards.[64]

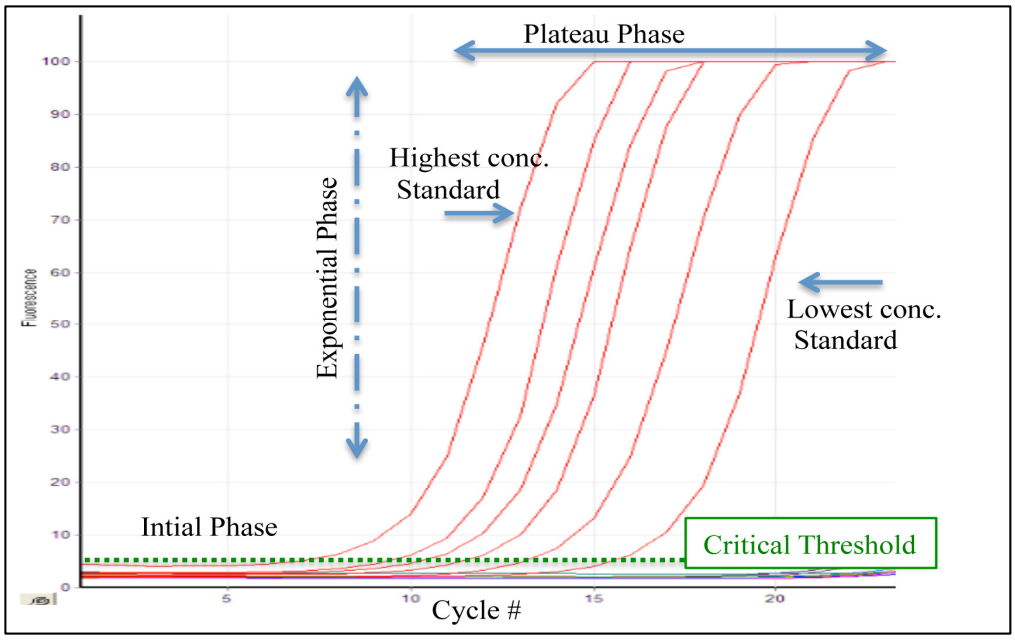


Figure 12: Real Time PCR overview. The initial phase where there is little product, the exponential phase where the amount of product is great and the plateau phase where reagents are consumed and the reaction completed. The critical threshold is the point at which the reaction goes from the initial to the exponential phase.

There are numerous ways in which the fluorescence monitoring can take place in real-time PCR (rtPCR). The most common methods for the quantification of forensic samples are done using an intercalating dye, Hydrolysis probes (Taqman) or Plexor® dye chemistry (Promega). Hydrolysis probes sometimes referred to as a Taqman probes, are ones in which the 5' end of the oligonucleotide is labeled with a reporter dye and 3' terminal end labeled with a quencher.* As the probe binds to its complimentary sequence and the Taq polymerase reaches the 3' end, it is endonucleolytically cut releasing the quencher molecule and hence a fluorescence signal observed. The most popular of this type of probe is the Quantifiler® Duo kit made by Applied Biosystems.

* The reporter and quencher molecules are close enough to prevent fluorescence until the quencher is removed by hydrolysis.

Intercalating dyes such as SYBR Green I and BOXTO allow for the monitoring of double stranded DNA without any specific binding site as it binds to the minor grooves of dsDNA. These dyes do not fluoresce when free in solution, because of their aromatic systems, which convert electronic excitation energy into heat.[65] As these dyes are bound to the minor grooves and their rotation is limited, an increase in signal intensity is observed. While this type of rtPCR monitoring may be susceptible to primer dimers, these do not interfere with the amplification and typically take place during the plateau stage.[66] Both Taqman and intercalating dye real time systems have been shown to be accurate and produce similar results.[67] The last type of probe commonly used in forensics is the Plexor quantification system designed by Promega Corporation. The Plexor system utilizes specific interactions between modified nucleotides isoguanine (Iso-G) and 5'-methylisocytosine (Iso-C). One primer is labeled with a fluorophore and an Iso-C at the 5' end, while the other primer is unlabeled. In the PCR master mix, modified dNTPs, iso-dGTP with dabcyl (a quencher) is used. As amplification takes place the specific interactions between the Iso-C and the dabcyl Iso-G are preferential at the complimentary position resulting in a quenching of the fluorescence signal (Figure 13). The latest model Promega Corporation kit is the Plexor® HY which like the Quantifiler® Duo kit incorporates specific Y-chromosome information for an estimated amount of male DNA that is being amplified in that sample.

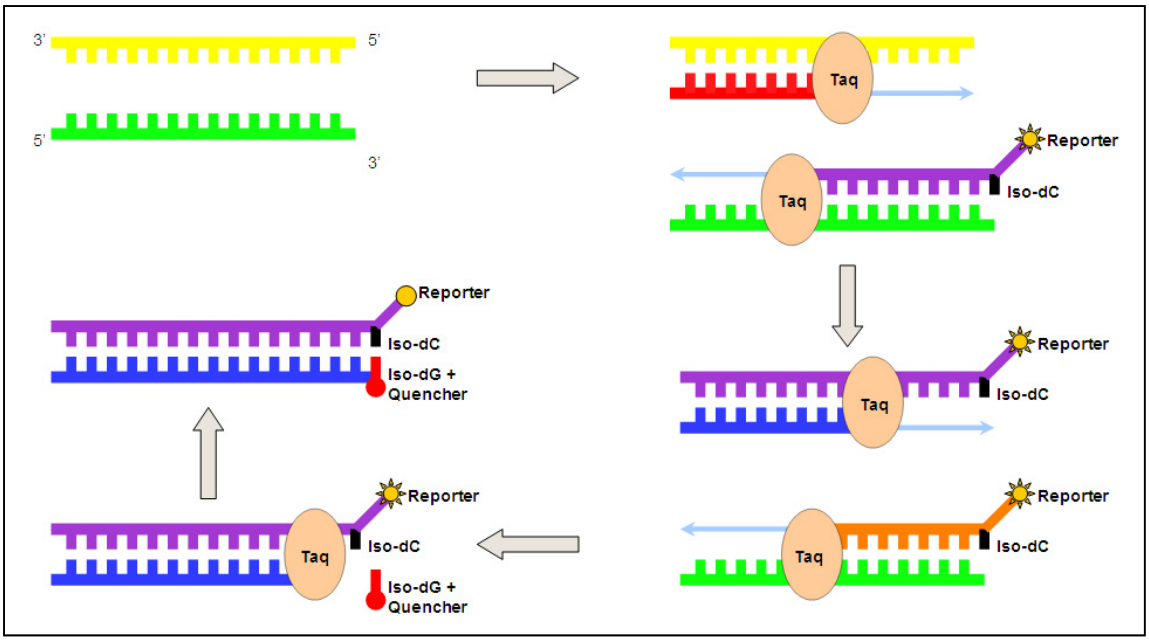


Figure 13: Overview of Plexor real time chemistry. As amplification takes place the reporter dye in quenched resulting in a decrease in fluorescence signal. The Ct values is then compared to the standards and the unknown concentration determined. Adapted from Thompson et al.

The type of rtPCR probe used has an effect on the sensitivity of the system and its ability to accurately quantify low copy number DNA. Multi-locus probes and those capable of detecting autosomal and Y-specific probes for male DNA have added advantages and are capable of detecting low amounts of DNA.[68] The use of primate-specific Alu short interspersed nuclear elements (SINE) found throughout thousands of positions in the human genome allows for a high sensitivity method with a wide range (2pg- 15ng) of detectable DNA.[69-71]

CHAPTER IV: SHORT TANDEM REPEATS (STRs)

a) STRs: Basics

Short tandem repeat markers exist throughout the human genome. These markers are highly polymorphic consisting of short sequence repeat motifs that vary in the length from one individual to the next. STRs are designated by the length of the core repeat unit, as well as, the number of times it is repeated in tandem. If the size of the repeat is between 10-100 base pairs, it is considered a minisatellite or VNTR. For example the D1S80 marker described earlier that contains a 16bp repeat falls into this category. Smaller repeat units, which are more commonly used in forensic genotyping, range from 2-6 base pair repeats and are called microsatellites. The most common microsatellite regions for forensic genotyping have tetrameric or pentameric repeat units, although dinucleotide repeats have been used for non-human and microbial samples.[72]

Most of the forensic microsatellite markers contain simple repeats. For example the sequence AGCTAGCTAGCT, contains a tetrameric or four base repeat, (AGCT). Compound repeats contain at least two simple repeats combined, for example AGAGACTAAGAGACTA. There may also be complex repeat units, which have a variable number length sequences and may contain intervening sequences. For example, AGCTACTGGCCCGATCGAGCTACTG.[73, 74]

It is also possible to have one or more bases deleted from a repeat unit. These are known as microvariants. The most common forensic marker that contains the deletion of one base from the motif is the TH01 locus. At this locus there can be nine complete AATG units and one ATG unit resulting in a 9.3 allele designation. The 9.3 allele is because one of the adenines in the motif is missing.

The STRs markers are well suited for forensic applications as a result of their high level of polymorphism, high sensitivity and high power of discrimination. Their small size permits the analysis of highly degraded samples. Since there are numerous microsatellites, STR markers can be chosen to safeguard against linkage disequilibrium to ensure accurate statistical calculations when combining these markers, and discrimination between close relatives. By selecting STRs specific to the Y-chromosome, male portions of the sample can be identified and used for the separation of male/female mixed samples.

b) Allelic Ladders

An allelic ladder is a reference standard that contains all possible alleles for a given locus within that human population. The reference ladder is used during amplification to determine the size of the amplicon and its allelic number designation based on comparison to the fragment size. In order to create an allelic ladder, a specific locus is amplified with all the possible alleles. The alleles for that locus are then separated using polyacrylamide gel electrophoresis and each allele cut out. Once this is done for all loci required, all the alleles are recombined to create the full ladder (Figure 14).[75] Diluting the original ladder and re-amplifying with the original primers can also remake the allelic ladder.[*76] However, most commercial forensic STR kits supply a high quality well-balanced allelic ladder for genotyping.

[*] Note: re-amplification of allelic ladders may result in peaks with poor balance or quality especially when using the same size amplicons as the original ladder.

Allelic ladders are not only important for designation of alleles, but also to correct for temperature and mobility shifts that are present instrument to instrument and lab to lab.

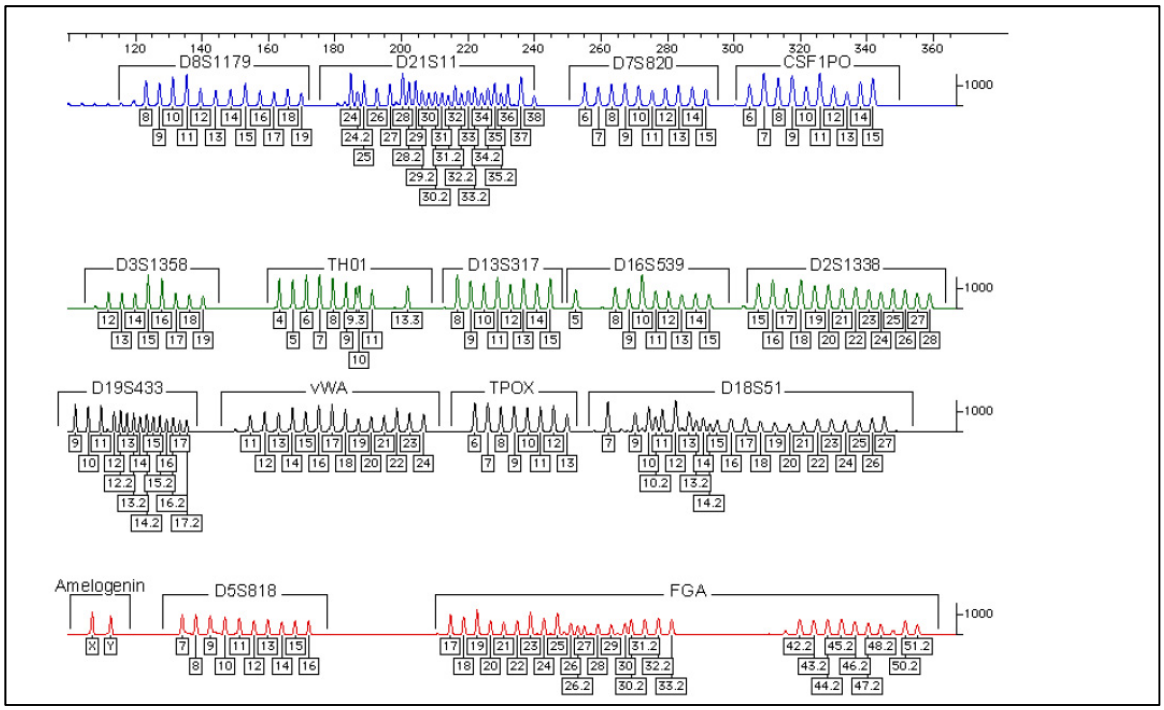


Figure 14: An electropherogram of the AmpFℓSTR® Identifiler® allelic ladder showing all allele for each locus.[17]

c) Commercial STR kits

Forensic laboratories prefer to purchase quality controlled STR kits rather than spend valuable time designing and validating their own because of the nature of forensics and the quality assurance and validations that are required for admissibility in the courtroom. These kits have evolved from containing 3-4 loci to as many as 21 loci multiplexed simultaneously using multicolor fluorescence detection in a single PCR. The two most commonly used forensic identity STRs kits in the United States of America contain 16 loci multiplexed of which 13 are core CODIS loci. Life Technologies,

formerly Applied Biosystems (ABI), manufactures the AmpFℓSTR® Identifiler® and Promega Corporation makes the PowerPlex® 16 kit. The latest versions of these kits, AmpFℓSTR® Identifiler® Plus and PowerPlex® 16HS, are slowly replacing their predecessor because improved sensitivity and more robust buffers (that handle inhibited samples during PCR) have lead to more reliable and accurate results without pre-PCR cleanup.

The standardized set of loci, which comprises the national DNA database, known as the Combined DNA Index System (CODIS) is common among commercial kits used for forensic human identity with some additional loci that give supplementary information and higher discrimination powers. The FBI selection group chose the 13 out of 17 loci recommended based on a number of factors and data evaluated. The loci included were TPOX, D3S1358, FGA, CSF1PO, D5S818, D7S820, D8S1179, THO1, VWA, D13S317, D16S539, D18S51, D21S11 and AMEL.[29]

TPOX, CSF1PO, D5S818, D13S317 and D16S539 contain simple repeat motifs while TH01, D18S51 and D7S820 are simple repeats that contain microvariants or non-consensus alleles. VWA, FGA, D3S1358 and D8S1179 are compound repeats with non-consensus alleles. The last locus D21S11 is a complex repeat unit. FGA, D21S11and D18S51 are the most polymorphic with TPOX having the least variations.

The European Network of Forensic Science Institute (ENFSI) in 2009 added 5 STR loci to the European Standard Set (ESS) of 7 core loci.The 12 core European loci are TH01, vWA, FGA, D8S1179, D18S51, D21S11, D3S1358, D12S391, D1S1656, D2S441, D10S1248, and D22S1045. The expansion and demand for more compatible systems between the US and Europe has lead to the formation of the CODIS Core Loci

Working Group sponsored by the FBI to recommend and expand the 13 core loci currently being used. In 2011, the addition of 8 loci was proposed which would allowed for an overlap of 14 loci with that of European kits such as AmpFℓSTR® NGM™ and PowerPlex® ESX 16. The loci suggested were D12S391, D1S1656, D2S441, D10S1249, D2S1338, D19S433, Penta E and DYS391. Amelogenin was also included as a required maker and the DYS391 chosen as a Y-chromosome marker to aid in the determination of male samples.[28]

Commercial kits include quality controlled PCR primer-mix, PCR buffer, DNA polymerase, positive controls, and the allelic ladder, which facilitates a validated out of the box amplification process. Multiple STR kits can also be combined to improve discrimination power. There are also specially designed kits for degraded DNA samples such as mini STR kits and large number STR loci kits for paternity testing.[76]

d) STRs: Chemical and Biological Artifacts

The analysis of STRs does have inherent limitations. These are mostly associated with the amplification, detection and analysis steps. These artifacts include stutter, dye blobs, microvariants, null alleles, drop-in and non-template addition, all of which can interfere with the interpretation of the genotype results and lead to misinformation if not carefully analyzed.

i) Stutter

The amplification of STRs often produces an additional amplicon that is one repeat unit smaller than the actual amplicon. These minor products are known as stutter

peaks. Because of the similarities of the sequences, strand slippage of the polymerase can occur during the primer binding stage of PCR causing the deletion of one or more repeat units.[77] Stutter products are commonly seen and forensic labs utilize threshold levels to determine the difference between low-level mixtures and stutter. Generally stutter peaks less than 15% are acceptable and easily distinguished from major peaks. The percent of stutter is calculated using Equation 3:

Equation 3: Stutter %

$$\frac{(stutter\_product\_peak\_height)}{(main\_product\_peak\_height)} * 100$$

Stutter peaks tend to decrease with the length of the repeat motif. As a result pentameric repeats exhibit less stutter than tetrameric repeats. Stutter also increases with the number of repeat units, as a result, alleles with fewer repeats produce lower stutter percentages than larger ones.[78]

Stutter peaks are explained as a result of slipped strand mispairing during PCR. (Figure 15) One of the two replicating strands can fold onto itself causing a loop structure. As a result, misalignment occurs producing an increase or loss in repeat number.[78] The result is a small amount of PCR product that is commonly one full repeat larger or smaller than the main product. Due to the nature of the amplification process, it is more common to see a loss of one repeat than an increase.

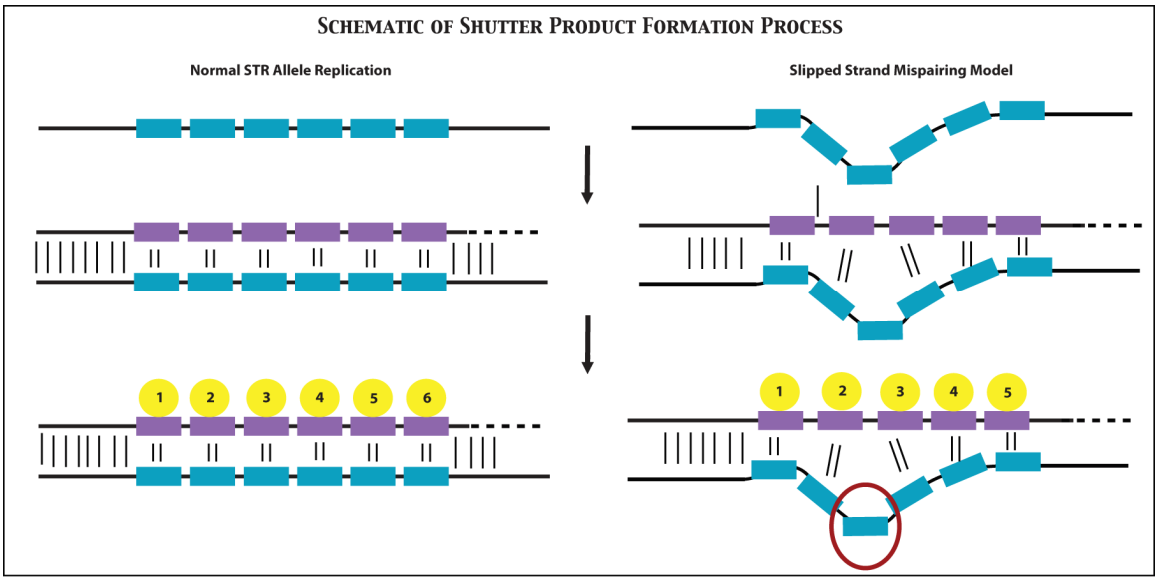


Figure 15: Schematic of the formation of stutter products during the PCR process. On the left shows a normal amplification without any stutter formation. On the right shows the slip strand model in which the primer is temporarily separated from the template. As it re-anneals it 'slips' resulting in a product that is one repeat short. Adapted from Walsh et al.[78]

The presence of stutter peaks must be carefully analyzed to avoid misinterpretation of mixtures and it is important that the height of the stutter peak fall below the threshold set by that laboratory.[79]

ii) Microvariants

Another issue observed with the use of STRs are microvariants. These are alleles that contain an abnormal repeat length because of the deletion of one or more nucleotides in the standard four or five base repeat motif. An example of one such locus is TH01. The locus contains an allele labeled as 9.3 which contains 9 repeats, plus a 10^{th} repeat unit one nucleotide shorter than the normal 4 bases. Extensive testing of the STR markers used in forensic identification in combination with population data sets has resulted in the documentation of the most common microvariants.[80] Microvariants are sometimes

referred to as “off-ladder alleles” since the fragment sizes do not correspond to those present in the allelic ladder.

iii) Null Alleles

The flanking and repeat regions of many target microsatellites are known to differ because of sequence variations and may be avoided through proper primer design. However, point mutations at a primer binding site can cause major problems with primer annealing during PCR and may result in one or both alleles not being properly amplified and detected.[81] The presence of such point mutations can be verified using primers with two different annealing positions and confirmed by sequencing.[82] Many of these variations have been well documented and multiplex kits have been designed to avoid primer binding at or near those locations of sequence variation. Furthermore, the closer the variation is to the 3’ end of the primer, the more likely drop out will occur. Some kits have used a second set of degenerate primers to overcome this issue by providing an alternate primer sequence at the variable site. In some cases the size of the amplicon may be altered by insertions or deletions of nucleotides between the primer binding sites causing a discrepancy from the original amplicon. It is important that STR kits used in forensic human identification be well designed and where possible areas of high primer binding site polymorphisms avoided. The presence of null alleles can result in inaccurate genotypes and the potential for false exclusions and inclusions.

iv) Non template addition

Non-template addition results from the tendency of the Taq polymerase to add an extra nucleotide at the 3'end of the PCR product. Adenine is the most commonly added nucleotide and hence non-template addition may be referred to as adenylation. The addition of an adenine (+A) is common. However, adenylation does no always on all the PCR amplicons. As a result split peaks are sometimes seen in electropherograms. To avoid the problem, primers can be designed to help promote full adenylation through the addition of a guanine nucleotide to the 5' end of the primer, or by adding a final soak step at 60°C or 72°C for 15-45 minutes to give the polymerase enough time to fully adenylate the product. Split peaks (+A/-A) are much more difficult to interpret especially if mixed samples are present. The major cause of incomplete adenylation is excess DNA template resulting in inadequate levels of polymerase in the reaction. Proper primer design can help reduce adenylation problems. For example, it has been found that an ATT on the 5' end of the unlabeled opposite strand primer can help promote adenylation.[83]

v) Stochastic Effects

When low levels of DNA template are present it is common to see uneven amplification between heterozygous alleles or drop out. This is known as stochastic amplification and results from an inadequate number of template strands leaving one or more alleles to be preferentially amplified. Typically samples containing 100pg or less of DNA are subject to these effects. More strict protocols must be used to avoid mistaken homozygous loci and loss of alleles when such samples are analyzed.[17, 84, 85]

Stochastic effects can be controlled by properly regulating the level of input template. To do this, forensic laboratories carefully quantify unknown samples, and establish minimum peak heights in resultant electropherograms, in order to avoid false homozygotes. Replicate analysis is also done to minimize problems with allele drop out.[86-88] It is important that laboratories have a well-documented stochastic and analytical threshold for each genetic analysis instrument.

CHAPTER V: STR MULTIPLEX DESIGN

a) Selection of Markers

Short Tandem Repeats (STRs) markers used in forensic genotyping are selected on the basis of their power of discrimination and ability to be multiplexed. Once a suitable set of STR markers have been selected, the primer binding sites must be chosen properly to ensure accurate amplification of the multiplex and electrophoretic separation.

When selecting markers for multiplexed systems, it is also important to make sure that the target regions are far enough apart to avoid any possible mis-priming and overlap between regions. With the ability to multiplex by both size and fluorescent dye generally a size difference of about 10bp between markers avoids overlap between adjacent markers. The markers used for forensic analysis have been well documented in terms of size, location and frequency. However, applications may occur in which there is a need for the re-design of these loci for improved detection and analysis. This is done primarily by primer re-design, as opposed to selection of different markers. Reference sequences can be obtained from STRBase (NIST) or GenBank (NCBI) website. Once the sequence is obtained it can then be imported into primer design software for the determination of useful and thermodynamically favorable primer sites.[89]

b) Primer Design

The ability to successfully amplify a sample for multiplex STR analysis is highly dependent on the primers used during PCR. The primers control the location of the target sequence and provide the initial point of elongation where the polymerase can attach and begin its process of adding new nucleotides. During PCR a forward and reverse primer

are required for each target region to permit binding to both the sense and anti-sense strands. The primer should be specific for its target and have a high efficiency for amplification success, without the formation of artifacts. Primer design is the first step in optimization of PCR and the parameters discussed below should be carefully analyzed and taken into consideration. With each additional primer added to a set of multiplexed PCR reactions, the complexity of the interaction increases between the individual primers and the importance of proper design more prominent.[90]

i) Primer Length

The ideal primer length that should be chosen for the design of a multiplex kit ranges from 18-25bp. The length of the primer controls the specificity of the binding, the hybridization stability and the cost.[54] Generally the longer the primer sequence the more unique it is. For each additional nucleotide added to the primer length, the possibility of finding that sequence in a random genetic sequence drops by a factor of 1/4. Therefore, the chance of finding a random primer sequence with 20 nucleotides is ($1/4^{20}$). Longer primer lengths are more specific and permit lower annealing temperatures, which in some cases can improve sensitivity.

On the other hand, the greater the length of the primer, the greater the chance of it binding to itself, forming secondary structures. Long primers take more time to break away from the primer-template complex requiring increased extension times that become longer with the addition of each nucleotide.[90]

ii) Primer Melting Temperature (T_m)

The primer melting temperature is the temperature at which half the DNA duplex dissociates and becomes single stranded and characterizes the stability of the duplex. Generally a range between 55- 60°C is best. All primers in a multiplex should have similar T_m values to ensure that the optimal temperature for annealing is close and that all primers are stabilized over the same temperature range. If primer melting temperatures vary greatly within a multiplex, the chances for mispriming are high and non-specific products may be amplified. If the melting temperature is too low even more non-specific products are observed and at temperatures that are two high, there can be a loss or complete drop out of allele peaks.

Most primer design software calculates the primer melting temperatures using the nearest neighbor method when suggesting primers for multiplex reactions. The melting temperature is calculated using Equation 4:

Equation 4:Primer Melting Temperature

$$Tm(^{\circ}C) = \left[\left(\frac{H}{S + R\ln(c/4)}\right) - 273.15^{\circ}C\right] + 16.6\log[salt]$$

Where T_m is the primer melting temperature, H and S is the enthalpy and entropy of helix formation, R is the molar gas constant (1.987 cal/°C mol), c is the DNA primer concentration in solution, 273.15 is the Kelvin to Degree Celsius conversion and [salt] is the concentration of salts present.[91-94]

iii) Primer annealing temperature (T_a)

The primer annealing temperature is the optimal temperature at which the primer will bind to the DNA template. The annealing temperature is generally a few degrees less than the lowest primer pair T_m. The optimal T_a can be calculated from Equation 5.[95]

Equation 5: Primer annealing Temperature

$$Ta = 0.3(Tm\_\mathrm{Pr}imer) + 0.7(Tm\_PCR\_\mathrm{Pr}oduct) - 14.9$$

At low $T_{a,}$ primers may bind to multiple sequences other than the target sequence resulting in non-specific products. At high $T_{a,}$ primers do not bind as easily and hence the amount of product may be reduced. Thus, the T_a must be optimized to ensure a specific product with high yield and no artifacts.

iv) GC content

When designing primers, the general rule is that the GC content should be between 40-60%. This allows for stronger annealing to the template as the GC pairing has three hydrogen bonds. The GC content directly affects the melting temperature, which is also critical to the specificity as described above. When designing a multiplex, all primers should have similar GC percentages as each other.[90] If primers GC % differ greatly, the primer length may be used to facilitate similar binding temperature and conditions.

v) Primer efficiency

The primer efficiency is described as the ability of the primer to bind to its target region specifically, with low false priming and formation of secondary structures. During

primer design, there are a number of precautions that are used to ensure a high efficiency of binding. For example, since the process of elongation during PCR starts at the 3' end of the primer, a GC clamp can be incorporated to increase binding and promote specific binding. However, no more than 3 G's or C's should be present within the last 5 bases at the primer 3'end.

vi) Secondary structures

When primers in a STR multiplex are not designed properly, the formation of secondary structures can be produced by inter or intra-molecular interactions. Interactions reduce the conformational changes and efficiency of primer binding. The three types of secondary structures formed are hairpins, self-dimers or cross-dimers. The stability of these structures depends on the primer sequence and its free energy of interaction with the nearest nucleotides.[93] Most primer design software takes into account these calculations depending on the enthalpies and entropies of the nearest nucleotides. If the free energy is greater than 0, then the secondary structure is too unstable to interfere with the reaction. Energies less than 0 can spontaneously form and greatly reduce the efficiency of the primer.

Hairpin secondary structures are formed as result of intra-molecular interactions of the primer sequence causing the primer to fold onto itself. These hairpin loops can be formed with as little as 3 nucleotides. The 3' end of the primer is most important. If the hairpin is at the 3' position, ΔG of about -2 kcal/mol or more is acceptable. If the hairpin is internal, a ΔG of about -3 kcal/mol or more is satisfactory.[96, 97]

Self-dimers are formed by inter-molecular attractions between two of the same primers. This means that the primer is homologous to itself, and if the dimers formed more readily than that of the primer binding to target, then the amount of product is greatly reduced and artifacts may be observed. (Figure 16) If the self-dimer occurs at the 3' position, a ΔG of about -5 kcal/mol is acceptable. If the self-dimer occurs along the primer sequence a ΔG of about -6 kcal/mol is satisfactory.[96, 97]

Cross-dimers are formed by inter-molecular attractions between two different primers. If two or more primers have similar sequences, especially in multiplex systems where the number of primers can be in excess of 30, they can form dimers between themselves and greatly reduce the amplification efficiency. (Figure 16) The use of appropriate software should be used to ensure primer compatibility when designing a system. If the cross-dimer occurs at the 3' position or along the primer sequence, the same self-dimer ΔG values are acceptable.[96, 97]

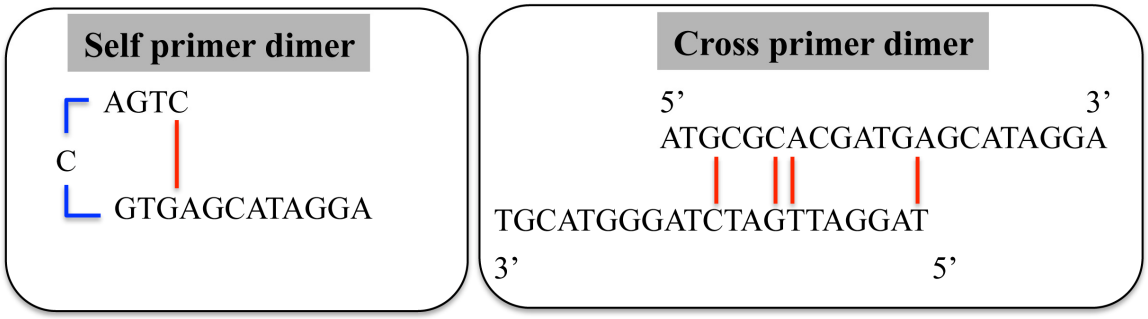


Figure 16: (Left) a primer self-dimer formed as a result of high ΔG values. (Right) a cross dimer formed between two different primers in a multiplex.

A basic local alignment search tool (BLAST) can be performed on the primers and checked against the human genome to see if any similarities that may be present.

This can be performed using the National Center for Biotechnology Information (NCBI) website. The more individual the primer sequence and the fewer secondary structures that are present, the more efficient and specific the product will be.

vii) Primer concentration

To ensure maximal binding, a relatively high concentration of primers is used. If the concentration of primer in the PCR master mix is too low then sensitivity and amplicons can be lost. If the primer concentration is too high, there is a greater chance for the formation of primer dimers or non-specific binding, which can result in undesired products.

Most PCR reactions have a primer concentration between 0.2-1μM. The primer concentration can also be a useful tool when designing STR multiplexes as different loci may amplify at different rates leading to peak height imbalance. The adjustment of the primer concentration of each locus is commonly used to maintain peak balance across loci in the multiplex.

CHAPTER VI: ANALYSIS AND INSTRUMENTATION

a) Capillary Electrophoresis (CE)

Capillary Gel Electrophoresis (CGE) is a powerful analytical technique that replaced the use of traditional slab gel-based electrophoresis for the separation of nucleic acids. Earlier systems of CGE used a UV-based detection method, which was later replaced by laser-induced fluorescence.[98] In 1995, the ABI prism 310 Genetic Analyzer was introduced. The ABI 310 system utilized multi-wavelength laser induced fluorescence detection, permitting the detection of a large number of multiplexed STR loci resulting in analyses with higher discrimination powers.

During capillary gel electrophoresis, a 50μm internal diameter silica glass capillary is filled with a viscous polymer matrix to achieve separation. The narrow channel permits the application of high electric field strengths without the problem of overheating, decreasing the run time when compared to slab gel electrophoresis.[99] The polymer used, coats the capillary wall reducing the electroosmotic flow (EOF) and filters the DNA depending on its pore or mesh size. The DNA molecule, which is negatively charged, migrates through the capillary under the influence of an electric field. The DNA fragments are then detected by laser-induced fluorescence through a window etched in the capillary wall.*

* The DNA has a constant charge to mass ratio thereby being separated by its size and ability to move through the pores of the polymer.

i) Sample Preparation

To prepare amplified samples for CE the PCR product is mixed with purified formamide. The dilution with formamide reduces the ionic strength of the sample relative to the buffer and helps denature the DNA. Past methods that involved the addition of water instead of formamide required rapid heating and snap cooling to help maintain the single stranded conformation and reduce the possibility of dsDNA peaks.[72, 100] The quality and conductivity of the formamide is very important. Break down products of formamide such as formic acid can interfere with the injection into the CE, negatively impacting the sample injection and resolution.[101] A formamide conductivity of 80μS or less should be maintained and constant freeze and thaw cycles should be avoided to minimize degradation.[99] An internal size standard is also added to the formamide/PCR product mixture. The software uses this standard to calibrate the system and determine the size of the unknown alleles.

ii) Injection

There are two methods of sample injection that can be performed in capillary electrophoresis, hydrodynamic or electrokinetic. Hydrodynamic injection involves the application of a pressure while the capillary end is submerged in the sample tube. While the volume of sample injected may be fairly constant it is not selective for the desired analyte. Instead for DNA analysis electrokinetic injection is more common. This injection mode involves the application of a voltage for a set period of time, to move charged molecules into the capillary. The amount of DNA injected can be estimated from Equation 6:

Equation 6: $DNA_{injection}$

$$DNA_{inj} = E_t(\pi r^2)(\mu_{ep} + \mu_{eof})\left[DNA_{sample}\right](\frac{\lambda_{buffer}}{\lambda_{sample}})$$

Where, E is the electric field, t is the injection time, πr^2 is the area of the capillary opening, μ_{ep} is the mobility of the sample, μ_{eof} is the electroosmotic mobility, λ_{buffer} is the ionic strength of buffer and λ_{sample} ionic strength of sample.*[99, 102]

Because PCR products are diluted in low conductive formamide, its ionic strength is reduced when compared to the CE buffer. As a result, a phenomenon known as field amplified injection or sample stacking can occur. Here, a narrow band of sample forms at the interface between the low conductive sample (high electric field) and the highly conductive buffer (low electric field) because of differences in mobility between the two zones.[99] The effect of sample stacking greatly improves the sensitivity of the injection.

iii) Separation

It is important to optimize the system for the highest possible resolution during separation because of the nature of forensic samples and the possible presence of microvariants and other artifacts. The sieving matrix, capillary and buffer can greatly affect the resolution and efficiency of the system.

1) Capillary

Capillary Gel Electrophoresis separations are performed in a hollow fused silica capillaries protected by polyimide coatings, which provide physical strength. Generally capillaries are used with internal diameters between 50-100μm and lengths of 25-75cm.

* This equation does not take into consideration the present of other ions that may be present in the solution changing the conductivity and mobility of the sample.

Most forensic CE systems use a 47cm long x 50 µm I.D. capillary. The small internal diameter (I.D.) allows for high voltages to be used with minimal joule heating, permitting fast separation. The uncoated capillary is greatly affected by electroosmotic flow (EOF) because of residual silanol groups on the silica surface. At pH values above 5, silanol groups are ionized to SiO^- and can interact electrostatically with the buffer cations.[103] These interactions cause problems with reproducibility due to the difference in separation velocities from run to run. In order to diminish the effect of the EOF, linear polymers within the sieving matrix are used to help coat the walls during separation (Figure 17).

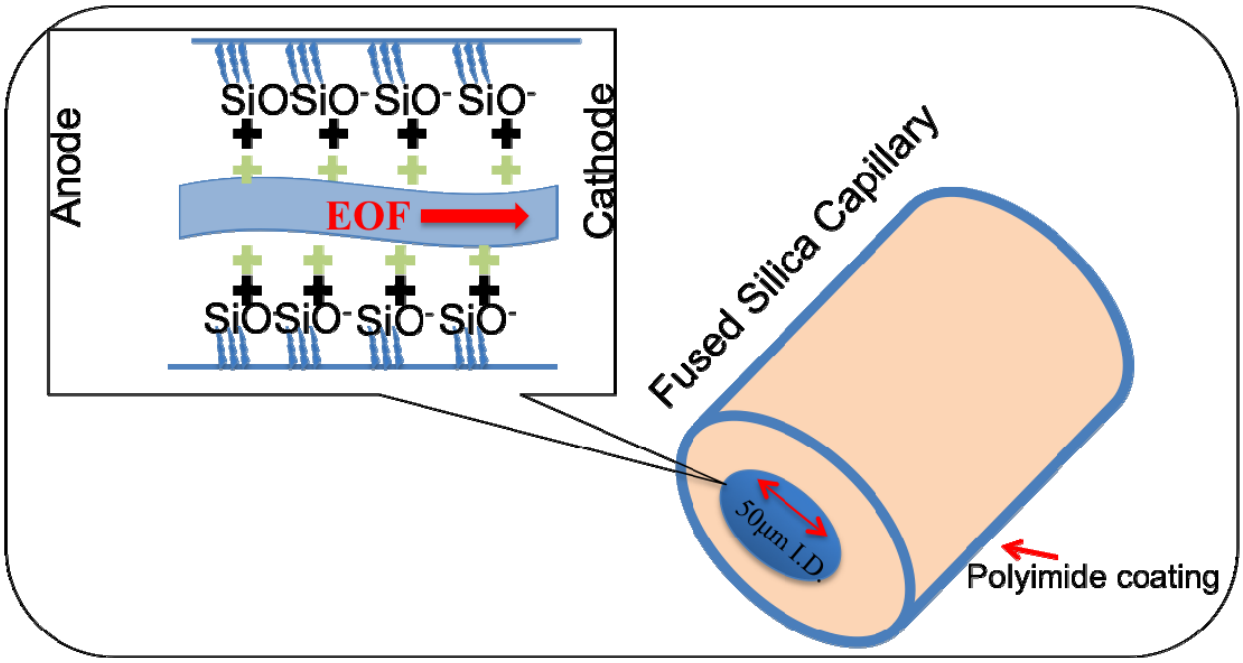


Figure 17: Diagram of fused silica capillary and the silanol groups along the wall surface. Coating of the wall surface can reduce the electroosmotic flow.

Fused silica capillaries dissipate heat very efficiently, permitting high voltages to be applied (up to 15-25kV) with minimal loss in resolution. For each capillary an optical

window is burned through the polyimide coating to create a transparent optical window, which allows the signal to be illuminated and detected via laser induced fluorescence.

2) Sieving Matrix

The sieving matrix is the key to successful high-resolution separation of biomolecules such as small DNA fragments. As a result of size independent mobility of the DNA, effective separation in free solution is not possible.[104] Early CE separation methods for the separation of nucleic acids involved the use of agarose or cross-linked polyacrylamide gels that were difficult to prepare and could not be reused.[105-107] The development of aqueous solutions of polymers in CE eliminated the need for cross-linked gels. The use of aqueous solution polymers for DNA separation was first demonstrated by Zhu et al. using a dilution solution of aqueous methyl cellulose to separate DNA fragments.[108] There have been numerous studies conducted to investigate the process by which these polymers are formed and how they interact with DNA fragments. However, these effects are highly complex and there are still some characteristic interactions that are not fully understood.

In 1958, at Oxford University, A.G. Ogston was the first to model the movement of spherical particles though a series of fibers.[109] Ogston's model was later used to describe the separation of DNA molecules through slab gel electrophoresis.[110] Ogston's theory predicts DNA to move through a polymer solution as spherical molecules with a radius of gyration. The smaller the DNA fragment, the smaller the radius of gyration and more mobile the fragment through the pores created by the polymer entanglement.[111, 112] The size of the mesh or pore created by the polymer strands in solution affects the

mobility of the DNA passing through it. The mesh size is a function of the concentration of the polymer in solution. In dilute polymer solutions the strands do not interact and no pores are formed. If the concentration of polymer is high enough, the individual polymer strands interact with each other and form a network of overlapping strands. This point is known as the entanglement threshold. The threshold is determined experimentally as a linear function of the natural log of specific viscosities at varying polymer concentrations versus the natural log of the weight fraction of polymer in solution. Alternative formulas to predict the entanglement threshold has also been described by Viovy and Duke using polymer physics.[111] They were able to generate an approximate value using Equation 7:

Equation 7: Entanglement Threshold

$$\Phi = \frac{M_w}{(N_A \, 4/3 \, \pi R_P^3)}$$

Where Φ is the entanglement threshold, M_w is the molecular weight, N_A is Avogadro's number and R_P is the radius of gyration for the polymer solution.

Using the Ogston model at low concentration of polymers, no entanglement should occur, no pores should be created and therefore no separation should be observed. This is not entirely true since longer polymers reach the entanglement threshold at lower concentrations than shorter polymers. It is possible for a shorter polymer to be at very high concentrations without being entangled. Therefore, the polymer concentration is important when modeled under the Ogston theory.

Another common theory used to describe the mobility of DNA through polymers is the Reptation model.[107] This model is used to described fragments that have a larger radius of gyration than the pore size of the entangled polymer. If the Ogston model holds

true, no separation would occur under this condition, as the larger fragments would not fit through the mesh and hence not be separated. Therefore the Reptation model is used to describe the mobility of fragments larger than the mesh size of the polymer. The reptation model predicts the DNA to unwind, and move through the pores in a snake like manner. The model relates the mobility to the length of the DNA fragment and its frictional forces. Equation 8 shows the relationship between mobility and DNA length.

Equation 8: Reptation model

$$\mu = 1/L$$

Where, μ is the mobility of the DNA molecule and L is a measure of the length of DNA. This equation can be further adapted to incorporate the effect of field strength on the DNA molecule. Higher field strength causes the DNA to be elongated and adopt rod like conformations.[111] Therefore, a more accurate equation can be described by:

Equation 9: Reptation with Electric field strength

$$\mu = \left(1/L\right) + bE^2$$

Where b is a function of the pore size and E is the electric field strength. Therefore with increasing electric field strength the size of the DNA becomes less critical for its mobility.

Both the Ogston and Reptation models seem to work well at predicting the mechanism of separation of DNA fragments through sieving matrices and their interactions with frictional forces and the polymer pores. Yet, they do not explain DNA separation in dilute polymer solutions. Barron et al. have described the use of a transient entanglement coupling mechanism that accounts for the separation of DNA in dilute

solutions.[113] In this model DNA collides with the polymer, dragging it through the solution increasing the frictional forces. The transient entanglement coupling mechanism results in a reduced electrophoretic mobility and separation of the DNA.[113, 114] Therefore, combinations of these models are needed to explain the mobility differences through entangled polymer solutions.

3) Buffer

The composition of the buffer used in the sieving matrix plays a critical role as it contains components that are responsible for, the solubility of the DNA, the ionic strength of the solution, its denaturing capability and its pH. At high buffer concentrations the current can be too high causing excessive heating and poor resolution. At low concentrations the electrophoretic flow may be too low resulting in long separation times with broad peaks and poor restoration of the capillary because of diffusion. During CE, buffer reservoirs at both the cathode and anode of the system produce a complete electrical circuit. This buffer is usually of the same or similar composition to that used in the sieving matrix. Over time and several runs, the buffer in these reservoirs should be changed as the concentrations between the cathode and anode may vary because of the flow and degradation of ions during the runs.

The main buffer composition used for most CE-based DNA analysis systems consists of 100mM 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid (TAPS),1mM ethylenediaminetetraacetic acid (EDTA) adjusted to a pH of 8 with sodium hydroxide (Figure 18). shows the chemical structure of these compounds. Some literature sources report the use of Tris-borate-EDTA (TBE) instead of TAPS but

this buffer can cause problems because of effects of temperature on the pH, which can affect reproducibility.[115] Additives such as urea and formamide also may be added to maintain strong denaturing conditions during the run.[116] The addition of reagents such as pyrrolidinone help to reduce viscosity and aid in surface coating of the capillary.[116] Lastly, CE systems are run at a temperature of 60°C to increase denaturing effects. Buffer components and additives must be stable at such temperatures.

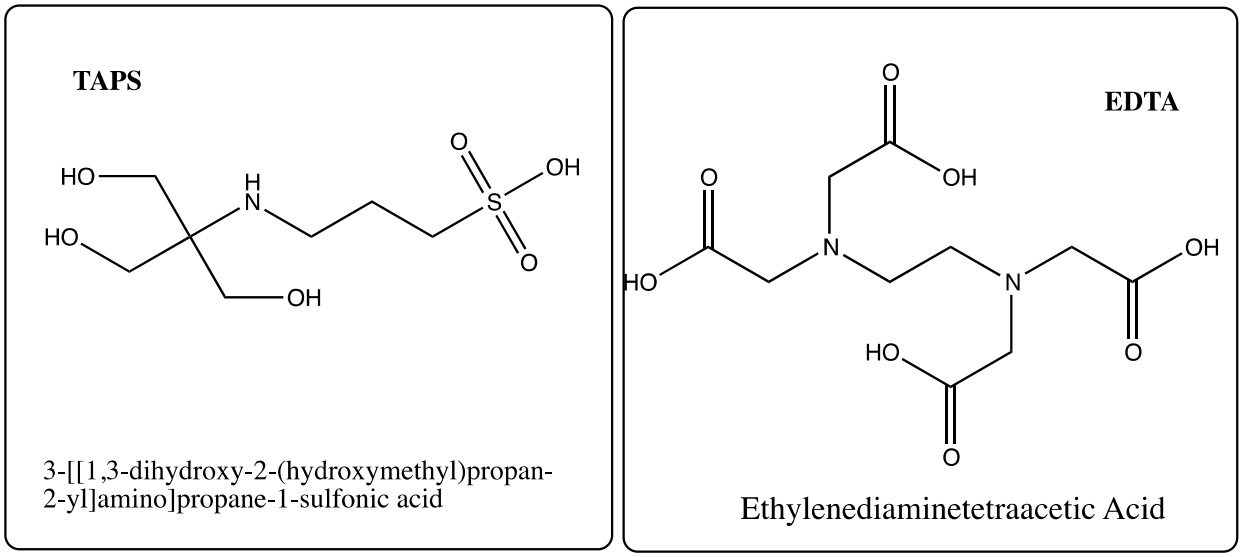


Figure 18: Chemical structures of TAPS and EDTA two major components in the buffer for separation of DNA on CE systems.

iv) Sample detection

Short Tandem Repeat primers used in human identification contain fluorescent dyes that are covalently bonded to the 5' end of the primer. The labeled DNA fragments are detected via laser-induced fluorescence (LIF) as they pass an optically clear window in the capillary. A single argon-ion laser at 488nm can be used to excite a range of fluorescence dyes, which emit from 520-680nm, permitting multi-wavelength detection that can be used to discriminate between different dyes.

The emission spectrum of the excited fluorophore is then recorded using a charge coupled device (CCD) camera.[99] The addition of a multi-wavelength CCD camera was a major breakthrough in CE analysis as it permitted the discrimination of large multiplex loci by both size and fluorescent label. Spectral discrimination is performed through the use of a matrix deconvolution by the fluorescent wavelength, which isolates the peaks into their respective dye lanes. However, the matrix calculation is dependent on the linear relationship between the fluorescence and concentration of DNA. If too much of one dye is present, it can also appear as a small peak in a neighboring fluorescent lane. This phenomenon is known as 'pull up'.

v) Interpretation

In CE, each amplicon's size is determined through the use of an internal lane standard (ILS). An ILS standard contains known DNA fragment sizes that can be used to determine the sizes of individual alleles. Either a Local Southern method or Global Southern method fit is used to determine allele size.[117] These two methods differ in that the local method uses an interpolation method by incorporating two size peaks before and two size peaks after the unknown calculated size, while the global method includes all size data to calculate the unknown peak size using a regression analysis.[117] The calibration process and designation of alleles are performed via software. These processes were originally performed using a software package from ABI in 2002 known as Genescan® and Genotyper®. The first was responsible for the sizing and the second for assigning allele calls. The software later developed into a single software package called

Genemapper® which encompassed all the required tools for the analysis of STR fragments and forensic human identification.[118]

Once the amplicons are sized and the allelic ladder analyzed, the amplicons are labeled using virtual bins created by the user. These bins are the size ranges in which each allele appears. They include sufficient width to account for slight mobility shifts and are usually compared with known allele sizes from a second external standard consisting of all known alleles (Allelic Ladder). If for some reason a major shift occurs in size of an allele relative to the allelic ladder it may be necessary to adjust the bin for the software to be able to call all alleles correctly. Once the bins are correct and the alleles designated, a genotype can be uploaded and compared to known profiles or a database for a possible match.

b) Microfluidic Electrophoresis

In the early 1990's a new type of chemical analysis emerged involving small microscale devices known as Lab-on-a-chip (LOC) or micro-total analysis systems (μTAS). These gained immediate interest by the forensic community especially in genome sequencing and genotyping.[119] The advantages of microchip analysis includes increased efficiency and decreased sample handling.[120] In addition, rapid analysis, reduced sample volume and reagent costs make microfluidics systems ideal for forensic DNA typing applications. The development of integrated microfluidic systems that includes the extraction, quantitation, amplification and separation, all on one device, has proven to be a challenge for the scientific community. These systems have complex

engineering and fluid manipulation and although smaller than traditional CE systems, are not truly portable.[120-124]

The design of the microchip channels and the material used in its manufacture are critical to the separation efficiency when analyzing DNA fragments through sieving matrices. The most common substrates used in the manufacture of microchips include borofloat fused silica, silicon, polydimethylsiloxane (PDMS) and polymethyl methacrylate (PMMA).[120] The type of substrate depends mostly on the application and cost of production. The channels are etched into the substrate depending on its manufactured material.[125] For example, wet chemical etching using hydrofluoric acid can be used for silica substrates, while hot embossing may be used on polymer type substrates.[126] The characteristics of the channel and the injection intersection can also increase the efficiency of the sample injection plug. Table 1 shows the general channel characteristics of common microchip devices reported in literature. Typical microchip dimensions are about 1-100μm in channel length, between 40-100μm wide and 10-30μm deep.[127] However, these designs may vary depending on the application and the amount of sample needed for detection.

Table 1: Microchip design parameters

Parameter of chip design	Approx. Average value
Area of chip	$10cm^2$
Length of channel	1-10cm
Diameter of wells	1-5mm
Velocity	1-5mm/s
Volume injected	10-500 pL

Common chip designs involve crossed-tee intersections or double-tee intersections shown in Figure 19.[128] The type of intersection is related to the injection scheme and amount of sample to be injected. Injection takes place by one of two schemes, "Gated" or "Pinched". Gated injections are used in cases where the sample injection needs to be continuous. These are performed using a perpendicular flow to push the sample into the waste well and away from the separation channel. In order to inject the sample, the perpendicular flow is stopped. Once the flow is stopped, the sample is allowed to fill the intersection and top of the separation channel. Once the perpendicular flow is resumed, the sample in the intersection moves into the separation channel and electrophoresis begins.[129] The amount of sample injected is controlled by the length of time the flow is stopped.

Pinched injection utilizes a 'push back' voltage that pulls the sample back towards the sample and waste wells while the sample is injected by applying a set voltage across the separation channel simultaneously. The use of a 'push back' voltage is important to decrease the sample leakage into the separation channel and reduce peak broadening

caused as a result of samples moving into the channel after the initial plug. The use of a double tee design can permit repeated injections as the design keeps the sample closer to the intersection. The double tee design also increases the sample volume, however, the injection plug volume remains fixed and independent of time.[129, 130]

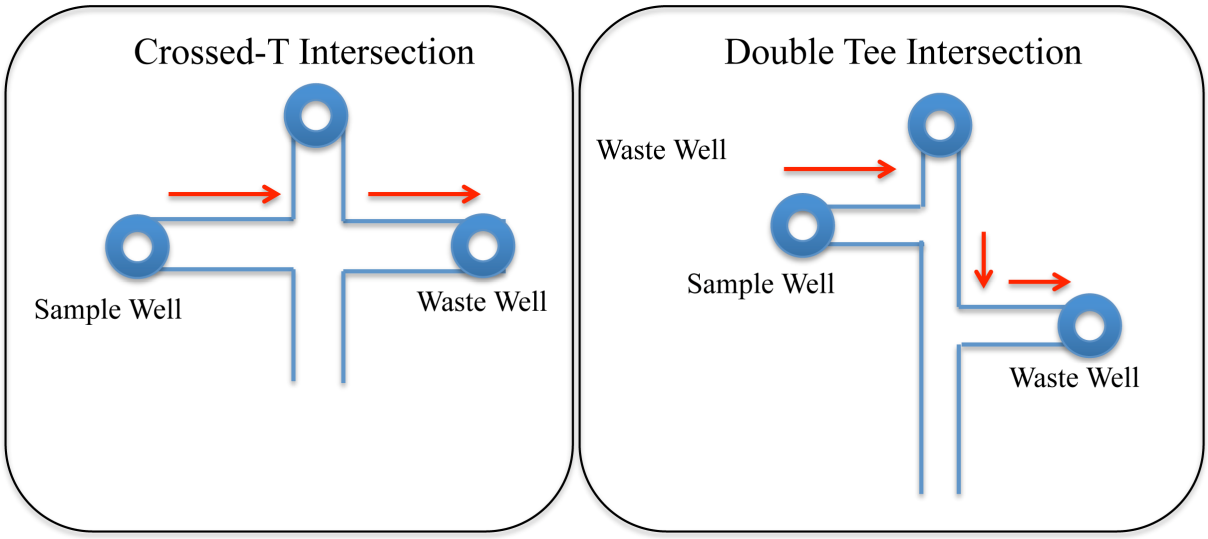


Figure 19: On the Left, a schematic of a Crossed-tee intersection most widely used in microchip designs. On the Right a Double Tee intersection design that allows for multiple rapid injections.

The process of microchip electrophoresis is similar to CE. First the separation channels are filled with the sieving matrix, then the sample and buffers are loaded into the appropriate wells an injected. The gel is loaded using a syringe or vacuum such that the entire channel is filled and no bubbles are introduced. Any bubbles trapped in the channel block the electric current used for injection and separation and results in a clogged channel. Due to the narrow channel size of these devices the viscosity of the sieving matrix is important to ensure efficient priming of the channels. Controlled

electrodes are used to manipulate the sample injection and perform electrophoresis. A laser induced fluorescence detector is used to detect the labeled DNA fragments. A schematic of using a pinched, crossed-tee injection is shown in Figure 20.

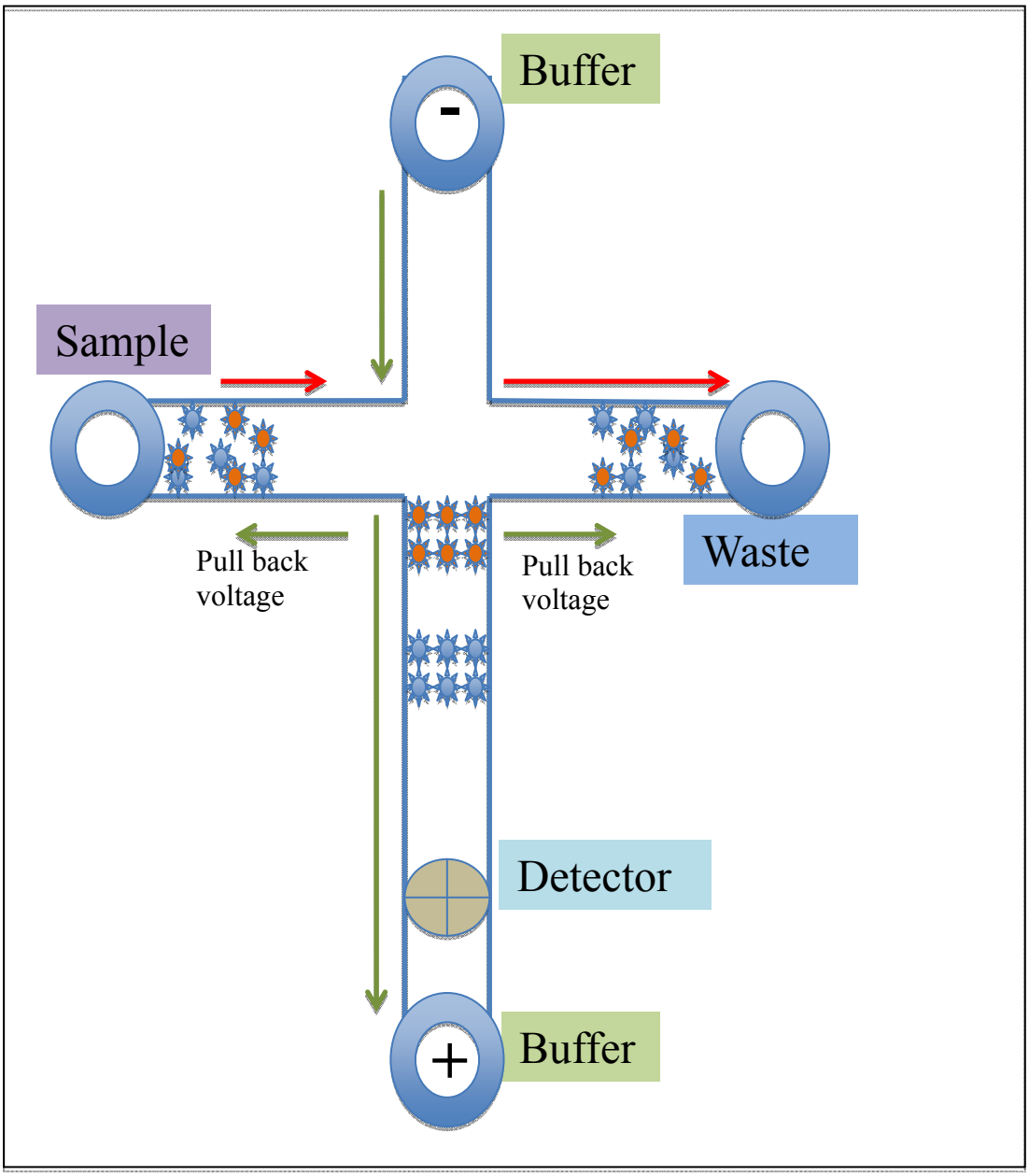


Figure 20: The schematic of a crossed-T microchip using a 'pinched injection' with pull back voltages. Once the sample is in the separation channel electrophoresis take place. The DNA is separated by size through an entangled polymer and detected using laser induced fluorescence.

CHAPTER VII: DEVELOPMENT OF MINI-PENTAMERIC STRs FOR USE ON PORTABLE RAPID MICROFLUIDIC SYSTEMS

a) Introduction

While the use of traditional capillary electrophoresis (CE) techniques has become standard for most forensic DNA analysis, there are some applications in which this large instrument is not suitable because of cost, complexity, lack of portability, and long sample run times (≥ 35 min per sample). Mass disasters in remote locations have emphasized a need for portable, user friendly systems for screening DNA evidence.[131, 132] In addition, many forensic laboratories are backlogged with DNA casework. Having a quick, reliable and accurate screening method would permit the analysis of mixed DNA evidence samples such as stains or allow investigators to piece together bone fragments and other similar forensic samples from a mass disaster. [133-137]

As a result of the portability limitations of conventional CE, research and development into the application of forensic based microchip electrophoresis have become widespread. These devices involve rapid separation of DNA in channels etched into glass chips followed by detection with laser induced fluorescence or amperometry. One of the first applications of DNA separation on microfluidic devices was demonstrated by Mathies and Woolley in 1995, where they were able to obtain single base pair resolution of DNA fragments between 150-200 bases in approximately 10-15 minutes. [138-142] The decrease in separation time was a result of the shorter length of the microchip and stronger electric field.[132] However, the DNA fragment sizes must be relatively small (100-350 bp) to achieve high resolution between fragments.

Microfluidic devices can increase the sample throughput as multiple separation channels can be implemented on the same chip. Because they can be mass produced inexpensively from glass or plastic, these devices may be the key for quick and portable forensic applications.[143-146] Maintenance and cross contamination problems are reduced due to the disposable separation platform. Microchip systems have been widely used in the analysis of a variety of different proteins and nucleic acids with good resolution and reduced sample run times. More specifically, a number of investigators have examined microchip based electrophoretic systems for the separation of STRs and mitochondrial DNA.[124, 142, 147-150] While these devices are still at the prototype stage, the approach is feasible and ready for implementation. It is also clear that integrated approaches involving extraction, amplification and detection can be implemented on microfluidic devices, further reducing sample handling. [151-154]

One major issue with microfluidic systems is developing devices with sufficient speed and resolution to compete with current capillary electrophoresis technology. The separation mechanism used in microfluidic devices (DNA sieving in entangled polymers) is the same as that required for standard capillary gel electrophoresis using 47 cm capillaries. While some gains in resolution may be obtained by microfluidic systems because of their unique crossed T injections, to obtain sufficient resolution (1-2 bp) over a size range of 100-500bp requires that these devices have separation channels nearly 20 cm in length. Such long channels are awkward in chip-based devices, making them less portable and/or requiring serpentine channels. [138, 142, 153] Truly portable devices should have much shorter channels to decrease the size and improve the analysis speed. Unfortunately, resolution in turn suffers. In previous research of the development of

miniSTRs, we have noticed that shorter amplicons (60-200bp) require much less stringent conditions for their separation and are particularly valuable in the analysis of degraded and inhibited samples.[135, 155] The issues with short separation channels could be alleviated through the use of a smaller set of short amplicons that do not require such long separation channels. Such a sample set would be ideal for application with microfluidic devices and would fit the need for a rapid onsite screening tool. [156]

The present research focused on the testing and adaptation of a commercially available microchip-based system for the analysis of miniSTRs on a novel set of pentameric short tandem repeats. The use of pentameric nucleotide repeat units has been shown to reduce the amount of stutter in the amplified sample, a useful characteristic when dealing with mixtures. In addition, pentameric STRs are highly polymorphic and have relatively few microvariants, which make them ideal for forensic analysis. A multiplex consisting of three different pentameric STR loci was developed and tested using both capillary electrophoresis and microfluidic systems for concordance.

b) Methods and Materials

i) Reference Sequences and Allele Range Information

Reference sequences for the penta STR markers Penta B, Penta C, Penta D and Penta E were obtained from GenBank through their accession numbers. The locations of the Penta B, Penta C and Penta E markers within the genome were obtained from the Promega Corporation, while primers for the Penta D marker were taken from previously designed miniSTR loci.[137]

ii) Primer Design

The PCR primers were designed for each marker using the Web based Primer3Plus interface software.[89] Typically the default primer parameters were used.[89] The primers were designed close to the target repeat unit to ensure that the smallest sized amplicons were used. An additional constraint on the primer design was their annealing temperatures (T_m), which had to be compatible to permit multiplexed PCR reactions. The resulting primers and their sequences are listed in Table 2.

Table 2: Penta Markers and Primer Sequences

Locus		miniSTR primer sequence 5' to 3')	Fluorescent Label (5')	Emission λmax (nm)	Excitation λmax (nm)	Tm (°C)	5' Tail added	Distance from Repeat (bp)
Penta B	F	GAG GCA ACA GTG CGA GAC	6FAM (blue)	517	494	62		16
	R	TTG AGC CTT GCA CTC CTA TT				63	GTTTCTT	
Penta C	F	CAG GGA TAT GCA CTG GTA ATA GA	Hex (green)	553	535	61		13
	R	CGC TTC TAG GGA CTT CTT CAG				62	GTTTCTT	
Penta D	F	GAG CAA GAC ACC ATC TCA AGA A	Ned (yellow)	575	553	63		11
	R	GAA ATT TTA CAT TTA TGT TTA TGA TTC TCT		635	680	58		19

iii) PCR primers and other reagents

Fluorescently-labeled (6-FAM, HEX and NED) primers, AmpliTaq™ Gold DNA polymerase and associated PCR buffers were obtained from Applied Biosystems (Foster City, CA). The forward primers were labeled with 6FAM, Hex and Ned dyes (Applied Biosystems) which permitted the use of compatible matrix standards with ABI Genetic Analyzers. Forward primers labeled with CY-5 were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and used for applications on the Agilent microchip system that has a red laser (680 nm). All unlabeled primers were ordered from IDT.

The design was intended to produce the smallest possible PCR amplicons for the three penta markers (Penta B, C, and D). Small PCR products are less affected by degradation, amplify more efficiently and can be better resolved than larger amplicons. The three primer sets were prepared from the accession numbers such that the target region included the penta-nucleotide repeats and the primers were placed as close to the target region to minimize excess flanking regions. A reduced size amplicon for the third marker, Penta D, had been previously developed and was not modified any further.[137] The fact that the three primer sets would be multiplexed was taken into consideration during the primer design phase of the research.

iv) Polymer Preparation

The PCR amplified STR products were separated using several different denaturing buffer systems. Commercial POP-4 (Applied Biosystems), POP-6 sieving buffer (Applied Biosystems) and a custom denaturing polymer (PVP/HEC) consisting of

a 3.5% (w/v) mixture of polyvinyl pyrrolidone (Polysciences inc., Warrington, PA) and hydroxyethyl cellulose (HEC) Aldrich Chemical (Milwaukee, USA) (20:80 ratio) were used for both the standard capillary system and the microchip assay.[157] A proprietary denaturing polymer obtained from Agilent Technologies was also utilized for microchip separations.

v) DNA samples

DNA standards from cell lines 9948 and K562 were purchased from Promega (Madison, WI) and diluted to 0.1 - 1.0ng/μL. These samples were used as the basis for the optimization of the STR multiplex. Anonymous buccal swab DNA samples were taken from a variety of subjects to provide a preliminary population study. These samples were approved for use through an Institutional Review Board and were extracted using a standard phenol-chloroform/isoamyl alcohol extraction protocol. (See Appendices)

vi) Quantification of DNA samples

All extracted samples were quantified using an ALU-based real-time PCR method with 0. 5x SBYR®-Green I dye (Molecular Probes, Eugene, OR) [30]. Quantification was performed in reaction volumes of 20μL using a Master Mix containing GeneAmp® PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5mmol/L $MgCl_2$, 200μmol/L deoxynucleotide triphosphates (Denville Scientific, dNTP's: dATP,dCTP,dGTP,dTTp), 1μM bovine serum albumin (BSA), TritonX 100 (10% solution), *Alu* forward and reverse primers and two units of RampTaq hot start Taq polymerase (5U/μL) (Denville Scientific, Metuchen, NJ). A series of 9948 DNA standard solutions of known concentration were diluted ranging from 10ng/μL to 0.1ng/μL and used to establish a

standard curve. All samples were run on a Corbett Robotics Rotor Gene 6000 instrument and the software used to calculate the critical threshold values and concentration of unknown samples.[158](Qiagen Corbett Robotics, Valencia, CA)[158](Qiagen Corbett Robotics, Valencia, CA)

vii) PCR Amplification

Amplification was performed in reaction volumes of 20µL using a Master Mix containing 1x GeneAmp® PCR Gold buffer (Applied Biosystems), 1.5mmol/L $MgCl_2$, 200µmol/L DeoxyNucleotideTriphosphates (Denville Scientific, dNTP's: dATP, dCTP, dGTP, dTTp), 1µM bovine serum albumin (BSA) and two units of AmpliTaq Gold® DNA Polymerase. Primer concentrations were adjusted from 0.5µmol/L to 1.5µmol/L to optimize the amplification reaction and obtain balanced peak heights.

Thermal cycling parameters, especially the annealing temperature, cycle number and extension times were altered to establish the optimum amplification conditions using the GeneAmp 9700 thermocycler (Applied Biosystems). Samples were run on an ABI Prism®310 Genetic Analyzer (Applied Biosystems) and then compared for peak height and quality. The optimized thermal cycling conditions are shown below. (* Denotes parameters that were varied during optimization)

PCR Cycling Parameters

- 95˚C for 10min; 96˚C for 1 min
- 32 cycles: 94˚C for 30sec,* 61˚C for 30sec, 70˚C for 45sec
- *60˚C for 60mins; 4˚C for 10mins
- 25˚C forever

The multiplexed amplification utilized 3 dye-labeled primers; 6FAM was used for Penta B, 6HEX for Penta C and NED for Penta D. A 5' -GTTTCTT- tail was added to the reverse primer of each locus to minimize possible problems with +/- A non-template addition. [137] In addition a final soak at 60°C for 60 minutes was added to the PCR cycle to minimize these effects.

viii) Analysis on ABI 310 (Single Capillary) Genetic Analyzer

The ABI Prism®310 Genetic Analyzer (Applied Biosystems) was used with filter set D to process the data from the four dyes 6FAM, 6HEX, NED and ROX after the matrix had been created using matrix standards from Applied Biosystems. Each sample was prepared by adding 1µLof PCR product to 12µL of Hi-Di™ formamide (Applied Biosystems) and 0.5µL GS500 ROX size standard (Applied Biosystems). Samples were then placed immediately into the instrument for analysis. Samples were injected for 5s at 15,000 V and separated at 15,000V for 26 min with a run temperature of 60°C. Standard electrophoretic conditions were used including POP™4 polymer, 1X Genetic Analyzer Buffer with EDTA (Applied Biosystems) and a 47-cm x 50-µm capillary (Polymicro Technologies, Phoenix, AZ). Certain samples were also analyzed using the PVP/HEC denaturing polymer buffer. Peak sizing was performed using the Global Southern algorithm.

ix) Analysis with Microfluidic Electrophoresis

Two different systems were used for the microfluidic analysis of the mini-penta STRs, a standard Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) for

use with native DNA and a beta version of the same system with an adjustable heat plate capable of performing denaturing analysis. Native DNA separations were performed using the standard 2100 system with the DNA 1000 Lab-on-Chip® Assay kit (Agilent Technologies) following the standard protocol provided. The chip was first primed with the gel dye mixture and each sample for analysis was prepared by adding 1μL of CY-5 fluorescently labeled PCR product and 5μL Agilent Marker (Agilent Technologies) to each of the 12 sample wells. The Agilent DNA 1000 ladder (Agilent Technologies) was used as a sizing standard. The results were interpreted using the expert system software provided with the system. Samples were run using denaturing conditions and analyzed in a similar fashion, except that a heated electrophoretic platform was used and the chips were modified with wider central channels to reduce problems with the increased viscosity of the buffer.

When prepared for analysis using the denaturing chip, the sample was diluted in formamide prior to analysis. The PVP/HEC polymer described above and a denaturing polymer supplied by Agilent Technologies were used for testing of the system and separation of alleles. The denaturing polymers were much more viscous than that of the existing DNA 1000 assay polymer, and required careful pipetting and priming of the chip to ensure that the polymer was distributed throughout all the channels. Sizing was performed using 2 standards; an 8bp lower marker and a 600bp upper marker. In addition, a specific sizing ladder provided by Agilent Technologies was used to calibrate the separation system.

c) Results & Discussion

The goal of this research was to develop a set of reduced size pentameric STR markers for use in microfluidic systems. The hypothesis was that mini STRs should provide enhanced sensitivity with these systems, permitting the rapid sorting and identification of forensic specimens in the field. In addition, since pentameric alleles are farther apart than the standard four base repeats used for the 13 CODIS markers, resolution of mixtures and heterozygous alleles should be less of a problem. Pentameric repeats also have fewer two base variants than tetrameric STRs and reduced stutter. These combined factors should make a pentameric multiplex ideal for rapid separations using short channel microfluidic chips.

i) Selection of STR Markers

The mini-pentameric multiplex was developed from STR locations provided by Promega Corporation. The sequences for each marker were obtained from GenBank via its accession number and the target region selected for input into primer design software. The accession number is a bookmark to that chromosome location and the corresponding genome sequence located on GenBank online database. Four pentameric STR markers were selected; Penta B, Penta C, Penta D and Penta E.

The primer sequences were selected using Primer 3 software and the PCR reaction conditions then optimized for each individual locus on the ABI 310 capillary electrophoresis system. [83, 89]

1) Penta B Marker Information

The Penta B STR marker is located approximately 33Mbp along the long arm of Human Chromosome 7 (7q33) shown in Figure 21. Penta B is a highly polymorphic marker that contains a wide range of alleles. The larger repeat distance (5bp) and its high variability make it useful for this application along with other inherent qualities of pentameric STR amplifications. However, current Penta STRs such as Penta D and Penta E are fairly large in size and pose resolution issues on short separation channel systems.

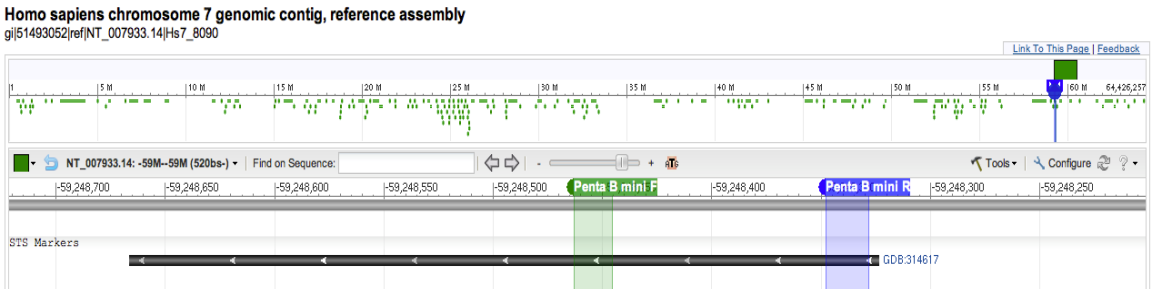


Figure 21: The position of Penta B STR marker on chromosome 7. The small dots along the top of the figure represent the position of genes (coding region) on that chromosome.

A small snippet of the mini-Penta B sequence on the antisense strand obtained from Genbank online database is shown below. The forward and reverse primers were designed as close to the target repeat region to ensure the smallest possible amplicon. Penta B contains a $[AAAAG]_n$ repeat motif.

GTCAAATCCAGAGAACACTGATCAATAATCTTTTGAATGAACACTGCA
TGACCATTAATTGTGATTAGTGAAGGTTTAAGAAAGGTATTGGGATAT
AAATTATCCATTGAATGCTCCATCTGTTATAAAATTATGTCTTTAAAAA
AGCCGTTGTGAGTAGAAAAAGAAAATCCATGAAGAGGTGAAGGCACA
ATTAAAATACTTGGATCACTGATAGAGAAAGTAATGATATACAACTTG
GTGATCCTGCATTCCTGAGAATGAAAAGCCAGCAATTAGACATGTATG
GTTCTGAGACCAGCCTGACCAACATAGTGAAACCCCGTCTCTACTAAA

AATACAAAGTTAGCCAAGCCTGGTGGTGGGTGCCTGTAATCCCAGCTA
C
ACGGGAGGCTGAGGCGGGAGAATCGCTTGAAGCTGGGAGACGGAAAG
TTGCAGTGAGCCGAGATCACGCCATTGCACTCCAGCC*GAGGCAACAGTG*
*CGAGAC*TCCAACTTGGGGGAAGA**AAAGA**AAAGA**AAAGA**AAAGA**AAA**
GAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGAAAA
GAAAAACACTCC*AATAGGAGTGCAAGGCTCAA*AGAAGCTCCATTTTAAG
TCTGTCTGCTGAGAAGCATTACAATATACACAAACCAAAATTTACCTA
GGGATCACTACTACTTATACGTCCTTACCCTGAAGAATTCAACATTTTT
GGATCTCCACTTTATTTCCAAATTACACTAAAGCAATGAGTACTCAATG
GCAACAATGTGTCTGGTGGCTCAAACAGCATCAGCCCACCCAAAGTAA
GAGAGCTTTAGGAAGATTGGAAACTGGGCTAGAAATCGTTGACCAGGC
AGAATTCATTTTTAATAAGGAAAGACATGTTGAAGAAAAAGACCTAAC
ATAAATAGAAAAACTAGAAGCATCCTATTTCAGGATGAGGGACAAAG
ATGACAAGTTACCTGGCATGACAGTACAGGACTGACAATTATAGCAGC
TACTAAGATGACATGCCCAAACCTGGAAATGGGAATGAGCCTGCATGC
CAGCACAGCTGGGTTATTAGATGTCTTAATTCAAATGGAGTCTGAGAT
ATCCCCAACACAATGACCAGGGCATCTCACAGCAGAATTTCAAGTTAC
ATACT

The position of the forward and reverse primers shown in yellow (italics) was chosen based on the selection parameters inputted into the primer design software. The start and end position of the designed mini-pentameric primer along the chromosome sequence is shown in Table 3.

Table 3: Penta B mini primer sequences start and end location on the chromosome 7.

	Penta B_m F	**Penta B_m R**
Accession No.	NT_007933.14	NT_007933.14
Start Location	59248446	59248329
End Location	59248463	59248348
Start Sequence	CCAAGTTGGAGTCTCGCACTG	ACTGTTGCCTCGGCTGGAGTG

2) Penta C Marker Information

The Penta C STR marker region located a little before the 38th millionth base on the Human Chromosome 9 is not as highly polymorphic as Penta B but contains enough alleles to aid in identity and be used for separation on short channel devices with good resolution. The location is shown in Figure 22.

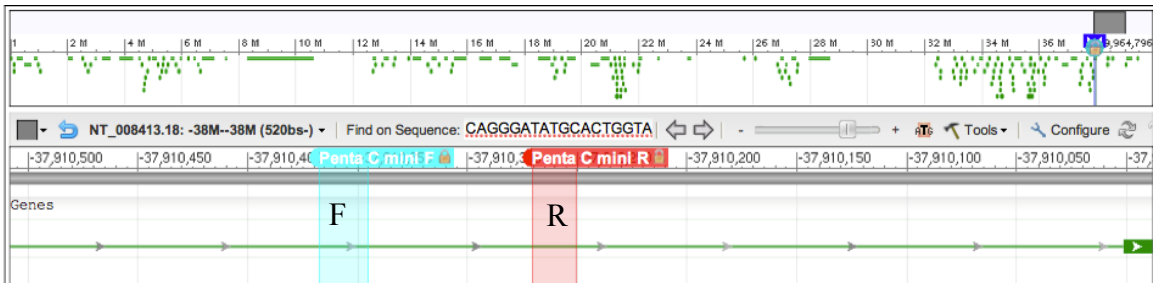


Figure 22: The position of Penta C STR marker on chromosome 7. The small green dots along the top of the figure represent the position of genes (coding region) on that chromosome. The light blue selection (F) represents the forward primer while the red selection (R) represents the reverse primer region.

The mini-Penta C sequence on the antisense strand near the repeat target region is shown below. Penta C contains a $[GTTTT]_n$ repeat motif.

AAGTACCTACAGTTCAGGAAGGCATGCGTGTCCTCAGTGCCACCAGAT
GAAGGAAGGAGCTCTGCCTGTGGGAGCCTGGAAAACCACCCAGCAGA
GGCAACACAGGCTGGACCTTGCAGGAGACAGGGTTTATAAAGAAGGG
CAGGTAGTACAGACTTTCTGCCCACCTTCCAGTGGCTAAGCCAAGTCA
CATGGCTAACTGCCTGCAAGGGAGGCTGGGAAATGTAGTCCCTGGCTG
GGTGGCCCCTTCCCAGTGACAGTTTCCTACTACTGAAAGGGAAGGACA
GATCACTTGCCATCCCTGCCACACAGTTTCCTCCTCTGGAAACTGGGGG
TGATGACCCCTGCCCTACCCACTTGTCATGGCATTGGGGACATGAACA
CACTTTGCACCTGTCAGGCAAGGCTTAAA*CAGGGATATGCACTGGTAATA*
*GA*AAAGAGGGACTAA**GTTTT**GTTTT**GTTTT**GTTTT**GTTTT**GTTTTGTTT
TGTTTTGTTTTGTTTTGTTTTGTTTTT*CTGAAGAAGTCCCTAGAAGCG*CTC
AGTGTTGGAATGCTCTCTTGTAGCAGTGGCGGCTGCTGCTGGTTCCGGG

TCAGATGCCGGAATTGGGGGTGCGCTTGGGTGCAGCTGCATTTCATCT
GGTCCTGGGCCTCGGTCCTGGCTTGGAGAGGTGCAGCTCACAGCCACT
TCATGGCTGGGATCCCTTCTGTCCCAGACAGCTGAGGAGACCCTTGGC
CTCAGCCTGAGTGTCAGGGGGGTAGTTCTGATAACTCTGTGTTTTGTTC
ACAGGAGCAACCAGGGTTTTATGCACATGAAACTGGCCAAAACCAAA
GAGAAATACGTTCTGGGTCAGAACAGCCCTCCGTTCGACAGTGTCCCG
GAAGTCATCCACTACTACACCACCAGAAAGCTACCCATCAAAGGGGCT
GAGCACTTGTCCCTCCTCTATCCCGTGGCTGTGAGGACCCTGTGAGCGG
ACCAGACCTGCCCTGCTCTGTGACAGAGCCTGAGACTTGGAGGTGCCA
GAGGCCCCCCACCAACCAGCCCAGCCACTG

The positions of the forward and reverse primers shown in yellow (italics) were chosen based on the selection criteria used in the primer design software. The start and end position of the designed mini-pentameric primers along the chromosome sequence are shown in Table 4.

Table 4: Penta C mini primer sequences start and end location on the chromosome 9.

	Penta C_m F	**Penta C_m R**
Accession No.	NT_008413.18	NT_008413.18
Start Location	37910346	37910251
End Location	37910368	37910271
Start Sequence	GTCCCTCTTTCTATTACCAG	CAACACTGAGCGCTTCTAGGG

3) *Penta D Marker Information*

The Penta D marker located on the long arm of human chromosome 21 shown in Figure 23 is not a new marker and has been previously described by Promega Corporation and used in their PowerPlex 16 STR kit. A reduced size Penta D marker has also been developed for use on degraded samples demonstrated by McCord et al.[76]

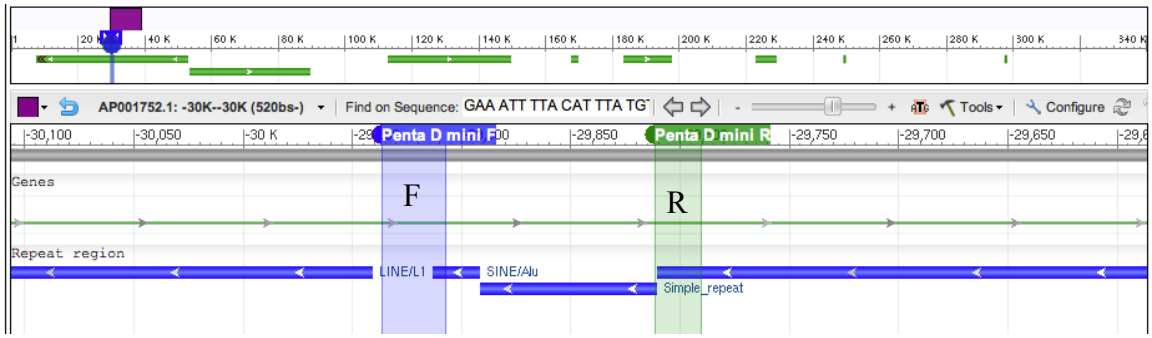


Figure 23: The position of Penta D STR marker on chromosome 21. The green lines along the top of the figure represent the position of genes (coding region) on that chromosome. The blue selection (F) represents the forward primer while the green selection (R) represents the reverse primer region.

Approximately 300 bases on either side of the mini-Penta D STR repeat is shown from the reference sequence located on the GenBank online database. Penta D marker contains a $[AAAGA]_n$ repeat motif.

AGGCTGAGGCAGGAGAATCGCTTGAACCCAGGAGGGGGCGACTGCAG
TGAGCCGAGATCGTGCCACTGCACTCCAGCCTGGGTGACAGAGCGAGA
CTCCATCTCAAAAAAAAAAAAAAAAAAACAGAATCATAGGCCAGGCA
CAGTGGCTAATTGTACCTTGGGAGGCTGAGACGGGAGGATCGAGACCA
TCCTGGGCACCATAGTGAGACCCCATCTCTACAAAAAAAAAAAAAAAT
TTTTTTTAAATAGCCAGGCATGGTGAGGCTGAAGTAGGATCACTTGAG
CCTGGAAGGTCGAAGCTGAAGTGAGCCATGATCACACCACTACACTCC
AGCCTAGGTGACA*GAGCAAGACACCATCTCAAGAA*AGAAAAAAAAG**AAA**
GAAAAGA**AAAGA**AAAGA**AAAGA**AAAGAAAAGAAAAGAAAAGAAAA
GAAAAGAAAAGAAAAGAAAAACGAAGGGGAAAAAA*AGAGAATCATA*
*AACATAAATGTAAAATTTC*TCAAAAAAATCGTTATGACCATAGGTTAGGC
AAATATTTCTTAGATATCACAAAATCATGACCTATTAAAAAATAATAA
TAAAGTAAGTTTCATCAAAACTTAAAAGTTCTACTCTTCAAAAGATACC
TTATAAAGAAAGTAAAAAGACACGCCACAGGCTAAGAGAAAGTACTT
CTAATCACATATCTAAAAAAGGACTTGTGTCCAGATTAAAGAATTCTT
ACACATCAATAAGACAACCCAATTAAAAATGGGCAAAAGATTTGAAG
AGATATTTAACCAAAGAAAACATATAAATGTGTCCGGGCGCGATGGTA
ATCCCAGCACTTTGAGAGGCCGAGGCAGGCGGATCACTTGAGG

The position of the forward and reverse primers are shown in yellow (italics) and no design was needed because of the already reduced amplicon size. The start and end position of the mini-pentameric primer along the chromosome are shown in Table 5.

Table 5: Penta D mini primer sequences start and end location on the chromosome 21.

	Penta D_m F	**Penta D_m R**
Accession No.	AP001752.1	AP001752.1
Start Location	29791	29908
End Location	29812	29937
Start Sequence	CTAGGTGACAGAGCAAGACA	GGGGAAAAAAGAGAATCATA

4) Penta E Marker Information

The last marker examined was Penta E. This marker is located approximately 26.2K along the long arm of Human Chromosome 15 (15q26.2) shown in Figure 24. The Penta E marker is used in the Promega Corporation PowerPlex16 STR kit. However, the amplicon size ranges are large and not suitable for this application. The Penta E was redesigned to give the smallest possible amplicons.

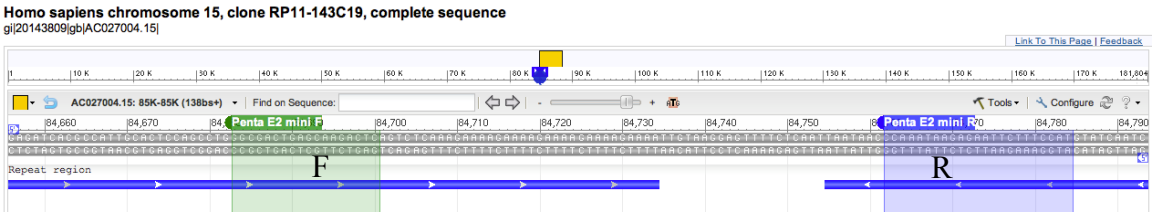


Figure 24: The position of Penta E STR marker on chromosome 15. The new forward and reverse primer region is shown in green(F) and blue (R) respectively.

The mini-Penta E sequence from the antisense strand near the repeat target region is shown below. Penta E contains a $[AAAGA]_n$ repeat motif. The position of the

redesigned forward and reverse primers to produce the smallest possible amplicon is shown in yellow (italics). The start and end position of the mini-pentameric primer along the chromosome sequence is shown in Table 6.

AGATCAAGACCAGCCTGGGCAACATGGTGAAACCCCGTCTCTACTAAA
ATACAAAAAATTAGCTGGGTGTGGTGGTAGGCACCTGTAATCCCAGCT
ACTCTGGAGGCTGAAACAGGAGAATCACTTGAACCCAGGAGGTGGAG
ATTGAAGTGAGCCGAGATCACGCCATTGCACTCCAGCCTG*GGCGACTG*
*AGCAAGACTC*AGTCTC**AAAGA**AAAGA**AAAGA**AAAGA**AAAGA**AAATTG
TAAGGAGTTTTCTCAATTAATAAC*CCAAATAAGAGAATTCTTTCCAT*GTAT
CAATCATGATACTAAGCACTTTACACACATGTATGTTATGTAATCATTA
TATCATGCATGCAAGGTAATGAGTATTATTTTCCTCATTTTATAAAAGA
GGAAACTGATGTTT

Table 6: Penta E2 mini primer location

	Penta E_m F	**Penta E_m R**
Accession No.	AC027004.15	AC027004.15
Start Location	84683	84762
End Location	84700	84784
Start Sequence	CTCCAGCCTGGGCGACTGAGC	AATTAATAACCCAAATAAGAG

The Web-based Primer3 + plus interface program was used to design PCR amplicons for the three pentameric STR markers Penta B, Penta C and Penta E.[89] A reduced sized Penta D marker was designed previously and therefore primer design was not needed.[76] The three new markers were designed such that the amplification region included the core pentameric nucleotide repeat without any excess flanking regions. It was important that the primers be as close to the repeat or target region, as to ensure that

the smallest possible amplicon would be generated from the PCR product because of the resolution limitations of the microchip system. The smaller the amplicon size, the faster the mobility of the DNA, thus limiting diffusion effects and increasing separation efficiency.

Once the target sequence was entered into the software, a few main primer parameters were adjusted so that the software would select an appropriate match. The primer length, annealing temperature, amplicon size and GC% were used to find a primer pair that would produce a specific amplicon with low chance of the formation of secondary structures. (Figure 25) The design process was repeated for all markers and the primers ordered from Integrated DNA Technologies (Coralville, Iowa) and tested. Figure 26 shows the results for one primer pair match of the Penta B loci using the Primer3 Plus software. The software reports the primer length, annealing temperature, GC%, amplicon product size and rating of the formation of primer-primer dimers or self-dimers.

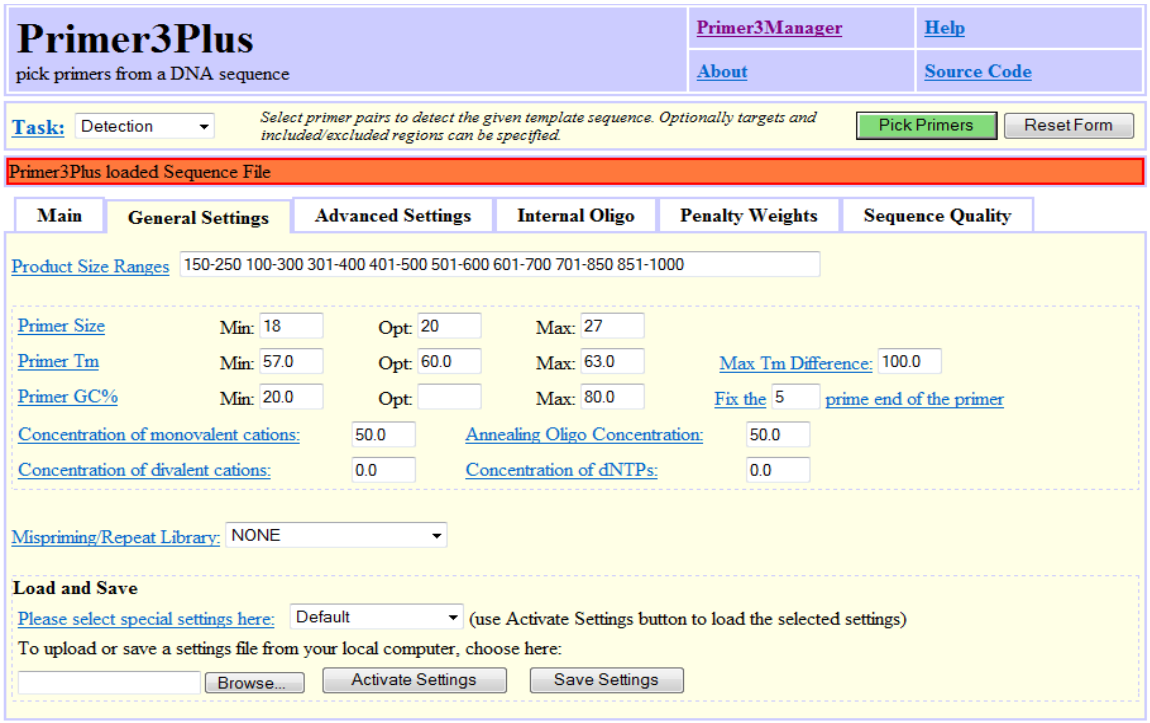


Figure 25: Primer3Plus parameter control settings for selection of primers. Primer length selection range between 18-27 bases, Primer annealing temperature of 60°C and >50 GC%. Parameters may be varied to improve primer pair matches to desired product.[89]

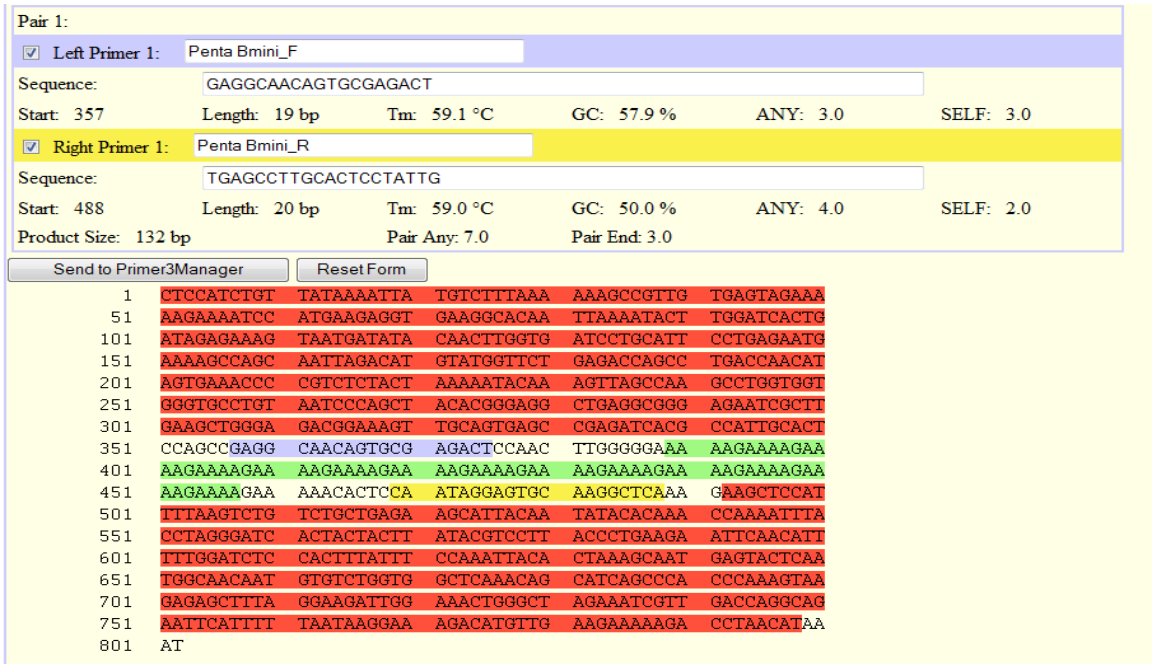


Figure 26: Primer 3 Plus Penta B mini primer match output view. The repeat unit is highlighted in green, forward primer in purple and reverse primer in yellow. Forward primer length 19bp, Tm 59.1°C, GC% 57.9, primer-primer score 3, self-dimer score 3. Reverse primer length 20bp, Tm 59°C, GC% 59, primer-primer score 4, self-dimer score 2.[89]

ii) Individual amplification of Pentameric STR Loci

The markers were amplified using each of newly designed primers under a range of conditions to achieve the highest sensitivity and specificity. The products were run on an agarose gel and visualized using ethidium bromide under a UV light. This was to ensure that the correct amplicon was being produced and without non-specific products. A gradient PCR was used to determine the best annealing temperature for each locus. The agarose gel in Figure 27 shows the optimized temperature PCR products for each of the pentameric loci. The newly designed mini-Penta E consistently showed non-specific products over a wide range of parameters and was determined that the primer pair was not desgined properly and discarded from the research.

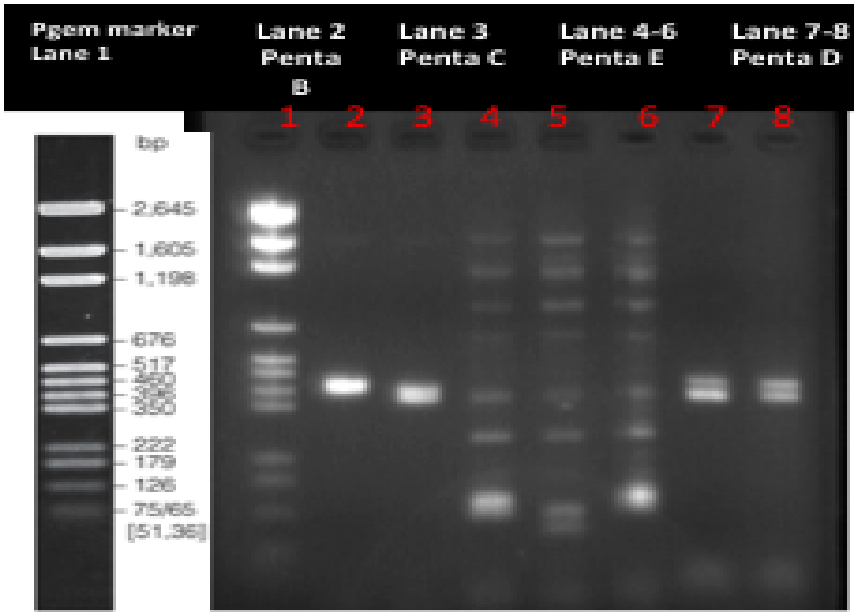


Figure 27: 2% Agarose gel with ethidium bromide showing the products amplified using the mini pentameric primers. Lane 1 contains a size ladder, Lane 2 Penta B mini, Lane 3 Penta C mini, Lane 4-6 Penta E mini at different annealing temperatures, Lane 7-8 Penta D mini duplicate.

Once the amplification showed highly specific and robust products on the agarose gel, new forward primers were ordered with 5' fluorescent tags. Penta E mini was no longer used and only three pentameric markers were examined. Penta B-mini was labeled using 6FAM dye, Penta C-mini with HEX and Penta D-mini with NED dye. Each marker was amplified and run on the ABI 310 to ensure the selectivity, sufficient resolution and sizing of the STR fragments. Figure 28 shows the results of the individual PCR amplification of each Penta locus.

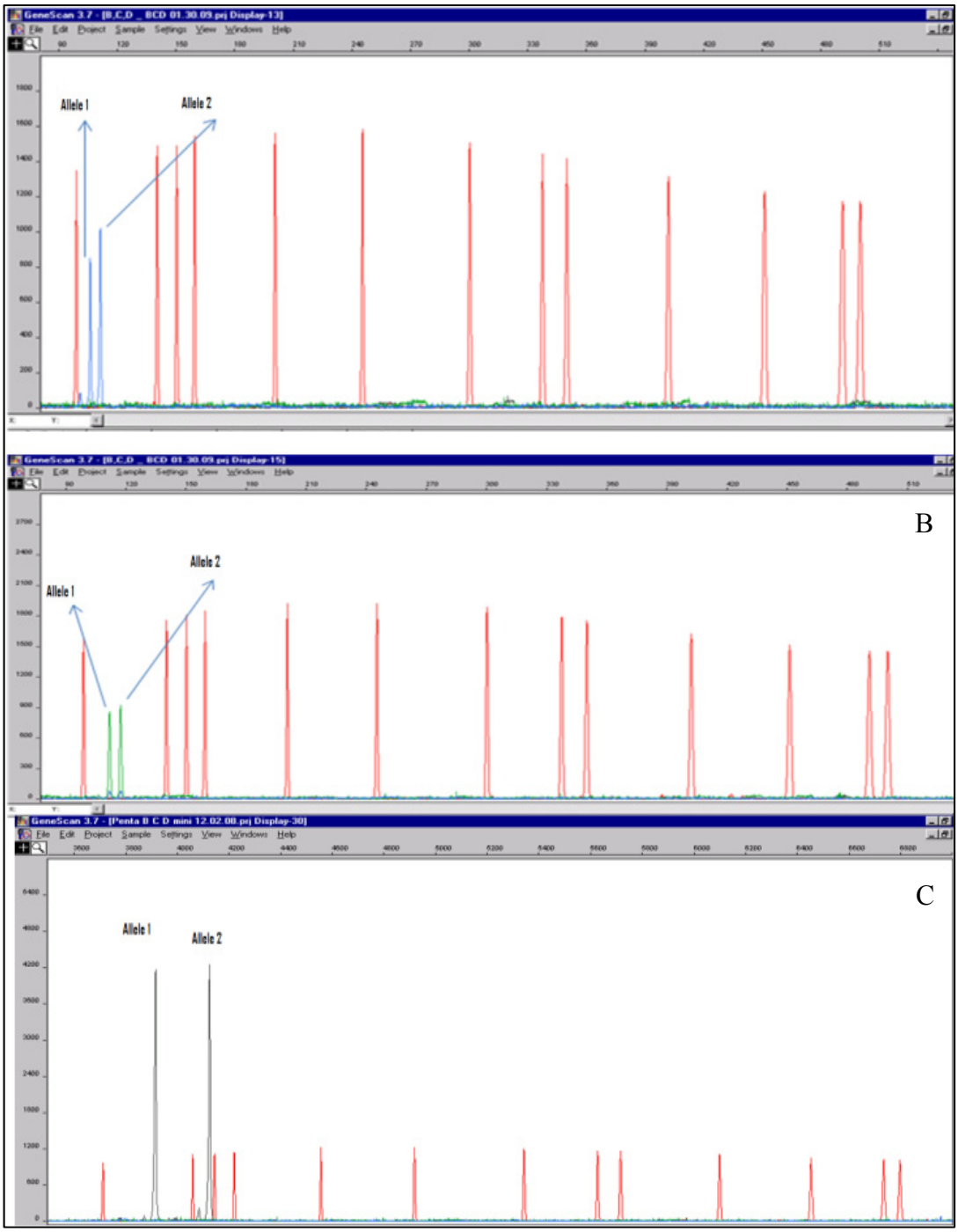


Figure 28: ABI 310 electropherogram of Individual mini Penta B, Penta C and Penta D loci with 9948 DNA standard using 0.5ng template @ 61˚C. The blue channel (A) or Bm labeled marker (6-FAM) shows the alleles present along with a GSROX-500 internal Standard. The Green channel (B) shows the Cm labeled marker (Hex) and the Yellow channel (C) shows the Dm labeled marker (NED).

iii) STR Multiplex Optimization

The separations performed in this research took place on a standard ABI 310 Genetic Analyzer with a 47 cm capillary and on an Agilent Bioanalyzer 2100, a commercially available DNA microchip system with a 1.5 cm separation channel. In its standard native DNA separation mode, the Agilent system provides resolution in the range of 6-12 bp depending on the DNA fragment size. [132, 159] While there have been a number of reports for the applications of STR separations on short channel microchips, the level of resolution obtained was insufficient for forensic DNA typing which contain 4 bp STR repeats, especially given the fact than many CODIS loci have common 2 bp variants.[83, 160] For this reason, the research focused on the use of pentameric STRs. To achieve maximal resolution of these loci, a new beta version of the Agilent 2100 chip system was tested with an on-board chip heater that permitted the use of denaturing buffers for enhanced resolution. It also should be noted that the detection system for this instrument is presently single channel, so multiplex detection based on different fluorescent dye labels is not possible. However, the chip system does provide a reliable platform to test assumptions about effective resolution and throughput of short channel microfluidic chips. The ability to amplify the three-pentameric markers simultaneously in a multiplex was demonstrated on the ABI 310 capillary electrophoresis system.

In order to optimize the pentameric STRs amplification conditions to ensure selectivity, reproducibility and balance, some parameters were examined, including, annealing temperature (55-65 °C) and primer concentrations (0.5µmol/L to 1.5µmol/L). On the basis of the theoretical primer melting temperature, it was determined that the best annealing temperature for the multiplex system was between 60-62°C. These conditions

minimized non-specific amplification and –A peaks. For the primer concentrations, the goal was to optimize sensitivity and peak balance. The results are shown in Figure 29. Experiments were performed examining the overall primer concentrations as well as individual concentrations within the different loci. The optimum and consistent multiplex conditions were observed at an annealing temperature of 61°C and final primer concentration of 1.5µM.

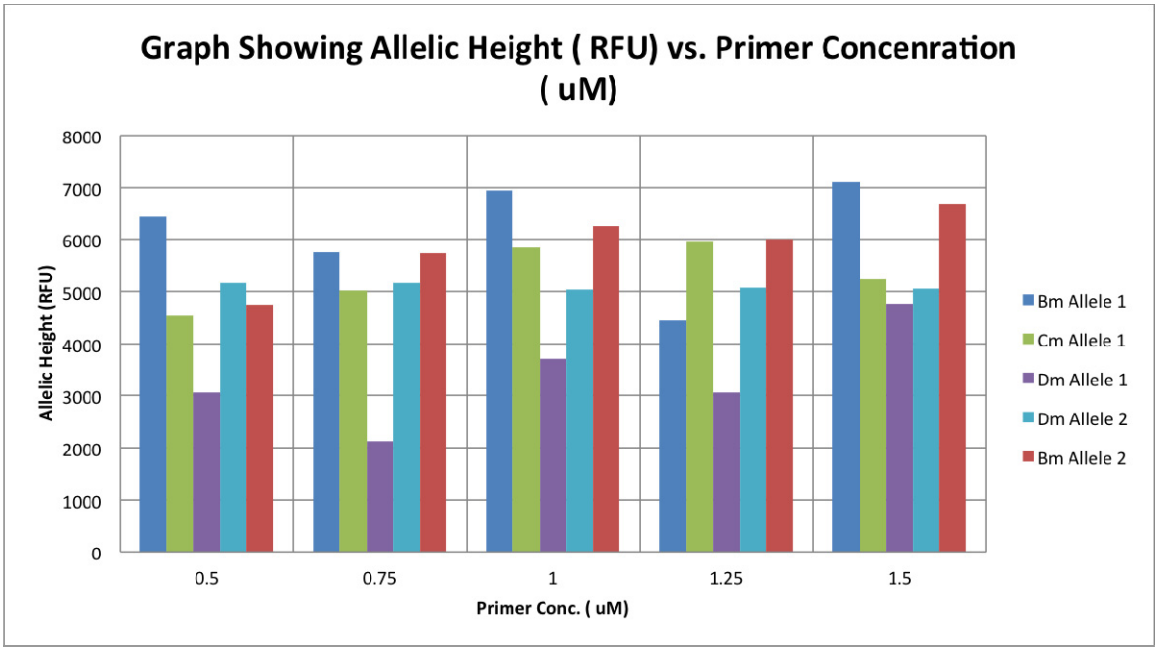


Figure 29: Graph of primer optimization showing the allelic height (RFU) vs. the final primer concentration (µM). The primer concentration with the highest peak heights and most balanced allele between all three loci can be seen at 1.5uM. Bm, Cm and Dm correspond to the respective mini-penta Markers.

The summary of the results obtained from the optimization of primer concentration is shown in Table 7. The reproducibility of the allele sizes for Penta B was seen with a standard deviation of ±0.08 bp from the average allele size while Penta C and Penta D had standard deviations values of ±0.15bp and ±0.13bp, respectively. These results were most probably due to small temperature fluctuations and slight mobility

shifts during each run on the ABI Prism®310 Genetic Analyzer. Figure 30 is an example of the multiplexed amplification using the K562 DNA standard under the optimum conditions. To test the validity of these conditions, a small set of 20 individuals was analyzed. The results showed consistent and reproducible amplification with the average stutter values of less than 6% for Penta B and under 1 % for Penta C and Penta D.

Table 7: The optimization of the Primer Concentration of the mini Penta B,C,D multiplex showing the Allele peak sizes, peak heights and stutter.

Locus	Primer Conc. (uM)	Allele 1 size (bp)	Allele 1 (RFU)	Stutter peak 1	Allele 2 size (bp)	Allele 2 (RFU)	Stutter peak 2
Bm	0.5	114.18	6432	407	139.03	4745	600
	0.75	114.24	5760	361	139.01	5732	634
	1	113.79	6933	490	138.9	6262	914
	1.25	113.82	4440	287	138.81	6014	851
	1.5	114.36	7108	416	139.07	6681	779
Cm	0.5	102.04	738	0	117.17	4540	303
	0.75	102.47	1220	0	117.11	5022	495
	1	101.98	922	0	117.9	5842	879
	1.25	101.96	560	0	117.83	5959	560
	1.5	102.84	1255	0	117.25	5236	432
Dm	0.5	90.42	3059	0	118.84	5157	0
	0.75	90.54	2133	0	118.55	5167	0
	1	90.12	3710	0	118.12	5030	0
	1.25	89.67	3055	0	118.06	5078	0
	1.5	90.62	4770	0	118.37	5054	0

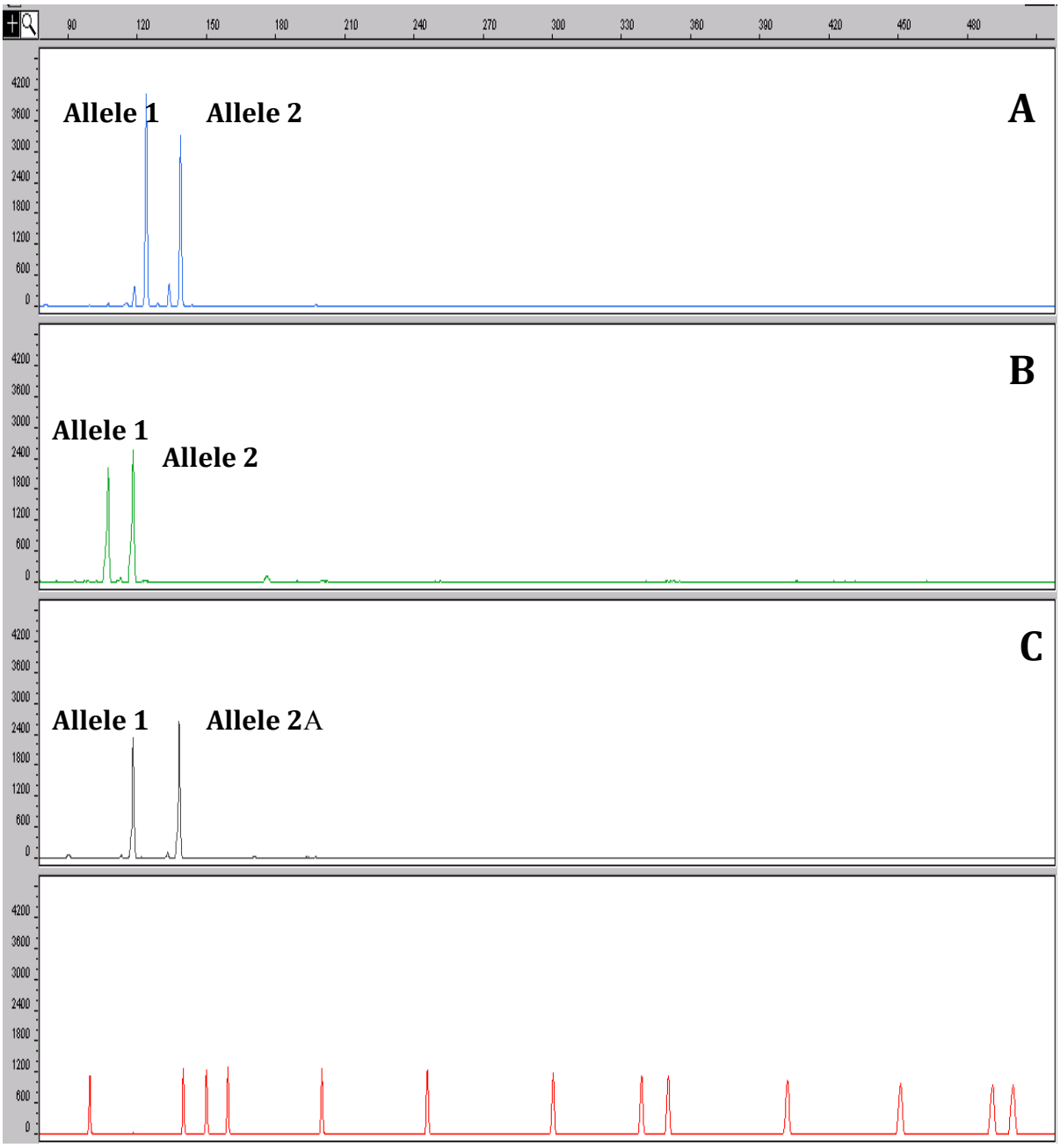


Figure 30: Electropherogram from ABI 310 Genetic Analyzer of 0.5ng template K562 DNA Standard, filter set D, injection 5s @15kV and run temperature of 61°C . Blue lane (A)- Penta Bm Marker Alleles at 124.06 bp and 138.86 bp with RFU of 4393 and 3572 respectively. Green Lane (B)- Penta Cm marker alleles at 107.55 bp and 118.35 bp with RFU of 2428 and 2778 respectively Yellow lane (black, C) - Penta Dm Marker Alleles at 118.35 bp and 138.79 bp with RFU of 2586 and 2859 respectively.

iv) Analysis by Microfluidic Chip

The currently available configuration of the Agilent 2100 Bioanalyzer involves fluorescent detection of dsDNA using an intercalating dye for fluorescence detection. Native dsDNA is inherently more difficult to separate because of its increased rigidity (45 nm persistence length) when compared to single stranded DNA (4 nm persistence length.) The difference in flexibility affects the mobility of the DNA as it passes through the sieving polymer matrix and hence its separation efficiency.[152] The resolution of this dsDNA chip (6-8 bp) is limited mainly to applications in which agarose gels are commonly used. Given this fact, it is very difficult to separate the 4 bp short tandem repeats commonly used in forensic DNA typing on these devices. However, separation of pentameric repeats was thought to be possible. Figure 31 illustrates the separation of the Penta D and the mini-Penta D marker under these conditions. The results demonstrate resolution of 12 base pairs for the large Penta D marker while the mini-penta D marker had a 9 bp resolution. When the larger Penta D marker and the mini-Penta D marker were amplified and run on the Agilent Bioanalyzer chip, there was an average 3 bp improvement in the resolution of the two peaks with the reduced Penta D amplicon. The modified system used a non-denaturing polymer allowing for dsDNA separation with an intercalating dye. The enhanced resolution of the smaller allele set is attributed to the fact that smaller DNA is less affected by orientation in the electric field and the dynamics of the entangled polymer matrix.[161]

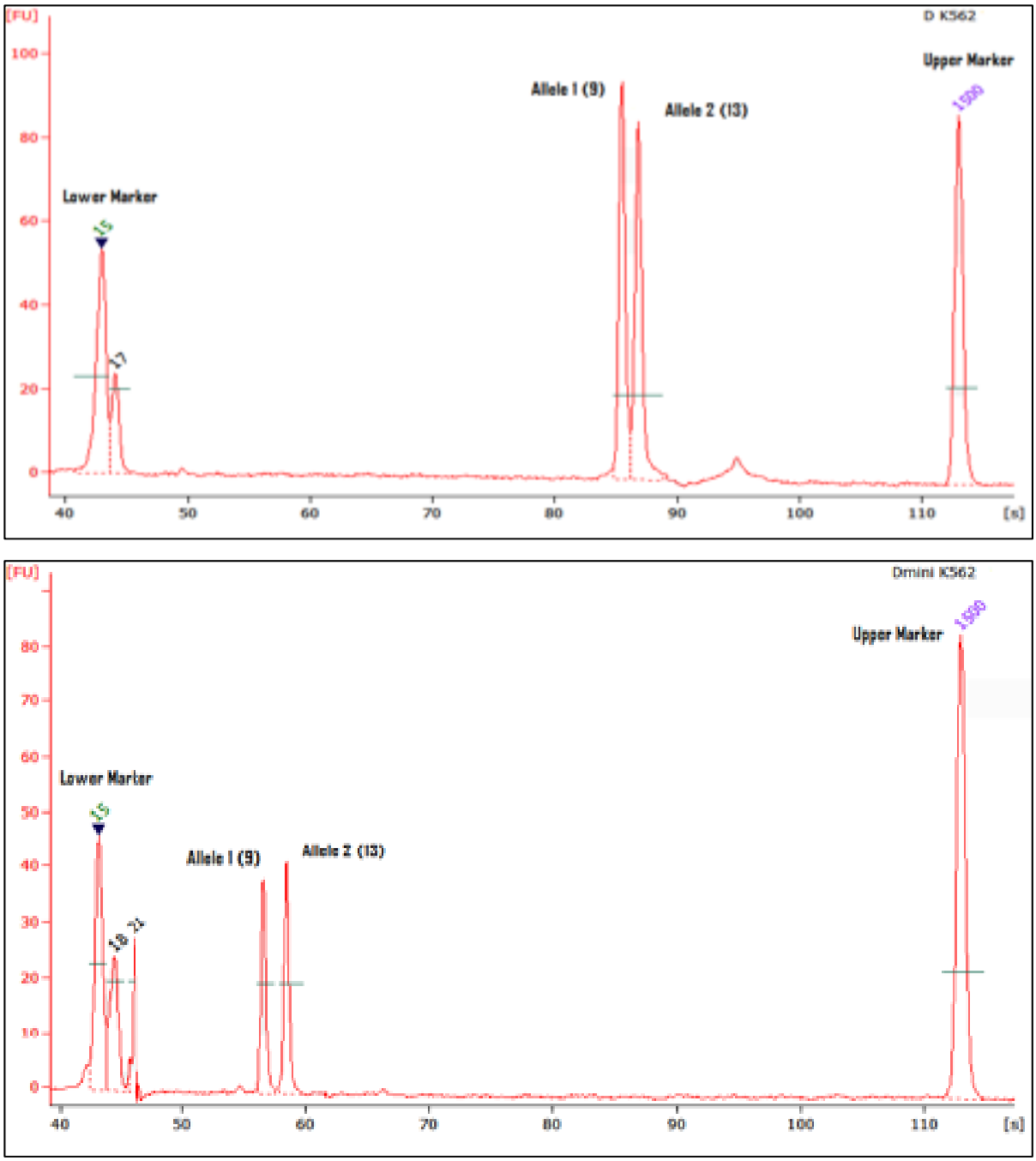


Figure 31: Result from Agilent Bioanalyzer 2100 electropherogram of a K562 DNA standard showing the penta D alleles 9 and 13 on 1.5cm separation channel length run at 350 V/cm. Top panel- Large Penta D marker with K562 standard alleles 9 &13 (20bp apart). Bottom panel - the mini-Penta D marker (Dm) with reduced amplicon sizes and improved resolution.

Although a significant improvement in resolution was achieved by using smaller sized PCR products with the native polymer, the resolution still was not sufficient to fully separate peaks differing by 5 base pairs. To improve overall resolution, the development of a denaturing DNA separation was undertaken using a mixed PVP/HEC polymer with 8M urea.[157] We also began working with a modified Agilent Bioanalyzer 2100 that contained an improved chip design and a heat plate. The added heat plate helped maintain the DNA sample in a denatured state. We then examined the system resolution under these new conditions using both the newly developed polymer and a standard POP-6 polymer obtained from Applied Biosystems. The combined effects of the smaller amplicon size, denaturing polymer and pentameric STR markers allowed for a system that could clearly distinguish adjacent alleles (5 bp) at baseline resolution (Figure 32). The average resolution for each loci of the dsDNA assay for Penta Bm, Cm and Dm were 7.5 ± 0.81 bp, 8.3 ± 0.76 bp and 8.5 ± 0.73 bp respectively while the resolution increased to 2.2 ± 0.74 bp for all three loci using the ssDNA assay. The resolution increased 70% for the ssDNA assay (Figure 33). The reproducibility of the system and sizing precision of the amplicons is shown in Figure 34 with precision of 0.15 bp. The shorter micro-channel also provided much faster separations than capillary systems with an overall run time of less than 2 minutes.

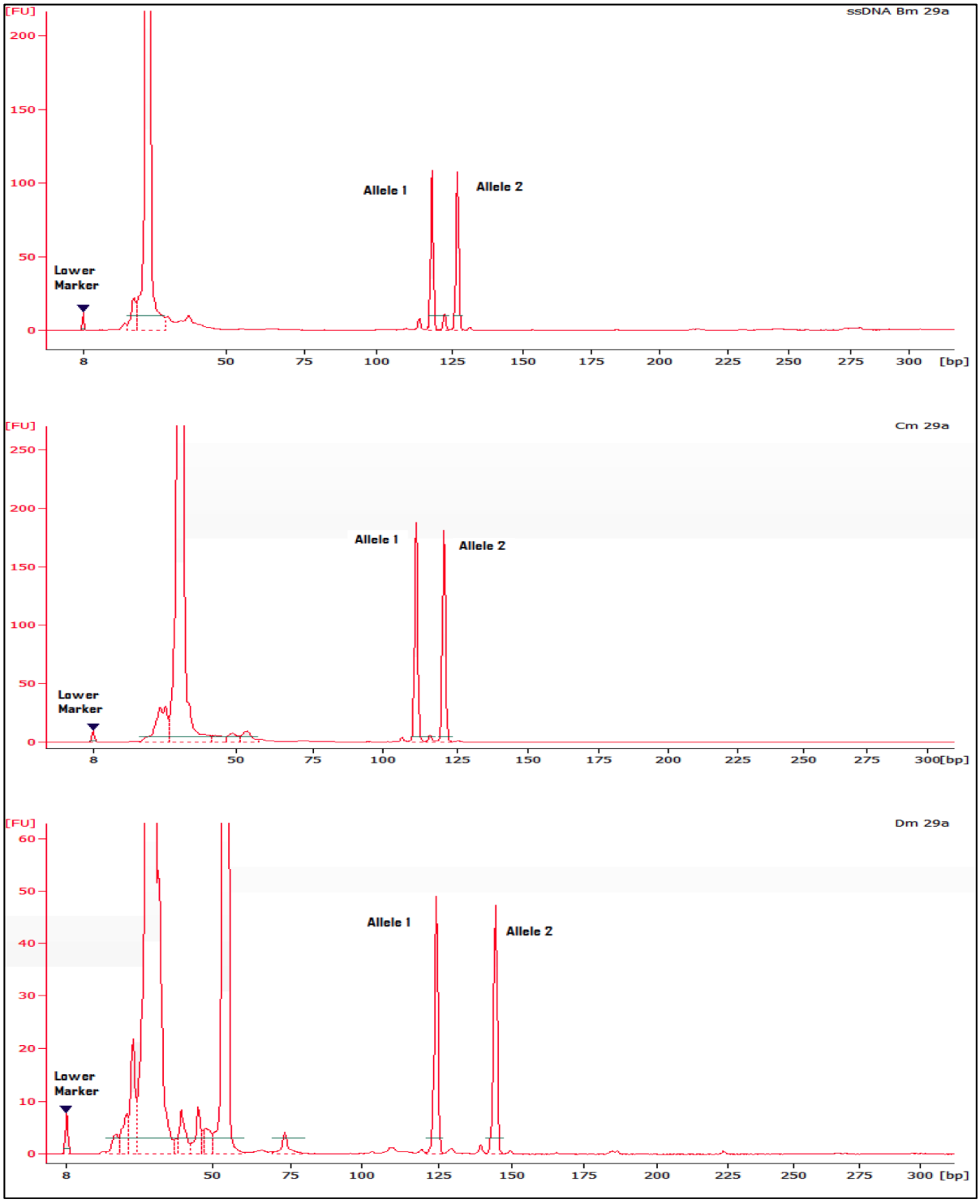


Figure 32: ssDNA assay showing Penta B, C and D Markers of an extracted DNA sample 29. Top- Penta Bm showings alleles at 118bp and 128bp with resolution of 2.2bp. Middle- Penta Cm showing alleles at 111bp and 121bp with resolution of 2.2bp. Bottom – Penta Dm showing alleles at 124bp and 144bp with resolution of 2.2bp.

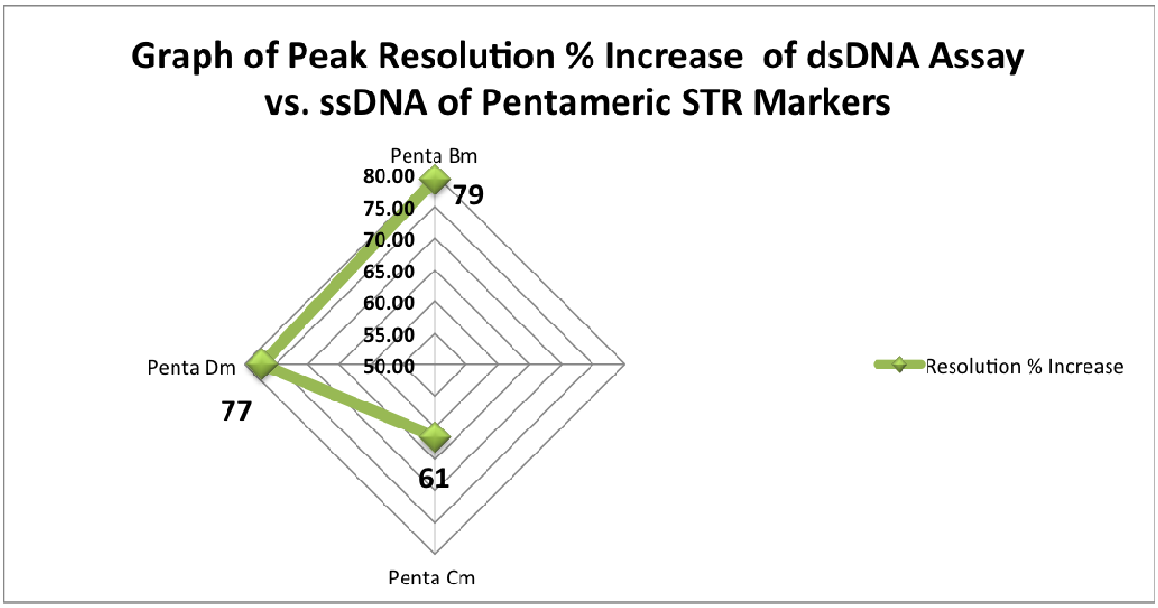


Figure 33: The percent increase in resolution for the single stranded assay when compared with the original native DNA separation. A 79%, 61% and 77% increase in resolution with the mini-Penta B, C and D markers

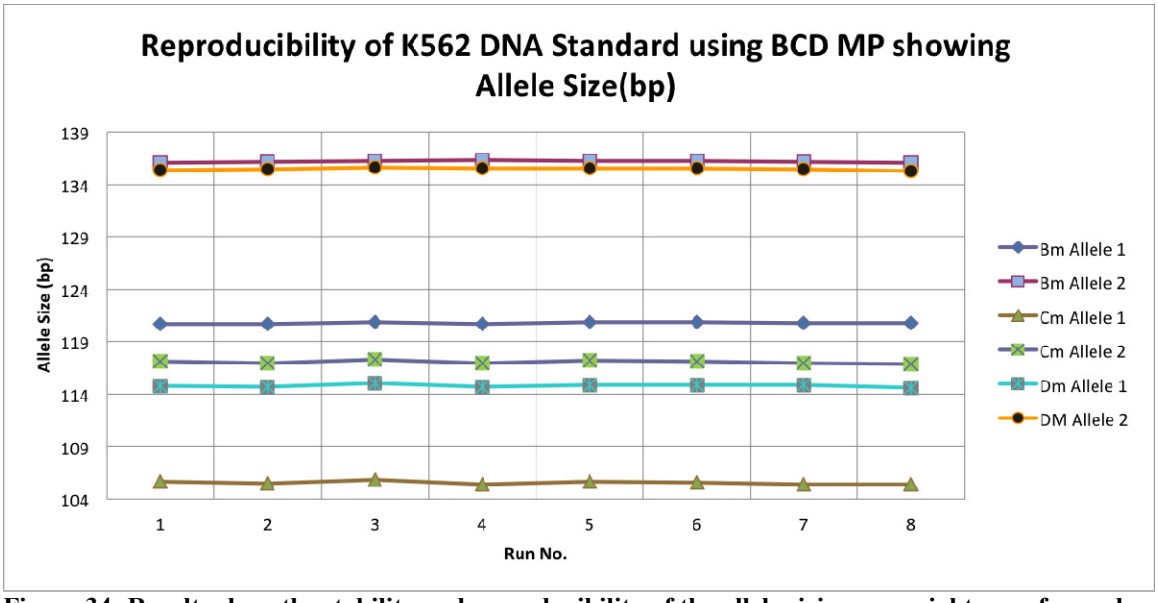


Figure 34: Results show the stability and reproducibility of the allele sizing over eight runs for each pentameric loci with a precision of 0.15bp.

d) Conclusions

It has been shown that by designing pentameric STR markers with smaller amplicon sizes and using a denaturing polymer it is possible to resolve pentameric STR systems on a short channel microfluidic device. The resulting separation can act as a quick onsite-screening tool for forensic DNA analysis. The utility of the method is demonstrated by an overall resolution increase of 70% between loci using the improved sieving matrix. The mobility of ssDNA and the redesigned pentameric repeats permits requisite resolution and provides an advantage of less stutter and fewer variant alleles. This system can be a valuable attribute especially when dealing with mixtures and degraded forensic samples.

CHAPTER VIII: THE DEVELOPMENT OF ENTANGLED SIEVING MATRICES FOR FORENSIC GENOTYPING ON MICROFLUIDIC SYSTEMS

a) Introduction

The purpose of the current project was to develop an entangled polymer solution that would improve the resolution of STRs when used with microfluidic instruments. It was hypothesized that a superior sieving matrix could be created through the optimization of a mixture of Polyvinyl Pyrrolidinone (PVP) and Hydroxyethyl Cellulose (HEC). The mixture of these two polymers provides both enhanced wall coating effects (PVP) and DNA sieving (HEC). By determining the appropriate ratio, concentration, and molecular weight of these two polymers, it was hypothesized that it would be possible to produce an acceptable separation of multiplexed short tandem repeats even on short channel (<2cm) microfluidic devices.

Certain sieving matrices are commercially available, such as the performance-optimized polymers (POP), POP-4, POP-6 and POP-7 by Applied Biosystems. These polymers were designed for sequencing and STR separation on 47cm fused silica capillary systems. The most important parameters associated with an efficient separation matrix are the polymer structure, molecular weight, viscosity and concentration. These parameters along with the separation voltage can greatly affect the resolution, which is, the most important parameter in optimizing DNA separations for microchip electrophoresis.

b) Methods and Materials

i) Reagents and materials

The polymer, hydroxyethyl cellulose, (HEC, 250,000 g/mol) was purchased from Aldrich Chemical (Milwaukee, USA). The second polymer, polyvinyl pyrrolidinone, (PVP, 1,000,000 g/mol) was purchased from Polysciences inc. (Warrington, PA, USA). In addition to those polymers, certified A.C.S Urea (60.06 g/mol) was purchased from Fisher Scientific (Fairlawn, NJ, USA). Amberlite MB-150, a mix-bed ion exchange resin, was purchased from Sigma (St. Louis, MO, USA). A Millex-LS hydrophobic syringe driven filter unit was purchased from Millipore Corporation (Bedford, MA, USA).

ii) Preparation of polymer solutions

In a 50 mL centrifuge tube, 25 mL of deionized water and 17.8 grams of urea was added (7.1 M). After stirring, the urea became dissolved in the water making a solution with a final volume of about 37.5 mL. Next, the two polymer powders were added to produce a final mixture at a specific weight % and ratio. For example, to prepare a 3.5 % solution with 20.4 % PVP relative to HEC, we weighed 0.29 g of PVP and 1.16 g HEC making a total of 1.45 g of the polymer mixture. The compounds were added to an Erlenmeyer flask containing a magnetic stirring bar and the urea solution. It was important to add the HEC/PVP powders slowly to the solutions to prevent the formation of large clumps of polymer. After the powders were added, the solution was allowed to stir overnight to make sure that the entire polymer mixture was dissolved. Then, 0.5 grams of Amberlite MB-150 mixed bed ion exchange resin was added to the polymer/urea solution and stirred for two hours. Ideally, the solution containing the

polymer mixture, urea, and resin was centrifuged at 7000 rpm for 5 minutes. However, it was also possible to let the solution stand in a 50 mL centrifuge tube for about three hours or until all the amberlite particles were deposited at the bottom. Thirty millilitres of the resulting supernatant liquid was then removed from the tube and returned to the Erlenmeyer flask with the stirring bar. 3.33 mL of 10X TAPS buffer was added to the Erlenmeyer flask containing the 30 mL of solution. It was stirred for 15 minutes. Prior to using the polymer solution, 5-micron Millex-LS hydrophobic Syringe tip filter (Millipore Corporation) were used to remove suspended Amberlite particles from the solution.

Table 8: Calculation values for development of Polymer depending on concentration (w/v%) and PVP relative to HEC ratio.

HEC Ratio	PVP Ratio	% Polymer Weight	Amount of HEC	Amount of PVP	% Required	Total polymer weight
0	100	1.0357	0	1.0357	3	1.24284
10	90		0.10357	0.93213	3.5	1.44998
20	80		0.20714	0.82856	4	1.65712
30	70		0.31071	0.72499	4.5	1.86426
40	60		0.41428	0.62142	5	2.0714
50	50		0.51785	0.51785	2.5	1.0357
60	40		0.62142	0.41428	2	0.82856
70	30		0.72499	0.31071		
80	20		0.82856	0.20714		
90	10		0.93213	0.10357		
95	5		0.983915	0.051785		
100	0		1.0357	0		

iii) Determination of the separation performance

Resolution (R) was calculated using the following equation:

Equation 10: Resolution

$$R = \sqrt{2\ln(2)}\,\frac{t_2 - t_1}{w_1 + w_2}$$

Where t and w are migration times and peak widths at half height of the electropherogram peaks 1 and 2, respectively. The chromatographic resolution was then divided by the distance in basepairs between the two peaks to get the resolution in basepairs.

iv) Viscosity Measurements

Viscosity measurements were performed with a falling ball viscometer (Gilmont Instruments, IL, USA). To perform the measurement, 5.0 mL of the polymer solution was placed in the measurement tube and the time for a steel ball to pass through the solution was determined. Each experiment was performed in triplicate and the viscosity (μ) in centipoise calculated from the following equation:

Equation 11: Viscosity

$$\mu = K(\rho_b - \rho_p)t$$

Where K is a constant (3.3), ρ_b and ρ_p are the density of the steel ball (8.02 g/mL) and the density of the polymer respectively, and t, the time (min) required for the steel ball to fall.

c) Results and Discussion

The goal for the present study was to adapt a previously developed entangled polymer matrix for use with a microfluidic chip. The current microchip system, the Agilent Bioanalyzer 2100 (Agilent Technologies) separates DNA fragments using a non-denaturing polymer combined with an intercalating dye to permit fluorescence detection. While the use of a dsDNA polymer separation is adequate for applications where resolution of 8-12bp is sufficient, most STR genotyping applications require resolutions of 2bp or better.

In the forensic DNA field, it is important that the STR alleles are well resolved and the results are as accurate as possible. If the system is not fully capable of resolving each allele it becomes difficult to distinguish the complete genotype. For this reason the composition of the sieving matrix used to perform DNA separations is critical. Most traditional forensic STR kits use denaturing buffers to permit optimum separations. Longer capillaries are also necessary for optimal resolution. Because the Agilent Bioanalyzer 2100 (Agilent Technologies) uses a very short micro-channel that is only 1.5cm long and performing separations with native sieving polymers, it was not possible to use this system to resolve forensic STRs. Thus we under took a project to redesign the system to permit short channel microscale separations.

The increased mobility of ssDNA opposed to dsDNA fragments through a sieving matrix could best be described as a result of the difference in rigidity and ability to move through the pores. The persistence length of dsDNA versus that of ssDNA is 45nm and 4nm respectively. The measure of rigidity makes it more difficult for dsDNA to move through the pores of the polymer and results in poor separation between peaks. These

effects on entangled polymers can be explained by Ogston sieving and Reptation models. The smaller the ssDNA strands, the more easily it moves through the polymer pores, while medium size fragments take a bit longer and large fragments move through the slowest. Therefore, with the right polymer and separation of ssDNA, a high resolution and selectivity can achieve separation even on very short separation channels.

To demonstrate the effect of the resolution that can be achieved on short channel microfluidic devices, three available denaturing sieving matrices were examined. Figure 35 shows a comparison of three denaturing polymers. The first one is a custom 3.5% PVP/HEC polymer that was previously been developed for the longer capillary instruments, the second one is the commercially available POP-6 polymer from Applied Biosystems and the third was a high resolution polymer provided by Agilent Technologies. While all three polymers are denaturing they differ in polymer chemistry and concentration.

The results from the initial study are shown in Figure 36. The 3.5% PVP/HEC denaturing polymer had better resolution than that of POP-6 for smaller size fragments (>300bp) while the POP-6 Polymer resolution was slightly better for large fragments (up to 500bp). The Agilent high-resolution polymer provided better separation at both smaller and larger fragments than the other polymers. We thus became curious about which specific parameters are most important in producing an optimum resolution across a wide range of fragment sizes on the shorter channel microchip system.

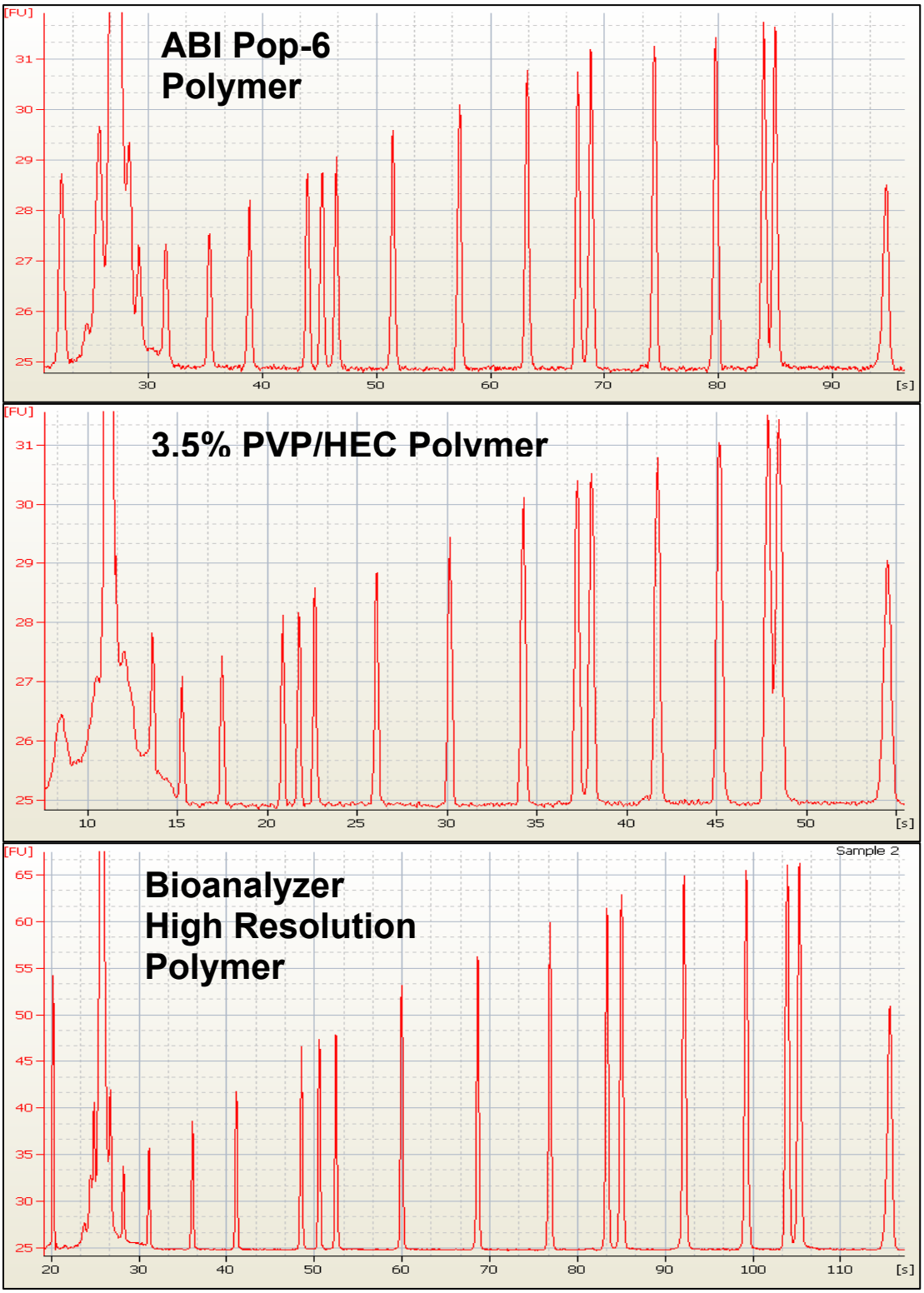


Figure 35: Results from Agilent Bioanalyzer 2100 comparing, ABI POP-6, 3.5% PVP/HEC custom denaturing polymer and Agilent denaturing polymer on a 1.3 cm separation channel length run at 350 V/cm. (Note that time scales (s) vary due to differences in polymer separation time)

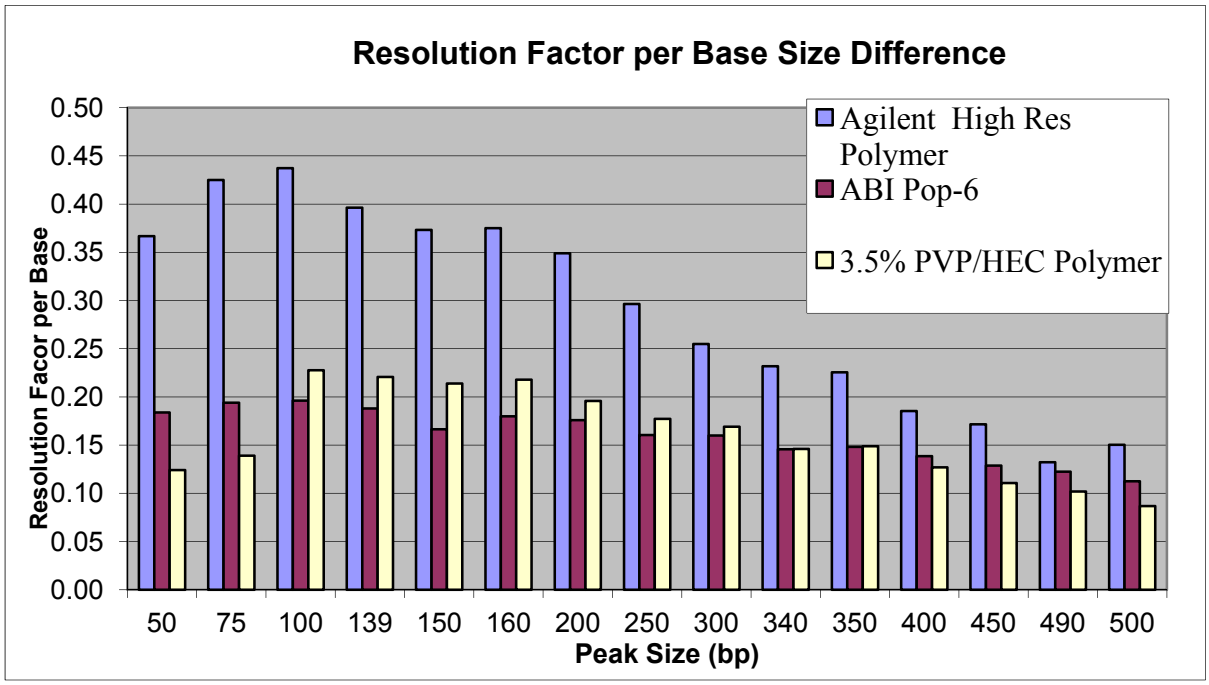


Figure 36: The comparison of three denaturing polymers on a short channel microfluidic system. The resolution was measured across a 500bp range.

i) Optimization of polymer

In order to optimize the sieving matrix for enhanced resolution on short channel microfluidic systems, a second order Doehlert design was developed to reduce the number of experiments needed while modeling the resolution and viscosity as functions of the polymer concentration (w/v) and ratio of PVP relative to HEC.[162] The goal was to achieve a polymer that produced the highest resolution possible over a range of 500bp, while maintaining a viscosity value as low as possible to ensure proper loading on the narrow channel microchips. It was also determined that the substrate of the chip played a critical role in the surface wall interactions, and hence the polymer ratio varied greatly depending on the chip surface, glass or plastic. The effect of the surface interaction is because the composition of the buffer consists of two components, the first PVP, a wall

coating polymer and the second HEC a polymer with a high sieving capability. Hence the use of a Doehlert Matrix model approach, allows for optimization of the polymer on different substrates without ample experimentation.

The range of parameters used in the design of the Doehlert Matrix is shown in Figure 37. The center polymer concentration used was 3.5% w/v and the center ratio of PVP relative to HEC was 50%. The concentration of polymer was increased incrementally by 0.5% w/v to achieve responses over a wide range.

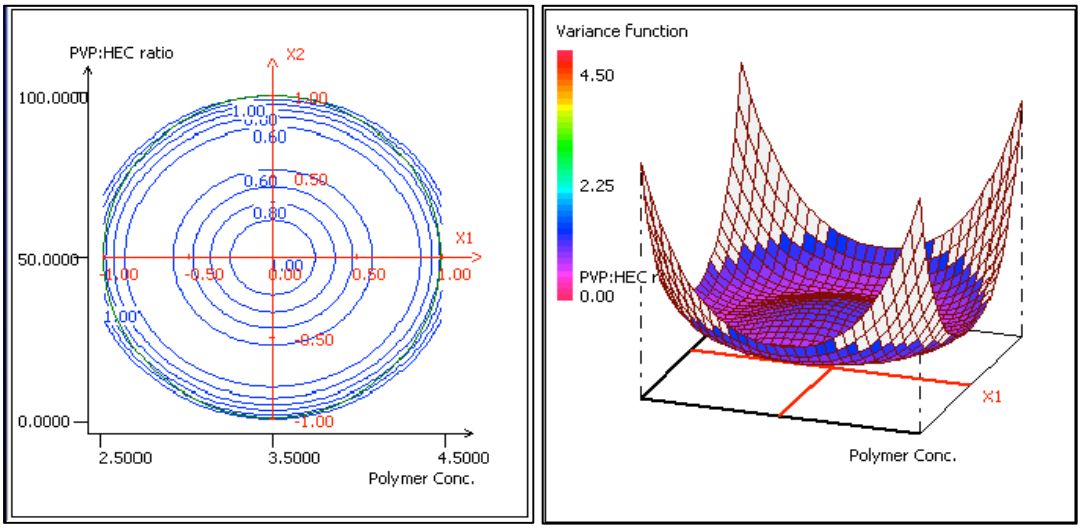


Figure 37: The figure on the left shows the range of PVP relative to HEC on the Y-axis and the range of polymer concentration (w/v%) on the X-axis used for the 2 factor Doehlert matrix. The figure on the right is a 3-dimentional representation.

A total of 7 polymers at different concentrations (%w/v) and PVP/HEC ratios were examined and tested based on the matrix coefficients calculated from the 2-factor Doehlert matrix in Table 9. The relationship between the matrix coefficient value and the actual polymer concentration and ratio made is given by Equation 12:

Equation 12: Doehlert matrix conversion

$$X_i = \frac{\chi_r - \chi_{rc}}{\Delta_{increment}}$$

Where X_i is the coded matrix value of an independent variable, χ_r is the real independent value, χ_{rc} is the center value and $\Delta_{increment}$ is the incremental increase.

The solutions were then tested on the microchip system using GeneScan LIZ 500 fluorescent ladder. The resolutions for the small size fragments (139-150bp) and the larger size fragment (490-500bp) peaks were examined. A quadratic formula was then used to model the response of the resolution, R_S and R_L values against the independent variables.

Table 9: 2-Factor Doehlert Matrix model use to create 7 polymers at different concentrations (w/v%) and ratios (PVP/HEC)

Experiment	Polymer Concentration, X_P	Actual Polymer Concentration	PVP/HEC ratio, X_R	Actual PVP/HEC ratio
1	1	4.5	0	50:50
2	-1	2.5	0	50:50
3	0.5	4	0.8	90:10
4	-0.5	3	-0.8	10:90
5	0.5	4	-0.8	10:90
6	-0.5	3	0.8	90:10
7	0	3.5	0	50:50

The result shown in Figure 38 shows the predicted relationship between the polymer concentration, polymer ratio and its effect on the resolution produced on a short separation channel glass microfluidic system. The model can be used to predict the expected resolution for a wide of range of polymer concentration and ratio on glass substrate microfluidic systems without the need for ample testing and optimization. The

optimum resolution observed from the model was between 20-25% PVP relative to HEC and between a 3.5-4.5% w/v polymer solution on the glass microchip. These values were different when compared to a plastic substrate where 5-10% PVP provided higher resolution separations.

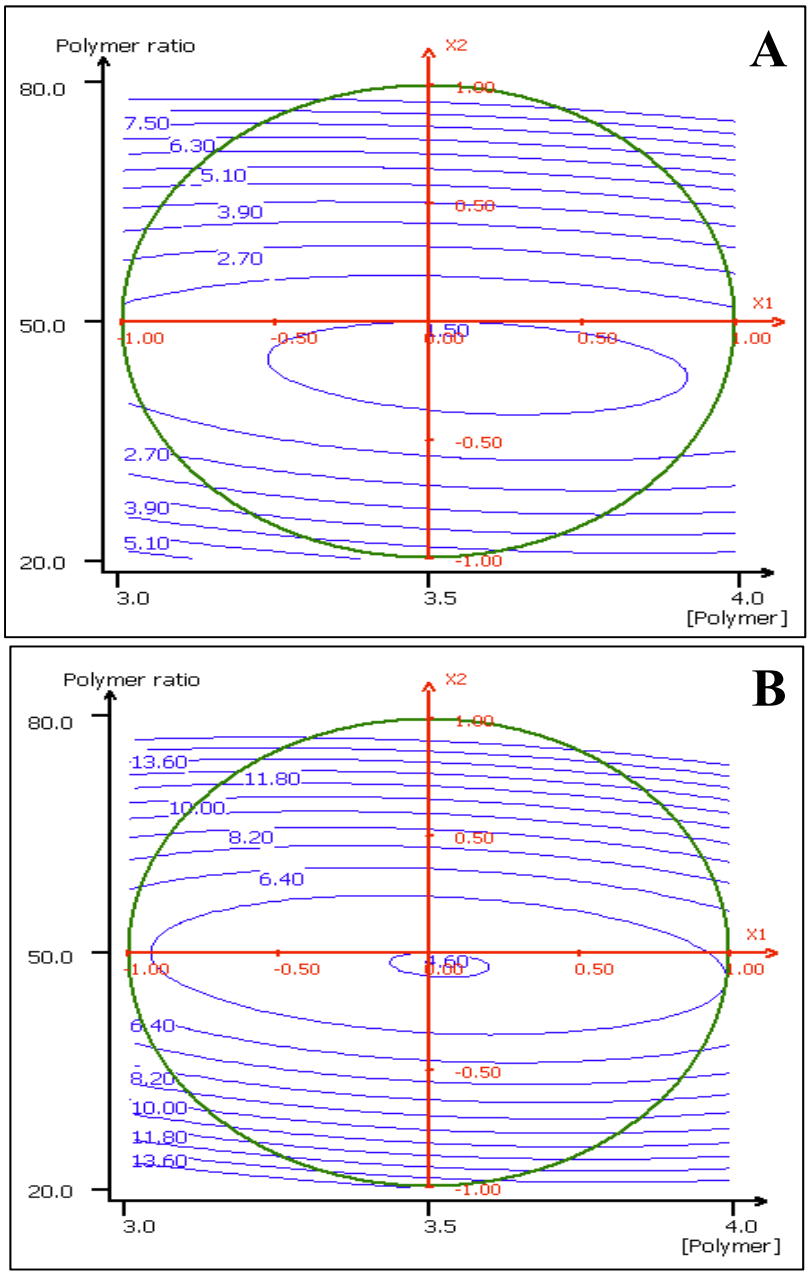


Figure 38: [A] the predicted resolution for small size fragments, R_S with respect to the Polymer Ratio (Y-axis) and Polymer w/v% (X-axis). [B] The predicted resolution for large size fragments, R_L with respect to the Polymer Ratio (Y-axis) and Polymer w/v% (X-axis).

ii) Viscosity study

The viscosity of the polymer solution is critical in applications where the separation channel is very narrow and force must be used to push the polymer into the

capillary or channel. The traditional ABI 310 and Agilent bioanalyzer systems utilize a glass tight syringe or pressure pump to load the polymer. Other microfluidic systems for forensic genotyping such as that developed by Arizona State University (Hurth et al., 2010) and in collaboration with the McCord's research group (Aboud et al., 2010) utilize a reverse vacuum to load the channel with polymer.[121, 163] It is apparent and especially critical for sieving matrices on microfluidic systems to have a low enough viscosity to allow for proper filling of the channels and efficient separation.

The calculated viscosities for the different polymer concentrations and ratio of PVP relative to HEC can be seen in Table 10. The response of polymer concentration and ratio of PVP related to HEC on the viscosity is model by Figure 39. With increasing polymer concentration and ratios of PVP to HEC viscosities of the polymer solution were lower. The optimum viscosity was around 300cP that could be loaded on the microchip with sufficient resolution.

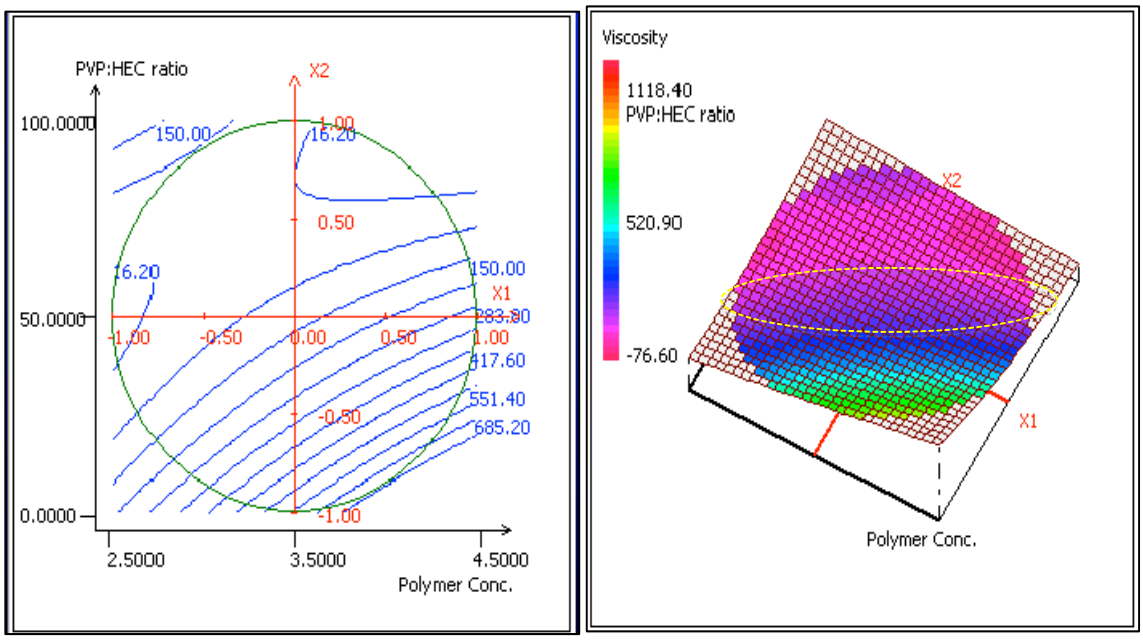


Figure 39: The response factor of viscosity over a range of polymer concentrations and ratios. The lower viscosities are observed at lower w/v % and higher ratio of PVP. The most optimum region of viscosity is between the pink/blue regions shown on the right figure.

While the viscosity of the polymer increased with increasing concentration (w/v%), we used a maximum of a 4% w/v polymer solution for our separations because of the increased back pressure that resulted from the narrow channels. Higher concentrations needed a longer priming time and extra care in filling the channels to avoid the formation of bubbles.

Table 10: Table showing the calculated viscosities from the polymer concentration and ratios used in the Doehlert matrix model.

Solution	Polymer Concentration (%)	Ratio (PVP:HEC)	Weight (1000uL)	Density	Time (min)	Viscosity	Average Viscosity	STDEV
1	2.4	50	1.0455	1.0455	1.934	44.52032585	49.24884928	4.1182
					1.967	52.0498		
					1.934	51.176422		
2	2.9	10	0.8813	0.8813	10.701	252.0792759	255.3551063	4.3667
					10.768	253.6733476		
					11.050	260.3126955		
3		90	1.0815	1.0815	0.683	15.6463175	16.15514083	0.4406
					0.717	16.4095525		
					0.717	16.4095525		
4	3.5	50	0.955	0.955	5.267	122.7897	124.6121168	1.7583
					5.417	126.2985323		
					5.351	124.748118		
5	4	10	0.8605	0.8605	31.633	747.380205	751.8495229	16.2175
					32.584	769.833113		
					31.251	738.3352507		
6		90	1.0507	1.0507	1.434	32.98778769	34.76763076	1.7257
					1.517	34.8813465		
					1.584	36.43375808		
7	4.6	50	0.9564	0.9564	11.235	261.8787318	265.1110352	3.1154
					11.384	265.3596739		
					11.501	268.0946998		

iii) Effect of field strength

The effect of separation voltage on the resolution was determined by analyzing the target 139/150 bp and the 490/500 bp peaks of an internal size standard Genescan Liz 500 (ABI). The measurements were made using a modified Agilent 2100 bioanalyzer with a 1.5 cm separation channel and a heat plate at 60 °C to improve analysis of denatured DNA. The results from the initial study, shown in Figure 40, indicated that an increase in field strength improved the resolution especially at the 490/500 bp peaks. The increase of field strength from 200 V/cm to 330 V/cm resulted in 10- 20 % increase in resolution.

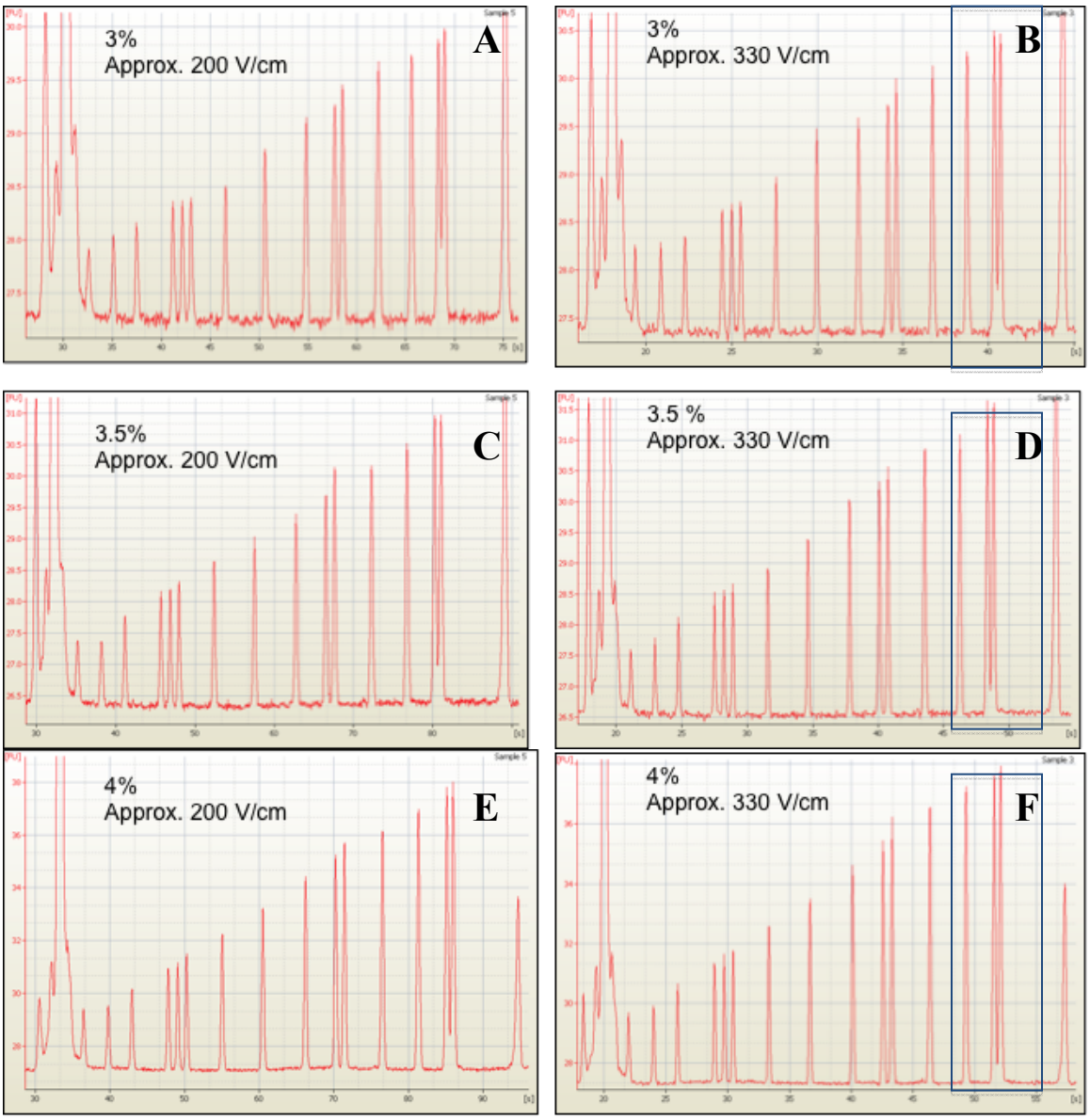


Figure 40: The effect of electric field strength to the separation of GeneScan Liz 500 size standard. An increase in resolution is observed, seen at the 490/500 bp peaks at higher voltages (330V/cm) over B, D & F.

After the results from the initial testing a more detail study was undertaken to characterize the effects of field strength on the resolution of both the small size DNA fragments and larger size fragments to determine the optimum separation voltage. The polymer concentration (w/v) was held constant during these tests. Figure 41 shows the

analyses of a 4.0 % polymer on the Agilent chip performed with a separation voltage of 350 V/cm. The resolution for the adjacent target peaks, 139/150 bp and 490/500 bp were calculated using Equation 10. The chromatographic resolution is converted to base pair resolution by determining the size difference between the two peaks and then dividing by the chromatographic resolution.

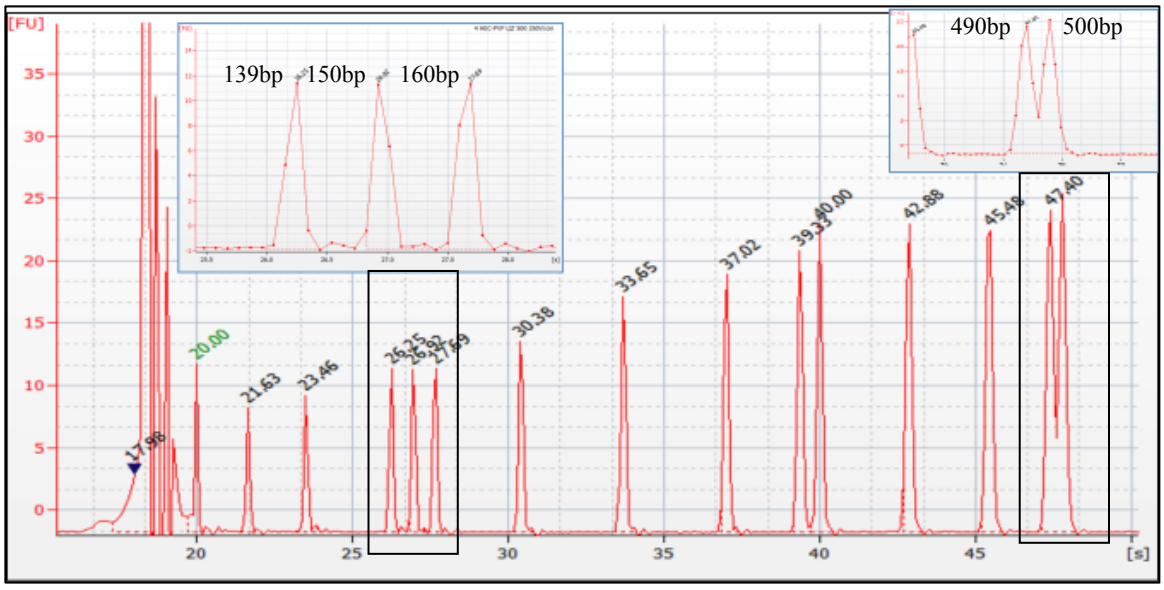


Figure 41: The separation of a LIZ 500 standard diluted 1/5 in formamide and analyzed on a 1.5cm Agilent 2100 microfluidic chip at 60 °C with 4 % HEC/PVP polymer. The separation was performed with electric field strength of 350 V/cm.

The average resolution for the separation performed at 350 V/cm, for the 139/150 bp and 490/500 bp peaks, were 4.62 ± 0.09 bp and 11.48 ± 0.04 bp, respectively. The separation performed at a voltage of 400 V/cm is shown in Figure 42. The resulting resolutions were calculated for the small and large target peaks and determined to be 3.67 ± 0.18 bp and 12.27 ± 0.09 bp respectively.

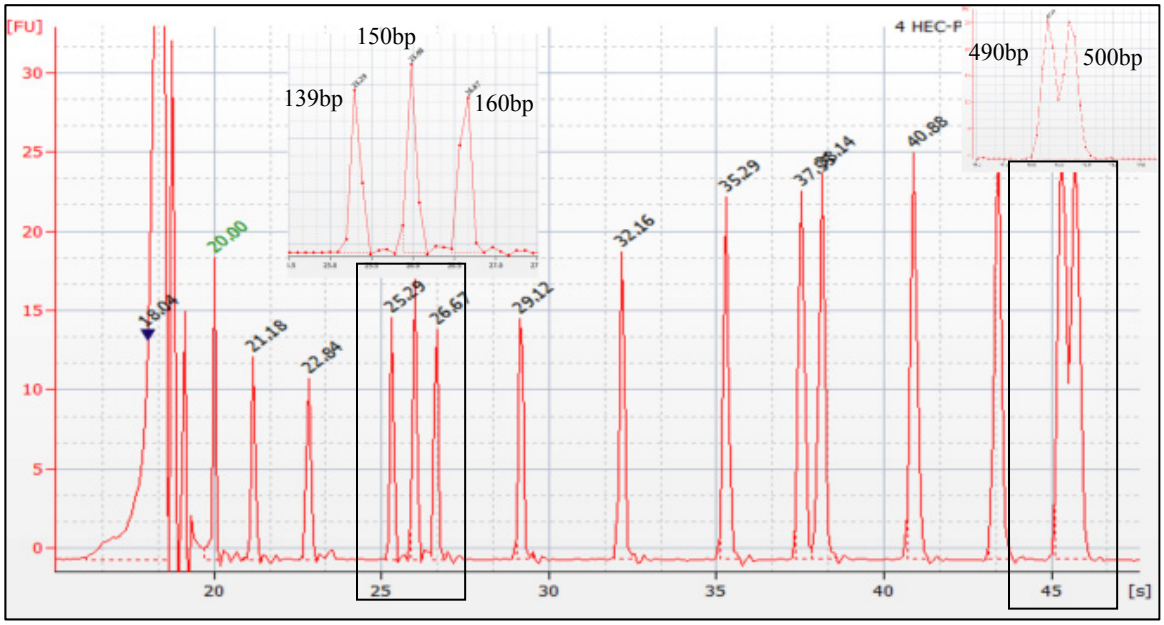


Figure 42: The separation of a LIZ 500 standard diluted 1/5 in formamide and analyzed on a 1.5cm Agilent 2100 microfluidic chip at 60°C with 4% HEC/PVP polymer. The separation was performed with field strength of 400 V/cm.

The electropherogram from the separation performed at 450 V/cm is shown in Figure 43.

The calculated resolution for the target peaks was approximately 5.7 ± 0.06 bp and 11.87 ± 0.15 bp respectively.

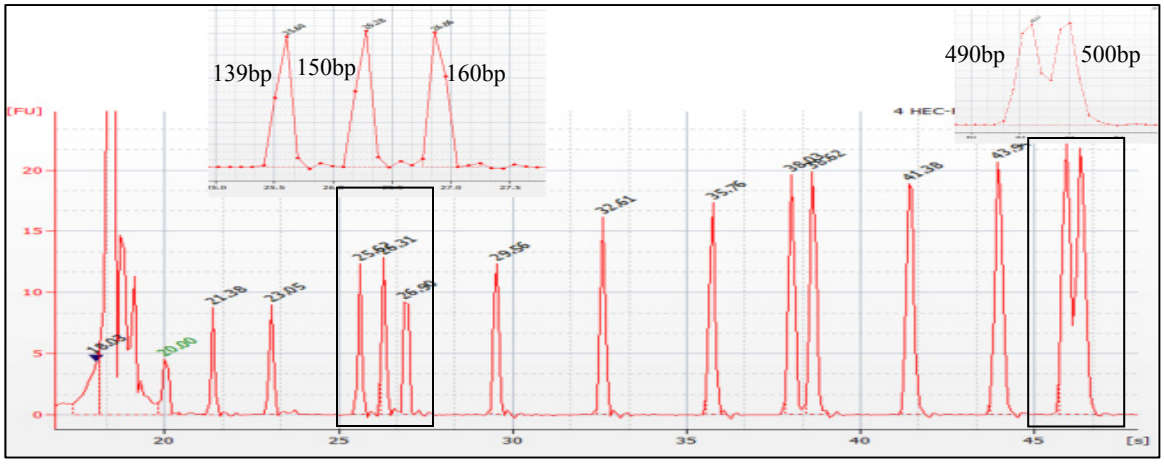


Figure 43: The separation of a LIZ 500 standard diluted 1/5 in formamide and analyzed on a 1.5cm Agilent 2100 microfluidic chip at 60°C with 4% HEC/PVP polymer. The separation was performed with field strength of 450 V/cm.

As the field strength was increased above 450 V/cm, reproducibility and resolution suffered and the separation efficiency was lost. The lost in resolution was likely because of high currents and joule heating effects causing the broadening of peaks, especially at the larger size fragments, which were subjected to the higher field strength over a longer period of time. Figure 44 shows results obtained from the polymer on the Agilent chip at 500 V/cm. The calculated resolution for these peaks was approximately 3.99 ± 0.36 bp and 11.71 ± 0.15 bp, respectively.

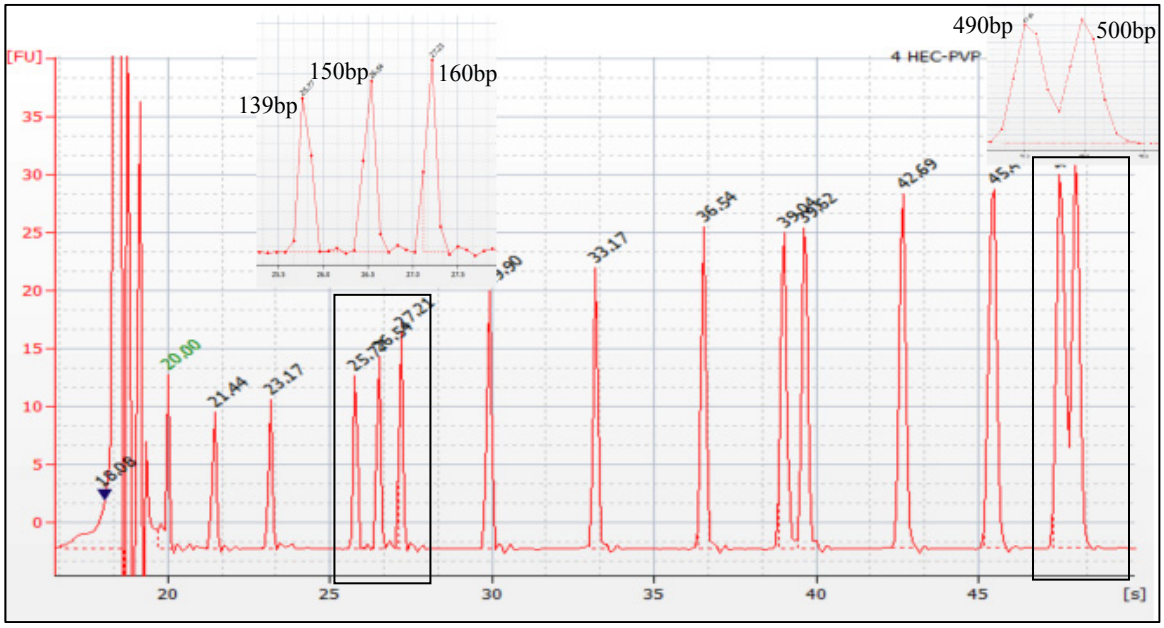


Figure 44: The separation of a LIZ 500 standard diluted 1/5 in formamide and analyzed on a 1.5cm Agilent 2100 microfluidic chip at 60°C with 4% HEC/PVP polymer. The separation was performed with field strength of 500 V/cm.

The summary of the peak resolutions calculated during the separation voltage study is shown in Table 11. It was determined that the optimum separation voltage on the short channel system was 350 V/cm. This was determined based on the higher resolutions

obtained at the larger size fragments. Figure 45 shows the plot of the overall comparison of the separation voltage versus the resolution obtained for the 139/150bp and 490/500bp peaks.

Table 11: Summary of peak resolution over a separation voltage range of 350-500V/cm.

Voltage	Avg. $R_{139/150bp}$	STDEV	Avg $R_{490/500bp}$	STDEV
350.00	4.62	0.09	11.48	0.04
400.00	3.67	0.18	12.27	0.09
450.00	5.70	0.06	11.87	0.15
500.00	3.99	0.36	11.71	0.15

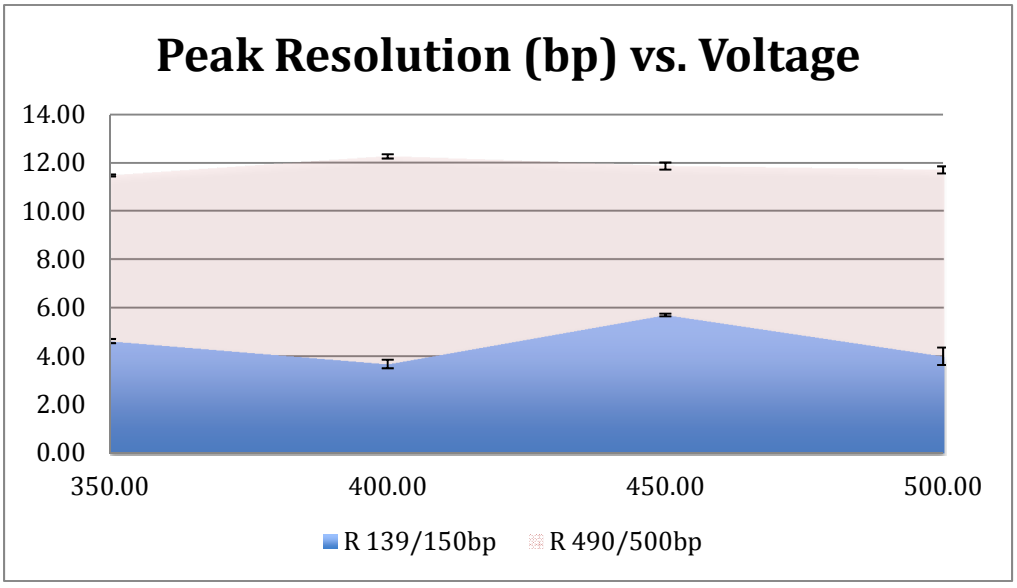


Figure 45: The 139/150bp and the 490/500bp peak resolution versus the separation voltage.

iv) Effect of PVP relative to HEC

Polyvinyl Pyrrolidinone (PVP) has been shown to be an effective polymer in coating the surface walls of fused silica capillaries by forming hydrogen bonds with its

carbonyl groups and the capillary wall.[164, 165] On the other hand, it is less effective at separating DNA when compared to other polymers such as Hydroxyethyl cellulose (HEC) or poly-methyldiacrylate PDMA. For this reason, combining PVP with HEC allows the user to take advantage of distinctive properties of both polymers to produce a polymer solution with high resolution and surface coating.[162] When examining a new separation mixture system it was important to determine effects of changing the ratio of PVP relate to HEC. Using the Doehlert matrix model (Figure 38), the optimization of the ratio greatly influences the resolution that can be achieved. However, for microfluidic systems the viscosity limits the ratio of PVP relate to HEC than can be used effectively. However, higher concentrations of HEC increased viscosity and resulted in incomplete separation on the microfluidic system. The summary of the effect of the ratio on the resolution is shown in Table 12. The ratio of about 50 % HEC provided a good separation and low viscosity. However, the best separation across both small DNA fragments and larger ones was observed at 80 % HEC. At this polymer concentration and percentage of HEC, the viscosity was low enough to fill the microchip channels efficiently.

Table 12: Effect of PVP relative to HEC on resolution

Polymer composition	Avg. $R_{139/150}$(bp)	STDEV ± bp	Avg. $R_{150/160}$(bp)	STDEV ± bp	Avg. $R_{490/500}$(bp)	STDEV ± bp
4% 20:80 (HEC/PVP)	3.41	0.23	3.39	0.22	6.74	0.35
4% 50:50 (HEC/PVP)	1.83	0.29	1.8	0.24	5.17	0.33
4% 80:20 (HEC/PVP)	1.82	0.27	1.96	0.43	5.07	0.36

v) Effect of Polymer Molecular Weight

Another factor that affects the resolution of the polymer is the molecular weight (MW) of the polymers being used. In previous work, the MW of the HEC polymer was 250K and the PVP, 1,000,000 MW.[157] It was thought that the molecular weight of the HEC polymer plays a more critical role in the separation process and that PVP helps reduce viscosity and with coating of the walls.[164] With this in mind, the molecular weight of the HEC polymer was examined and the highest possible MW of PVP used and kept constant. The experiment permits the observation of the effect of the molecular weight of the HEC component on the resolution.

In this experiment, we tested 3 MW's of the HEC polymer, 150K, 250K and 720K. The polymers were tested on both the ABI 310 Genetic Analyzer and the Agilent Bioanalyzer microchip system to detect any differences between the two platforms. The results from the separation performed with the 150K MW HEC were poor when compared to that of the 250K MW HEC polymer. However, the 720K MW HEC showed slight improvements in resolution when the polymer solution could be loaded. These higher viscosity solutions resulted in problems with the injection and filling of the separation channel. The 720K MW HEC polymer solutions were not reliable on both the microchip system and the ABI 310. Figure 46 shows the results from a 3.5% w/v PVP/HEC polymer solution using the 720K MW HEC polymer. A small increase in the peak resolution of +0.5 bp was observed at small DNA fragment size and about +4 bp at larger fragment sizes with the higher MW. However, loading of the chip and capillary was not always successful. Table 13 summarizes the resolution calculated from the results that were obtained in Figure 46.

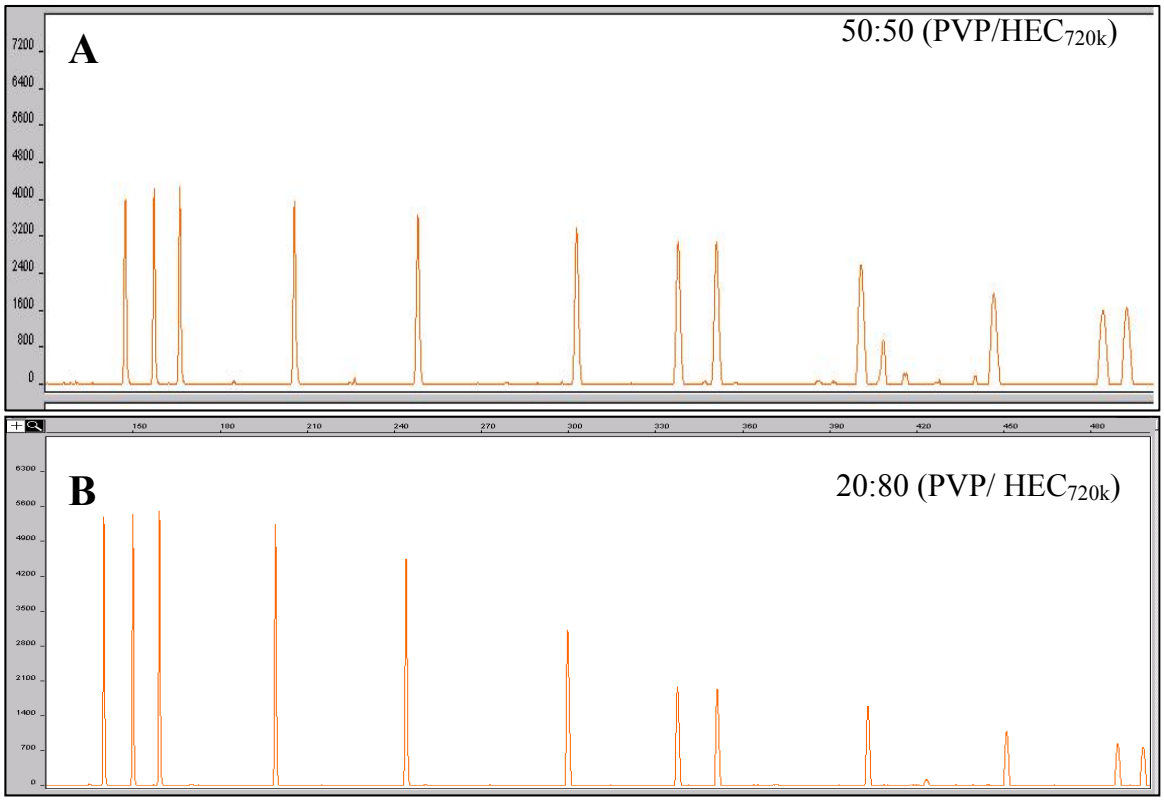


Figure 46: Electropherogram showing the separation of Liz 500 on the ABI 310 Genetic Analyzer using 720K MW HEC. A small increase in resolution can be seen from the figure above (50:50) PVP:HEC to the figure below (80:20).

Table 13:Summary of resolution obtained using 720k MW HEC polymer.

3.5% Polymer ratio (PVP/HEC)	$R_{139/150}$	STDEV (+/-)	$R_{150/160}$	STDEV (+/-)	$R_{490/500}$	STDEV (+/-)
50:50	2.01	0.34	2.19	0.39	8.21	0.32
80:20	1.53	0.28	1.45	0.33	4.58	0.40

d) Conclusion

The composition of the sieving matrix plays a critical role in obtaining maximum resolution especially on short channel microfluidic devices. The increase separation voltage provided a faster separation, however the resolution of the larger fragments

suffered likely as a result of joule heating and band broadening. The optimum conditions for highest resolution on the microchip system was at 350 V/cm using a 3.5-4 % w/v polymer solution with 80-85 % HEC relative to PVP, and with viscosities lower than 300 cP. Higher molecular weight polymers provided greater separation, however, the high viscosities of these solutions proved difficult in filling the narrow separation channels. Lastly, the type of material the chip is made from affects these parameters. Both glass and plastic chips exhibited different optimum ratios of PVP relative to HEC required for the best separation.

CHAPTER IX: DEVELOPMENT OF RAPID GENOTYPING ON SHORT CHANNEL MICROFLUIDIC SYSTEMS

a) Introduction

There are situations in which it is important to quickly and positively identify an individual. Examples include suspects detained in the neighborhood of a bombing or terrorist incident, individuals detained attempting to enter or leave the country, and victims of mass disasters. Systems utilized for these purposes must be fast, portable, and easy to maintain. DNA typing methods provide the best biometric information yielding identity, kinship and geographical origin, but they are not portable. Currently, DNA typing is performed by large-scale sequencers using multichannel fluorescent capillary array electrophoresis. Complex robotic extraction and PCR processing create economies of scale, permitting large numbers of samples to be efficiently processed. Unfortunately this process is not flexible enough for many applications in the field and is not quick on a per sample basis.

The proposed alternative, microfluidic DNA typing holds great promise, but constraints on resolution and problems with coupling inline extraction, inline PCR and multicapillary analysis make these systems highly complex. Integrated systems require a complicated amalgamation of engineered components making them highly vulnerable to clogging, misalignment, and voltage leakage. The issues with large-scale integration of extraction, amplification and DNA electrophoresis also make these systems less than portable. While there is no doubt that the technological issues may someday be solved, there are alternative modular approaches to perform this task that do not require extensive

engineering and do not require complete system integration. Such modular systems are easily repaired in the field and can be quickly switched out if problems occur.

Another advantage of microfluidic genotyping is the speed and portability of the instruments. These attributes could be of great value in situations such as mass disasters permitting rapid identification of victims. Events such as the September 11 terrorist attack on the World Trade Center demonstrate the need for portable, user-friendly systems for the analysis of DNA. Such instruments have also been envisioned as a tool for the processing of suspects at police stations, detecting bacterial spores in food contamination, and serving homeland security applications.

Current forensic DNA typing kits use a set of universal core loci established by the FBI that have been catalogued in their CODIS database system. These 13 core loci contain highly polymorphic tetranucleotide repeats that can be used to discriminate between individuals for forensic identification. Unfortunately, many of these loci have complex sequence variants that require single base resolution with the need for extended separation channels to fully resolve all alleles. This need for longer separation channels has resulted in the development of less portable devices in order to encompass the full chip dimensions. An alternative approach discussed permits faster and more accurate genotyping on smaller microfluidic devices through the use of less variant pentameric STRs. However, upon further evaluation of our microfluidic system, and the excellent resolution obtained for the pentameric STR separation, it was hypothesized that with an optimized sieving matrix, a small subset of the full cadre of DNA markers could be used as a quick screen to permit rapid identification of detained individuals. To provide the necessary precision, a slight re-engineering of the system was required to permit a second

detection channel that would provide accurate, precise, and robust separation of the tetrameric STRs used in forensic identification. Therefore, we set out to develop a rapid DNA genotyping system, using a short channel microfluidic chip, that would be capable of high discrimination using a subset of current CODIS STR markers.

b) Methods and material

i) DNA Samples

DNA standards from cell lines 9948 (male) and K562 (female) were purchased from Promega (Madison, WI, USA). These samples were primarily used for the development and optimization of the 7plex STR kit for short channel separation. Buccal swab DNA samples were taken from a variety of subjects, extracted, quantified and then genotyped using optimal conditions. These samples were then used to provide a small population data set for evaluation of the genotyping accuracy along with the precision, robustness and efficiency of the system.

ii) Extraction and Quantification of DNA samples

All samples were extracted using a phenol-chloroform/isoamyl alcohol (PCIA) protocol.[166] The samples were then quantified using an ALU-based real-time PCR method with 0. 5x SBYR® -Green I dye (Molecular Probes, Eugene, OR, USA).[71] Quantification was performed in reaction volumes of 20 μL using a Master Mix containing GeneAmps PCR Gold buffer (Applied Biosystems), 1.5 mmol/L MgCl2, 200 mmol/L deoxynucleo- tide triphosphates (Denville Scientific, dNTP's: dATP, dCTP, dGTP, dTTP), 1 mM BSA (Sigma-Aldrich, St. Louis, MO, USA), Triton X-100 (10% solution), ALU forward and reverse primers and two units of RampTaq hot start Taq

polymerase (5U/mL) (Denville Scientific, Metuchen, NJ, USA). A series of 9948 DNA standard solutions of known concentration were diluted ranging from 10 to 0.1 ng/mL and used to establish a standard curve. All samples were run on a Corbett Robotics Rotor Gene 6000 instrument (Qiagen Corbett Robotics, Valencia, CA, USA). The instrument software was used to calculate the critical threshold values and concentration of unknown samples.

iii) PCR amplification and Optimization

Amplification was performed in reaction volumes of 20µL using a Master Mix containing 1x GeneAmp® PCR Gold buffer (Applied Biosystems), 8mmol/L $MgCl_2$, 300µmol/L deoxynucleotide triphosphates (Denville Scientific, dNTP's: dATP,dCTP,dGTP,dTTP), 1µM bovine serum albumin (BSA) and 2.5 units of AmpliTaq Gold® DNA Polymerase. During the initial stages of the STR 7-Plex optimization, primer concentrations were adjusted from 0.5µmol/L to 1.5µmol/L to determine the effect on amplification efficiency and peak height balance. The input DNA template was varied from 0.1ng- 2ng/µL. Thermal cycling parameters were taken from the manufacturers recommendations for the Promega PowerPlex 16® STR kit, and tested over a range of annealing temperatures (58°C-62°C) and cycle numbers (28-32), in order to obtain the optimal amplification conditions.

The final PCR cycling parameters were as follows: 95°C (11 min), 96°C (1 min), 10 cycles with 30 sec at 94°C, 30 sec at 60°C, and 45 sec at 70°C, 22 cycles with 30 sec at 90°C, 30 sec at 60°C, and 45 sec at 70°C, 60°C (30 min), 4°C (8 min), 25°C (20 min).

Table 14: The MP7 Locus information, Primer Sequences and Allelic Range

Marker	Chromosome position	Genbank Acc No.	Common Repeat Unit	Primer Sequence 5' to 3'	Allele Range
Amel				F CCCTGGGCTCTGTAAAGAATAGTG R ATCAGAGCTTAAACTGGGAAGCTG	X-106bp Y-112bp
CSF1PO	5q33.1	X14720	AGAT	F CCGGAGGTAAAGGTGTCTTAAAGT R ATTTCCTGTGTCAGACCCTGTT	5 (317bp) 16 (361bp)
D16S539	16q24.1	G07925	GATA	F GGGGGTCTAAGAGCTTGTAAAAAG R GTTTGTGTGTGCATCTGTAAGCATGTATC	4 (260bp) 16 (308bp)
D7S820	7q21.11	G08616	GATA	F ATGTTGGTCAGGCTGACTATG R GATTCCACATTTATCCTCATTGAC	5 (211BP) 15 (251bp)
D13S317	13q31.1	G09017	TATC	F ATTACAGAAGTCTGGGATGTGGAGGA R GGCAGCCCAAAAAGACAGA	5 (157bp) 17 (205bp)
D5S818	5q23.2	G08446	AGAT	F GGTGATTTTCCTCTTTGGTATCC R AGCCACAGTTTACAACATTTGTATCT	6(115bp) 18(163bp)
Penta D	21q22.3	AP001752	AAAGA	F GAAGGTCGAAGCTGAAGTG R ATTAGAATTCTTTAATCTGGACACAAG	1.1(370bp) 18(454bp)

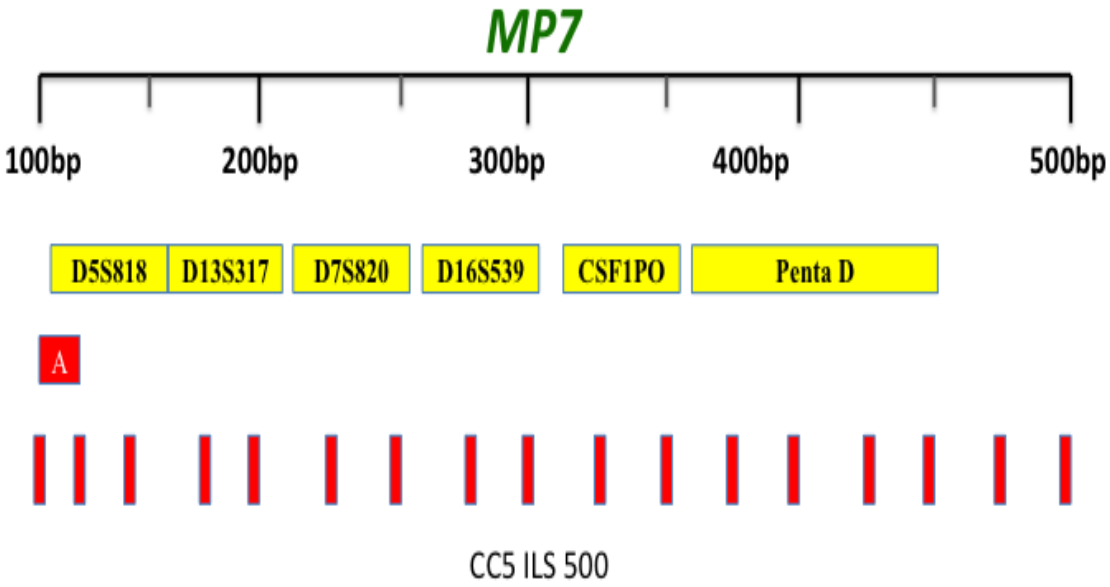


Figure 47: MP7 a single lane multiplex designed for rapid microchip electrophoresis. The multiplex contains 6 STR markers in the yellow lane and amelogenin a sex determining marker in the red lane.

iv) Microfluidic system and electrophoresis

The prototype Bioanalyzer 2100 system used in the separation of STRs was provided by Agilent Technologies (Agilent Technologies, R&D, Waldbronn, Germany). The system obtained for this research varies from the commercially available Bioanalyzer in that it incorporates a heat plate to keep the samples and system at 60°C during the run, as well as dual lasers capable of detecting multiple fluorescent dyes. This modified platform permits the analysis of single-stranded DNA (ssDNA) and improves sizing precision through the use of an internal lane standard (ILS). A multiplex system, MP7 was developed using certain markers present in the Promega PowerPlex 16 kit. Markers were utilized that gave a high statistical power of discrimination for a single lane and had minimal single base variants.

The chip was first primed with the polymer so that all the channels were filled with the sieving matrix. Next a size standard was prepared by adding 0.5μL of GeneScan™ 500 LIZ(Applied Biosystems) or CC5 Internal Lane Standard 500 (Promega corporation) to an Agilent buffer with HiDi® formamide in a 1:1 mixture. Five microliters of the above solution was then pipetted into each of the 12 samples wells. One microliter of the sample was added and electrophoresis performed. The results were interpreted using the software provided with the instrument.

c) Results and Discussion

The goal of the present work was to develop a rapid separation of a subset of CODIS STRs suitable for quickly screening unknown suspects at police stations, points

of entry and mass disasters. Three key points were clear in order for us to achieve our goal. First, we needed a rapid, highly precise microfluidic system. Secondly, since the number of dye channels was limited to two, the multiplexed loci would have to fit within a single lane with the second lane reserved for the sizing standard. Lastly, the STR loci would have to be chosen such that all alleles were resolved with minimal variant alleles.

i) Development of Precise and Accurate DNA Sizing on Microchip System

The presence of variant alleles creates a necessity for highly accurate size determination. With the capability of the instrument to simultaneously detect two fluorescent signals using a single excitation wavelength at 532 nm, and with emission wavelengths of 575 nm and 670 nm, we were able to add an internal size standard, which greatly increased the precision and accuracy of fragment size determination. The selection of the internal size standard dye label was based on the optimal laser detection wavelengths. The goal was to minimize spectral interference between the two detection wavelengths. Two different standards were examined, CC5 ILS500 (Promega Corporation) and GeneScan Liz 500 (ABI). The CC5 ILS500 size standard gave slightly higher signal intensities, and therefore, was used for most of the experiments. The samples were labeled using a Tetramethylrhodamine (TAMRA) dye.

The precision of the system was demonstrated using a 400 bp DNA ladder MapMarker®(BioVentures,TN). The precision of system for fragments over 300 bp was less than ±0.08 bp with smaller fragments having a precision value of ±0.02 bp. The separation of the CC5 ILS500 and 400 bp MapMarker® fragments can be seen in the decomposed data Figure 48. The resolution between peaks were deemed important in this

study as the peaks were on average ten or more base pairs apart and were very well resolved.

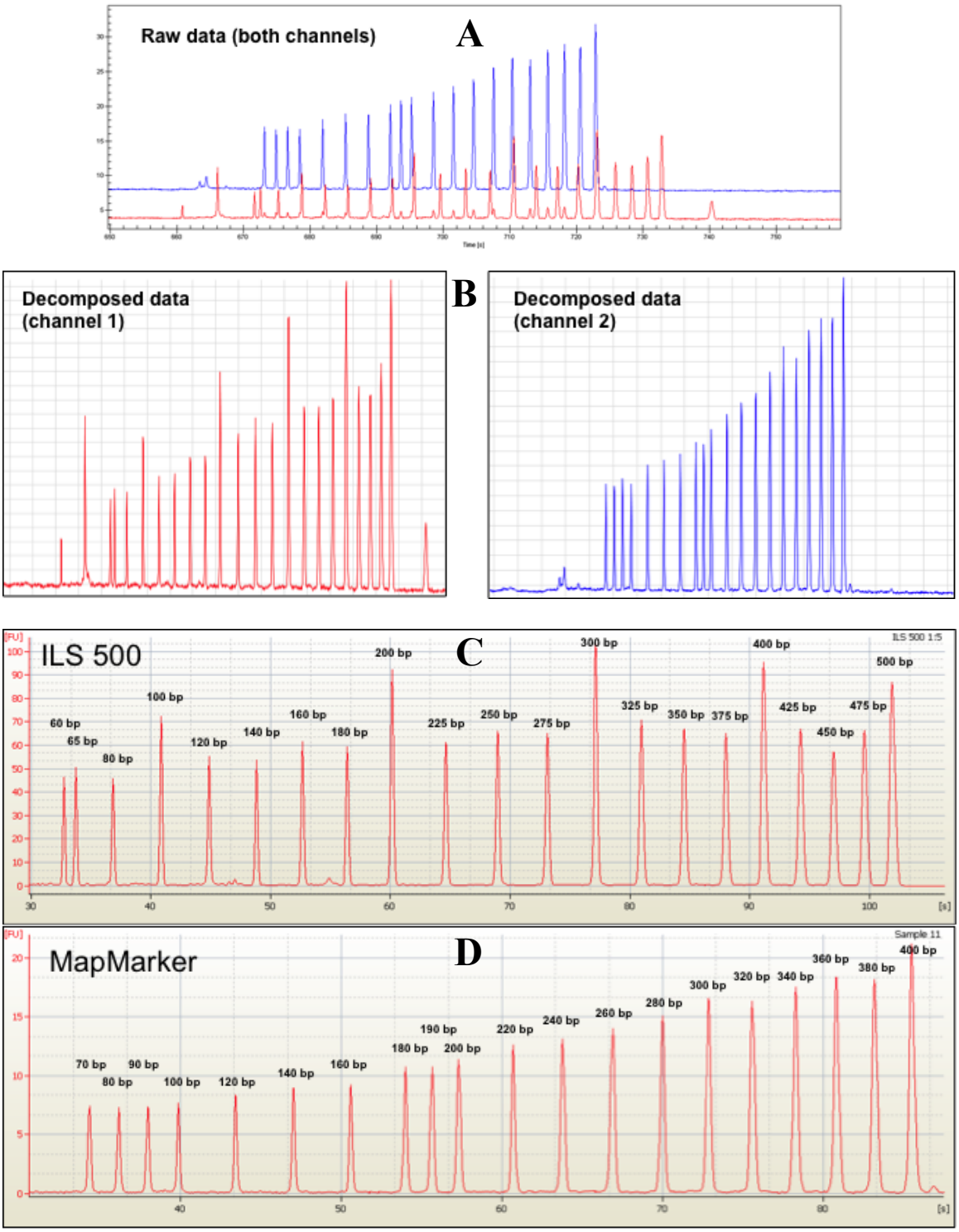


Figure 48: Electropherogram showing the use of an internal size standard (CC5 ILS500) for increase precision in sizing of a 400bp ladder. A) The raw data showing both the CC5-ILS and TMR-labeled 400bp Ladder. B) The decomposed signals of each dye. C) The CC5-ILS 500-size standard with the sized fragments. D) The TMR-labeled MapMarker 400bp ladder with sized fragments. Runs were performed using conditions described in the methods section.

Traditional DNA electrophoresis systems use a Local Southern Size Algorithm to establish a calibration curve using a nearest neighbor approach.[99] However, for this system a Global Southern Size Algorithm was used to enhance precision over the entire range of fragments. The Global method of sizing provides a more accurate result especially if there are mobility and temperature shifts within a run.[117] The data in Figure 49 show the correlation between the calculated experimental DNA fragment size and the actual values of the 400 bp MapMarker® ladder. The maximum deviation observed from the true size was 0.54 bp and the overall precision of the system was under 0.21 bp with all but the four largest alleles showing a precision of better than 0.17 bp. These results indicate the resolution required for an overall sizing precision (approx. 3 times the precision, 0.17 bp) of 0.5 bp or better across most loci with slightly reduced precision for the larger size loci. This level of precision is more than adequate for determining genotypes of the 7-loci multiplex. The variations may be a result of small mobility shifts that result from room temperature fluctuations. However, they are very small changes and do not affect the accurate calling of alleles.

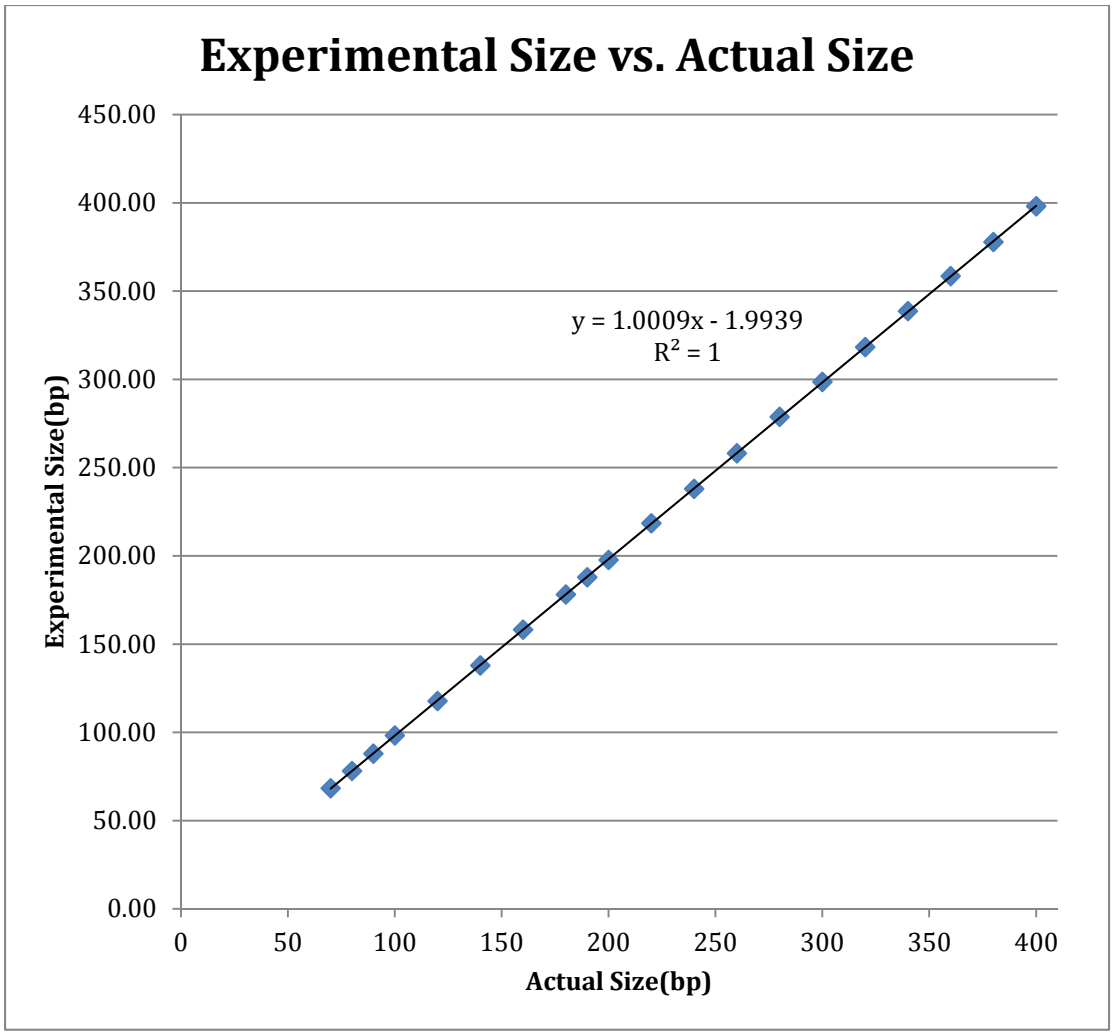


Figure 49: Graph showing the relation between the separation DNA fragment size against the actual fragment size in basepairs. The correlation between the experimental and actual data is shown with a R^2 value of 0.99. The runs were performed with a separation voltage of 350V/cm with total run time of under 2minutes.

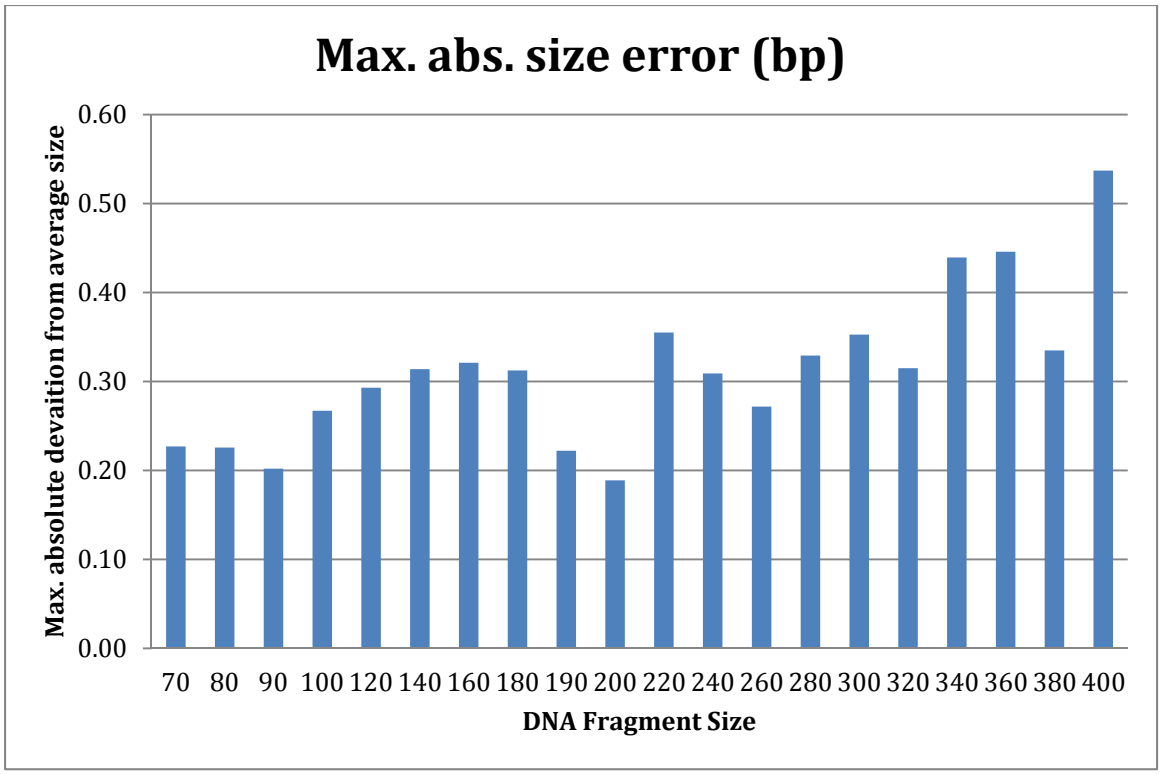


Figure 50: The maximum deviation from the calculated size to the actual size observed during the separation of a 400bp MapMarker® ladder on a 1.5cm separation channel microfluidic system. The average deviation is less than 0.3bp. The runs were performed with a separation voltage of 350V/cm with total run time of under 2minutes.

ii) Reproducibility

The nature of the injection process used in microfluidic systems makes it possible to produce a more compact sample injection than that used in capillary electrophoresis systems. This is accomplished using a pinched injection with a cross-T sample interface. The injection technique produces a consistent and reproducible sample plug without sample leakage. The reproducibility of the system was also tested using the 400 bp MapMarker ladder Figure 51. The ladder was separated and the fragment sizes calculated for one hundred runs. The results are summarized in Table 15. The highest deviation

reported was 0.21 bp at the largest sized fragments greater than 350 bp. Fragments between 150-350 bp had deviations on average of 0.15bp and the fragments below 150 bp had an average 0.1 bp deviation.

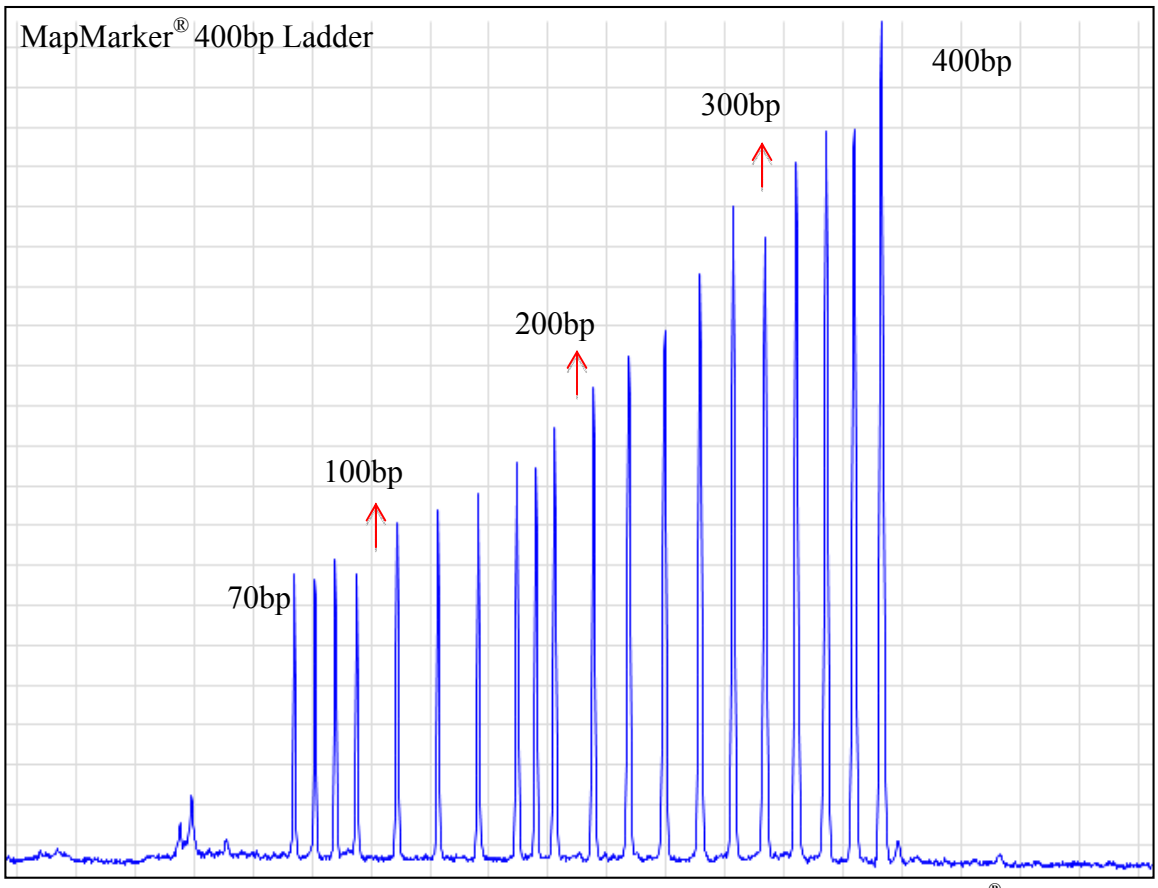


Figure 51: Electropherogram showing the separation of TMR labeled MapMarker® 400bp DNA ladder on a 1.5cm separation channel Bioanalyzer system. The run was performed with a 350V/cm separation voltage with a sample run time less than 2 minutes.

Table 15: The Precision and Reproducibility Data for 100 runs of a 400bp DNA ladder separated on a 1.5cm channel microfluidic system in under 2minutes.

Target Size	Average	STDEV	%CV	Max. neg. Size Error	Max. pos. Size Error
70	68.38	0.09	0.13	-0.11	0.23
80	78.14	0.11	0.14	-0.12	0.23
90	87.98	0.1	0.11	-0.2	0.19
100	98.3	0.08	0.08	-0.27	0.08
120	117.79	0.12	0.1	-0.29	0.24
140	137.92	0.12	0.09	-0.24	0.31
160	158.23	0.11	0.07	-0.32	0.28
180	178.13	0.13	0.07	-0.25	0.31
190	187.91	0.13	0.07	-0.22	0.21
200	197.73	0.14	0.07	-0.19	0.18
220	218.48	0.13	0.06	-0.27	0.36
240	238	0.12	0.05	-0.18	0.31
260	258.15	0.13	0.05	-0.27	0.18
280	278.8	0.16	0.06	-0.33	0.31
300	298.59	0.15	0.05	-0.35	0.35
320	318.33	0.15	0.05	-0.31	0.24
340	338.67	0.21	0.06	-0.39	0.44
360	358.51	0.18	0.05	-0.45	0.36
380	377.85	0.21	0.06	-0.3	0.33
400	398.12	0.21	0.05	-0.4	0.54

iii) Development of MP7

To create the MP7 STR kit the markers D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D were selected from the information in Table 14. These loci were selected to yield good discrimination with minimal variant alleles. To permit sex typing the amelogenin XY marker was inserted in the same channel as the internal size standard. This could be done without affecting the sizing curve, as this locus contains only two alleles, which are at 106bp and 112bp respectively and do not overlap with any of the

sizing peaks. The resulting 7 STR marker multiplex, MP7, had discrimination powers of $1x10^6$ or greater (Figure 47).

iv) Optimization of MP7

The main parameters examined in the optimization of the MP7 kit were the concentration of magnesium, the concentration of dNTPs and the primer annealing temperature. These parameters were tested and their effects on the efficiency of amplification observed by examining the peak height and balance of each locus. The first parameter tested was the magnesium ion concentration, which ranged from 2mM to 10mM. Magnesium, a cofactor to the Taq polymerase, is directly related to the efficiency of amplification. It was observed that 6mM to 8mM Mg^{2+} produced the best combination of peak intensity and balance across loci (Figure 52). At 10mM [Mg^{2+}] the smaller sized loci continued to increase in height, however, the larger size fragments showed decreased sensitivity and non-specific artifacts were observed.

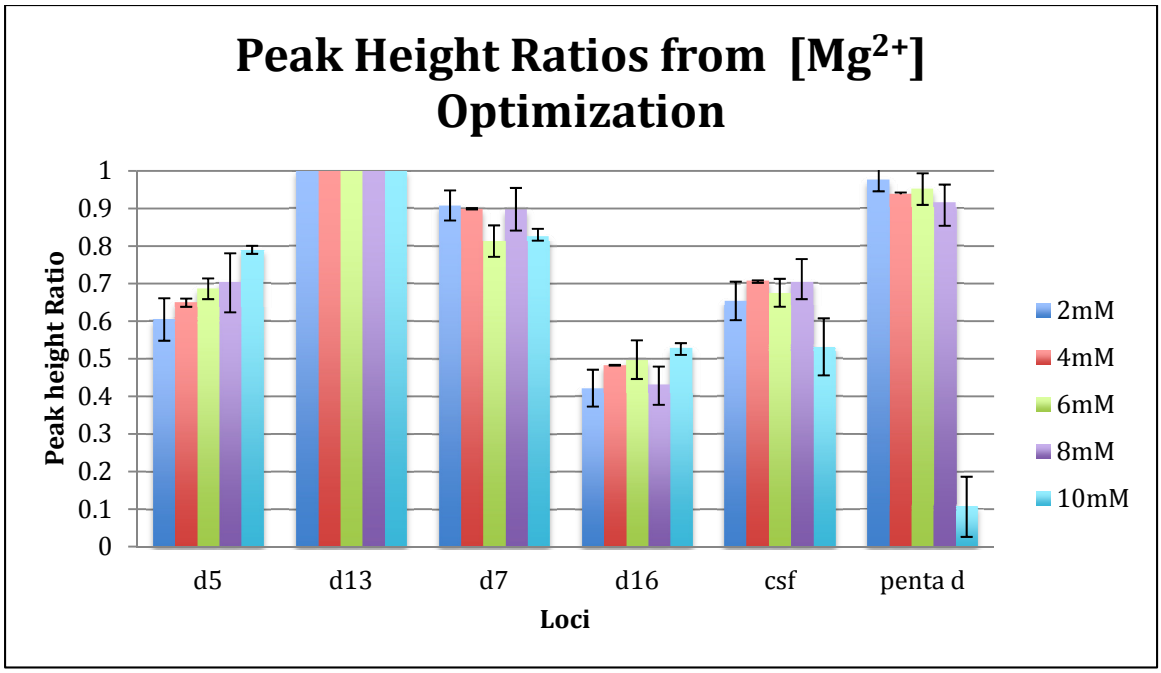


Figure 52: The optimization of [Mg^{2+}] at each locus ranging from 2mM to 10mM run in triplicates. The peak height ratios were calculated from the height of allele 1 versus that of allele 2 at each locus. The optimum value of magnesium ion was determined to be between 6mM and 8mM. The highest PHR deviation was 0.08 at 10mM Penta D

The overall concentration of magnesium is also related to the concentration of the dNTPs in the reaction as they chelate Mg^{2+} ions. Therefore, a balance between the Mg^{2+} concentration and the dNTP concentrations is important for an efficient amplification. The concentration of dNTPs examined ranged from 200µM to 500µM. Concentrations of dNTPs at 200µM each showed the best balance across all loci, with the exception of Penta D, which had that poor peak balance at this concentration (Figure 53). As a result of this, a final concentration of 300µM was chosen to provide the best balance across the entire range of the multiplex.

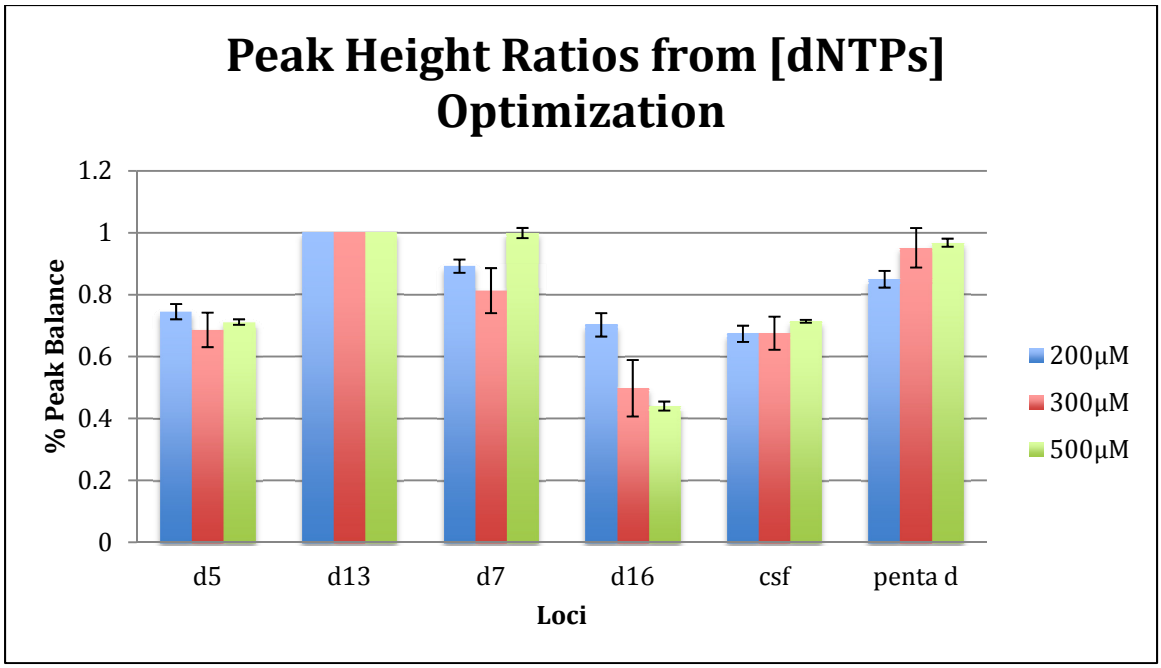


Figure 53: The optimization of [dNTPs] at each locus at 200µM, 300µM and 500µM. The Peak Height Ratios calculated from triplicate runs with a maximum standard deviation of 0.08. The optimum value of dNTP concentration was determined to be 300µM.

The effect on the PCR parameters on the success of the amplification was also measured by analysis of the peak resolution. In order to calculate the chromatographic resolution, a software was used to model the electropherogram data and report the resolution between the adjacent peaks. (Figure 54) The chromatographic resolution was then converted to base pair resolution (Equation 10). Figure 55 shows the resolution obtained with varying concentration of magnesium. The efficiency of the amplification at a concentration of 8mM magnesium produced the best resolution of peaks for the entire multiplex.

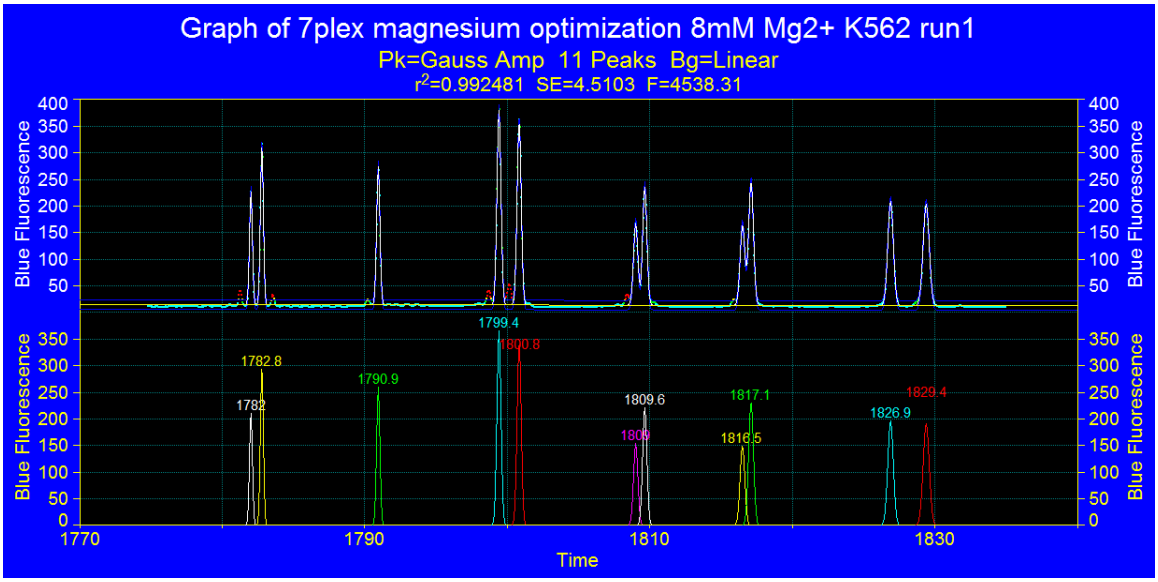


Figure 54: Peak fit software used to fit the data from an electropherogram of a DNA K562 standard amplified using a PCR mix containing 8mM [Mg^{2+}], 300µM dNTPs, 2.5U Taq using cycling condition from the method section. The chromatographic resolution was then calculated between adjacent peaks.

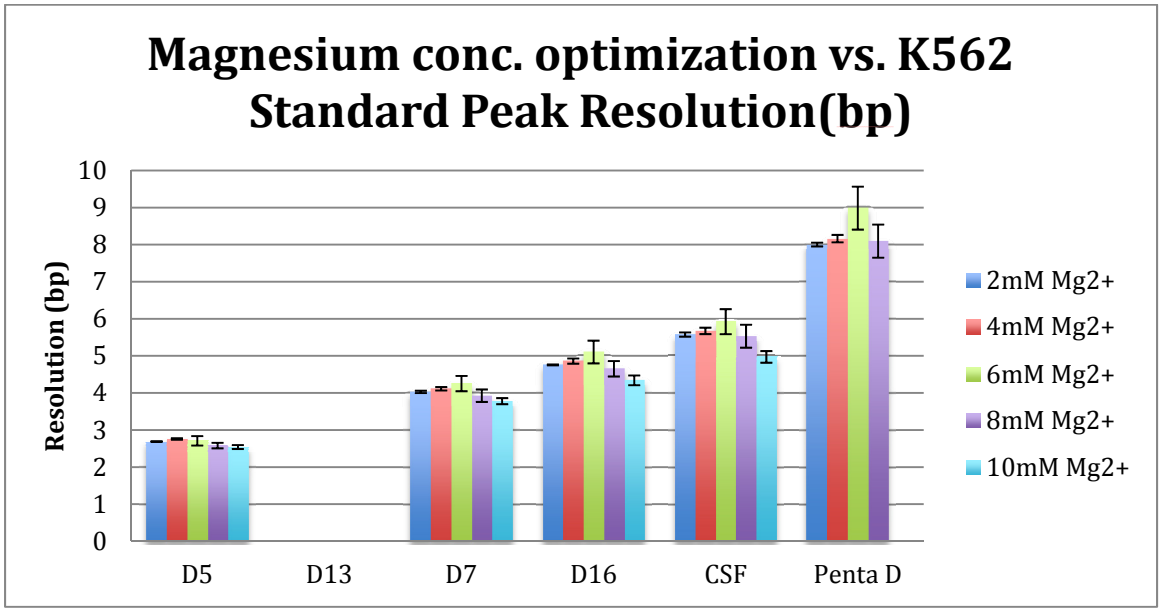


Figure 55: The Effect of [Mg^{2+}] on bp resolution of a DNA standard K562 amplified using 8mM [Mg^{2+}], 300µM dNTPs and 2.5U Taq under standard cycling conditions. The smaller size Markers D5 and D7 show similar resolution across the range of magnesium. The larger markers D16, CSF and Penta D show greater differences with 8mM being the best. Note at D13 there is no resolution calculated because the sample is homozygous at the locus.

The optimum PCR conditions that yielded the highest sensitivity and balance both within and between loci were achieved using an amplification performed in reaction

volumes of 20µL, using a Master Mix containing 1x GeneAmp® PCR Gold buffer (Applied Biosystems), 8mmol/L $MgCl_2$, 300µmol/L deoxynucleotide triphosphates (Denville Scientific, dNTP's: dATP,dCTP,dGTP,dTTP), 1µM bovine serum albumin (BSA) (a common PCR additive which helps overcome inhibition and increase PCR efficiency) and 2.5 units of AmpliTaq Gold® DNA Polymerase. The overall results can be seen from the electropherogram in Figure 56 showing the separation of the DNA standard K562 (Promega Corporation). The results of the standard are consistent with the known sample profile with alleles 11,12 at D5S818, 8,8 at D13S317, 9,11 at D7S820, 11,12 at D16S539, 9,10 at CSF1PO and 9,13 at Penta D.

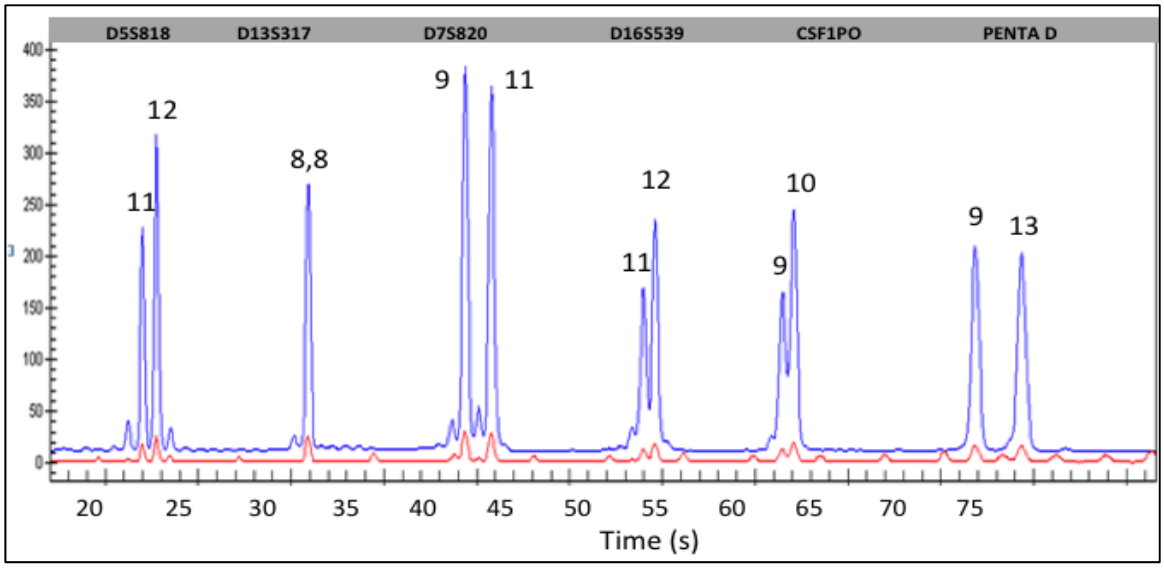


Figure 56: Electropherogram of DNA Standard K562 amplified using MP7 under optimized conditions. The results shows a well balance inter and intra-locus amplification with sufficient resolution to resolve between a single 4bp repeat observed at the CSF1PO locus.

To demonstrate the performance of the system with real samples, a standard PCIA extraction was performed from buccal swabs, amplified and injected on the microfluidic system. The resulting electropherogram can be seen in Figure 57 showing the genotype

for that individual and the red channel data containing the amelogenin marker and the internal size standard. The overall genotype observed was calculated to be 1 in 1.13 x10^6 or higher using the OmniPop 150.5 software over 160 population data sets.

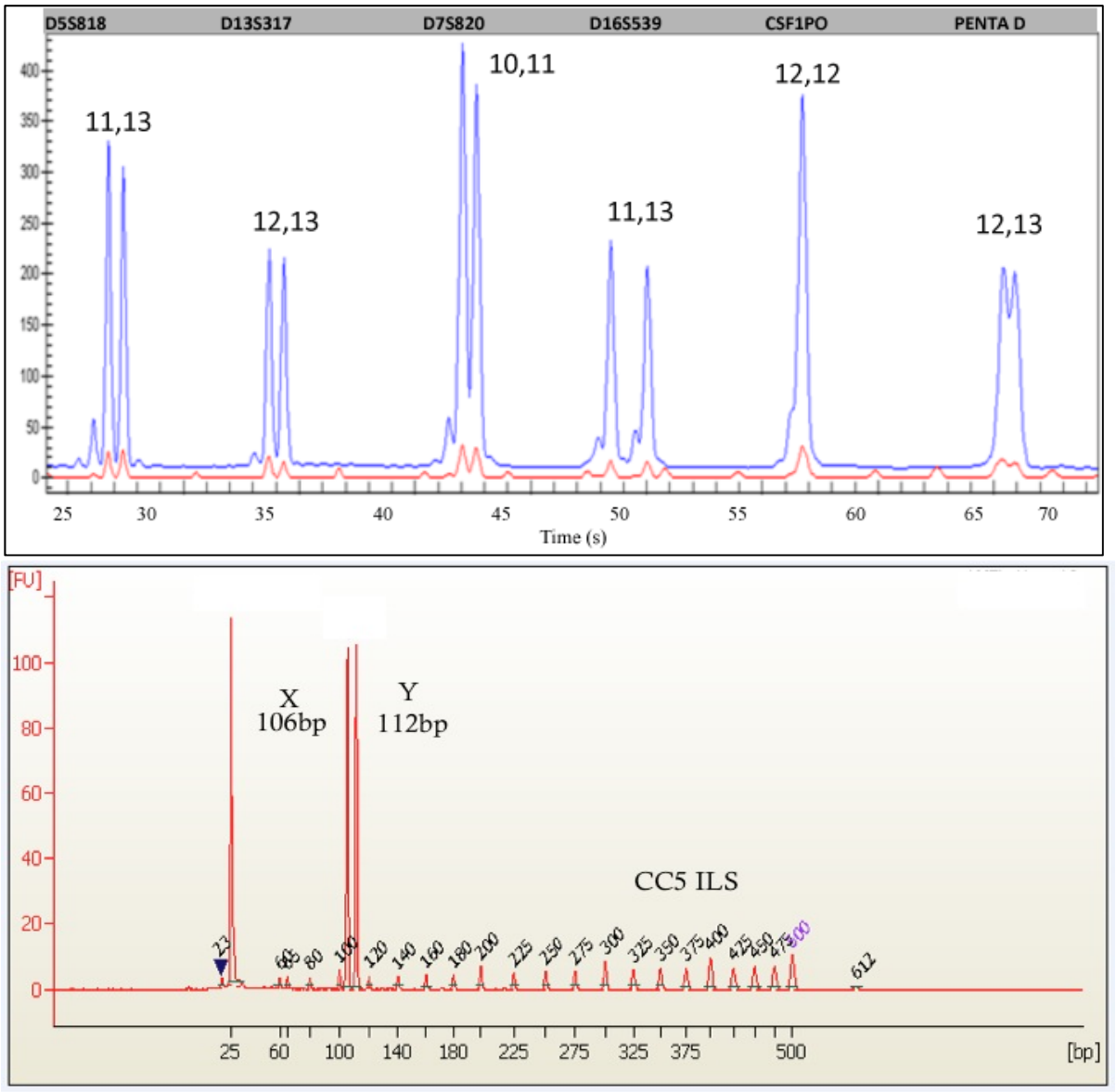


Figure 57: Above: Electropherogram of extracted buccal swab sample amplified using the MP7 kit. The resulting genotype was obtained in under 80s with a power of discrimination of 1 in 1.13x10^6. Below: Electropherogram data from the red lane of an extracted buccal swab showing the internal size standard CC5 ILS 500 and the amplification of the amelogenin a sex-determining marker. The presence of a Y allele at 112bp represents the sample being of male origin. The sample was amplified using the MP7 STR kit with 8mM [Mg^{2+}], 300µM dNTPs, 2.5U Taq under standard cycling conditions.

The careful design and development of the MP7 STR kit on a dual laser short channel microchip system allowed for rapid (<80 s), precise (better than 0.5 bp) and accurate sizing of alleles with a discrimination power greater than 1 in 1 $x10^6$. While baseline resolution was not achieved over the entire range of the multiplex, the system was capable of resolving individual alleles at high precision even at the largest fragment size in the Penta D locus. The well-established loci and with the minimal microvariant alleles permits confident designation of all alleles in the multiplex. In order to get an accurate designation of alleles from the calculated size, an allelic ladder was run with each chip. The use on an internal lane ladder not only allowed for precise allele calling but also demonstrated the robustness of the system by permitting the examination of all possible alleles for each locus within a single run. The overall run time of the allelic ladder was under 80 seconds and is shown in Figure 58. The analysis time is significantly faster than that of traditional CE systems at 40 minutes and is also the fastest genotype of a 7 loci multiplex ever reported. The allelic ladder showed clear separation of all alleles with adequate resolution over the entire 450 bp range.

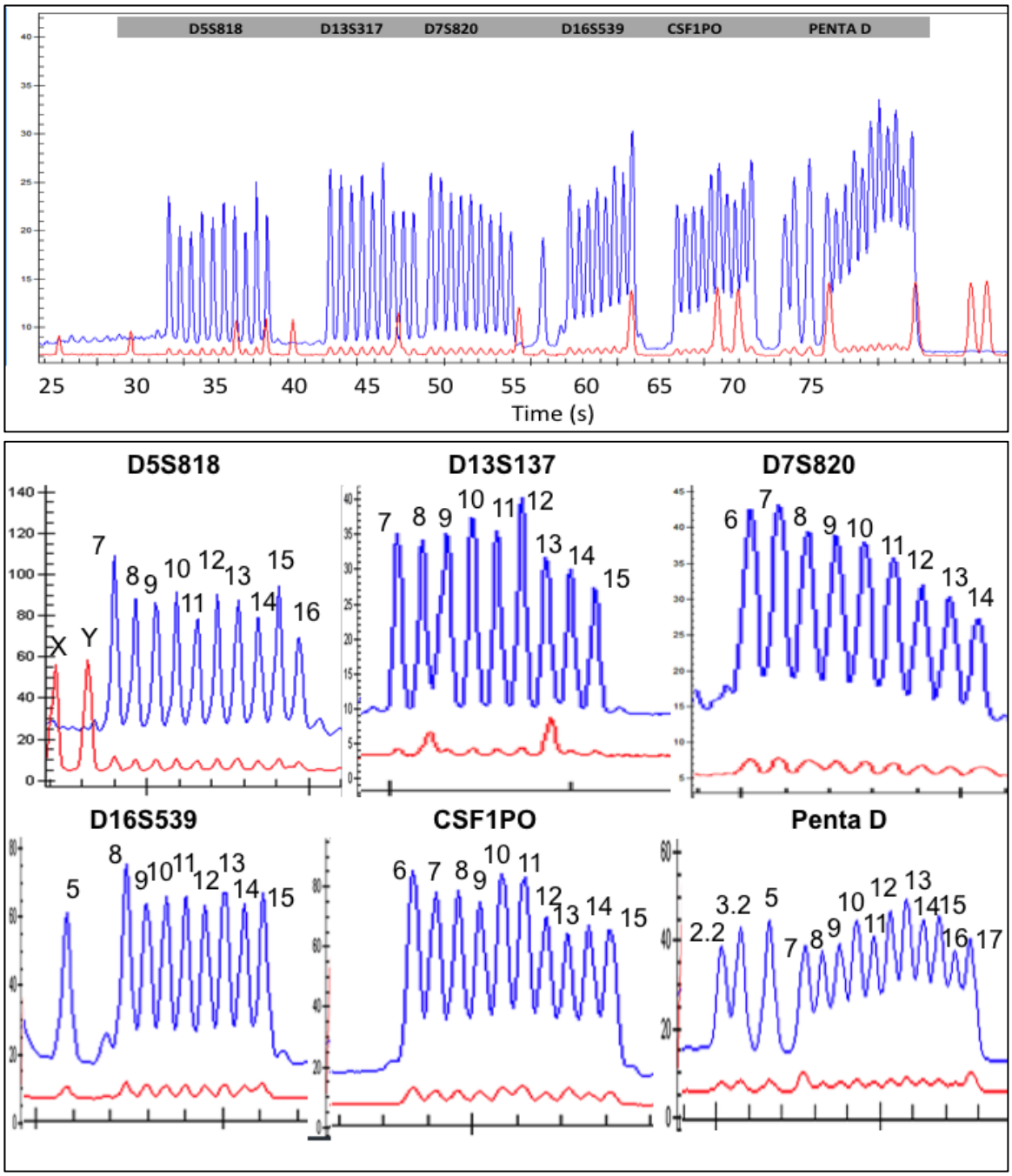


Figure 58: Electropherogram of the MP7 Allelic Ladder showing the separation of all possible allele for the given markers. The largest size adjacent alleles can be clearly differentiated. The ladder was obtained from Promega Corporation and run at the standard microchip run condition of 350V/cm with a denaturing sieving matrix. The ladder run time was performed in under 80 seconds.

d) Conclusions

The goal of this project was to develop a rapid system capable of separations on short channel microfluidic devices with the required resolution needed for microsatellite analysis in forensic DNA samples. This was accomplished with the use of enhanced sieving matrices under denaturing conditions and the addition of an internal size standard for increased precision in a second emission channel. The resulting system was capable of performing separation on a 1.5 cm channel with sizing precision of better than +/- 0.5 bp in less than 80 seconds. Furthermore, the development of a subset of CODIS STR markers (MP7) for the system provided discrimination powers of 1 in $1x10^6$ or higher. The overall system is capable of rapidly identifying suspects and other persons of interest detained at border crossings and police stations.

CHAPTER X: ULTRA-FAST DIRECT PCR FOR FORENSIC GENOTYPING

a) Introduction

Forensic DNA typing not only provides scientific evidence in the courtroom but can also be used as an investigative tool for the elimination of suspects and for confirmation of identity at places such as border crossings or airports. However, such applications require a small, rapid, easy to use system that can provide an answer onsite within the holding time limits permitted by the law. The processing of DNA samples for STR analysis involves extraction, quantification, amplification and separation of DNA. The sample to answer workflow requires a relativity long analysis time to achieve a genotype. While technology has advanced significantly since the discovery of the polymerase chain reaction, the processing of DNA prior to electrophoresis has changed very little. Even though automation is widely used for the extraction process, the chemistry involved has remained fairly consistent. Many laboratories still use organic extractions such as PCIA, while other have upgraded to automated systems that use magnetic beads or similar solid phase extraction kits. The process workflow involves numerous wash steps along with relatively long heating steps required to lyse the cells and expose the DNA template. Further purification steps may also be required to remove proteins, RNA and other possible inhibitors that may affect the amplification process downstream.

Current analytical systems using multicapillary sequencers and robots are well adapted for large-scale high throughput processing of criminal offenders from blood or buccal swabs. However, the potential for high-speed analysis of a single individuals'

DNA type has yet to be realized. Microfluidic systems offer the promise of improved speed because of shorter channel lengths and narrow sample injection zones. However, coupling current extraction processes to existing microfluidic devices results in a fairly complex device with numerous design challenges.[140, 151, 167, 168]

However, there is a simpler solution to this problem. The engineering problems associated with in-line extraction can be bypassed by amplifying a sample directly from a paper punch, removing the need for any extraction at all.[120, 169, 170] Results can be further speeded up by upgrading the chemistry of the Taq enzyme to permit high-speed amplification of DNA, reducing the overall reaction time from 2 ½ hours to under 15 minutes.[123, 171] Lastly, STR analysis using short channel microfluidic systems such as the system developed in Chapter 9 can be achieved in less than 2 minutes. Combining these three processes would result in an ultra-fast, sample to genotype result in about 20 minutes. The overall process would be modular requiring no additional engineering. Such a system would also be more robust because system breakdowns or clogging in one module would not affect the downstream results. The system would also have a small footprint, requiring only two lunch box sized devices and a small laptop.

The key advances that permit such rapid processing are the advent of direct PCR and rapid thermal cyclers.[172] Direct PCR utilizes special buffers and enzymes to amplify DNA directly from a paper punch of FTA paper containing a blood or salvia stain.[173] The user would swab an individual's mouth, wipe the swab on specially treated paper (the paper lyses the cells and eliminates infectious agents), and punch out a small piece of the paper containing the saliva. The saliva sample would then be placed in a tube with PCR

reagents and amplified directly.[45] No special washing steps are required and minimal sample waste is produced. The resultant PCR product can then genotyped.

By using high-speed thermal cyclers like the Streck Philisa or Analytik Jena Speed Cycler[2] amplification ramp rates as fast as 15 degrees per second can be used resulting in full sample amplification in 10-15 minutes. Thus, this fast amplification when coupled with a 2-minute microfluidic separation can produce a complete genotype in under 20 minutes. With such protocols a laboratory could simply purchase a fast thermal cycler with appropriate enzymes to easily perform a rapid direct DNA amplification. Small, dedicated laboratories at borders, police stations and mass disaster sites could perform the task in under 20 minutes using this chip based microfluidic system and a small thermal cycler. Even without the chip system a full profile is possible in 45 minutes or less.

b) Methods and Materials

i) DNA samples

DNA standards K562, 9948 and 9947A were obtained from Promega Corporation and used as positive controls during the amplification and optimization process. Two simulated reference control samples M and O were obtained from swabbing the inside of the cheek of two individuals and transferred to FTA paper. Samples M and O were also extracted using phenol-chloroform-isoamyl alcohol and amplified using PowerPlex16 HS (Promega Corporation) under standard manufacturer conditions and used as a known reference samples.[174] A small population was also obtained from 18 individuals by

swabbing of the inside of their cheeks and transferring to the FTA paper, samples were then analyzed using the MP7 kit. The samples collected and used for this project were approved for use through an Institutional Review Board #091510-00 at FIU.

ii) Fast Thermal Cyclers

Two Ultra fast thermal cyclers were obtained and used in the development of rapid direct amplification. The SpeedCycler$^{2®}$ from Analytik Jena (Germany) and the Philisa® from Streck Inc. (Nebraska, US) were both used and demonstrated ultra-fast amplification. The Philisa on average performed about 3 minutes faster than the SpeedCycler$^{2®}$ under the same protocol. This was attribute to its faster cooling rates.

The SpeedCycler$^{2®}$ system contained a replaceable 36 well low profile rapid (LPR) block made from gold-plated sterling silver. The system was capable of maximum heating rates of 15°C/s and cooling rates of 10°C/s. The lid of the system is also heated at temperature up to 120°C to avoid condensation at the lid of the tube that can result from heating and evaporation in the PCR tube. The systems overall dimension was 280 x 290 x 250mm, producing a relatively small footprint. The system operation was controlled by a built in touch screen. Special 20µL LPR PCR tubes were constructed using ultra thin walls to ensure efficient heat transfer to the liquid. The tubes were sealed using a plastic film that was secured over the tube strips.

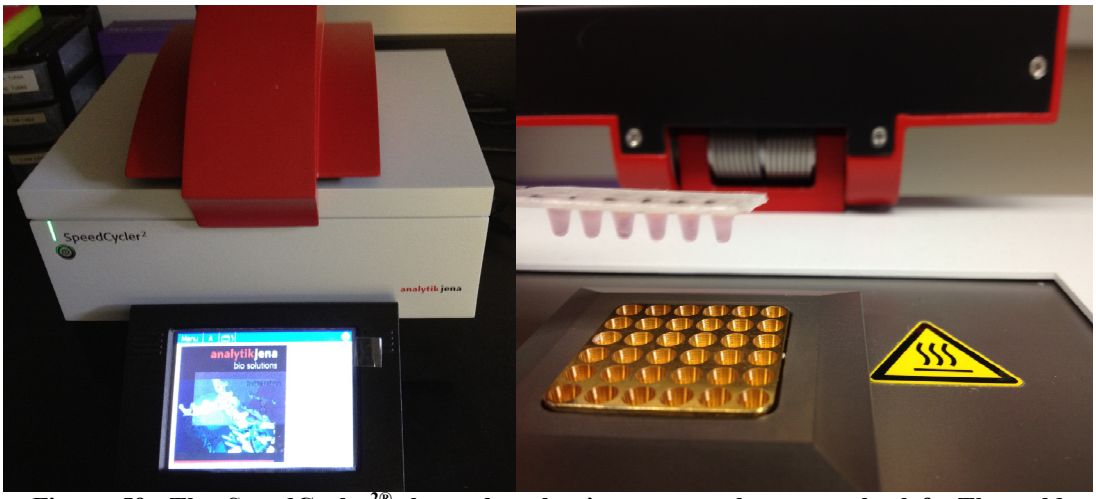


Figure 59: The SpeedCycler[2®] thermal cycler instrument shown on the left. The gold plated heated block and sample tubes shown on the right.

The Philisa® thermal cycler contained a 8 well low mass silver sample block with maximum heating rates of 15°C/s and cooling rates of 12°C/s. These high rates were achieved by means of a dual Peltier module. The instrument is controlled by a small netbook laptop and had dimension 285 x 202 x 215mm with a weight of 7.5lbs. This system uses a thin walled, flat polypropylene tube that can hold a maximum volume of 50µL. This design increases the surface to volume ratio and allows for more efficient heat transfer to the sample mix.

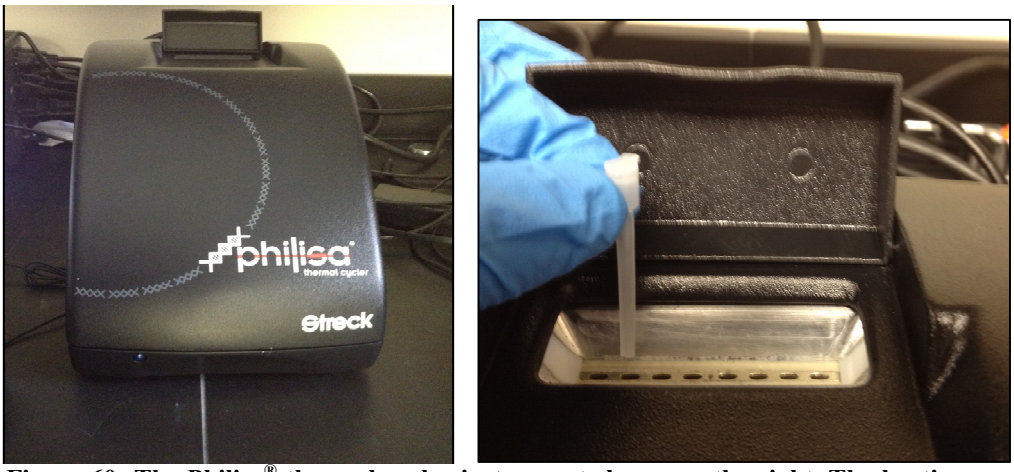


Figure 60: The Philisa® thermal cycler instrument shown on the right. The heating block and thin walled polypropylene pcr tubes shown on the right.

iii) Rapid Polymerases

DNA polymerases capable of rapid incorporation of bases were tested to determine which enzyme would yield the fastest amplification, highest fidelity and compatibility with a direct buffer. The enzyme chosen from the initial tests and used for all further experiments was, Z-Taq polymerase (Takara Bio Inc.). The enzyme was supplied with a 10x Z-Taq Buffer and 2.5mM dNTP mixture. The other rapid polymerases tested are listed in Table 16.

iv) Direct Buffers

A number of direct buffers were examined for compatibility with rapid polymerases listed in Table 16. The buffers were tested for both effectiveness in amplification and compatibility with FTA paper without elution and further extraction. The AnyDirect F buffer mix (Bioquest, Korea) was combined with the rapid polymerase and used for all further amplifications, optimization and validation of this technique.

Table 16: List of Rapid Polymerases and Direct Components

	Hot Start	Kit Components	5'-3' exo	3'-5' exo	Speed	Inhibition Resistant
AnyDirect F Buffer Mix (BioQuest)	Chemical (95,15')	• AnyDirect Buffer F • HotTaq-fx (5U/µL)	Yes	No	--	Yes
Terra Direct (Clontech)	Ab (98,2')	• Terra PCR Direct Polymerase Mix (1.25U/µL) • 2X Terra PCR Direct Buffer (Mg2+ 2mM & 400µM dNTPs)	No	---	--	Yes
Phusion Human Direct (Finnzymes)	Affibody (98,1')	• Phusion DNA Polymerase (2U/µL) • 2X Phusion Human Specimen PCR Buffer • Dilution Buffer • DNARelease Additive • Control Primers	Yes	Yes	15-30s/kb	Yes
KAPA2G (Kapabiosystems)	Ab (95,1~3')	• 2X KAPA2G Fast HotStart DNA Polymerase Master Mix (1U Polymerase, Mg2+ 3mM and 0.2mM dNTPs)	Yes	No	15s/kb	--
Phire II (Finnzymes)	Ab (no reactive)	CHAPTER I: Phire Hot Start II Polymerase CHAPTER II:5X Phire Reaction Buffer (Mg2+ 7.5mM) CHAPTER III: DMSO (GC rich template)	Weak	--	10-15s/kb	Yes
Z-Taq (Takara)	No	• Z-Taq DNA Polymerase (2.5U/µL) • 10X Z-Taq Buffer (Mg2+ 30mM) • dNTP mixture (2.5mM)	Yes	Yes	5xTaq	--
SpeedSTAR (Takara)	Ab (94,1')	• Z-Taq DNA Polymerase (5U/µL) • 10X fast Buffer I(Mg2+ 30mM) • 10X fast Buffer II(Mg2+ 20mM) • dNTP mixture (2.5mM)	Yes	Yes	10 s/kb	--

v) Rapid Direct STR PCR amplification

The MP7 multiplex containing the STR markers AMEL, D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D was amplified in a reaction volume of 12.5µL using a final PCR master mix containing 1X Z-Taq Buffer (Takara Bio Inc.), 1X AnyDirect F Buffer (BioQuest, Korea), 432µmol/L each dNTPs (Takara Bio Inc.), 4mmol/L $MgCl_2$, 0.08µg/µL bovine serum albumin (BSA), TAMRA fluorescently labeled primers at concentrations 0.5µM for D5S818, D13S317, 0.6µM for D7S820, CSF1PO and AMEL, 0.8µM for D16S539, and 1µM for Penta D. A final concentration of 1.25 Units of Z-Taq Polymerase (Takara Bio Inc.) was added to the master mix referred to as the RD-MM from this point on.

A 1.2mm punch of the sample from the FTA paper was directly added to the Rapid Direct (RD) Master Mix and amplification performed in 16 minutes on the Philisa thermal cycler or 19 minutes using the SpeedCycler[2]. The cycling conditions are listed in Table 17.

Table 17: Cycling Conditions Total time = 16min

98°C	5 seconds
60°C	5 seconds
72°C	15 seconds

X 30 cycles

vi) STR Analysis

Following PCR amplification 1µL of the sample was added to 5µL of a HiDi® formamide (Applied Biosystems) and Agilent's proprietary marker buffer (Agilent Technologies). The microchip was then primed with the sieving matrix and 1µL of the

PCR product in the marker/formamide mixture was added to each sample well. STR Analysis was performed on a modified Agilent Bioanalyzer containing a heating plate, denaturing polymer and dual lasers. The analysis was performed in about 80 seconds for each sample. Genotyping was performed from the sizing results obtained using an excel software macro developed in house that compared the sizes of individual alleles with a previously run allelic ladder.

c) Results and Discussions

The goal of this project was to develop a rapid genotyping method for buccal swabs that required no extraction step. In order to achieve ultra-fast STR amplification two major areas were investigated; rapid thermal cycling and high-speed polymerases.

i) Fast Thermal Cycling

Typically most thermal cyclers use sample heating blocks with a cooling fan coupled to peltier systems while others utilize air driven systems that cycle temperature directly at suspended sample tubes. Infra-red (IR) radiation or lasers pulses have also been used to heat samples.[171] Generally, the use of cycling blocks made from highly conductive alloys permits a more rapid transfer of heat. Most high-speed thermal cyclers use this technique, as it is particularly effective for high-speed cooling. To further improve heat transfer, a reduced volume reaction mix is prepared in specialized thin walled tubes that conform to the sample wells providing maximum contact with the heating block. This greatly increases the speed at which amplification can occur. However, these physical changes to the instrumentation must also be accompanied by

higher processivity enzymes that are capable of maintaining amplification fidelity and specificity at such rapid rates.

A number of authors have explored the use of high-speed amplification. Early work by Idaho Technology in the 1990s produced amplification rates permitting 30 cycles in under 20 minutes using a hot air based thermal cycler.[175] In a subsequent report the same group utilized a capillary tube based thermal cycler capable of performing 30 PCR cycles in 7 ½ minutes.[176] In 2006 Neuzil et al. reported amplification rates of 40 cycles in 6 minutes using a 100nL reaction volume on a mineral oil drop using IR radiation heating on a microchip.[177] Vallone et al. in 2008 produced amplification of 16 STR loci in 35 minutes using a mixture of two rapid polymerases on a thermal cycler with 4°C/s ramp rate.[172] Wheeler et al. in 2011 used an in house system which was capable of performing 30 cycles in 2 minutes 18 seconds at ramp rates up to 45°C/s utilizing convective fluid flow for faster heating and cooling.[171] In this research we have utilized two commercial state of the art thermal cyclers; the SpeedCycler^2 (Analytik Jena, Germany) and the Philisa thermal cycler (Shreck, New England USA). Both systems can perform rapid heating and cooling with heating rates of 15°C/s and cooling rates of 10°C /s and 12°C /s respectively.

ii) Enzymes

A variety of enzymes were tested with respect to efficiency and effectiveness in the amplification of MP7 STR kit. Many of these enzymes contain antibodies or chemical modifications to block amplification prior to initiation of the reaction. These so-called hotstart enzymes are useful as they limit the amplification of artifacts that sometimes

occurs prior to the initiation of fast PCR cycling. However, some required at least 1-5 minutes at elevated temperatures in order to be activated. Newer hotstart enzymes such as the rapid polymerase, P2 utilize antibodies attached to the polymerase that blocks amplification. These enzymes required a few seconds to denature the antibody and activate the polymerase making them applicable for thermal cycling at high speeds. Manufacturers of these polymerases reported amplification times of as little as 10 minutes, however, these times were achieved with single, small DNA fragments averaging 150bp in size.

Table 18 summarizes the results from the testing of the rapid polymerases. From our initial study we determined that three rapid enzymes produced a peak heights above 15 RFUs and peak height ratios greater than 0.6 when amplified under the rapid cycling conditions. Note, enzymes were used outside of manufacturers protocol guidelines and which in some cases can result in inefficient amplifications. Two enzymes gave good initial results under rapid amplification in less than 14 minutes without optimization and use of additives. This amplification protocol was determined from varying the length of time at each amplification step, starting at 1s until amplification of all loci was completed. The electropherograms from the rapid amplifications using polymerase-1 (P1) and polymerase-2 (P2) are shown in Figure 61.

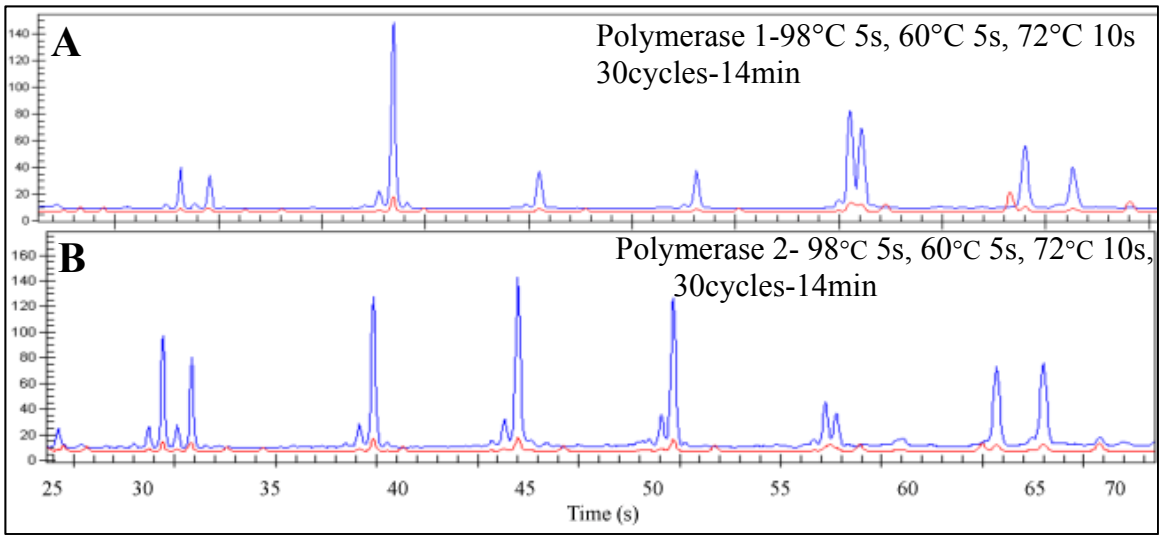


Figure 61: Electropherogram showing the comparison of the amplification of DNA standard 9948 performed in less than 14 minutes. (A) Rapid amplification using P1 (B) amplification performed with P2. The number of PCR cycles was held constant at 30 cycles and using 1ng DNA template.

Table 18: The summary of the rapid polymerase and direct buffer tests performed under rapid conditions. Cycling conditions were, 98°C for 5s, 60°C for 10s, 72°C for 10s. A total 30 cycles was completed in 14minutes.

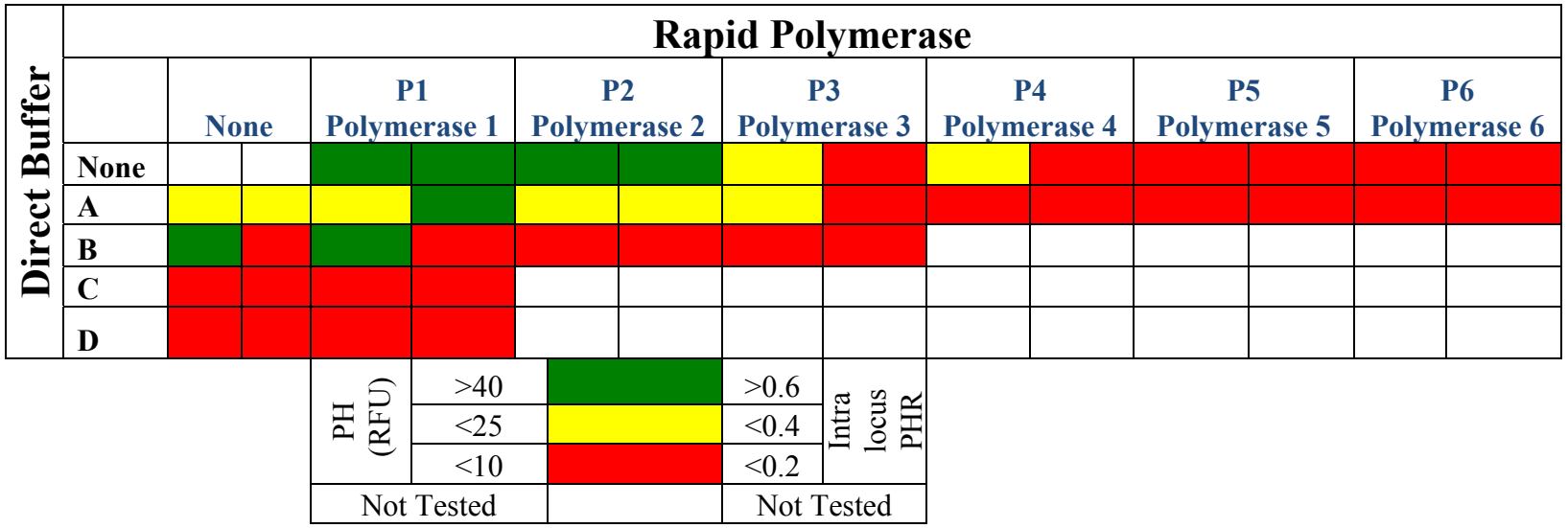


Table 19: P1 vs. P2 amplified using MP7 cycled 30 times at 98°C for 5s, 60°C for 5s, 72°C for 10s with a total amplification time of 14mins.

Polymerase	D5S8181		D13S317		D7S820		D16S539		CSF1PO		Penta D	
	PHR	Stutter %	PHR	Stutter %	PHR	Stutter %	PHR	Stutter %	PHR	Stutter %	PHR	Stutter %
P1	0.91	14	na	8	na	8	na	8	0.89	8	0.98	6
P2	0.79	20	na	14	na	14	na	18	0.74	10	0.98	4

Rapid amplification of DNA standard 9948 (Promega Corporation) using the MP7 kit showed a well-balanced amplification in 14 minutes for the P1 and P2. The 14-minute amplification performed with polymerase 2 on average produced peak heights 100% higher than with polymerase 1. On the other hand, P1 produced peak height ratios (PHR) that were increased at the D5S818 and CSF1PO markers from 0.79 to 0.91 and 0.74 to 0.89 respectively. The percentage of stutter were also calculated and decreased on averaged by 6% using P1. Table 19 summaries the peak height ratio and %stutter obtained using the two enzymes amplified in 14 minutes cycled 30 times at 98°C for 5s, 60°C for 5s, 72°C for 10s.

The ability of the enzymes to accurately amplify products at such fast rates may be partly attributed to the addition of a proofreading or 3'-5' exonucleases which is not present in traditional Taq enzymes. This allows for a higher fidelity polymerase. The speed or processivity of these enzymes allow them to incorporate nucleotides as fast as 100 bases per second. The mechanism that allows the polymerase to bind more rapidly is somewhat guarded. However, previous work suggests that the use of small proteins that bind with dsDNA help facilitate the rapid delivery of the enzyme to the template.[178, 179]

iii) Direct amplification from paper punches

Direct amplification involves the use of a cellulose-based paper (FTA paper) that contains a weak base, chelating reagents, anionic surfactants and uric acid that lyses cells and denatures proteins upon contact. The paper punch is directly added to the PCR mixture without the need for a prior extraction step. One key issue is the development of a specific buffer to solubilize and elute the DNA off of the paper punch while

concomitantly supporting amplification. This buffer should also contain components capable of binding to and reducing PCR inhibitors. In this research, as much as possible, existing chemistry was used to permit implementation and validation in a relatively a short time frame.

One of the first direct PCR methods without extraction was described by Mercier et al. in 1990 using whole blood samples.[180] Park et al. was able to demonstrate direct amplification using AmpFℓSTR® Identifiler® STR kit (Applied Biosystems, Foster City, CA) from FTA paper using with a 15 minute elution step and replacement of the kit buffer with AnyDirect buffer (BioQuest, Korea) in 2008.[181] A few years later, 2011, Wang et al. was able to eliminate the need for an elution step prior to amplification using AmpFℓSTR® Identifiler® Direct PCR kit in 1 ½ hrs.[45] Verheij et al. in 2012 reported a direct amplification of the AmpFℓSTR® SGM Plus™ STR kit in 47 minutes using a fast thermal cycler and rapid enzyme.[182]

While many of the components used in the formulation of a direct PCR buffer are proprietary, the ability to perform direct amplification often depends on the buffer composition, pH and salt content. The use of zwitterionic amino acids such as tricine, bicine, ACES, CHES, TAPS and HEPES that contain both an acid and base region facilitate more physiological ionic strengths and permit improved solubilization of the DNA into the buffer.[181, 183, 184] The pH of the buffer is adjusted depending on the enzyme being used, values between 7.5 and 9 are typical. In addition the use of non-reducing carbohydrates such as glycol, polyglycols and polyglycerols and non-reducing sugar derivatives such as sorbitol, trehalose and mannitol have been shown to help overcome

inhibitors that may be present.[185] Other PCR additives such as BSA, polyamines and surfactants can help with the efficiency of amplification without purification.[56]

The results from a variety of different direct buffers are listed in Table 18. The buffers are coded with a letter as they were tested outside the manufacturers suggested protocols. Results are reported in terms of peak height and peak balance between loci directly amplified from a paper punch. The initial direct amplification tests were performed using the conditions obtained with only the rapid polymerase. This starting point was chosen, as it was the fastest possible amplification that was obtained using high quality extracted DNA samples without the addition of the direct buffer. The direct buffer and rapid polymerase combination that produced amplifications under these conditions was then chosen and optimized. The initial cycling conditions were 98°C for 5s, 60°C for 5s, 72°C for 10s a total of 30 times with an overall amplification time of 14 minutes.

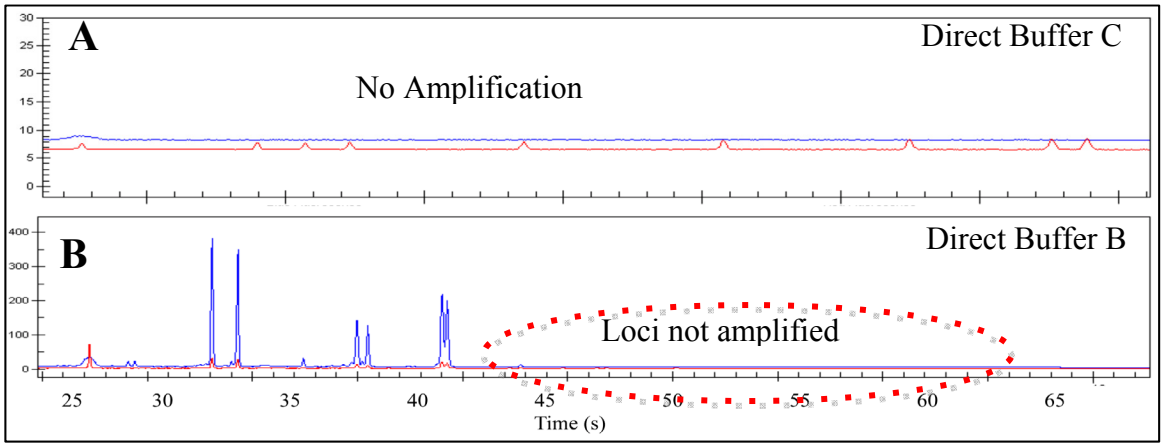


Figure 62: Electropherogram showing the comparison of two direct PCR kits performed under rapid cycling conditions from an FTA punch. The top panel A shows no amplification using the Direct buffer C. Panel B shows partial amplification with three loci dropped out of the MP7 using the Direct B. Both samples were amplified using polymerase 1.

The rapid polymerase 3 and direct buffer A combinations yielded no results once the amplification was sped up from about 40 to 14 minutes. (Figure 63) For this reason

these enzymes were rejected for further study. The rapid enzymes that permitted the amplification of DNA directly from a FTA punch were polymerase 1 and polymerase 2. However, it was observed that the polymerase 1 yielded better amplification especially at the larger loci when combined with the direct buffer A. (Figure 63) However, while all 7 loci were amplified the intra locus peak balance was still poor and a "ski slope" effect observed with the larger fragments. To help improve the balance of the larger loci a longer extension time (15s) was added to permit more balanced amplification. This changed the amplification time from 14 minutes to 16 minutes.

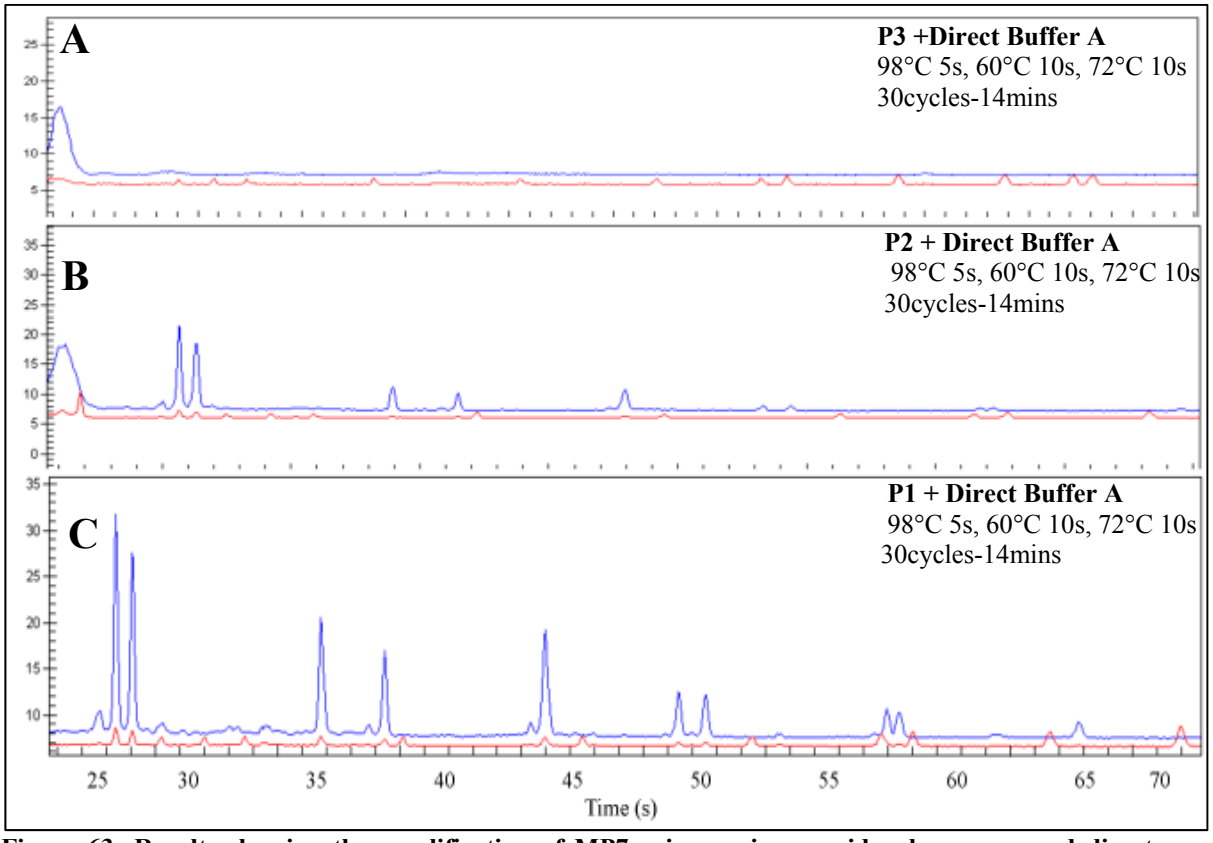


Figure 63: Results showing the amplification of MP7 using various rapid polymerases and direct buffer combinations. A show the results using rapid polymerase P3 with direct buffer A. Panel B shows the results using rapid polymerase P2 with direct buffer A. Panel C shows using rapid polymerase P1 and direct buffer A. The amplification was performed at 98°C for 5s, 60°C for 10s, 72°C for 10s and a total of 30 cycles with a total time of 14minutes.

iv) Rapid Direct PCR optimization

In order to optimize the use of rapid direct (RD-PCR) the following parameters were examined to develop robust, reliable results. All parameters were optimized using the Z-Taq Polymerase (P1) and AnyDirect F buffer (direct buffer A) using the cycling conditions listed in Table 17. Tests were performed to monitor the effect of additional quantities of enzyme, Mg^{2+}, dNTPs and BSA. The first parameter examined was the Mg^{2+} ion concentration. Magnesium acts as a co-factor to the Taq polymerase, increasing the concentration of Mg^{2+} improves the processivity of the enzyme.[16] However, excess levels of Mg^{2+} can result in a high error rate and increased incorporation of mismatched nucleotides resulting in non-specific products. The effects of increased Mg^{2+} concentrations on the rapid and direct amplification of the MP7 STR kit is shown in Figure 64. Overall inter locus ratios were calculated between the first and last loci (D5S818 and Penta D, respectively). Table 20 shows the results calculated, a value of 1 represents perfect balance between two alleles. The inter locus ratio remained fairly constant at 0.9 with increased Mg^{2+} concentrations at the both loci with exception at 12mM Mg^{2+} where the Penta D locus amplified poorly with an inter locus balance of 0.67. The peak height for the last allele decreased an average 87± 5% and 96 ± 6% for D5 and Penta D respectively, at 12mM Mg^{2+}. A final concentration of 4mM magnesium the highest sensitivity and inter/intra locus balance. At this concentration no additional Mg^{2+} needed to be added to the commercial buffer.

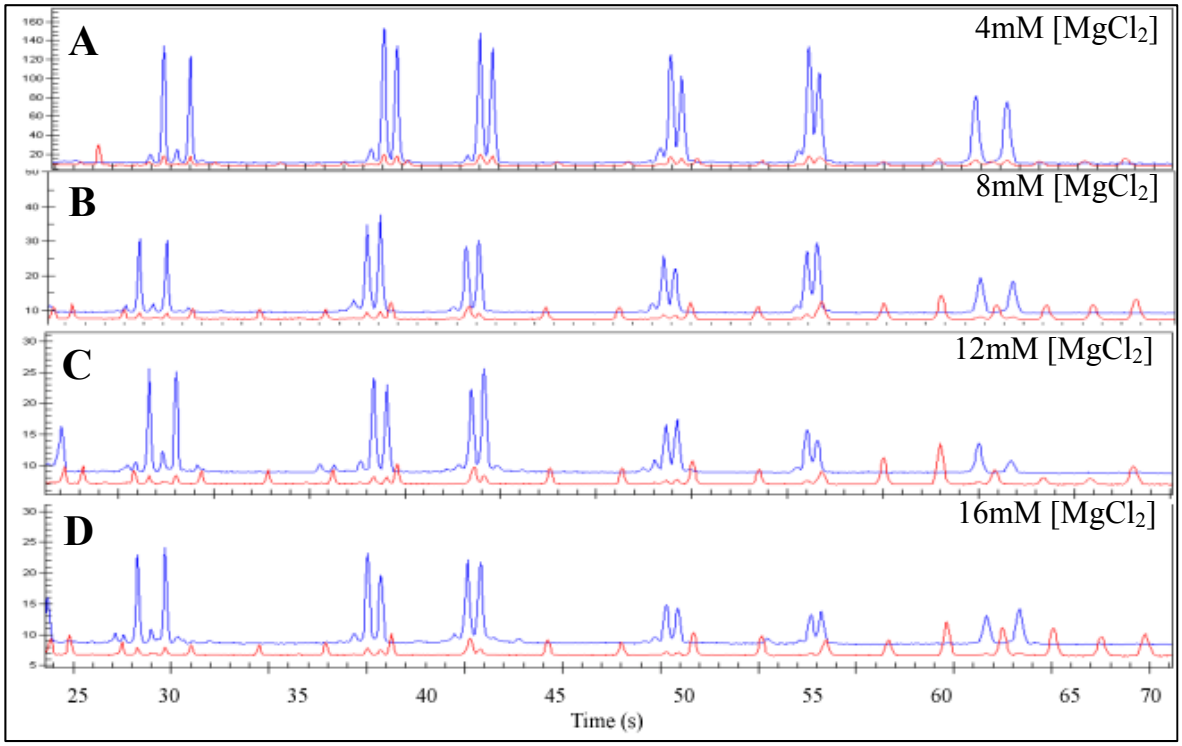


Figure 64: The electropherograms from the optimization of $MgCl_2$ for RD-PCR using MP-7 in a 16-minute amplification. Starting from the top, no additional $MgCl_2$ from buffer concentration 8mM, 12mM and 16mM. After 8mM $MgCl_2$ the effects of unspecific amplification and peak balance are observed. The best overall amplification is observed at 4mM [$MgCl_2$].

Table 20: Rapid-Direct amplification of MP7, Magnesium concentration study. MP7 amplifications were performed at 98°C for 5s, 60°C for 10s, 72°C for 15s, 30 cycles with a total time of 16minutes.

Final [Mg2+]	D5 PHR	D5 Peak Height Decrease	Penta D PHR	Penta D peak Height Decrease
4mM	0.93	--	0.91	--
8mM	0.97	83%	0.89	86%
12mM	0.94	87%	0.67	96%
16mM	0.93	88%	0.81	94%

A second factor that can affect the PCR is the concentration of dNTPs. dNTPs are the source of nucleotides that are used in the extension of bases along the DNA template. Excess dNTPs can inhibit the PCR and reduce the effect of the magnesium ions. While

lower concentrations can result in incomplete primer extension and premature termination of the PCR process.[186] Experiments were performed to examine the effect of the additional dNTPs on the rapid and direct amplification. Concentrations ranging from 0- 300µM extra dNTPs were tested. An addition of 300µM of each dNTP resulted in 150 ± 5% improvement in peak intensity for the largest alleles. These results may indicate that during rapid amplification the accessibility of dNTPs may be a limiting step in the primer extension especially for larger alleles.

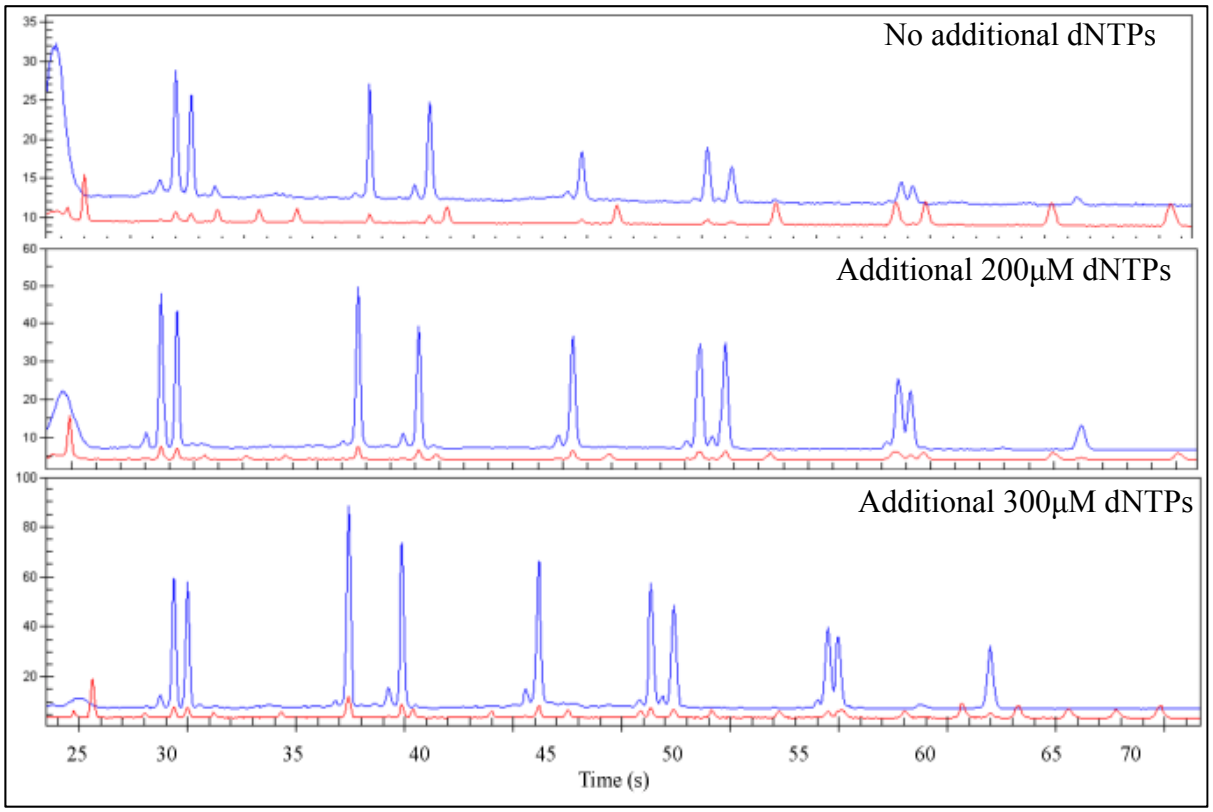


Figure 65:The effect of additional dNTPs on the amplification of RD-PCR using MP7. The addition of 200µM of each dNTP yielded better peak balance of larger size loci. MP7 amplifications were performed at 98°C for 5s, 60°C for 10s, 72°C for 15s, 30 cycles with a total time of 16minutes

Table 21: Rapid-Direct amplification of MP7, dNTPs concentration study. MP7 amplifications were performed at 98°C for 5s, 60°C for 10s, 72°C for 15s, 30 cycles with a total time of 16minutes.

Alleles	D5 A1	D5 A2	D13 A1	D13 A2	D7	D16 A1	D16 A2	CSF1PO A1	CSF1PO A2	Penta D
PHR at 200µM [dNTP]	0.9		0.8		--	0.99		0.91		--
PHR at 300µM [dNTP]	0.9		0.9		--	0.99		0.94		--
Peak Height Increase %	106	136	233	200	261	205	212	186	185	150

A variety of additives can be used to help improve the efficiency of the PCR reactions and reduce the effects of inhibitors. These additives include various detergents, buffer salts, denaturants and BSA. Mitigation of the effects of inhibitors is important as the sample from the FTA punch may contain a variety of environmental contaminants that will be added into the PCR mix without purification. A common additive that is used in most PCR reactions is BSA. BSA helps minimize absorption of DNA onto the walls of the PCR tube and helps stabilize the enzyme, minimizing the effects of inhibitors such as humic acid and hematin.[62, 187]With this in mind, we examined the effects of the addition of BSA from final concentrations of 0.02 to 0.08µg/µL on the rapid and direct PCR amplification. As shown in Figure 66, the increase in BSA concentration resulted in a 200 ± 4% and 500 ± 6% increase in peak height at D5S818 and Penta D respectively. (Table 22) The inter locus balance was also increased from 0.23 to 0.93 at the largest locus with 8 times BSA. A final total concentration of 0.08µg/µL BSA yielded the best overall amplification. Further addition of BSA did not produce additional improvements in peak height most likely due to the fact that elevated levels of BSA can be inhibitory. Table 22 summarizes the results from the BSA study.

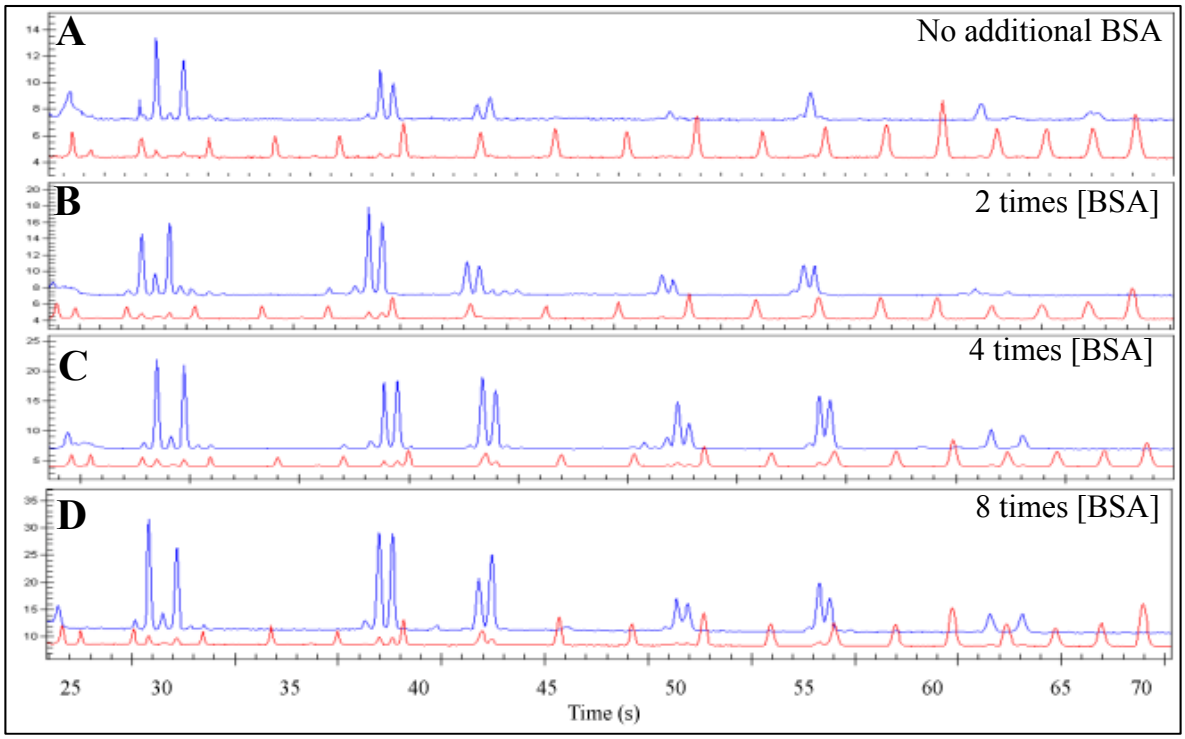


Figure 66: Electropherogram showing the effect of the addition of BSA to the amplification of RD-PCR. The increase of BSA leads to a higher yield and a more balance amplification across all loci.

Table 22: Rapid-Direct amplification of MP7, BSA concentrations study. MP7 amplifications were performed at 98°C for 5s, 60°C for 10s, 72°C for 15s, 30 cycles with a total time of 16minutes.

[BSA] Increase Factor	D5 PHR	D5 Peak Height Increase	Penta D PHR	Penta D Peak Height Increase
0	0.93	--	0.23	--
2	0.91	138%	0.88	165%
4	0.93	228%	0.7	304%
8	0.92	232%	0.93	516%

An increase in the concentration of the enzyme can also affect the sensitivity of the amplification. High polymerase concentrations can result in easier access of the enzyme complex to the template binding regions, improving the processivity of the Taq enzyme. However, high levels of enzyme can produce non-specific amplification, while

low levels may reduce the amplification efficiency.[16, 186] Many commercial kits are designed to use the smallest amount of polymerase possible to maintain a low cost per reaction. During the amplification of MP7 under rapid and direct PCR conditions, it was found that 1.25U of polymerase was sufficient and provided a well overall balanced amplification. Doubling the polymerase concentration (2.5U) increased overall peak height for the reaction, up to 90 ± 4%. However, due to cost concerns a final concentration of 1.25U was used for further samples.

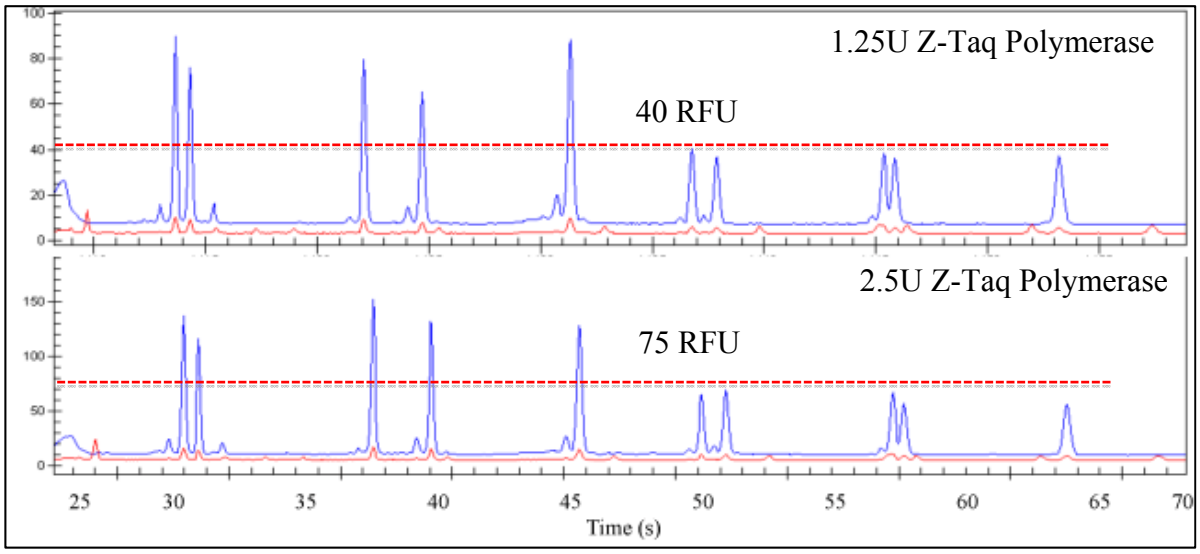


Figure 67: The effect of enzyme concentration on the amplification of MP7 using RD-PCR. The sensitivity of the amplification is almost doubled with double the amount of enzyme.

v) Thermal cycling parameters

Cycling parameters were also investigated to determine the minimum length of time required at each stage in the PCR process that still produced a balanced electropherogram across the entire multiplex with acceptable peak heights.

The first parameter examined was the denaturing time. It is believed that denaturation occurs instantaneously above a certain temperature.[175] Thus, an increase in time above this minimum should not affect amplification. Denaturation times from 1 to 30 seconds were examined. At denaturation times between 1 and 4 seconds, a small decrease in amplification peak heights were observed. This decrease was likely due to small differences between the temperature of the heating block and the sample itself resulting in slightly less enzyme activity. After 5s no increase in peak height or balance was observed. A denaturation time of 5s was found to be optimum to maintain rapid amplifications times.

The second parameter examined was the annealing time. During this process the primer needs sufficient time to properly bind to the target region. Most of the primers within the MP7 multiplex kit are less than 24 bases in length. These are relatively short sequences and when designed properly can bind specifically to the target region in less than 1 second.[175] However, annealing times of less than 5s resulted in non-specific peaks. These non-specific peaks associated with shorter annealing times were attributed to non-specific binding during the primer extension process. When the annealing time was increased to 5s, the non-specific peaks disappeared. At 5s annealing, good specificity of primer binding occurred while maintaining rapid amplification.

The most important cycling parameter is the extension time. During this process the polymerases must have sufficient time to incorporate all nucleotides necessary to fully complete extension of the target region. The smaller amplicons are more quickly completed, resulting in a ski slope effect in which large amplicons are progressively less well amplified if the time is too short. The processivity of the P1 enzyme is

approximately 67 nucleotides per second requiring at minimum of 7.5 seconds to complete the extension of a 500bp amplicon. However, due to number of loci in the STR kit we anticipated the need for slightly longer extension times due to the nature of the reaction and competition of reagents in solution. In order to overcome this issue from amplicons that span over a wide size range, the enzyme must have sufficient time to complete the full extension of all target regions. Extension times were examined from 5 to 25 seconds. A minimum time of about 10s was needed for the MP7 multiplex to amplify, however, under these conditions, the larger loci such as the CSF1PO and Penta D had a 72 ± 4% decrease in peak height when compared to the shorter ones. The most optimum extension time for rapid amplification was at 15 seconds. Under these conditions both inter and intra loci balance was achieved.

vi) Validation and Concordance

In order to establish the robustness and accuracy of the rapid direct PCR system, a validation study was performed on a small population of 18 individuals. Each person provided a buccal swab and their DNA was transferred onto FTA paper. The sensitivity, effects of reaction volume, effects of inhibitors, effects of degradation and the stability were all examined. The rapid and direct PCR using the MP7 STR kit was also tested for concordance with the commercial PowerPlex16 HS kit (Promega Corporation).

1) Sensitivity

The amount of input DNA template was varied from 0.625ng to 5ng. (Figure 68) The peak heights were compared using the smallest size locus (D5S818) and the largest sized locus (Penta D) on the microchip system. The peak heights for the rapid and direct

amplification using 5ng template DNA were 220 and 143 RFU at the smallest and largest amplicons respectively. (Table 23) At 0.625ng input DNA template, the peak heights decreased by a factor of six, to 36 ± 4 and 25 ± 1 RFU respectively. At 0.625ng template DNA the amplification maintained balance across all loci with a signal to noise ratio of 300 ± 20. (Figure 69) While the system is well capable of detecting lower levels of DNA, it is not typical for single source reference samples to contain less than 0.5ng of DNA.

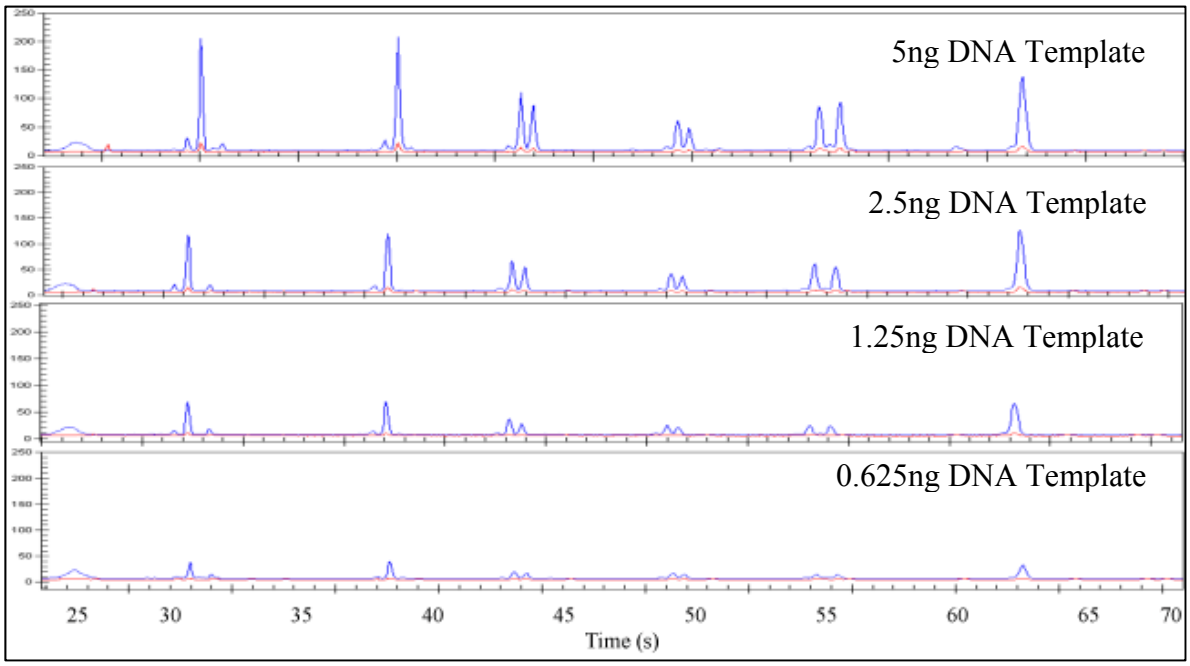


Figure 68: Electropherogram showing the results from the sensitivity study. From the top 5ng, 2.5ng, 1.25ng and 0.625ng input DNA template (Figure 69) respectively. The peak heights of the alleles decrease by a factor of 1.5, 2.5 and 6 respectively.

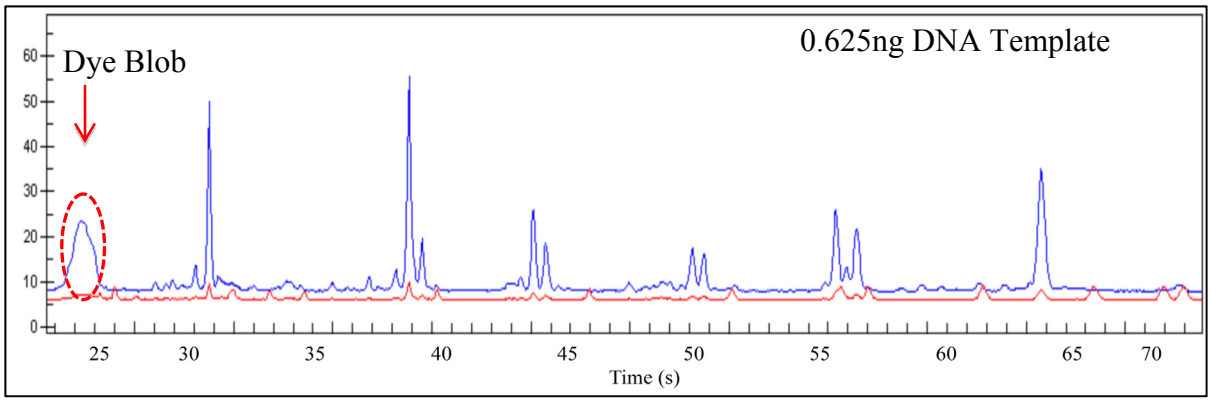
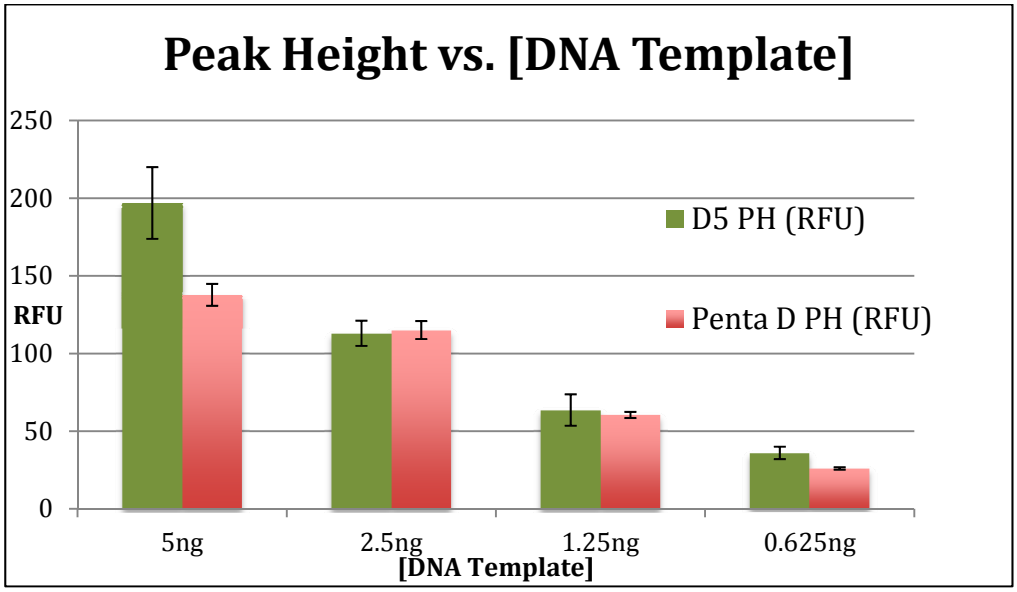


Figure 69:Electropherogram showing a close up of the amplification with 0.625ng DNA template. At the smallest size locus, D5S818 the allele has a peak height of 36.09 RFU. At the largest size locus, Penta D the alleles has a peak height of 25 RFU.

Table 23: The results from the RD-MP7 Sensitivity Study showing the allele peak heights at D5S818 and Penta D along with their decrease factors. Below is the graph of the peak heights versus the amount of input template DNA.

[DNA Template]	D5 PH (RFU)	STDEV	Decrease factor	Penta D PH (RFU)	STDEV	Decrease factor
5ng	197.0	±23.1	NA	137.8	±7.1	NA
2.5ng	113.1	±8.1	1.7	115.2	±5.8	1.2
1.25ng	63.7	±10.1	3.1	60.5	±2.0	2.3
0.625ng	36.1	±4.0	5.5	26.1	±0.8	5.3



2) Reaction volume

The reaction volume of the RD-PCR master mix was examined from 12.5µL to 25µL. It has been reported that the use of higher reaction volumes may help overcome inhibition by dilution.[188, 189] However, the results from this study showed no differences in peak height with the larger 25µL reaction volume or its efficiency in helping to overcome inhibition during amplification. Therefore, a final reaction volume of 12.5µL was found optimum with added benefits of reduced cost per reaction.

3) Effects of inhibitors

In order to simulate real life samples and effects of inhibitors that may be found in buccal swab samples; coffee, 40% alcohol/volume 1919 premium rum (Angostura,Trinidad) and Coca-Cola soda were examined. Subjects who had drunk a glass of one of the three substances were buccal swabbed 1 minute later. The samples were then transferred to FTA and analyzed using MP7 kit under RD-PCR conditions. The results in Figure 70 show the effects of coffee, soda and alcohol on the amplification. The Coca-Cola soda did not affect the amplification. However, coffee and the alcohol samples produced a 50± 6% decrease in peak heights and a number of non-specific amplification peaks. These results may suggest that inhibition of the enzyme is taking place. The exact mechanism in which these two substances affect the amplifications needs to be examined in future work.

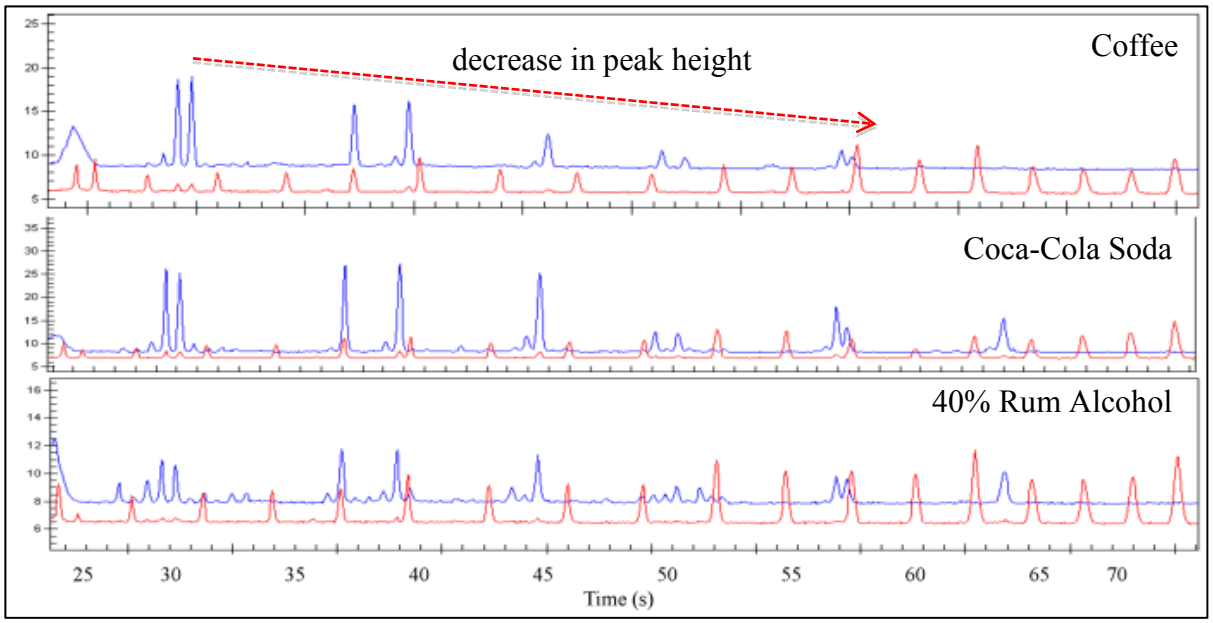


Figure 70: The effects of Coffee, Soda and Alcohol on the amplification directly from a FTA punch. Coffee and Alcohol shows a decrease in peak heights and increase of non-specific peaks.

4) Degradation

The ability of rapid and direct PCR to analyze degraded DNA samples was examined by exposing FTA paper containing a buccal swab under an Ultralum UVC-508 cross-linker for 10 to 300 seconds. After 10s exposure at the highest intensity UV setting, the sample could still be genotyped accurately. However, small non-specific fragments were seen between 100-130bp. After 30s of exposure, degradation of the amplicons was observed with a 30% decrease in peak height. After 300s the alleles were not recognizable and non-specific peaks observed.

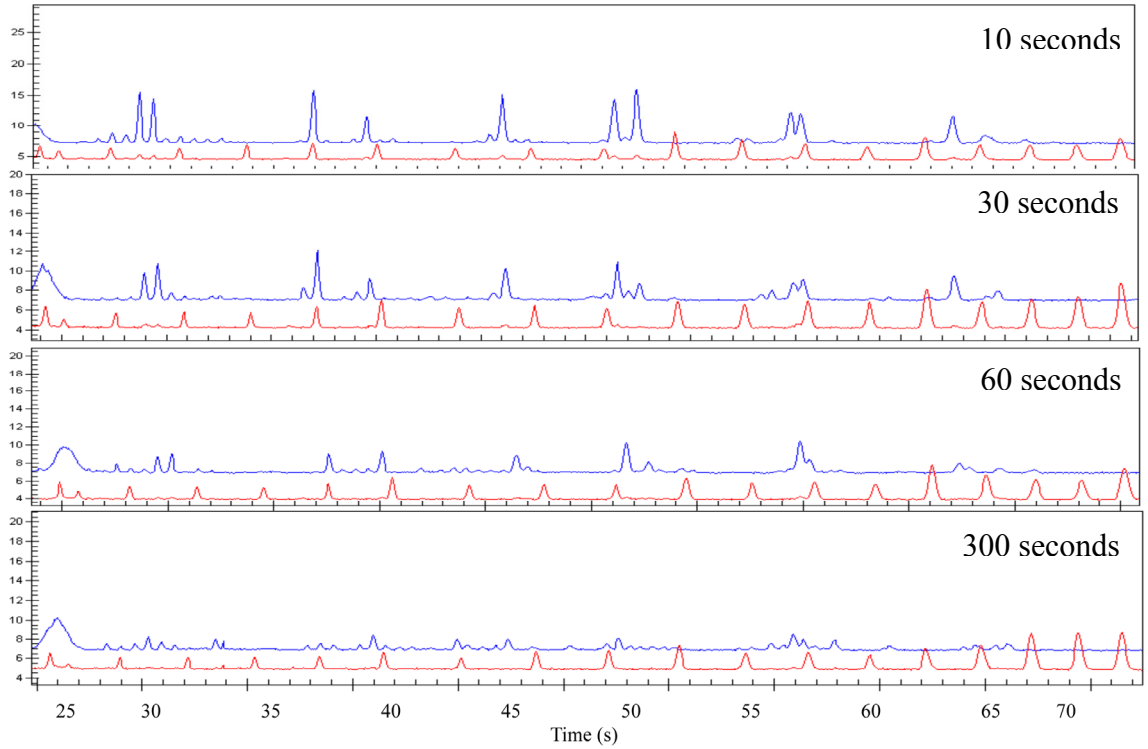


Figure 71: The effect of UV radiation at various exposure time intervals from 10s to 300s. A steady decay in the sample can be seen starting at the largest size fragments to the smallest sized fragments.

5) Stability

We also examined the stability of the sample on the FTA paper over a set period of time. The samples were examined at 3 months and 6 months following collection and amplified. Prior to 3 months the sample shows no decrease in peak height and balance. Figure 72 shows the results obtained from 3months versus 6 months. At 6 months, a 80± 4% decrease in peak heights were observed across all loci. The results at 3 months showed a similar loss in peak height.

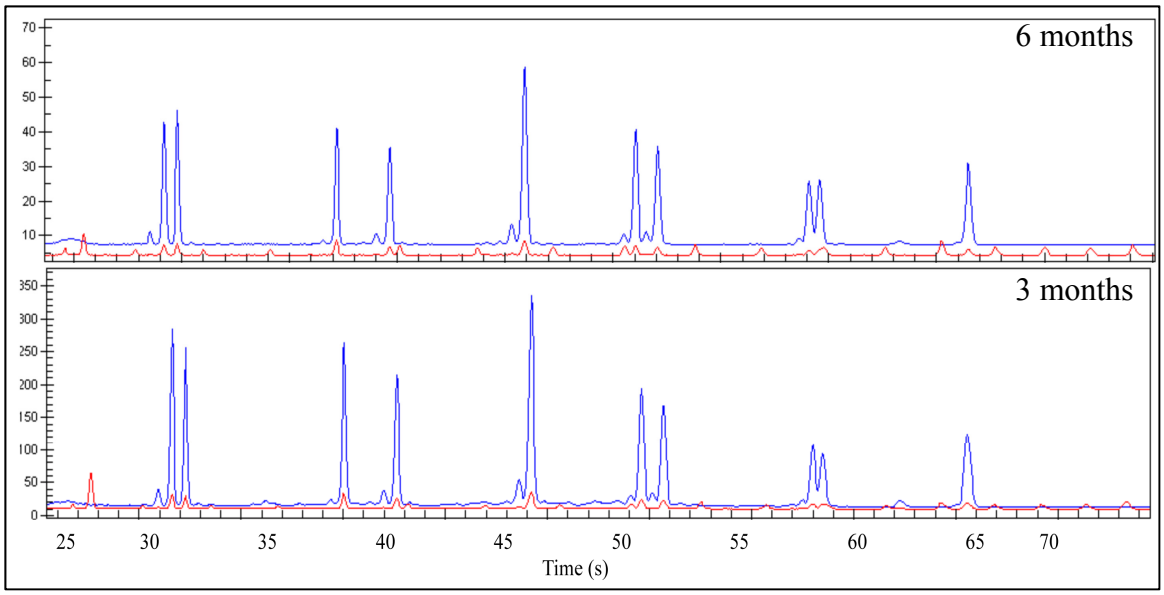


Figure 72: Stability of samples on FTA paper then directly amplified using the MP7 kit at RD-PCR conditions. At 3 months samples remain stable with peak heights above 150 RFUs. At 6 months peak heights decrease average 80%. The peak height ratios remained constant.

6) Concordance

A small concordance study of 18 individuals was performed using a comparison between the rapid and direct amplification of the MP7 multiplex kit and a traditional amplification using PowerPlex 16 HS (Promega Corporation) on the ABI 310 Genetic Analyzer. The results are summarized in Table 24. The samples showed a 99.5% concordance of total alleles (Table 25). Only one samples had an allele drop out at the largest size Penta D locus. This sample showed 80% overall lower peak heights when compared to the other samples. This was most likely as a result of an insufficient input of DNA template from the swabbing. The electropherograms from two of the samples used in the concordance study are shown in Figure 73 and Figure 74. The reproducibility of the system was demonstrated by the use of multiple allelic ladder runs showing precision value of approximately 0.08bp across all fragments (Table 26).

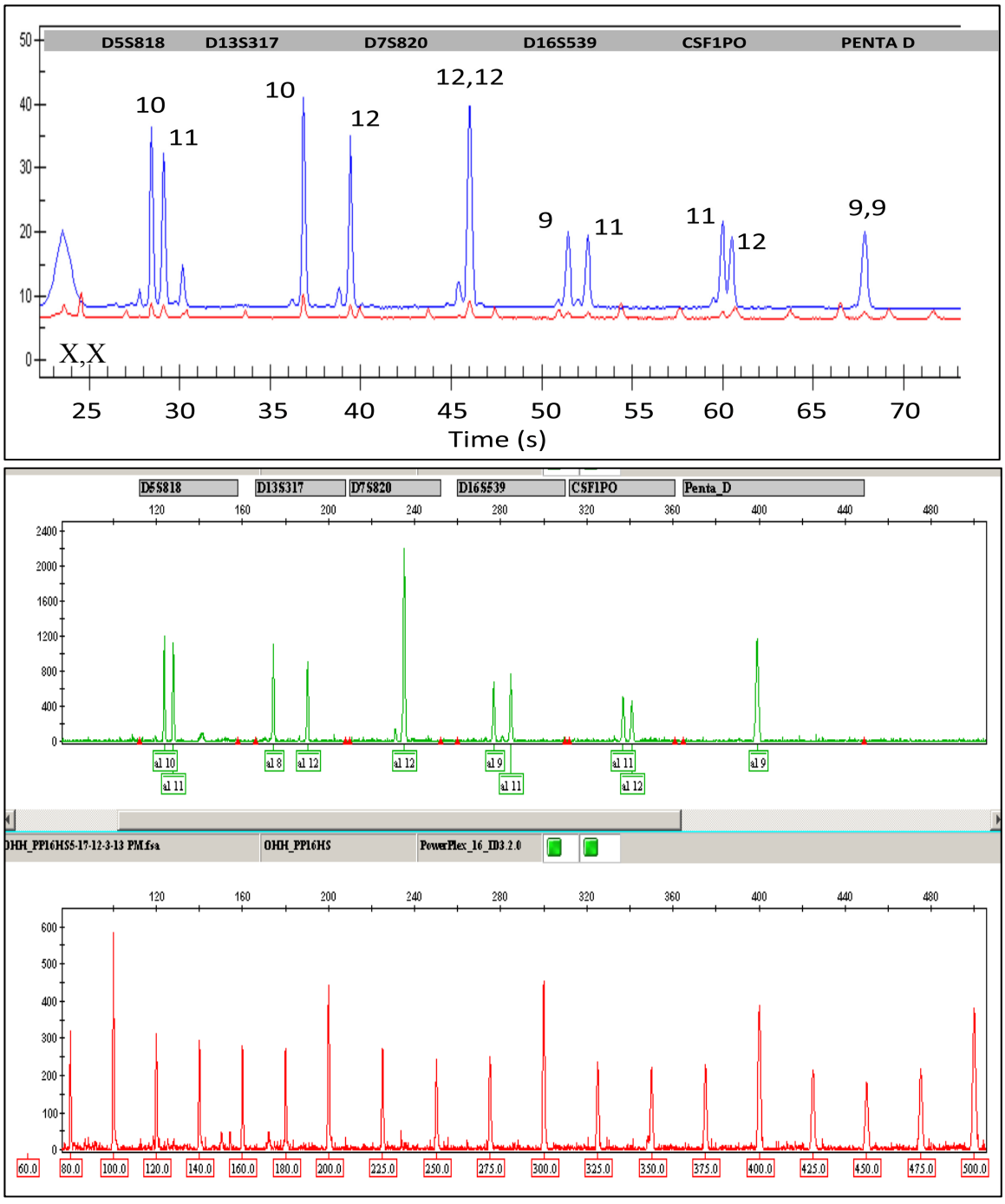


Figure 73: The electropherogram of sample 1 using RD-amplification of the MP7 STR kit (TOP) and the PP16HS kit on an ABI 310 Genetic Analyzer (BELOW).

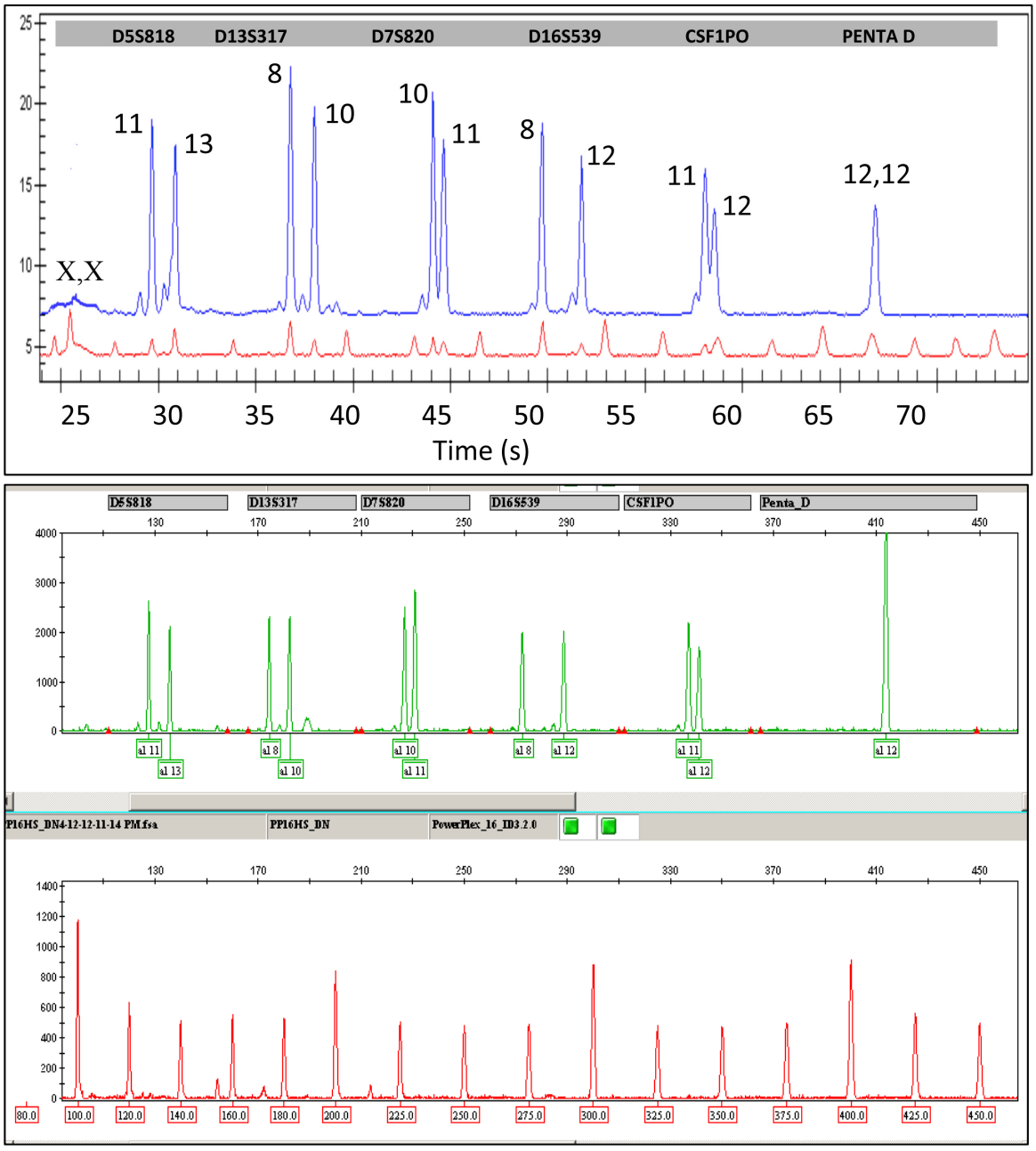


Figure 74: The electropherogram of sample 7 using RD-amplification of the MP7 STR kit (TOP) and the PP16HS kit on an ABI 310 Genetic Analyzer (BELOW).

Table 24: Genotype Concordance of 18 individuals from RD-PCR compared to PP16 HS

Sample No	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D
1	10,11	8,12	12,12	9,11	11,12	9,9
2	10,13	11,13	11,11	11,13	12,12	12,13
3	12,12	10,12	10,12	9,12	11,12	12,12
4	9,11	8,12	11,12	12,12	10,11	11,11
5	7,11	8,11	11,12	11,12	10,10	9,12
6	7,10	8,12	11,12	11,12	10,11	9,9
7	11,13	8,10	10,11	8,12	11,12	12,12
8	9,11	11,12	8,9	12,13	11,12	10,13
9	9,11	9,14	11,12	10,12	11,13	9,9
10	7,1	8,9	11,11	11,12	10,12	11,13
11	10,13	8,12	8,12	12,13	12,12	12,13
12	9,11	12,12	10,12	11,11	10,10	10,13
13	12,12	11,13	9,12	11,11	11,12	10,13
14	7,11	8,11	11,12	9,10	9,12	12
15	11,13	9,14	8,10	11,13	10,12	10,12
16	13,13	11,11	10,13	12,13	12,12	10,11
17	11,13	12,13	10,11	11,12	12,12	12,14
18	12,12	11,12	10,12	9,13	10,11	11,12

Table 25: Summary of detectable alleles using RD-amplification on microchip

No. of individuals	18
Total alleles	216
No. of alleles present	215
No. of alleles absent	1
Allele % observed	99.5%

Table 26: Allelic Ladder sizing reproducibility

Loci	Allele	Ladder 1	Ladder 2	Ladder 3	Ladder 4	Ladder 5	Avg value	STDEV
D5	**7**	115.04	114.96	114.88	115.05	114.82	114.95	0.10
	8	119.03	118.96	118.9	119.05	118.84	118.96	0.09
	9	123.05	122.98	122.9	123.07	122.88	122.98	0.09
	10	127.08	126.99	126.91	127.06	126.86	126.98	0.09
	11	131.07	131	130.9	131.07	130.89	130.99	0.09
	12	135.1	135.03	134.94	135.1	134.9	135.01	0.09
	13	139.15	139.05	138.97	139.14	138.98	139.06	0.09
	14	143.14	143.08	142.98	143.15	142.97	143.06	0.09
	15	147.13	147.06	146.99	147.15	146.96	147.06	0.08
	16	151.18	151.11	151.02	151.17	151	151.10	0.08
D13	**7**	175.08	175.05	175.02	175.08	175.02	175.05	0.03
	8	179.23	179.21	179.14	179.22	179.14	179.19	0.04
	9	183.31	183.3	183.24	183.34	183.22	183.28	0.05
	10	187.43	187.41	187.37	187.47	187.36	187.41	0.04
	11	191.53	191.5	191.48	191.57	191.46	191.51	0.04
	12	195.6	195.6	195.56	195.63	195.55	195.59	0.03
	13	199.68	199.62	199.61	199.69	199.56	199.63	0.05
	14	203.77	203.76	203.7	203.78	203.69	203.74	0.04
	15	207.91	207.83	207.82	207.91	207.79	207.85	0.05
D7	**6**	214.66	214.6	214.5	214.67	214.51	214.59	0.08
	7	218.77	218.69	218.68	218.81	218.62	218.71	0.08
	8	222.9	222.83	222.74	222.94	222.72	222.83	0.10

	9	227.03	226.96	226.91	227.09	226.89	226.98	0.08
	10	231.14	231.09	231.04	231.21	230.99	231.09	0.09
	11	235.3	235.23	235.2	235.3	235.13	235.23	0.07
	12	239.41	239.35	239.31	239.42	239.27	239.35	0.06
	13	243.55	243.52	243.47	243.57	243.46	243.51	0.05
	14	247.68	247.64	247.6	247.7	247.59	247.64	0.05
D16	**5**	261.53	261.47	261.41	261.56	261.46	261.49	0.06
	8	273.37	273.37	273.3	273.45	273.37	273.37	0.05
	9	277.42	277.41	277.36	277.52	277.39	277.42	0.06
	10	281.34	281.27	281.27	281.41	281.33	281.32	0.06
	11	285.33	285.27	285.21	285.38	285.27	285.29	0.06
	12	289.25	289.22	289.17	289.33	289.17	289.23	0.07
	13	293.13	293.14	293.16	293.26	293.09	293.16	0.06
	14	297.11	297.02	297.05	297.17	297.04	297.08	0.06
	15	301.04	300.99	300.99	301.12	300.98	301.02	0.06
CSF	**6**	322.26	322.19	322.26	322.3	322.28	322.26	0.04
	7	326.42	326.38	326.31	326.48	326.46	326.41	0.07
	8	330.64	330.54	330.51	330.68	330.65	330.60	0.07
	9	334.73	334.75	334.67	334.84	334.76	334.75	0.06
	10	338.95	338.88	338.82	338.97	338.91	338.91	0.06
	11	343.09	343.02	342.99	343.07	343.04	343.04	0.04
	12	347.17	347.11	347.12	347.25	347.17	347.16	0.06
	13	351.36	351.27	351.21	351.33	351.19	351.27	0.07
	14	355.41	355.39	355.3	355.46	355.36	355.38	0.06
	15	359.43	359.46	359.35	359.53	359.44	359.44	0.06
Penta D	**2.2**	376.9	376.83	376.89	376.94	376.89	376.89	0.04
	3.2	382.09	382.09	382.07	382.18	382.06	382.10	0.05
	5	390.14	390.22	390.2	390.39	390.22	390.23	0.09
	7	400.26	400.39	400.39	400.4	400.33	400.35	0.06
	8	405.57	405.52	405.58	405.76	405.59	405.60	0.09
	9	410.5	410.51	410.48	410.63	410.6	410.54	0.07
	10	415.55	415.67	415.57	415.72	415.67	415.64	0.07
	11	420.46	420.53	420.54	420.66	420.57	420.55	0.07
	12	425.48	425.48	425.65	425.61	425.56	425.56	0.08
	13	430.76	430.82	430.85	430.96	430.83	430.84	0.07
	14	436	435.9	436.08	436.09	436.02	436.02	0.08
	15	441.14	441.13	441.31	441.32	441.25	441.23	0.09
	16	446.12	446.15	446.23	446.31	446.3	446.22	0.09
	17	451.35	451.45	451.53	451.51	451.52	451.47	0.07

d) Conclusions

The development of a rapid and direct PCR amplification was achieved by use of the combination and optimization of, a high-speed thermal cycler, a rapid polymerase, and a direct buffer. A number of enzymes and direct buffer were examined outside of manufacturers' protocols to permit short cycling times. The rapid polymerase, Z-Taq combined with the direct buffer, AnyDirect F buffer, showed the best balanced electropherogram and was optimized for use with the MP7 multiplex kit. The rapid and direct system was capable of performing amplification of the 7 STR loci (MP7) directly from a paper punch without an extraction in 16 minutes.

The system was then validated using a small population of 18 individuals. The sensitivity of the system was capable of amplifying a full STR profile with 0.625ng or lower input DNA template. The rapid and direct amplification coupled with the microfluidic separation demonstrated a robust, reliable genotyping with a 99.5% accuracy of alleles called. The entire process is rapid and reliable, reducing the time from sample to genotype from 1-2 days to under 20 minutes.

CHAPTER XI: CONCLUSIONS

The overall goal of this project was to develop an ultra-fast and direct PCR amplification of a subset of CODIS STR markers for forensic genotyping. The system developed was able to quickly identify persons of interest and could easily be deployed to remote locations for rapid in-field analysis. In this project, several new pentameric and tetrameric STR multiplexes were prepared and adapted for use with a short channel microfluidic instrument. To further improve the speed of analysis, a number of direct PCR procedures were identified, adapted and optimized for use with ultra-high speed thermal cycling. The overall result was an analytical procedure capable of producing a forensic genotype in under 20 minutes.

Prior to this work, short channel microfluidic devices were not capable of producing sufficient resolution to permit the analysis of STR markers of forensic interest. To overcome this problem a set of 3 pentameric STRs were designed with small amplicon sizes to permit optimal resolution. These loci also had a larger size repeat unit than the more common 4 base STRs and in addition had fewer microvariants and reduced stutter. To further improve resolution a denaturing sieving matrix was applied to the microfluidic device resulting in a 70% increase in resolution from the traditional native separation, permitting baseline separation of all loci even when using channel lengths as short as 1.5 cm.

The denaturing sieving matrix consisted of a combination of HEC and PVP linear polymers in a TAPS buffer. It was determined that an optimal polymer composition of 3.5-4% w/v and with a ratio of 80-85% HEC relative to PVP was optimal for the glass

microchip system. Higher molecular weight polymers further improved the resolution, but increased viscosity to an unacceptable level, causing loading issues with the chip due to excessive backpressure. The optimal viscosity that could be reliably loaded was approximately 300cP using a 3.5% w/v mixture of 80% HEC/PVP. An electric field strength of 350V/cm provided fast separations with minimal joule heating effects and peak broadening.

The addition of an enhanced denaturing sieving matrix for ssDNA separation coupled with the addition of a second detection channel permitted the use of an internal size standard which resulted in a sizing precision better than ± 0.2bp across a 450bp size range in under 80 seconds. A subset of CODIS STR markers (MP7) was developed for use with this system that provided discrimination powers of greater than 1 $x10^6$ and was capable of fully resolving all alleles. Run times were under 80 seconds for a 7 loci STR multiplex.

The development of a rapid and direct amplification using the MP7 multiplex kit was achieved by combining a rapid polymerase (Z-Taq), with AnyDirect Buffer F and a high-speed thermal cycler that permitted rapid and reliable results. This system reduces the time from sample to genotype from 1-2 days to under 20 minutes. Operation requires minimal equipment and can be easily performed with a small high-speed thermal cycler, reagents, and a microfluidic device with a laptop. The system was optimized and validated using a number of test parameters and a small test population. The overall precision was better than 0.17 bp.

The overall system reduced the time from sample to genotype from 1-2 days to less than 20 minutes. The small footprint of the system allows for easily deployment at places such as police stations or boarder crossings and permits the rapid identification.

CHAPTER XII: FUTURE WORK

Future work involved with this project may include a more detailed validation and quality assurance study on a variety of forensic samples including, low copy number and mixtures. This would help characterize the robustness of the method as well as address issues commonly encountered with the quality of forensic evidence. Further expansion may also be done to develop several STR multiplex kits to permit the analysis of all 13 CODIS loci. This would help increase the power of discrimination and its usefulness in the courtroom. Alternatively, the development of multicolor microfluidic systems that have more than two channels may also permit larger multiplex sizes and increase the number of STRs that could be analyzed simultaneously.

An investigation into the kinetics of the rapid polymerases and interactions with the chemicals directly involved with permitting the direct amplification may also be further characterized. The use of a rapid-direct PCR combined with fast separations can also be applicable to other scientific fields such as, the detection of diseases for medical purposes, to track animals, food contamination concerns and environmental applications.

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APPENDICES

Appendix 1

Stock Solutions

0.5M EDTA, 500 mL: 93.05 g Ethylenediaminetetraacetic acid di-sodium salt dehydrate to 400 mL ddH2). Adjust pH to 8.0 with 10N NaOH. Adjust final volume to 500 mL.

20% SDS, 500 mL: Dissolve 100 g sodium dodecyl sulfate in 400 mL ddH_2O. Heat solution to dissolve SDS completely. Adjust final volume to 500 mL.

1M Tris-HCl, pH 8.0, 500 mL: 60.55 g Trizma base in 400 mL ddH_2O. Adjust pH to 8.0 with HCl. Adjust final volume to 500 mL.

10 N NaOH, 100 mL. 40 g NaOH to 70 mL ddH_2O. Adjust final volume to 100 mL. Store in plastic bottle.

Buffers:

TE (10mMTris-HCl/ 0.5 mM EDTA, pH 8.0), 500 mL: Add 5 mL of 1 M Tris-HCl and 100 uL of 0.5 M EDTA to 395 mL ddH_2O. Autoclave.

310 Buffer 10X (1M TAPS, 20 mM EDTA pH 8.0), 100 mL: Add 24.33 g TAPS and 4 mL 0.5M EDTA to 70 mL ddH_2O. Adjust pH to 8.0 with 10N NaOH. Adjust final volume to 100 mL.

Hair Extraction Buffer (HEB) (10mM Tris HCl, pH 8.0, 100 mM NaCl, 5 mM CaCl2, 2% SDS, 39 mM dithiolthreitol (DTT)), 500 mL: 5 mL Tris HCl, 50 mL 20% SDS, 2.922 g NaCl, 0.2775 g CaCl2 to 350 mL ddH_2O. Adjust final volume to 500 mL. Add 6 mg/mL DTT when ready to use. Keeps for 2 weeks in the fridge after DTT is added.

Differential Lysis Buffer (DEB) (100 mM NaCl, 10 mM EDTA, 0.4% SDS), 500 mL: 2.92 g NaCl, 10 mL 0.5 M EDTA, 10 mL 20% SDS. Adjust final volume to 500 mL with ddH_2O.

Stain Extraction Buffer (SEB) (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 2% SDS, 38 mM DTT), 500 mL: Dissolve 2.92 g NaCL in 250 mL ddH_2O. Add 5 mL 1M Tris, 10 mL 0.5 M EDTA, 50 mL SDS. Titrate to pH 8.0 with HCl. Adjust final volume to 500 mL with ddH_2O. Add 6 mg/mL DTT when ready to use. Keeps for 2 weeks in fridge after DTT is added.

Appendix 2

Protocol:
Buccal Swab Extraction

I. Purpose

To extract and purify human DNA from buccal swab samples.

II. Safety

All laboratory safety procedures will be complied with during this procedure.

III. Reagents, Supplies, and Equipment

Reagents

PCR ddH_2O
Stain Extraction Buffer
Dithiothreitol
Proteinase K (600U)
Phenol/Chloroform/Isoamyl Alcohol (25:24:1) (PCIA)
5% Bleach solution
ddH_2O

Supplies

Wire cutters
Weighing papers or boats.
100-1000 µL pipettor
10-1000 µL barrier pipet tips
10-100 µL pipettor
10-100 µL pipet tips
0.5-10 µL pipettor
10 µL pipet tips
2 mL spin basket compatable tubes and spin baskets
Microcon YM-30 spin columns
15 mL capped conical bottom tubes
gloves
Kimwipes

Equipment

Temperature-controlled water bath set at 56ºC
Sample tube holder
Microcentrifuge
Analytical balance

IV. General

1. Procedure will be used for extracting and purifying human DNA from buccal swab samples.
2. Procedure will be used as necessary for research.
3. Gloves should be worn at all times.

IV. Procedure

1. Preparation of samples

   a. Use the wire cutters to cut the end of a buccal swab.
   b. Place the cut swab in the bottom of a 2 mL tube.
   c. Rinse wire cutter blades with ddH2O, bleach, and again with ddH2O between samples. Use a new weighing paper for each sample.

2. Preparation of stain extraction buffer (SEB).
   a. Weigh out 6 mg of Dithiolthreitol (DTT) per mL of SEB to be used.
   b. Add DTT to filtered SEB in 15 mL tube, and mix. May be stored up to two weeks in the refrigerator.

3. Add 300µL of SEB to each swab sample and to one reagent blank.
4. Add 2 µL of Proteinase K to each sample and to the reagent blank.
5. Make sure caps are secure, and spin tubes down to ensure swab is covered by the SEB solution.
6. Place tubes in tube holder, and place tube holder across water bath well so that the bottom of the tubes is covered by the water in the water bath.
7. Incubate samples at 56ºC for 2-4 hours.
8. Remove tubes from holder, wipe dry with Kimwipes, and spin down in microcentrifuge.
9. Remove swab sample from tube and place in spin basket. Place spin basket back in tube, cap, and spin in microcentrifuge for 1 minute at 5000 rpm.
10. Remove spin basket from tube and throw away.
11. Add 300 µL PCIA to each sample. Mix and spin at 5000 rpm from 3 minutes.
12. Assemble Microcon filters by placing filter cup, blue side up, into 2 mL tubes. Label with sample name/number.
13. Remove aqueous (top layer) from sample tube with 100 µL pipettor and tips. Transfer to filter cup of Microcon (2-3 transfers). Make sure not to remove any of the organic layers.
14. Cap tubes and spin at 13000 rpm for 12 minutes.
15. Dispose of filtrate. Add 200 µL PCR H_2O to filter cup, cap tubes, and spin at 13000 rpm for 12 minutes.
16. Prepare collection tubes by removing caps and labeling with sample name/number.

17. Add 60 µL PCR H2O directly to filter cup, and invert cup into collection tube. Allow to sit for 3-5 minutes, and spin at 5000 rpm for 2 minutes.
18. Remove filter cup and discard. Cap tubes at store at 4ºC overnight before quantification.

Appendix 3

Chelex DNA Isolation

You will be isolating DNA from cheek cells scraped from the inside of your mouth. The **Chelex extraction** method will be used to isolate the DNA from your cells.

I. PRINCIPLE

Chelex® 100 or InstaGene® (BioRad, Hercules, CA) is a chelating resin that binds cations, removing them from the solution. The Chelex resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions that act as chelating groups. Chelex, during boiling, prevents the degradation of DNA by chelating metal ions that may otherwise catalyze the breakdown of DNA subjected to high temperatures in low ionic strength solutions. The basic procedure consists of a wash to remove some of the contaminants (e.g., heme and proteins) followed by boiling the sample in a 5% Chelex solution. A fraction of the supernatant is added to the PCR reaction. This extraction produces single stranded DNA and is most applicable to downstream PCR applications.

II. General Safety Requirements

1. Always wear lab coat and gloves.
2. Do not pipette by mouth.
3. All appropriate MSDS sheets must be read prior to performing this procedure.
4. Treat all biological specimens as potentially infectious. Follow Universal Precautions.
5. Avoid direct exposure to ultraviolet light when using the germicidal lamp in the biological hood or the transilluminator.
6. The heat plate can become very hot. Be careful not to touch the heating surfaces while in operation.
7. Use proper protective equipment to prevent burns when handling boiling water or hot solutions.
8. Distinguish all waste as general, biohazard, or Sharps and discard appropriately.

III. SPECIMEN

Any biological sample believed to contain nucleated cells. The extraction can be prepared from oral swabs, filter paper or gauze.

IV. ESSENTIALS

(A) Reagents

Bleach, 5% (sodium hypochlorite solution)
Centricon-100 microconcentrator or Microcon-100 microconcentrator
Chelex® 100, or InstaGene 5% or 20% suspension, (Bio-Rad)
Forceps, disposable

Milli-Q Type I water
Scalpel, disposable
Tris-EDTA (TE) buffer, (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
PBS
Weigh boats

(B) Supplies

Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettes)
Microfuge Tubes, (1.7 ml, or 1.5 ml)

(C) Equipment

Heat block or boiling water bath
Eppendorf Thermomixer
Boiling Water Bath
Freezer, -20^{o}C
Dry Block Incubators (37^{o}C, 56^{o}C)
Magnetic Stir bars
Magnetic Stirring Plate
Microcentrifuge (e.g., Eppendorf)
Microcon 100 Concentrators
Refrigerator, 4^{o}C
Vortex

(D) Extras

Ice
Kimwipes
Waste containers for dry and wet waste
Sharps Container
NOTE: For sperm samples, will also need Proteinase K (20 mg/ml) and 1.0 M DTT, and SPIN-EZE tubes with baskets

IV. FRESH REAGENT PREPARATION

1. Prepare a 5% and 20% suspensions of Chelex® 100 must be made fresh weekly using Milli-Q water.
2. pH the suspension daily before use. The pH must be between 9.0 -10.0. Do not attempt to adjust the pH.

V. QUALITY ASSURANCE

1. In order to prevent contamination, all steps in this procedure should be conducted in the proper laboratory hoods when appropriate.

2. Any laboratory workspace and all pipettes and racks to be used in this procedure must be cleaned with 5% bleach and thoroughly dried before beginning. When using a laminar flow hood, turn on the ultraviolet light for a minimum of 10 minutes after cleaning.

3. Before use, forceps, scissors, etc., must be cleaned with 5% bleach, rinsed in ethanol, and dried thoroughly. In addition, they should be cleaned in the same manner following the processing of each individual specimen (when used).

4. No reagents or supplies used for the extraction of DNA from specimens will be allowed in a post-amplification room.

5. Any equipment taken from a post-amplification room to a pre-amplification room **must** be sterilized with 10% bleach immediately before removal from the post- amplification room and immediately upon arrival in the pre-amplification room. Reagents should never be transferred from a post-amplification room to a pre-amplification room.

6. Only one evidence specimen will be open at any one time.

7. Change pipette tips between each transfer or addition of sample or reagent, unless otherwise noted.

8. No aliquot of any reagent may be returned to the original stock container.

9. A minimum of one reagent blank must be carried throughout the extraction procedure and assayed in parallel with the evidence samples.

NOTE: An **Extraction Reagent Blank Control** must be initiated for each set of extraction tubes. The volume of reagent blank amplified must be no less than the volume of evidentiary sample extracted. This control will regulate for the presence of contamination in the extraction reagents. The reagent blank should be the first tube setup during purification. The extraction reagent blank must be carried through the remaining analyses.

VI: PROCEDURE: EXTRACTION OF DNA FROM BUCCAL SWABS (CHEEK CELLS)

1. Wipe down work area with 5% bleach. Use gloves and aerosol pipette tips to minimize contamination. Change or clean gloves and instruments between each sample.

2. When pipetting Chelex solutions, the resin beads must be distributed evenly in solution. This can be done by gentle mixing with a stir bar or mixing well before using. Use a pipette tip with a large bore (P-1000 µl).

NOTE: DNA extracted with Chelex is single-stranded and should be quantified by slot blot or real time PCR since it is unsuitable for quantitation methods that use ethidium bromide.

3. Dissect swab in the weigh boat, cut into small pieces (approx 1/3) and place in a labeled tube.

4. Add 200 µl or more of 5% Chelex to submerge the entire substrate.

5. Incubate at 56^{o}C for 30 minutes in heat block or Thermomixer.

6. Vortex for 5-10 seconds.

7. Incubate in a boiling water bath or Thermomixer for 8 minutes.

8. Vortex for 5-10 seconds.

9. Spin for 3 minutes at maximum speed.

OPTION: If FURTHER DNA purification is needed, extracts may be concentrated using Concentration of DNA with Microcon-100 and Centricon-100 protocol per manufacturer.

Store at 2-8^{o} C for short-term storage. Freeze at -20^{o} C (or colder) for long-term storage. To use after long-term storage on Chelex beads, repeat steps 4 through 7.

This protocol was adapted from: the manufacturer's protocol and DNA Typing Protocols: Molecular Biology and Forensic Analysis, Biotechniques Books.

Appendix 4

Real time quantification using Alu primers

I. PURPOSE

To quantify human DNA using Real Time PCR, Alu primers, and SYBR Green I dye.

II. SAFETY

All laboratory safety procedures will be complied with during this procedure. SYBR Green Dye is a mutagen/carcinogen. Appropriate handling procedures should be followed.

III. ESSENTIALS

(A) Reagents

SYBR Green I dye
DMSO
PCR ddH_2O
10* ABI Buffer I
$MgCl_2$ (25 mM)
dNTPs (2.5 mM)
Amplitaq Gold or RampTaq hot start Taq polymerase (5U/µL)
Triton X100 (10% solution)
Nonacetylated BSA (20 mg/mL)
Alu primers, AluF and AluR82 (or AluR124) (100 pmol/µL)
9947A 10 ng/uL DNA standard

(B) Supplies

0.2 mL flat cap PCR tubes
2 mL flat cap microcentrifuge tubes
10 µL pipet tips
20 µL pipet tips
gloves
white paper

(C) Equipment

0.2 –2 µL pipettor
0.5 – 10 µL pipettor
10-100 uL pipettor
Corbett Rotor Gene 3000 Real Time PCR instrument, 36 sample rotor

IV. GENERAL

1. Procedure will be used for quantifying extracted human genomic DNA samples.
2. Procedure will be used as necessary for research
3. Gloves should be worn at all times

V. PROCEDURE

1. Preparation of 0.5 % working solution of SYBR Green I (Molecular Probes, Eugene, OR)
    a. Take 1 µL of 10,000X concentrated Sybr Green I and add 199 µL of DMSO. Prepare aliquots for future use.

3. Prepare DNA standards or use standards from Quantiblot kit.
    a. Use 9947A DNA standard, dilute to 1, 0.5 and 0.1 ng/µL

4. Prepare DNA samples to be quantified: spin down before opening tubes.

5. Prepare flat-top tubes, label them on the cap. Label two sets of standards and 1 NTC (no template control).

6. Prepare and vortex Alu Mix for 36 samples:
    a. For 36 samples: Mix 528.0 µL PCR H_2O, 84.0 µL 10* ABI Buffer I, 67.0 µL dNTPs, 50.0 µL $MgCl_2$, 8.4 µL Taq polymerase, 8.4 µL *Alu* Primer 1F(forward), 8.4 µL *Alu* Primer 124R (large) or 82R (small) (reverse), 8.4 µL TritonX 10%, 8.4 µL SYBR green solution, 8.4 µL BSA in 2 mL tube.
    b. Spin down before opening tube.

7. Pipette 19 µL of Alu Mix into labeled PCR microtubes.

8. Add DNA
    a. Add 1 µL DNA sample to each properly labeled tube; add 1µl of each diluted standard to marked tubes.
    b. Add 1 µL ddH_2O to NTC tube.
    c. Vortex and mix.
    d. Remove bubbles and spin down.

9. Turn on PC and turn on the Rotor Gene instrument
    a. Clean accessible optics with cotton Q-tip and ethanol.

b. Place tubes on the appropriate wells of the carousel and place ring on top. Align carousel in the chamber. Screw in the cap with the red dot on top. Close cover.

10. Open RotorGene program.

11. Select SYBR Green (I) and click on New.

12. Select 36 well rotor, click on "No domed tubes" box, and click Next.

13. Type name in Operator field, and any notes in Notes field. Select 20 uL volume and click Next.

14. Make sure FAM/Sybr is highlighted and click Next.

15. Click Start Run.

16. Save As window will pop up. Name file as DateNameProject (e.g.061906KerryTest). Click Save and instrument will start.

17. Sample window will appear shortly. Label standards, NTC, and samples under Name, and then under Type (select Standard, NTC, or Unknown from pull down menu). For standards, enter known concentration and scale (e.g. ng/uL). Change color boxes on far left, if desired. Click Finish.

18. When experiment is completed, click Analysis-Quantitation-Show. New windows will appear and a box in the middle – click Cancel.

19. Fill:

Slope Correct ON
Eliminate cycles before 5
Threshold default (0.03)
When the box comes up, click OK.

20. The standard curve, fluorescence threshold cycle (Ct) and concentrations of samples will be calculated. The standard curve should have an efficiency and *r* value close to 1.00. You can choose to exclude those standard samples that cause give errors.

21. Click "reports" in the upper left of the Quantitation window – Full Report - Send to Word and save or print.

Appendix 5

Gel Analysis of PCR Products

I. PRINCIPLE

To visually determine if the genomic DNA extraction or PCR reactions have yielded any DNA/product. An agarose gel separates DNA by size, the higher the molecular weight the slower the DNA moves through the pores of the gel; therefore the smaller molecular weight DNA will travel through the gel faster compared to high molecular weight DNA. Ethidium bromide is an intercalating dye that has a minimum detection limit of 1-5ng/band.

II. GENERAL SAFETY REQUIREMENTS

1. Always wear lab coat and gloves.
2. Do not mouth pipette.
3. Ethidium bromide is a potential mutagen.
4. UV light is carcinogenic: ALWAYS WEAR FACE SHIELD, GLOVES AND LAB COAT to protect your eyes and skin.

III. ESSENTIALS

(A) Reagents
Agarose (e.g., Seakem®, cat no. 50000)
Double distilled water or UV treated ddI water
6X gel loading dye (Promega, cat. no. G190A, lot no. 13687907)
1kb ladder (Promega, cat. no. G171A, lot no. 14297901)
100bp ladder (Promega, cat. no. G2101, lot no. 191437)
Ethidium bromide (Fisher, BP1302-10)
TBE (Tris, borate, EDTA) buffer (Fisher, BP1333-1)

(B) Supplies
Pipettes and tips (20µl, 200µl, 1000µl)
Erlenmeyer flask
Ziploc Container
Weighing boat

(C) Equipment
Microwave (Ewave)
Agarose gel running equipment (Fisher, FBSB-2318)
Transilluminator (Fisher BioTech, model FBTIV-614)
Digital camera (Kodak, EDAS 290)
Scale

(D) Extras
Digital camera & software

IV: PROCEDURES

1. Preparation of work bench and supplies

1.1 Remove used bench cover
1.2 Wipe down all horizontal surfaces with 5% bleach
1.3 Wipe down all horizontal surfaces with double distilled water
1.4 Cover all surfaces with a clean bench cover

2. Reagent preparation
2.1 Make 10X TBE buffer

a. Weigh out:

108g Tris base

55g Boric acid

9.3g EDTA

Add to 800 ml of double distilled water; mix and adjust to 1 liter with double distilled water

3. Agarose

3.1 Put weighing boat on scales and press zero button

3.2 Weigh out agarose as appropriate for detection level

3.2.1 Use 1% gels for high molecular weight after extraction procedure (detects 0.5-10kb); use 1.5% gels for lower molecular weight DNA after PCR procedure (detects 0.2-3kb)

3.3 Measure 100 ml of 1X TBE buffer.

3.4 Combine the 1X TBE with the agarose in an Erlenmeyer flask and gently swirl to mix.

3.5 Place the flask in the microwave and heat for a minute on high.

NOTE: make sure the agarose has dissolved. Additional heating may be required but caution should be taken that the solution does not bubble over and produces steam that can burn.

3.6 The solution should be left to cool until it reaches 50-60^{o}C; this normally takes about 15-30 min depending on the volume of gel mix.

NOTE: the solution must not be cooled so long that is solidifies, to prevent this the solution can be kept in a water bath that is set to 50-60^{o}C

3.7 The gel apparatus must be set up with a comb that will allow wells of appropriate size for the volume of liquid to be loaded.
Note: refer to manufacturer's instructions

3.8 When the solution is cool to touch the outside of the flask, the solution should be poured into the mold.
NOTE: the solution should be poured until it covers the bottom of the mold and comes about a third of the way up the comb.

3.9 The gel should be left for approximately 30 min-1 hour to cool and solidify.

3.10 Once solid the gel to be transferred to a gel running apparatus.

3.11 The apparatus should be filled with 1X TBE until it covers the gel and there is roughly 1mm of solution above the gel.

3.12 The samples should be prepared by combining 5µl of sample to 3µl of 6X dye solution.

3.13 The gel should then be loaded with 8µl of each sample in a separate well.

3.14 The ladder should be prepared and loaded in the same way except only use 1-2 µl of ladder. A 1 kb ladder should be used for high molecular weight DNA and a 100bp ladder should be used with lower molecular weight DNA.

3.15 A positive and negative lead should then be attached to the gel box and to a power supply.
NOTE: the black (negative) lead should be located closest to the wells; the red (positive) lead should be located furthest from the wells.

3.16 The gel should then be run at 5.0 V per cm (measure distance from anode to cathode poles) or until the 1st color of the dye reaches near the end of the gel. This can be run at 5V/cm (gel size from pole to pole = 43 cm, means the gel can be run at 215V).

4. Detection of DNA (if bands are faint or not visible)

4.1 A container should be filled with 200 ml double distilled water and 20µl of ethidium bromide should be added.

4.2 The gel should then be carefully lowered into the container.
NOTE: do not splash the water, as ethidium bromide is a potential mutagen

4.3 The container can be left on a rocker for at least 30 min.

4.4 The gelis then be placed in another container with water only for 10 min to remove the residual ethidium that has not intercalated into the DNA.

4.5 The gel should then be placed on a transilluminator (302nm).

4.6 A hood should be placed over the transilluminator and a digital image should then be taken of the gel.

4.7 The gel can then be visualized on a computer program such as Kodak ID 3.6.

4.8 The bands on the gel can be compared to the known molecular weight bands of the ladder and a determination of size can be made.

Appendix 6

STR AMPLIFCATION

I. PURPOSE: To generate Human (Miniplex) STR profiles

II. SAFETY: All laboratory safety procedures will be complied with during this procedure.

III. ESSENTIALS

(A) Reagents

PCR ddH_2O
Miniplex master mix consisting of:
10* ABI Buffer I
dNTPmix (2.5 mM each)
Amplitaq Gold Taq polymerase (5U/µL)
Nonacetylated BSA (20 mg/mL)
Miniplex primers:
BigMini: TH01, CSF1PO, TPOX ,FGA, D21S11, D7S820
(100 pmol/µL)
9947A DNA standard, 0.25 ng/µL

(B) Supplies

0.2 mL PCR tubes
0.2 mL domed caps
0.6 mL flat cap microcentrifuge tubes
20 µL pipet tips
10-100 µL pipet tips
10-100 µL pipet tips
gloves
bench paper

(C) Equipment

0.2-2 µL pipettor
0.5-10 µL pipettor
10-100 µL pipettor
PCR 9700 Thermal cycler
Flat tray (red)

IV. GENERAL

1. Procedure will be used for DNA typing using the Miniplex STR kits
2. Procedure will be used as necessary for research
3. Gloves should be worn at all times

V: PROCEDURE

1. Preparation of reaction mix

    a. Mix 200 µL dNTP mix, 250 µL 10* Buffer, and 50 µL ddH2O for 500 µL reaction mix. Vortex and spin down.

2. Prepare primers for individual loci.
    a. Use 5 µL of reverse primers and 5 µL of forward primers for 50 µL of mix. Dilute to 50 µL with ddH_2O.
    b. Mix each loci separately.

3. Prepare primer mix for number of samples required plus extra for pipetting error (1 extra per 10 samples, depending on number of samples).
    c. 0.4 µL each of THO1 and CSF1PO per sample
    d. 0.5 µL TPOX per sample
    e. 0.6 µL each of FGA and D21S11 per sample
    f. 0.8 µL of D7S820 per sample

4. Add 5 µL per sample of reaction mix to primer mix.

5. Add 1 µL per sample of 1: 20 dilution non-acetylated BSA to primer mix.

6. Add 0.4 µL per sample of Taq.

7. Prepare 0.2 µL tubes in strips for ladders to be amplified. Prepare 1 reagent blank and 1 positive control (0.250 ng/µL 9947A).

8. Pipette 9.7 µL of primer mix into tubes for each sample.

9. Add ddH_2O to each sample to make a final volume of 24 µL (1 µl will be the DNA you add for a final volume of 25 µl.

10. Cap tubes, flick to remove bubbles and mix, and spin down.

11. Place tubes in flat red tray and place tray in Thermal cycler.

12. Go to User (example: kerry).

13. Select program mini (or mini 33).

    a. Program is: 95° C for 10 minutes warm up; cycle 94° C for 1 minute, 55° C for 1 minute, 72° C for 1 minute; 60° C for 45 minutes; 25° C for 1 minute. Use 33 cycles.

14. Press “Run”

15. Use 25 for reaction volume

16. Press “Start”

17. After run has ended, remove tubes from thermal cycler.

Appendix 7

ABI 310 GENETIC ANALYZER

I. **PURPOSE**: Operation of the ABI 310 Genetic analyzer.

To document the procedures used to prepare and operate the ABI PRISM 310 Genetic Analyzer for the purposes of sequencing or fragment analysis. The objective of this procedure is to successfully complete sample analyses.

II. SAFETY

All laboratory safety procedures will be complied with during this procedure.

1. Always wear gloves
2. Wear eye protection when handling polymer (POP-4 or POP-6) close to eyes

III. ESSENTIALS

(A) Reagents

Ethanol, 95%
Water, double distilled
10X Genetic Analysis Buffer with EDTA
Performance Optimized Polymer (POP) 4 or 6
Highly Deionized (Hi-Di) Formamide

(B) Supplies

0.5 tubes
1.5 tubes
Pipette tips
Septa
Ice
Buffer and water vials

(C) Equipment

48 or 96 sample tray
ABI 310 genetic analyzer with software
Computer
Pipettes
Heat block
Vortex
Centrifuge

IV. PROCEDURE

1.0 Work Space Preparation

1.1 Wipe down laboratory work space with 5% bleach.
1.2 Wipe down laboratory work space again with double distilled water
1.3 Place clean bench cover on laboratory bench. Tape in place.

2.0 Prepare 310

2.1 Turn on 310 with switch at the back left halfway up. **(ALWAYS TURN ON 310 BEFORE OPENING SOFTWARE!** If the 310 software runs with the machine off it will have nothing to communicate with and everything will have to be reset)
2.2 Open the 310 Data Collection Software
2.3 Pull down Window menu, choose Manuel Control
  2.3.1 In Function pull down menu choose Syringe Home: execute
  2.3.2 In Function pull down menu choose Autosampler Home X-Y Axis: execute
  2.3.3 In Function pull down menu choose Autosampler Home Z Axis: execute

3.0 Clean Electrode (if it was touched, if it is new, or if buffer solutions are replaced)

3.1 Push Tray button on left side of machine inside doors

3.2 Carefully wipe electrode with lint-free lab wipe with sterile distilled deionized water, dry with lab wipe

3.3 Push Tray button to return tray

4.0 Polymer and Buffer

4.1 Take Polymer (POP-4 or POP-6) out of refrigerator to acclimate to room temperature

4.2 Prepare 1X Buffer from 10X Buffer

4.2.1 For a single run: 1.3 mL 10X Buffer and 11.7 mL Sigma deionized water

4.2.2 For stock: 5mL 10X Buffer and 45 mL Sigma deionized water

5.0 Gel Block

5.1 Screw top and bottom ferrels in firmly, **lightly screw** capillary ferrel in; DO NOT TIGHTEN.

5.2 Align steel shafts on 310 with two large holes in block
5.3 Align buffer valve with activator arm
5.4 Slide gel block in

6.0 **Fill Buffer and Water Vials**

6.1 Fill glass vial to the black line with 1X Buffer and mark B
6.2 Fill other glass vial to the black line with deionized water
6.3 Cut lid off of a 1.5 mL Eppendorf tube

7.0 **Fill and Install Syringe**

7.1 Mix polymer by inverting, then let sit for at least 5 minutes
7.2 Draw 1.0 mL sterile deionized water slowly into syringe and then dispense into waste (Always pull plunger straight up)
7.3 Draw 0.15 mL polymer slowly into syringe, invert syringe tip up, slowly pull the 0.15 mL polymer through entire syringe
7.4 Still holding tip up, dispense polymer into a lint-free lab wipe
7.5 Carefully fill syringe at least 0.2 mL more than desired volume
7.6 Hold syringe tip up to eye level against a white background and tap sides to raise any air bubbles. Slowly push them out with plunger into lab wipe.
7.7 Move syringe to 0.2 mL more than needed for samples
7.8 Wipe and blot syringe tip with lab wipe
7.9 Install syringe using the steel hub to screw into place, NOT THE GLASS

8.0 Install Capillary (Green 47cm capillary for fragment analysis, pink 61cm capillary for sequencing

NOTE: Capillary should not be out of polymer or water for more than 30 minutes, steps 8.0, 9.0, and 10.0 should be completed within that 30 minutes

8.1 Carefully wipe capillary window with lint-free lab wipe with 95% Ethanol (Capillary window is only part of capillary that is very breakable)

8.2 Open door in front of heat block

8.3 Gently insert capillary, window end, into capillary ferrel in gel block. Position so the tip of capillary is just entering first gel channel intersection. Tighten ferrel (tightening ferrel will move capillary forward slightly so adjust initial positioning to accomodate).
8.4 Open laser detector door

8.5 Carefully position capillary in track with the window centered over the laser and the color mark aligned with the top edge of the laser detector plate.

8.6 Close the door to secure laser, do not move capillary

8.7 Thread capillary into electrode thumbscrew so that capillary tip protrudes past the electrode about 0.5mm

8.8 Tape capillary to heat plate to secure in place

8.9 Close heat plate door, make sure capillary has not moved.

9.0 Fill Gel Block

9.1 Go to Window, Manuel Control, Function menu and select Buffer Valve Close

9.2 Manually open waste valve below the syringe

9.3 Press syringe plunger until polymer has gone into waste valve (uses about 0.1mL polymer)

9.4 Close waste valve manually

9.5 From Function menu select Buffer Valve Open

9.6 Press syringe plunger until polymer fills gel block channel with no bubbles (uses about 0.1mL polymer)

9.7 From Function menu select Buffer Valve Close

10.0 Calibrate Autosampler (if electrode is moved or cleaned, if capillary is moved or changed, if capillary touches septa caps, if the memory has been reset, or if prompted by the instrument).

10.1 Remove sample tray and Eppendorf tube in position 3 (better to remove vials in postions 1 and 2 as well)

10.2 From Instrument menu select Autosampler Calibration, click Start and follow instructions

10.3 Use arrow keys on the screen or computer to move autosampler, press the shift key with an arrow to move half steps

10.4 Align dot on front of tray platform with capillary so that it is almost touching, click Set

10.5 Repeat for dot on the back of the tray platform, click Set

10.6 Follow instructions to complete calibration, when tray is presented place buffer vial marked B in position 1, water vial in position 2, and the 1.5 Eppendorf tube with water in position 3 (if samples are ready place sample tray in autosampler, if not see 11.5)

10.7 From Function menu in Manuel Control (Window) select Autosampler to Position, type in 1 and execute

10.8 From Function menu select Autosampler Up, type in 450 and execute (capillary is now in lubrication at both ends)

11.0 Prepare Buffer and Syringe

11.1 Fill shot glass (anode buffer reservoir) to the red line and install on bottom left of gel block (push up and twist right)
11.2 Move syringe toggle to the right over syringe
11.3 From the Function menu in Manuel Control select Syringe Down, start with 100 steps
11.4 Continue to bring syringe drive down to meet top of plunger, lowering steps (the last few executions should only be in steps of 1)
11.5 If sample tray has not already been loaded, from Function menu select Autosampler Down 450 steps, execute
11.6 Press Tray button on left side of instrument to present tray and load sample tray
11.7 Press Tray button to return tray, from Function menu select Autosampler Up 450 steps, execute
11.8 Close doors

Creating a Sample Sheet

12.1 From the File menu select New and click on appropriate sample sheet icon (example: for fragment analysis with a 48 tube tray select GeneScan Smpl Sheet 48 Tube)
12.2 Select 4 Dyes or 5 Dyes in upper right corner
12.3 Enter sample names according to their positions on the tray
12.4 Fragment
  12.4.1 Click next to the dye color in the Std column on each sample to specify size standard (4 Dye : Red, 5 Dye: Orange)
  12.4.2 Sample Info and Comments columns are optional, but may be useful for certain applications
12.5 Sequencing
  12.5.1 Set appropriate DyeSet/Primer file for each sample (see page 6-19 of ABI PRISM 310 Genetic Analyzer User Guide)
  12.5.2 Select appropriate Matrix file for each sample
12.6 Collection name is optional

12.7 From the File menu select Save As, name the sample sheet and save

13.0 Preparing the Injection List

13.1 From the File menu select New and click GeneScan Injection List for fragment analysis or Sequence Injection List for sequencing
13.2 Select the sample sheet from the pull-down menu
13.3 Enter your name as the operator
13.4 Add multiple injections by highlighting a row and selecting Insert from the Edit menu, copy the sample name in the new cell
13.5 Select a module from the Module drop down list
13.6 Fragments
  13.6.1 Select the matrix in the Matrix column
  13.6.2 Check the Auto Anlz box to automatically analyze with GeneScan, but DO NOT check the Auto Prt box
13.7 Change any specific parameters needed for particular samples
13.8 When finished click Run to start the run

VI. ABI 310 Breakdown

1.0 Preliminary

1.1 Check Status window to make sure electrophoresis, laser, and temperature are off. If not, go to Manuel Control in Window menu, in Function menu select:
  1.1.1 Electrophoresis Off then Execute
  1.1.2 Laser Off then Execute
  1.1.3 Temperature Off then Execute
1.2 Open doors
1.3 Push Tray button on left side of machine to present tray
1.4 Dispose of used samples, dispose of 1.5 eppendorf tube, and wash two glass vials in positions 1 and 2 and white caps with distilled water (dispose and use new vials every two weeks). Dispose of septa (always use septa only once)

2.0 Capillary Removal and Storage

2.1 Open heat block door
2.2 Carefully slide capillary up and out of electrode
2.3 Open laser block door, remove capillary, close door
2.4 Unscrew capillary ferrel just enough to slide capillary out
2.5 ALWAYS keep capillary lubricated once polymer has gone through it
  2.5.1 Store capillary with ends in 0.5 tubes filled with deionized water and capped with septa
  2.5.2 Remember to check and refill if needed the water in the tubes

3.0 Syringe

3.1 Go to Manuel Control in Window menu, in Function menu select Syringe Home, Execute
3.2 Move syringe toggle to left
3.3 Unscrew syringe by the steel base (NOT THE GLASS)
3.4 Dispense any leftover polymer into a lab wipe and dispose
3.5 Pull up warm deionized water all the way into the syringe, dispense into waste, repeat
3.6 Blot end of syringe with dry lab wipe and wipe clean with slightly damp lab wipe
3.7 Store in syringe box

4.0 Gel Block

4.1 Remove anode buffer reservoir (shot glass): pull down and twist left, rinse with deionized water
4.2 Slide gel block off steel shafts, be careful of activator arm and buffer valve pin (gel block is a little tough to slide off)
4.3 Wipe inside of 310 with a lab wipe dampened with Sigma water
4.4 Rinse gel block with warm tap water
4.5 Unscrew ferrels and rinse, squirt distilled water through to rinse inside
4.6 Squirt distilled water through all the channels in the gel block. Block and unblock holes to push water through all channels
4.7 Repeat flushing all channels and ferrels with warm sterile water with a 1mL pipette
4.8 Wipe outside dry with lab wipe
4.9 Dry all channels and ferrels by pushing air through with a 10cc syringe or canned air. Block and unblock holes to push air through all channels
4.10 Wipe all outer surfaces of block with lab wipe (including holes for steel shafts
4.11 Slide back onto 310

5.0 Shut Down

5.1 Store clean shot glass, two glass vials and caps, and gel block ferrels on white tray under gel block in 310
5.2 Close doors
5.3 Close 310 software
5.4 Turn off 310 with switch in back on left halfway up (NEVER TURN OFF MACHINE WITH 310 SOFTWARE RUNNING)

Appendix 8

Principle to analyze STR output with GeneMapper

To analyze human DNA samples and identify the alleles present at STR loci and the gender marker amelogenin, all of which are amplified using the AmpFLSTR® kits and run on the ABI PRISM® 310 Genetic Analyzer.

I. GENERAL SAFETY REQUIREMENTS

None.

II. ESSENTIALS

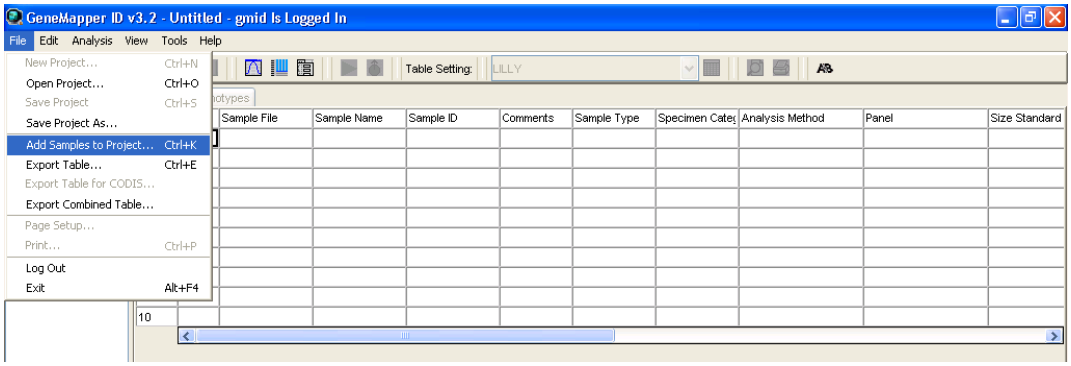
Computer with GeneMapper software installed.

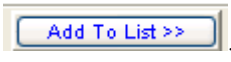
III. PROCEDURE

1. Generating a project

1.1. Open GeneMapper program.
1.2. A new project sheet should automatically open.
Note: New projects can be opened at any time by clicking on file and scrolling down to new project.

1.3. Click on file and scroll to Add samples to the project. Under the file drop down menu, select **Add Samples to Project**



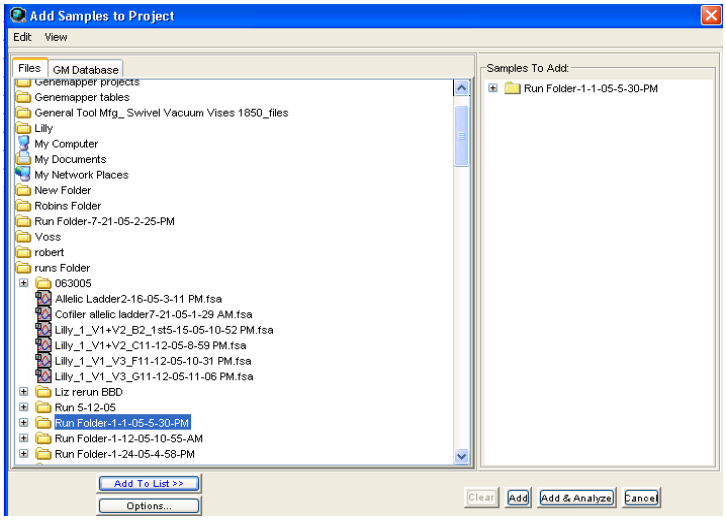
1.4. Select the folder that contains the samples to be analyzed and click on 

. The folder will move to the right column (Samples to Add). Click on the 

button in the bottom right corner of the menu.

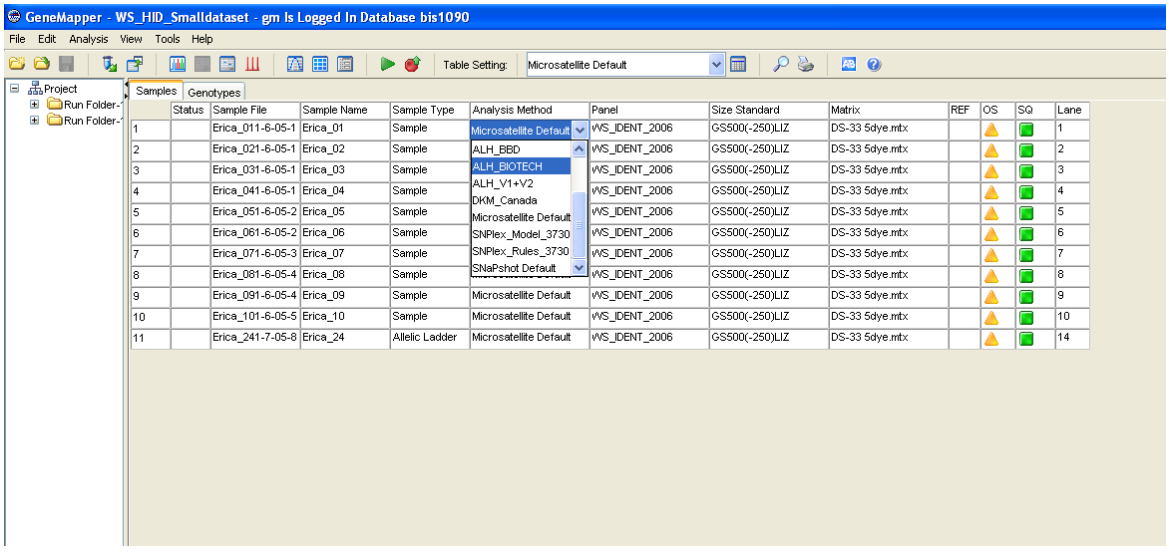
1.5. Select the sample you want to analyze from the files.

1.6. Click Add to List.
1.7. Repeat steps 1.4 and 1.5 until you have all the sample files required.
Note: An allelic ladder must be included in the list of files requiring analysis.
1.8. Click Add. The samples will be listed in a spreadsheet.



2. Selecting analysis criteria

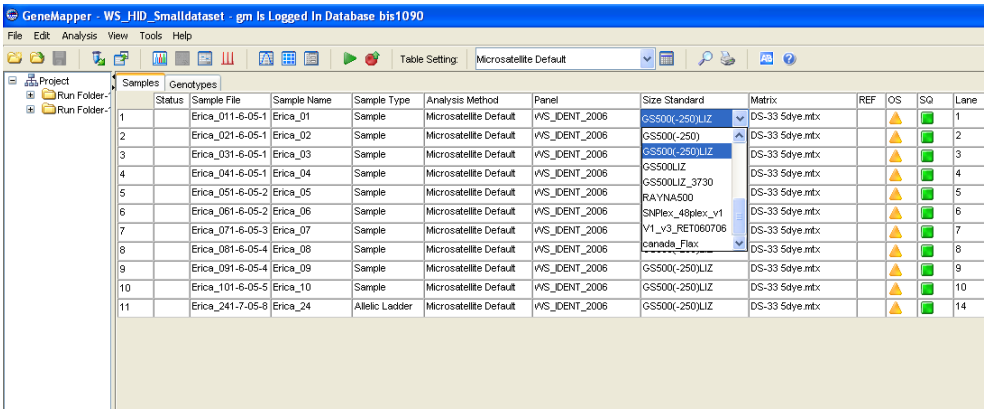
2.1. Select the sample type. Assign the allelic ladder, positive control, and samples.



2.2. Select the analysis method (example: Microsatellite default; HID Advanced, etc.)

Note: To apply a setting to all samples click the bar naming the column and press Ctrl + D.

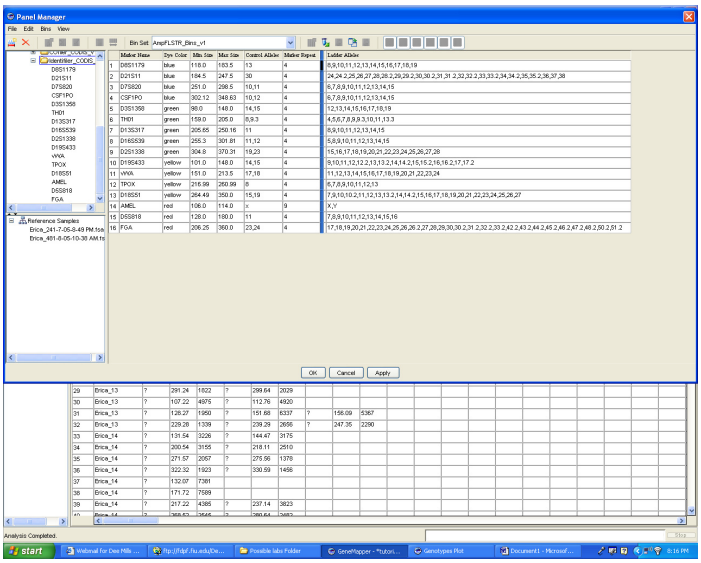
2.3. Select the **Panel**. Double click on AmpFLSTR_Panels and select appropriate panel for your kit (Ex: Identifiler _CODIS _v2; Cofiler_CODIS_v1, etc)

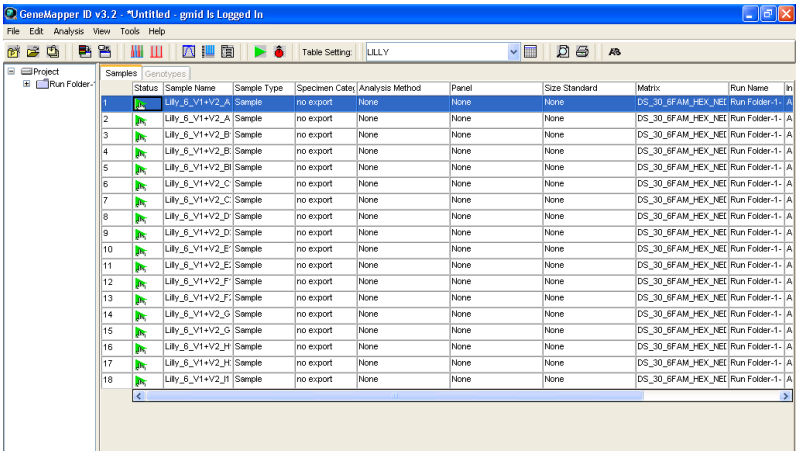


2.4. Select the size standard (Ex: GS 500(-250)LIZ; CE_F_HID_GS500, etc.)

2.5. If matrix has not been assigned during the run, select the matrix (ex: DS_32_5FAM_JOE_NED, etc). If analyzing the GeneMapper example data, do not change.

2.6. Click analyze. Name the project and click OK. Samples should now appear in the Genemapper spreadsheet. At this time changes can be made to sample names and sample order, in addition, non-working samples can be deleted.





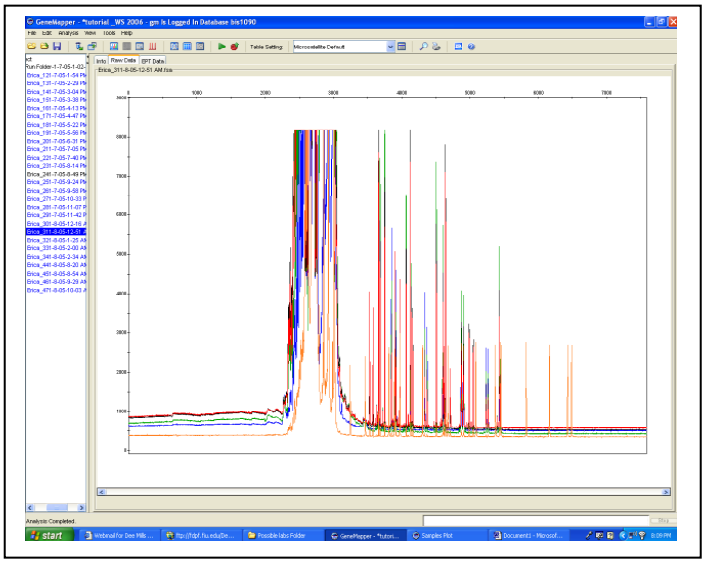
2.6.1. To change a sample name double click on the current sample name and change it to the new one.

2.6.2. To change the order of the samples go to the edit drop-down menu, select sort and reorganize your data.

2.6.3. To delete a sample, highlight the row of the sample to be deleted, go to the edit drop-down menu and select Delete from Project. Click ok.

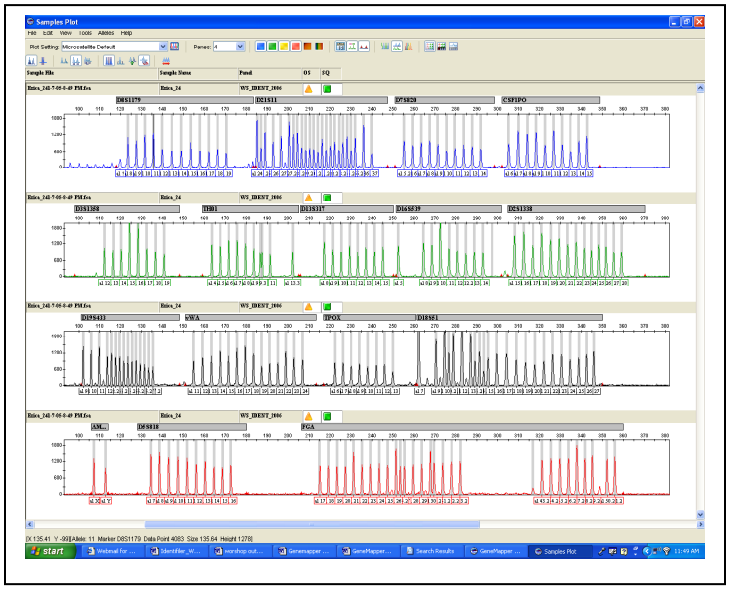
3. Analyzing samples

3.1. Select all samples and the rows will be highlighted blue. Click view and



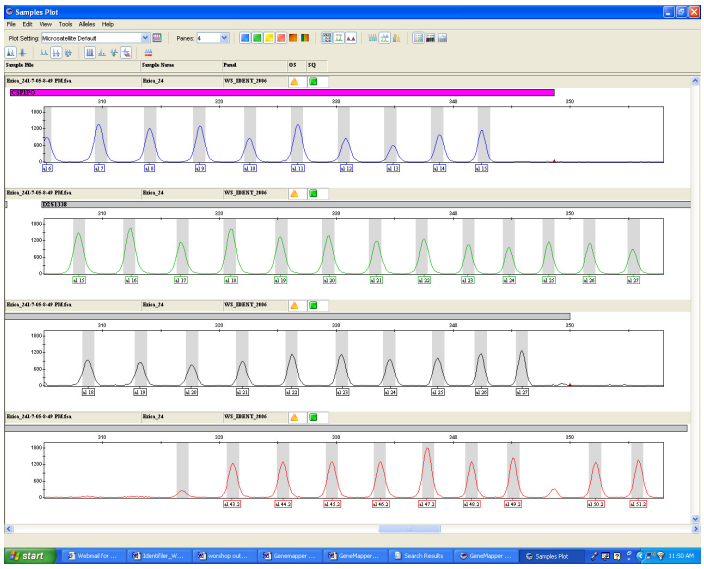
scroll down to raw data and check that the samples ran properly then close raw data.

3.2. Click Display Plots button. This shows all of the dyes in one window.
3.3. Click Separate Dyes button. The loci are now separated and the



genotypes of the samples at each locus are shown.

3.4. To view the peaks at one locus, click on the locus name so it is highlighted pink. Then place the cursor over the top line of the box



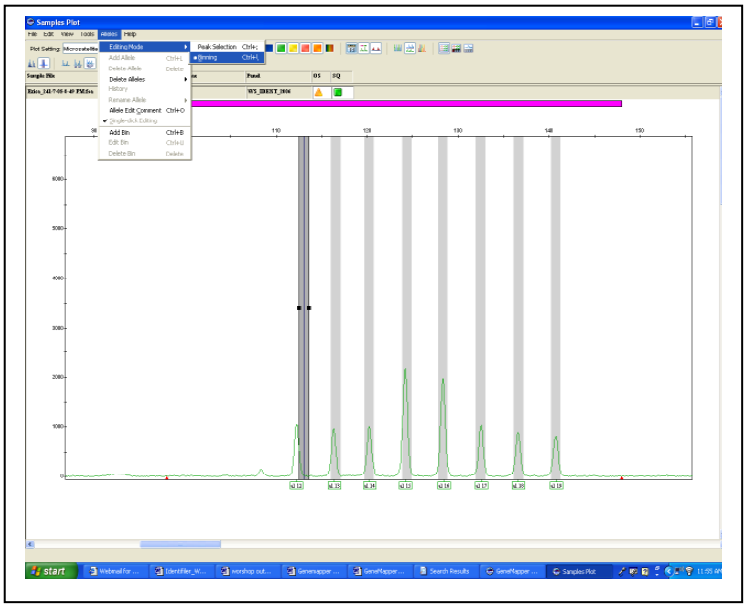
surrounding the data, and click and drag the cursor to zoom in on the peaks.

3.5. To zoom back out click the full button view. Now the genotypes of the samples can be examined.

4. Binning and analysis

4.1. If the bins are not correct on the allelic ladder, your sample peaks will not be called.

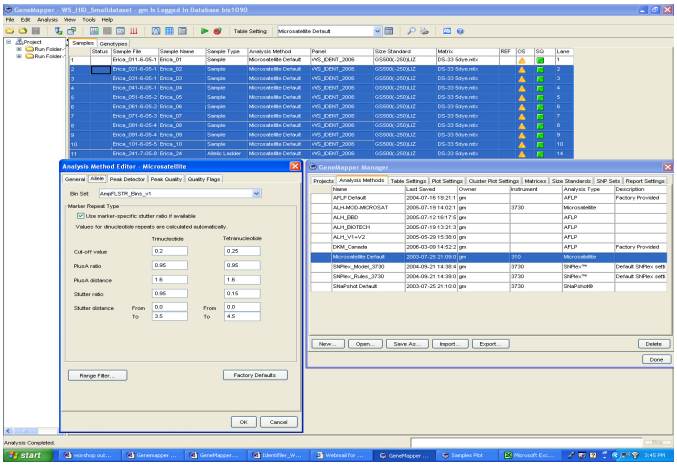
4.2. To edit the bins, go to the sample plot window, zoom in on marker and go to alleles, edit, binning. This will allow you to click and drag the bin over the peak in the correct position. (see picture).

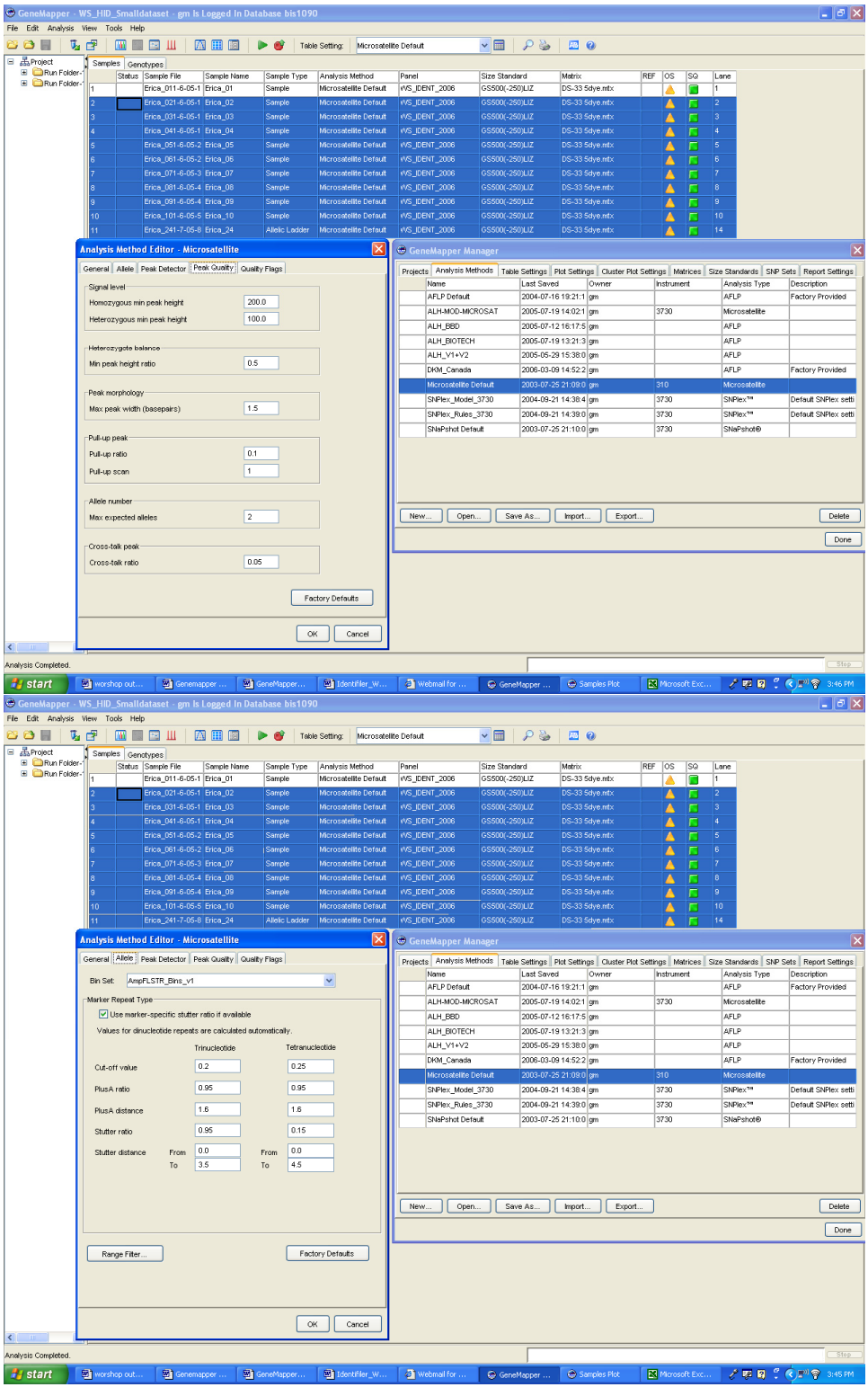


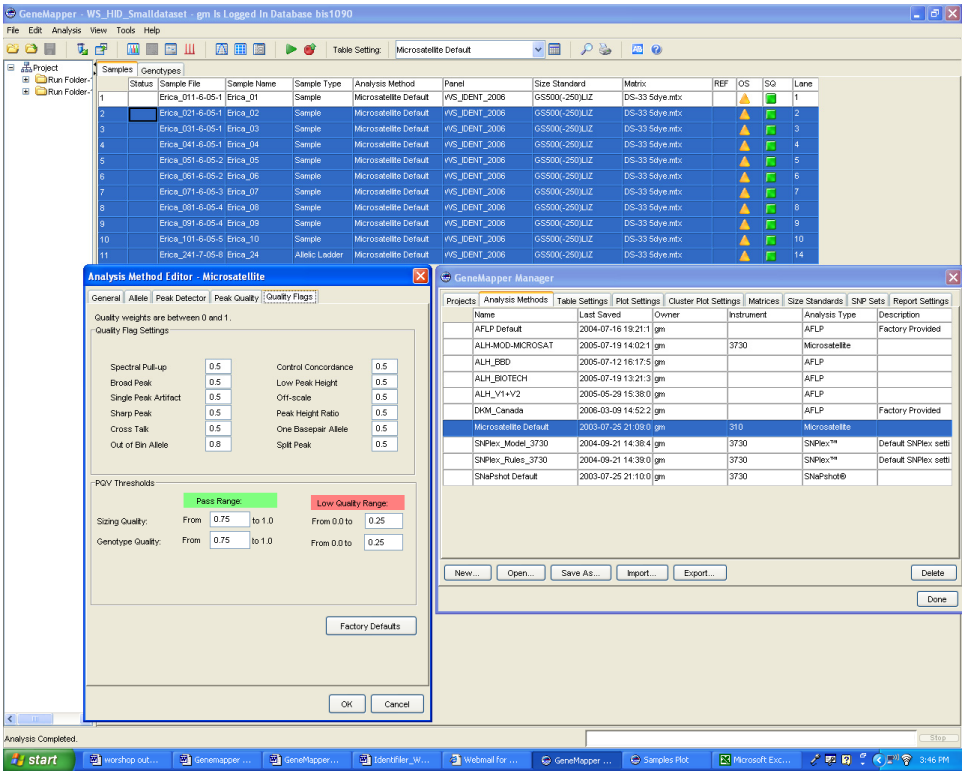
4.3. When finished, save the panel with a new name (save as) and exit. Click on the  button to re-analyze the samples.

4.4. Analysis parameters need to be set according to your laboratory SOP. For demonstration purposes, the default settings were used here.

4.5. Double click on the analysis method to see parameters.

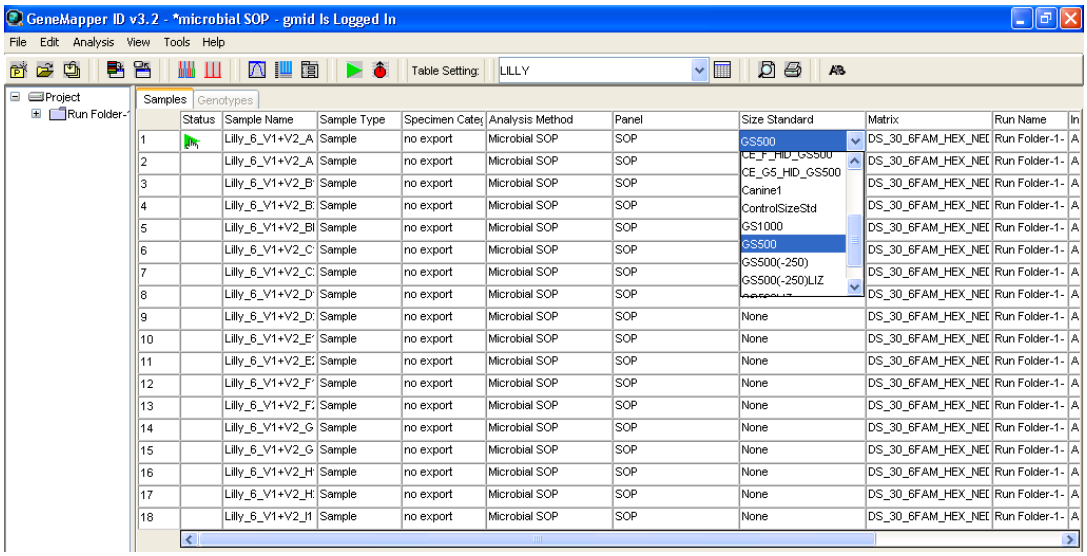



GeneMapper - WS_HID_Smalldataset - gm Is Logged In Database bis1090
Analysis Method Editor - Microsatellite
GeneMapper Manager
Analysis Completed.



5. Calling a size standard

5.1. From the size standard column select GS500, for example.



5.2. Copy the size standard for the entire data set as described before. All samples should now show the green flag in the status window indicating that they need to be reanalyzed.

5.3. Reanalyze the samples by clicking on the  icon.

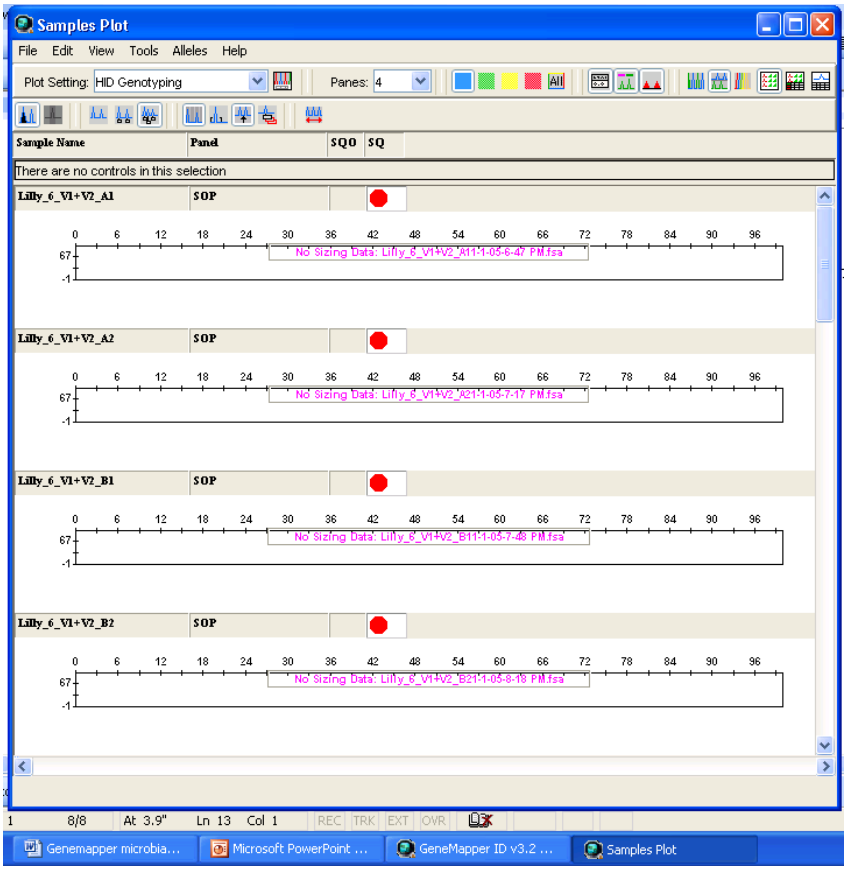
5.4. Verify that the size standard migrated correctly by highlighting all samples and then clicking on the  icon.

5.5. Click on  and then  , this will allow you to see only the results from the fluorescent dye you attached to the samples. You can scroll down to see the remaining of the samples.

5.6. If the panel says **No Sizing Data** or looks like the picture below, proceed as follows.

5.7. From the spreadsheet, highlight the samples that showed no sizing data and click on the icon.

5.8. A screen with the sample size for that sample will appear. Click on the button X in the upper left corner of the screen to clear the size



standard.

IF using the GS 500 standards, starting with the leftmost peak of the triplet, left click on the peak to select it and then right click on it to choose the correct size for the peak. Peak sizes will be 35, 50, 75, 100, **139, 150, 160**, 200, 250, 300, 340, 350, 400, 450, 490, 500.

NOTE: Each size standard may call its peaks differently; check the manufacturer's literature to find out what sizes are assigned to each peak.

5.9 Once all the peaks have been re-labeled click **Apply** then **OK.** Repeat procedure with other samples as needed.

5.10 The green flag should now appear next to those samples whose size standard was changed. Click on the icon to reanalyze the samples. Verify that all

the plots are visible by clicking on the  icon and scrolling down through the samples.

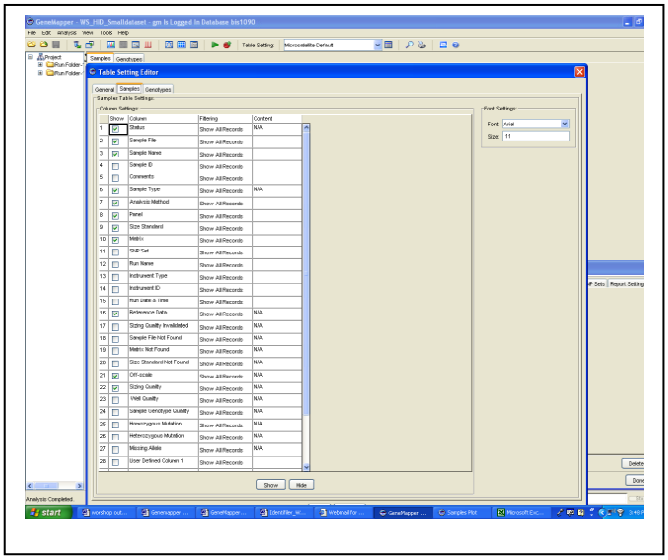
6. Table settings editor and genotype table export

6.1. Go to Tools, Table setting editor. The samples tab has the settings that you can change and will display the sample spreadsheet with the selected column headings.

6.2. The genotype table is the table information you want to export.

6.3. Check Sample name, marker, and allele; Check show 2 alleles and keep alleles, etc together. This is the table you will export.

6.4. Go to File, export table. Save as .txt file. Open Excel, open .txt file and follow the Excel wizard to import data into Excel. Save. This is your Genotype table output that reports the individual STR genotypes.



6.5. Save project and exit GeneMapper.

Example of Genotype Table in Excel

	#_01	#_01	#_01	#_01	#_01	#_01	#_01	#_01	#_01
Marker	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
Allele 1	12	27.2	9	12	16	7	11	9	20
Allele 2	15	32		13	17	8	12	11	
	#_01	#_01	#_01	#_01	#_01	#_01	#_01		
Marker	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
Allele 1	14	16	8	19	X	11			
Allele 2		17		20	Y	12	22		
	#_02	#_02	#_02	#_02	#_02	#_02	#_02	#_02	#_02
Marker	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
Allele 1	13	29.2	10	12	16	8	11	11	16
Allele 2	15				18	9		12	20
	#_02	#_02	#_02	#_02	#_02	#_02	#_02		
Marker	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
Allele 1	15		8	14	X	12			
Allele 2	16.2	17		18	Y		23		

VITA

MAURICE J. ABOUD

Experience

FLORIDA INTERATIONAL UNIVERSITY, MIAMI, FL — Research Assistant, 2009 to Present

- Research Assistant in McCord's DNA Research Group

INTERNATIONAL FORENSIC RESEARCH INSTITUTE, FIU — Academic Workshop Assistant, 2009-2010

- Advanced DNA typing analyses and population statistics, National Institute of Justice, 2008-DN-BX-K19

FLORIDA INTERNATIONAL UNIVERSITY — Teaching Assistant, 2007-2009

- Teaching Assistantship in Chemistry and Forensic Science

WESTCHESTER COUNTY FORENSIC SCIENCE LABORATORY — Internship, 2006

Education

FLORIDA INTERNATIONAL UNIVERSITY, MIAMI, FL — PhD. Chemistry emphasis in Forensic Science, 2012; MSc. Forensic Science, 2009

UNIVERSITY OF NEW HAVEN, WEST HAVEN, CT — Double Major BSc. Forensic Science & Chemistry, Summa Cum Laude Honors, 2007

Honors & Achievements:

- Florida International University
  - Doctoral Evidence Acquisition Fellowship
- University of New Haven –
  - Presidential Scholarship, 2003-2007

- Alpha Phi Sigma national Criminal Justice Honor Society, 2007
- University of New Haven Men's Faculty Award, 2005
- The National Dean's List 27th and 28th edition Volume 1, 2005

Publications

- Aboud, M. J., Gassmann, M., McCord, B. R., The development of mini pentameric STR loci for rapid analysis of forensic DNA samples on a microfluidic system, Electrophoresis 2010, 31, 2672-2679

- Cedric Hurth, Jian Gu , Maurice Aboud, Matthew D. Estes, Alan R. Nordquist, Bruce R. McCord , and Frederic Zenhausern, Direct loading of polymer matrices in plastic microchips for rapid DNA analysis: a comparative study, Analytical Chemistry

Presentations

- Aboud, M.; Oh, Hye Hyun.; McCord, B. 2012.; Gassmann, M. "Rapid and Direct CODIS STR screening using short channel microfluidic systems". Poster presentation. American Academy of Forensic Sciences (AAFS) 64th annual scientific meeting, Atlanta, GA, USA.
- Aboud, M.; McCord, B. 2011. "Forensic DNA Screening using short channel microchip electrophoresis". Poster presentation. 17th Latin-American Symposium on Biotechnology, Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis and Microchip Technology, Hollywood Beach , FL, USA
- Aboud, M.; McCord, B. 2011.; Gassmann, M. "A Rapid Pentameric STR screening using short microchip capillary electrophoresis". Exhibit. American Academy of Forensic Sciences (AAFS) 63rd annual scientific meeting, Chicago, IL, USA and Promega twenty-second International Symposium on Human Identification, National Harbor, MD, USA
- Aboud, M.; McCord, B. 2011. "Forensic DNA screening using short microchip capillary electrophoresis". Poster presentation. Florida International University 1st Graduate Week symposium, Miami, FL, USA.
- Aboud, M.; McCord, B. 2010.; Gassmann, M. "A Rapid Penta STR Screening Method by Microchip Capillary Electrophoresis". Exhibit. Promega twenty-first International Symposium on Human Identification, San Antonio, TX, USA and American Academy of Forensic Sciences (AAFS) 61st annual scientific meeting, Denver, CO, USA. Oral presentation. The 17th International Symposium on Capillary Electroseparation Techniques, Baltimore, MD, USA.