Sizing and Quantitation of Polymerase Chain Reaction Products by Capillary Electrophoresis for Use in DNA Typing

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ABSTRACT

Capillary electrophoresis has many attributes which are desirable in DNA separations, namely, speed, high resolution, direct quantitation, and complete automation. The goal of this work has been to develop a CE system to rapidly evaluate forensic DNA samples, principally those derived from the polymerase chain reaction. Factors affecting resolution, sensitivity, and precision have been studied in order to obtain a CE system which performs in a rapid, reliable, and reproducible manner. Separations were found to be primarily dependent upon the concentration and length of the cellulose sieving medium. Sequence dependent binding of DNA intercalators is also described.

This work describes several improvements in the separation of DNA fragments by capillary electrophoresis, including (a) new methods for injection of DNA from highly ionic matrices, (b) significant reduction in separation time without diminishing resolution, and (c) stable operating conditions for routine analysis. The use of a single internal standard for precise quantitation of PCR products and dual internal standards for precise sizing and accurate genotyping of PCR-amplified DNA is also shown. Peak area precision of 3% R.S.D. and peak migration time precision of $\leq$0.2% R.S.D. may be obtained with appropriate internal standards and conditions. In addition, direct comparisons to conventional methods of PCR product analysis have been made to demonstrate the validity of the CE results.

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‡ individual chapters contain more detailed listings

ACKNOWLEDGEMENTS

This work was funded by a grant from the National Institute of Justice and greatly facilitated by the aid of numerous individuals at the FBI Laboratory's Forensic Science Research Unit. First and foremost, I wish to express my appreciation to Dr. Bruce McCord, under whose tutelage I was introduced to the field of capillary electrophoresis. The opportunity to work daily with an ethusiastic scientist is a memory I will treasure forever. I am indebted to Bruce for allowing me to present our research at major conferences. Most importantly, Bruce was a friend as well as a fellow researcher.

The comments of Bruce Budowle in his critical reviews of my manuscripts improved both me and my writing. I enjoyed the challenge of convincing him that DNA typing with "no gels" was feasible. I wish to also recognize the previous work of Janet Jung Doyle, in whose footsteps I followed when investigating the use of CE for mitochondrial DNA analysis and short tandem repeats. Jan taught me the techniques of the polymerase chain reaction and tried to answer my incessant questions regarding DNA typing. To the many others at Quantico, Rena Merrill and Barbara Koons in particular, I thank you for your encouragement and enthusiasm.

Finally, I thank Professor Ralph Allen for opening the doors to me for a wonderful graduate experience and a promising future. His faith in my abilities to pursue a graduate education gave me the opportunity to fulfill a dream of working in the FBI Laboratory.

DEDICATION

To my parents

who fostered my love for learning,

and to my wife Terilynne

who tolerates it.

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EXPLANATORY NOTES

Chapter Table of Contents. At the beginning of each chapter, a detailed outline has been included to guide the reader.

Footnotes. Additional material, which clarifies but may digress from the flow of the text, has been arranged as footnotes following the final chapter. These footnotes are designated in the text by the chapter number followed by sequential lettering.

Appendices. Three appendices are included which may benefit the operational use of this technique. The first appendix is a survey of the CE/DNA literature and evaluates the DNA samples, CE instruments, conditions, and detection schemes used by researchers in the field. The second appendix contains a troubleshooting guide for the Beckman CE instrument, in which common problems are discussed with effective solutions. The third appendix includes ideas and materials which will be useful as this technique is prepared for admission to the courtroom.

Literature Survey. A current literature survey has been included in the order in which I discovered them. References in the text are made to this database listing. As capillary electrophoresis and DNA typing are both rapidly expanding fields, these references can provide a valuable footing for future researchers.

Chapter 1

DNA Typing and the Polymerase Chain Reaction

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I. Introduction

With the high rate of violent crime, there has been a growing need to effectively identify the perpetrator. For example, in 1991 alone, over 100,000 rapes were reported in the United States and almost 750,000 violent crimes were committed [548]. In many of these cases, biological material from the criminal was left at the crime scene. Since each person has a unique DNA sequence within the nucleus of their cells that makes them genetically different from everyone else (except, of course, an identical twin), techniques which examine the DNA structure are the ultimate source of information about the identity of an individual (**1A**). Unfortunately, determining the complete sequence of an individual is currently prohibitive in terms of both time and cost. Instead, techniques, such as DNA typing or profiling, have been developed to examine differences between individuals at various locations within variable regions of the human genome. This has brought about a dramatic advance in law enforcement and the criminal justice system since DNA evidence can be used in so many violent crimes [548].

Besides being useful in directly relating crime scene evidence to a suspect, the DNA profiles of violent criminals may be utilized in much the same manner as fingerprints have been in the past: to identify an unknown suspect who is a repeat offender. The importance of such information is illustrated in the fact that an estimated 60% of released felons are rearrested in less than 3 years [548]. Many states have established DNA data banks with the profiles of convicted felons which can aid in the identification of a criminal who leaves biological material at a crime

scene or the exclusion of a falsely associated individual [343,548].

The large number of samples that could be potentially analyzed emphasizes the need for fast, efficient, and automated means of DNA typing. For example, the state of Virginia receives between 1,500 and 2,000 samples per month from convicted felons, but can only process about 3,000 per year using traditional restriction fragment length polymorphism techniques [343]. The Department of Defense (DOD) has an even greater problem. They have been collecting DNA samples from all service members for use as "DNA dog tags." The collected DNA profiles will help confirm the identities of those soldiers killed in accidents or on the battlefield. The DOD estimates that samples will be obtained from over 2.5 million U.S. service personnel by the end of the century [343]. The technology necessary to process such a large database of samples in the event of war or mass disaster is not available. For this reason, more automated and rapid procedures are needed to process this information. The use of capillary electrophoresis has the potential to alleviate this problem. The goal of this research has been to develop a greater understanding of the processes and practices involved in the application of CE to DNA typing.

II. DNA Typing and RFLP Methods

DNA is composed of a four letter alphabet which spells out the genetic blueprint of an individual. While most of the human genome is exactly alike between individuals, variations (polymorphisms) do exist, principally in non-coding regions. DNA typing examines the variability in DNA length or actual base sequence among individuals at discrete locations in the genome. Two principle methods for DNA typing are currently used: the restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR).

Alec Jeffreys of Leicester University in England is credited with the discovery of hypervariable regions in DNA [212] and their subsequent application to forensic DNA typing [178,292] (**1B**). DNA typing was first used in a forensic context to solve a murder/rape case in Narborough, England in 1987 [680]. Since DNA typing crossed the Atlantic Ocean in the late 1980s, commercial laboratories such as Lifecodes (Stamford, CT) and Cellmark (Germantown, MD) have used it in paternity testing and later criminal cases. The FBI Laboratory began processing criminal cases involving DNA evidence in December 1988 after several years of research and evaluation at the Forensic Science Research and Training Center (FSRTC), FBI Academy, Quantico, Virginia [685]. Since that time, numerous state and local forensic laboratories have begun DNA testing.

The most common DNA profiling technique is restriction fragment length polymorphism (RFLP) analysis using variable number of tandem repeat (VNTR) probes [343,347]. This particular application of RFLP makes use of the fact that

sequence and/or length variations in non-coding regions of DNA can be used to distinguish among individuals in a population. As these VNTR regions of DNA are inherited in a Mendelian fashion, they can be used in paternity testing as well as forensic cases [428]. The RFLP process involves many technical steps, which are time-consuming and not easily automated (**Figure 1-1**). In fact, this entire process can take from several days to a week to complete. Additional loci can be typed after removing the previous probe and repeating the hybridization process (see **1C**). Since six probes are usually hybridized sequentially in a forensic case, the entire process can take 6-8 weeks to obtain results (if radioactive probes are used).

From an analytical standpoint, RFLP presents several challenges. First, a large number of alleles may occur due to variations in the length of the VNTR or the location of restriction sites. To produce a frequency estimate of an allele, the individual bands must be placed into a particular size range or bin based upon a comparison to markers run in neighboring lanes [684]. Second, the resolving capability of the agarose gel system is not uniform over the entire range. For example, alleles which differ in size by 100 bp may be resolved at 1000 bp (e.g. 1000 & 1100) but not at 10,000 bp (e.g. 10,000 & 10,100). Resolution between RFLP bands can vary due to the band widths, which is a function of the quantity of DNA in the sample, size of the fragment, size of the repeat sequence, and the exposure time of the autoradiographic film [684]. The large quantities of intact high molecular weight DNA needed for RFLP are sometimes not available in forensic situations.

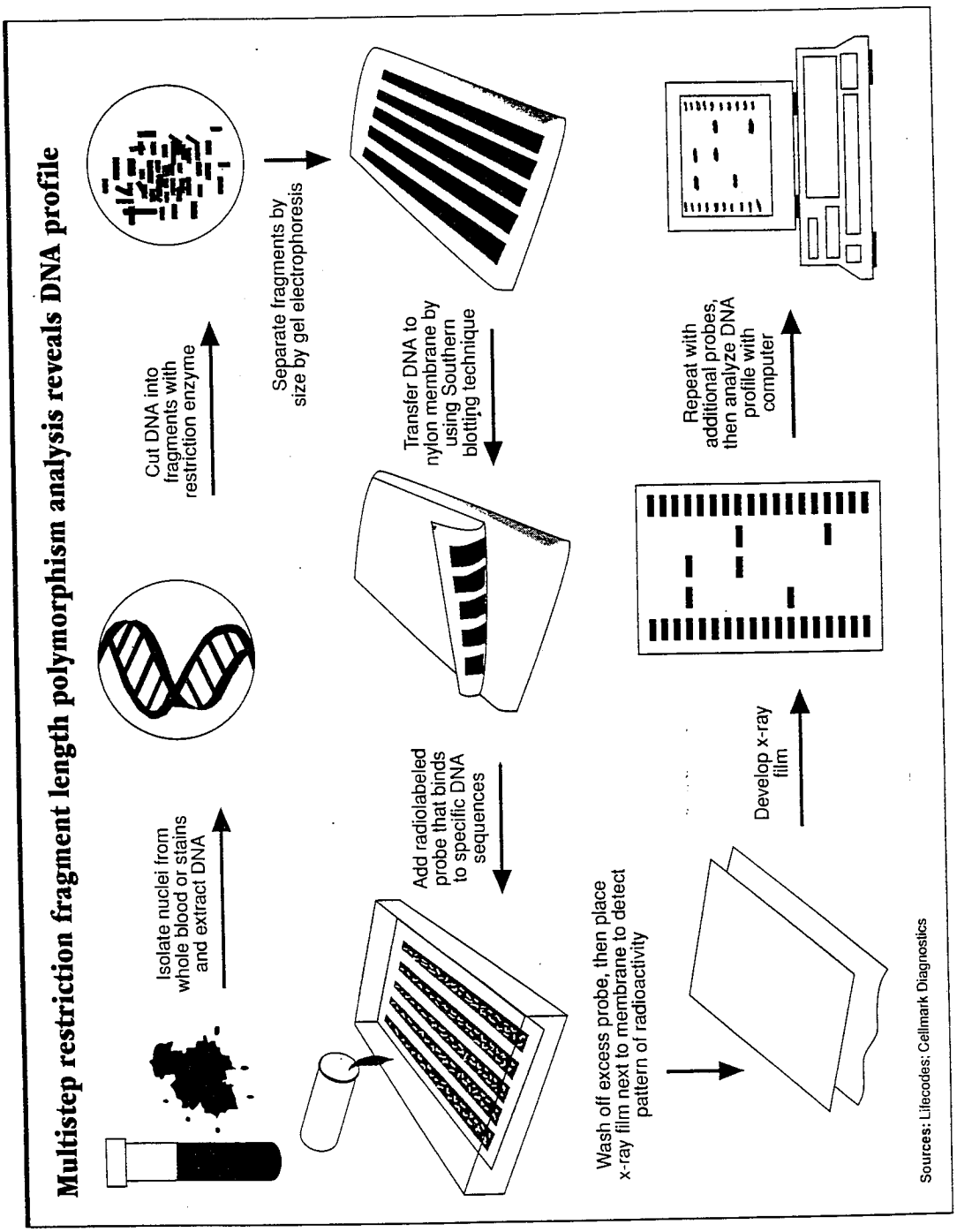

Figure 1-1. The restriction fragment length polymorphism (RFLP) process. Adapted from [343]. See 1C for a detailed description of RFLP.

While RFLP has proven reliable and an effective means for high degrees of discrimination among individuals [343,516,668], it remains a lengthy and laborious process. Detection with radioactive probes is becoming increasing unpopular in the wake of more stringent requirements of those dealing with radioactive materials and their disposal. Many labs have converted to chemiluminescent detection of the VNTR probes [260]. Still others are looking toward new technologies, such as the polymerase chain reaction, which offer great sensitivity and speed [152,323].

III. PCR-BASED METHODS

A. THE POLYMERASE CHAIN REACTION (PCR)

Since its inception in the mid 1980s, the polymerase chain reaction (PCR) has revolutionized the field of molecular biology [168,173,174,184]. The impact of PCR has been such that its discoverer, Kary Mullis, shared the 1993 Nobel Prize in Chemistry, less than 10 years after its introduction. With PCR, specific regions of a small quantity of DNA molecules can be amplified to a readily detectable level. It is generally believed that many of the perceived limitations of RFLP will be overcome by PCR. The popularity of PCR is such that new applications are constantly being developed in the fields of medical diagnostics, forensic science, and molecular genetics [171,178,180,181].

1. The PCR Process

PCR might be considered a molecular xeroxing process. With this *in vitro* reaction, nanogram quantities of genomic material are copied repeatedly in a three-step cycle [173]. A solution containing a DNA template, two oligonucleotide primers, the four deoxyribo-nucleotide triphosphate building blocks (dNTPs), and a thermal stable DNA polymerase (from the thermophilic bacteria *Thermus aquaticus*) [168] is prepared (**Figure 1-2**). These reagents are all combined in 50 mM KCl, 10mM Tris-HCl, and 1.5 mM $MgCl_2$ buffer, usually in a 50 μL volume. This DNA "cocktail" is then placed in a thermal cycler device which can rapidly and precisely change the temperature of the solution (**1D**). In theory, with 32 temperature cycles, a

billion copies of a single DNA template can be amplified in a matter of hours [181].

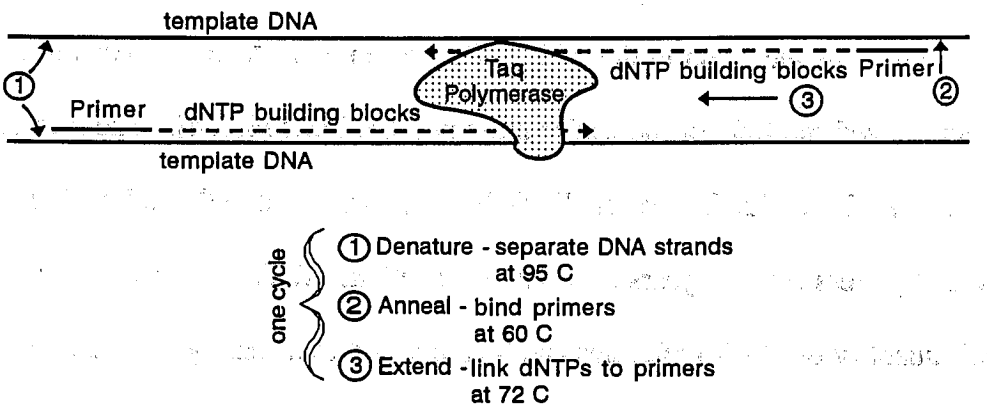

Figure 1-2. Components of the polymerase chain reaction and the effects of temperature cycling.

2. Detection of Amplicons and Determination of Product Size

Agarose gel electrophoresis with ethidium bromide staining is the standard technique for amplicon (PCR product) detection in most molecular biology laboratories [321] (**1E**). The conventional method of determining DNA fragment size is by comparison to a standard in an adjacent lane. Since multiple lanes can be run on each gel, this side-by-side comparison is possible. If quantitative information is desired, the gel is scanned using a densitometer, and results are compared to reference standards. When higher resolution is desired (than can be afforded by agarose), cross-linked polyacrylamide gels, containing smaller pores, can be used [241]. An alternative for PCR product sizing is capillary electrophoresis [152,559]. The development of this technique will be discussed in the following chapter.

B. THE USE OF PCR IN DNA TYPING

The speed, sensitivity, and specificity afforded by use of the polymerase chain reaction, along with the ability to examine degraded DNA, have made PCR-based methods the next generation of DNA typing techniques for forensic laboratories [323]. Analysis times are on the order of days rather than the weeks required for RFLP. In addition, alleles are formed in discrete sizes and thus can be evaluated without determining their base pair size. Most of the forensic DNA community is investigating the use of PCR for the examination of length polymorphisms and sequence polymorphisms (**Figure 1-3**). With length polymorphisms, the number of copies of a repeated sequence can differ among individuals. Whereas with sequence polymorphisms, the actual nucleotide sequence may differ. In either case, for PCR methods to work, relatively constant regions on either side of the sequence or length variable region must exist to allow binding of the PCR primers (**Figure 1-3**).

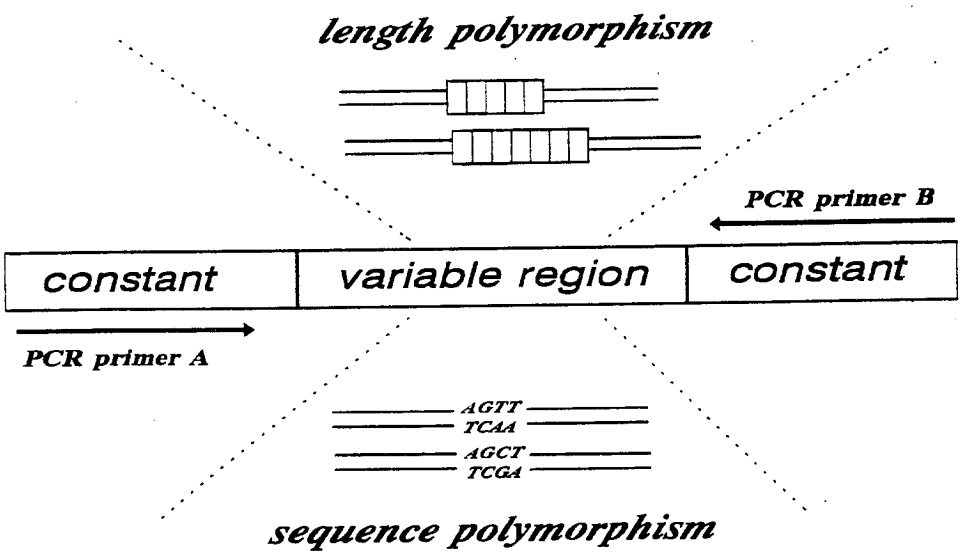

Figure 1-3. The flanking DNA sequences of length or sequence polymorphisms must be relatively constant between individuals to allow reliable primer annealing sites for PCR.

1. Length Polymorphisms

Tandemly repeated DNA sequences (e.g., VNTRs or STRs) are the primary length polymorphisms examined in DNA typing methods. VNTR loci are usually classified into two groups depending on the length of the core repeat sequence: (1) minisatellites (VNTRs) with 9-80 bp repeats and (2) microsatellites (short tandem repeats, STRs) with 2-7 bp repeats [664]. Both VNTRs and STRs are widespread throughout the genome. In fact, it has been estimated that the human genome contains approximately 500,000 STRs [417]. Often these STR loci are associated with genes characteristic for genetic disorders. As a result, study of VNTR/STR loci has become important in several fields including genetic mapping, linkage analysis, genetic disease, and mutational studies, as well as forensic and paternity testing [159] **(1F)**.

Variable Number of Tandem Repeats (VNTRs)

Two common PCR-amplified length polymorphisms include the VNTRs apoB [258,340] and D1S80 [241,259]. These VNTRs contain repeat sizes of 14 and 16 bp, respectively, and fragments in the size range of 350-1000 bp when amplified by PCR. Dozens of alleles can exist for many VNTR loci. A heterozygosity number is often used as a measure of the possible number of alleles and their frequency distributions. Essentially, the heterozygosity is an indicator of the polymorphic nature of the system and its usefulness in distinguishing among individuals **(1G)**.

Typing of VNTRs (or STRs) is usually done by comparison of an individual's

PCR-amplified DNA with a standard which contains a majority of the possible alleles in the population at large. This standard, termed an allelic ladder, is formed by mixing PCR-amplified DNA obtained from individuals who are known to possess the desired alleles [241,419,645].

When PCR primers straddle a VNTR region, the length of the PCR product generated for each allele is equal to the number of repeat units times the repeat unit length plus the size of the flanking regions. Thus, in order to resolve alleles differing by a single repeat unit, a separation system must demonstrate resolution equal to the length of the repeat unit over the size range of possible alleles.

Several difficulties with large VNTRs have encouraged some researchers to look for loci with smaller repeats. "Allelic dropout," a situation where the larger allele of a heterozygous individual fails to amplify because it is not as efficiently extended by the polymerase, has raised some concern with the use of large PCR products [164]. In addition, the wide size range of alleles at VNTR loci can limit the the number of VNTR systems which may be simultaneously amplified and detected, due to overlap in fragment sizes [164]. These difficulties may be overcome with short tandem repeats, which can have repeat units ranging from 3-5 bp.

Short Tandem Repeats (STRs)

Short tandem repeats have several advantages over the larger VNTRs including: narrow allele size ranges which reduce preferential amplification [163], the ability to coamplify several regions simultaneously [163,619], great abundance

throughout the genome [159], and small allele sizes (generally <300 bp) which permit degraded DNA to be more successfully analyzed [325,619]. Degraded DNA, where the genomic material has been fragmented into smaller pieces, can be a factor in forensic cases, especially when the biological evidence has been exposed for long periods of time to chemical or physical forces in the environment [619].

Though numerous STR loci have been reported in the literature, many do not fit the characteristics needed for identity testing: faithful amplification, interpretable and accurate results, and independence from other STR loci [270]. For example, dinucleotide repeats often form "shadow bands" from strand slippage or polymerase stuttering [270,325]. Therefore, the less common tri-, tetra-, and pentanucleotide repeats are preferred because they amplify more reliably and provide more easily interpretable results (i.e., N and N+1 alleles are further apart) [270,325].

Some STRs may exhibit sequence variance within the repeat unit itself (**1H**). When an allele from such a system is identified, it is designated by the number of complete repeat units and the number of base pairs of the partial repeat, separated by a decimal point [480]. Two common examples come from the tetranucleotide repeats TH01 and F13A01 (**TABLE 1-1**). TH01 contains a common allelic variant that is 1 bp shorter than 10 repeat units, and is therefore referred to as a 9.3. The smallest allele for the F13A01 locus is 2 bp shorter than 4 repeats, or a 3.2 [421]. **TABLE 1-1** compares the characteristics of several STRs systems which are commercially available for human identity testing [681]. STRs are particularly useful in forensic DNA typing as they can be efficiently amplified by PCR, even with degraded DNA,

and the subsequent PCR product size or allele can be precisely and accurately determined.

TABLE 1-1. Commercially Available STR Typing Systems. From Ref. [681].

Locus	Chromosome	Repeat	Known Alleles	Heterozygosity‡
TH01	11p15.5	AATG	5-9,**9.3**,10,11	0.81
F13A01	6p24-25	AAAG	**3.2**,4-16	0.67
vWF (VWA)	12p12-pter	AGAT	11,13-21	0.83
FES/FPS	15q25-qter	AAAT	7-14	0.71
CSF1PO	5q33.5-34	AGAT	7-16	0.73
TPOX	2p23-2pter	AATG	6-13	0.66
F13B	1q31-q32.1	AAAT	6-11	0.66
LIPOL	8p22	AAAT	7,9-14	0.71
HPRTB	Xq26	AGAT	6-17	0.66
AMEL	Xp22.1 & Y	N/A	212bp/218bp	N/A

‡ Estimated from a Caucasian database.

Multiplexed STRs

The desire for more information, coupled with the need to limit consumption of a DNA sample where its availability is limited (e.g., evidence from a crime scene), has led to the coamplification and typing of multiple STR systems (**1J**). Multiplex PCR, which involves adding more than one set of PCR primers to the reaction in order to target multiple loci, is an ideal technique for DNA typing because the probability of identical alleles in two individuals decreases with an increase in the number of polymorphic loci examined [165] (see **1K**).

Researchers have simultaneously amplified three [163,324], four [344,163, 619], and even seven [163,473] or eight [641] STR loci using PCR. By using an octoplex system, sex determination (see below) can be incorporated along with a combined probability of matching between two unrelated individuals of 1×10^{-9} or better, a value which is in excess of that produced by four single locus RFLP probes [641].

Sex-Typing

The ability to designate whether a sample originated from a male or female is useful in rape cases, where distinguishing between the victim and the perpetrator's evidence is important. Gender identification has recently been demonstrated using PCR [426,544,642]. In one study, primers were designed to flank a 6 bp deletion within intron 1 of the amelogenin gene on the X homologue [544]. The amplification of this area results in 106 bp and 112 bp PCR products from the X and Y chromosomes, respectively [544]. The Promega Corporation has since designed different primers which yield a 212 bp X-specific band and a 218 bp Y-specific band (see AMEL in **TABLE 1-1**) [681]. Mannucci *et al.* [544] were able to detect as little as 20 pg (~3 diploid copies) and sample mixtures where female DNA was in 100-fold excess of male DNA. More recently, the amelogenin sex-typing marker has been used in combination with other STR loci [473,619,641].

2. Sequence Polymorphisms

Several hypervariable regions, including the control region of mitochondrial DNA and the second exon of the HLA gene DQα (**1L**), exhibit enough sequence variability among individuals to be useful for human identity testing. In order to distinguish among alleles which are the same length, but differ internally, one of two methods is typically used: (1) hybridization to known oligonucleotides (**1M**) or (2) direct sequence analysis of each base by Sanger sequencing [506]. Multiple sequence polymorphisms can also be simultaneously amplified (**1N**).

Mitochondrial DNA (mtDNA)

All of the previously mentioned markers are found in DNA housed in the cell nucleus. Another form of DNA, mitochondrial DNA (mtDNA), is also being investigated for use in forensic DNA typing. Mitochondria have an extranuclear DNA genome which encodes several of the transfer and ribosomal RNAs used for translation of mitochondrial genes. Human mtDNA consists of a circular molecule containing 16,569 bp [505] (**TABLE 1-2**). The first reported sequence, known commonly as the Anderson sequence, is used as a reference to which all subsequent analyses have been compared [190,505].

Mitochondrial DNA has potential forensic value because it can be efficiently amplified from limited biological material. In cases where the amount of extracted DNA is very small, as in tissues such as bone, teeth, and hair, the probability of obtaining a DNA typing result from mtDNA is higher than that of polymorphic

markers found in nuclear DNA, primarily because there are more copies of mtDNA per cell [190]. In addition, mitochondrial DNA is more robust than nuclear DNA and will often survive extreme environmental conditions [256,543]. However, because mtDNA is maternally inherited with no genetic recombination between generations, it is not as polymorphic as many nuclear (RFLP) markers. The lack of editing functions in the mtDNA polymerase does introduce variation through a relatively high mutation rate.

TABLE 1-2. Characteristics of Nuclear and Mitochondrial DNA.

	Nuclear DNA	Mitochondrial DNA
Size	~3 billion bp	16,569 bp
Copies per cell	2	can be >1000
Structure	linear	circular
Inherited from	father & mother	mother
Generational recombination	yes	no
Mutation Rate	low	10-50X nuclear
Sequence known	Genome Project goal	described in 1981

The most extensive mtDNA variations between individuals in the human population are found within the control region, or displacement loop (D-loop) [254] (**1P**). Two hypervariable regions in the D-loop, known as HV1 and HV2, are normally examined for polymorphisms [190,247]. Following PCR amplification of the HV1 and HV2 regions, their base compositions are normally determined by direct

sequencing [190,247,254] or sequence-specific oligonucleotide probes [427,507] (see **1M**).

Typing of mitochondrial DNA has proved useful in identifying human skeletal remains from the Vietnam War [255] and the family of the last Russian Tsar, Nicholas II [256,621]. MtDNA is also being sequenced from human hairs shafts, which usually fail to yield typeable results from nuclear markers [247,253].

C. GENERAL ACCEPTANCE OF PCR-BASED DNA TYPING

DNA typing methods offer a powerful technique to law enforcement for connecting suspects to, or excluding them from, a crime scene or identifying human remains at an accident. The last decade has seen numerous advances in the field of forensic identification, and the future will probably hold many more. While the advantages of PCR methods over RFLP can clearly be seen in **TABLE 1-3**, many laboratories and the courts are slow to accept new procedures. Laboratory validation and court acceptance of PCR-based methods is proceeding, but at a slower pace than might be expected given the current state of research (**1Q**). The general acceptance and use of the faster and more sensitive PCR processes for DNA typing are anticipated.

TABLE 1-3. Comparison of RLFP and PCR Methods Used for DNA Typing.

	RFLP	PCR
Time Required	can take 6-8 weeks	1-2 days
Amount of DNA needed	50-500 ng	0.1-1 ng
Condition of DNA for typable results	high MW, intact	may be degraded
Hazardous Materials	radioactive probes	none
Multiple Probes	sequential	simultaneous
Allele Identification	binning required	discrete alleles
Discriminating Power	$>10^{-9}$ (6 loci)	$>10^{-9}$ (8-12 loci)
Automatable	no	yes

Chapter 2

Capillary Electrophoresis as a Tool for DNA Analysis

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I. Introduction to Capillary Electrophoresis

A. THE VALUE OF CE FOR DNA ANALYSIS

The electrophoretic separation of DNA is an indispensable technique in a modern molecular biology laboratory and in DNA typing [675]. While slab gel electrophoresis has been a proven technique for 30 years, there are several advantages to analyzing DNA in a capillary format: the injection, separation, and detection can be automated; only minute quantities of sample are consumed; high voltages are permitted as a result of better heat dissipation; qualitative and quantitative information are available; and the time at which any band elutes is precisely determined (2A). While CE has many advantages over traditional gel electrophoresis, its use is not widespread due to several problems including: difficulty of injecting detectable levels of DNA from high salt matrices, and the lack of procedures for accurate sizing and quantitation of DNA fragments. This work describes solutions to these problems and demonstrates the reliability of CE for DNA analysis, specifically DNA typing of PCR products.

B. FUNDAMENTALS OF CE

To properly relate the advantages of CE over gels, it is important to understand how electrophoresis works in general and how the high voltages used in CE can improve the speed and efficiency of a separation. Electrophoresis is the movement of an ion under an applied electric field. The velocity (v) at which the ion moves can be described by the equation

$$v = \mu_e E = \mu_e (V/L) \quad (2\text{-}1)$$

where μ_e is the electrophoretic mobility (cm^2/Vs), E is the electric field strength (V/cm), V is the voltage applied across a gel or a capillary, and L is the gel or capillary length (cm). The μ_e of the ion may be further defined in terms of its charge and Stokes radius

$$\mu_e = \frac{q}{6\pi\eta r} \quad (2\text{-}2)$$

where q is the charge of the ion, η is the viscosity of the buffer, and r is the radius of the ion. Empirical determination of μ_e (cm^2/Vs) for a solute may be accomplished with knowledge of the peak migration time (t_m,s), the capillary length (L_t,cm), the length to the detector (L_d,cm), and the applied electric field (V,volts) using the relationship,

$$\mu_e = \frac{(L_d)(L_t)}{(V)(t_m)} \quad (2\text{-}3)$$

The electrophoretic mobility of an ion, as determined by Eq. 2-3, is independent of the separation conditions (voltage, column length, separation time).

In a separation technique, such as CE, the ability to resolve the various components in a mixture is important and is usually judged in terms of column efficiency or peak resolution. The optimum conditions for a separation are usually those in which a particular minimum resolution can be achieved in the shortest period of time. There are two parameters which are important to consider for a separation: peak width (efficiency) and peak separation (resolution). In this work, separation

efficiency (N, number of theoretical plates) was defined as

$$N = 5.54\left[\frac{t_1}{HW_1}\right]^2 \qquad (2\text{-}4)$$

while the resolution (R) between two peaks was determined by

$$R = (2\ ln2)^{1/2}\frac{t_2 - t_1}{HW_1 + HW_2} \qquad (2\text{-}5)$$

where t_1 and t_2 are the migration times of each peak and HW_1 and HW_2 are the full widths at half-maximum of peak 1 and 2 [126]. Several factors influence CE separations and the ability to resolve sample components in a timely and reproducible manner: electroosmotic flow, Joule heating, and band broading. The above equations can be used to evaluate the effect of these factors on the overall separation.

Electroosmotic Flow

In fused silica capillaries, where negatively charged silanol groups line the inside of the capillary wall, a double layer can form from cations in the electrophoretic buffer being attracted to the silanol groups. Upon application of an electric field, the mobile cations in the diffuse double layer migrate toward the cathode which pulls solute molecules in the same direction and generates electroosmotic flow (EOF). This EOF is highly dependent on environmental parameters, such as pH, temperature, voltage, and buffer viscosity [16]. Unless EOF is carefully control or eliminated, obtaining reproducible separations of DNA will be difficult [3,12]. Coating the inner wall of the capillary, to cover the charged silanol

groups, is a solution preferred by many researchers [231,280,283].

Joule Heating

As seen in Eq. (2-1), the speed of the separation increases in direct proportion to the voltage applied (**2B**). However, there is a limit to the applied voltage which can be absorbed by the system. The movement of ions in an electric field generates heat. In order to determine at what electric field strength the system becomes unable to dissipate this Joule heat, the applied voltage is plotted against the current in what is commonly referred to as an Ohm's law plot (**Figure 2-1**). The point at which this plot deviates from linearity (inset arrow) is the conventional method for determining the optimum separation voltage in CE [402].

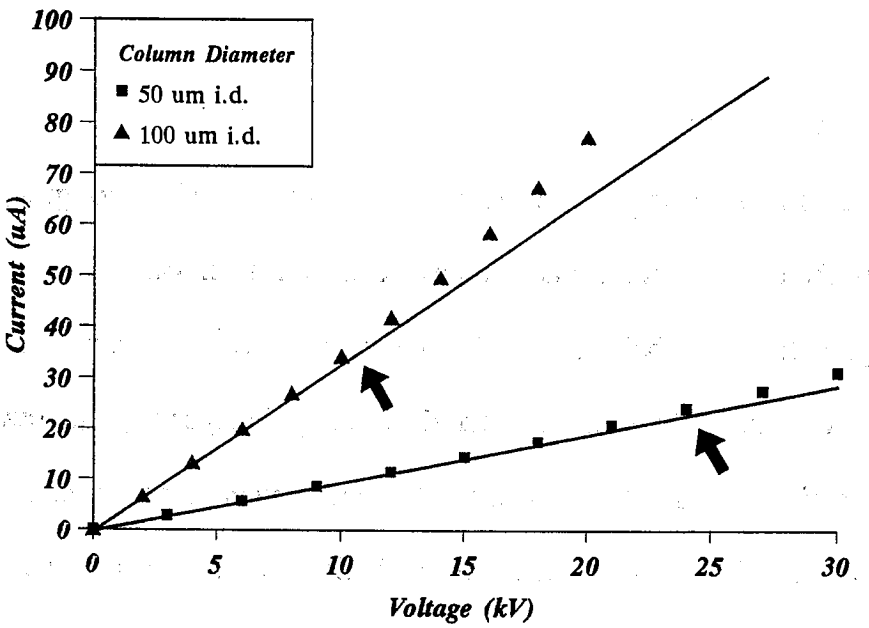


Figure 2-1. Ohm's Law plot showing linearity between voltage and current while Joule heat is dissipated. The arrows indicate the optimum separation voltage. The values in the plot were determined by stepping through the voltages indicated and measuring the current. These measurements were made with a 37 cm DB-17 capillary and a 1% HEC, 100 mM Tris-borate-EDTA buffer (see Ch. 4).

Notice that the narrower capillary (50μm i.d.), which has a higher surface area-to-volume ratio, can dissipate heat more efficiently at higher voltages and hence the operating voltage can be higher (**Figure 2-1**). With higher separation voltages, less longitudinal diffusion can occur (see below) and peak efficiency will improve.

Band Broadening

In any chromatographic process, keeping the analyte zone narrow throughout the separation is important for high efficiency and resolution. The main band broadening factor in CE is longitudinal diffusion, which is kept to a minimum due to the high electric fields and rapid run times (**2C**).

II. Capillary Electrophoresis Parameters

Figure 2-2 illustrates the principal elements of a typical CE instrument used in DNA analysis. A narrow capillary, with an internal diameter of 20-100 μm (375 μm O.D.) and a length of 25-100 cm, connects two buffer vials (**2D**). After the sample is injected onto the column, electric fields on the order of 2,000-20,000 volts are applied across the narrow bore capillary to separate the components. Samples are separated as they move through a viscous polymer solution or a cross-linked gel and are detected by laser-induced fluorescence or UV absorbance as they flow past an etched window in the polyimide coating of the capillary (**2E**).

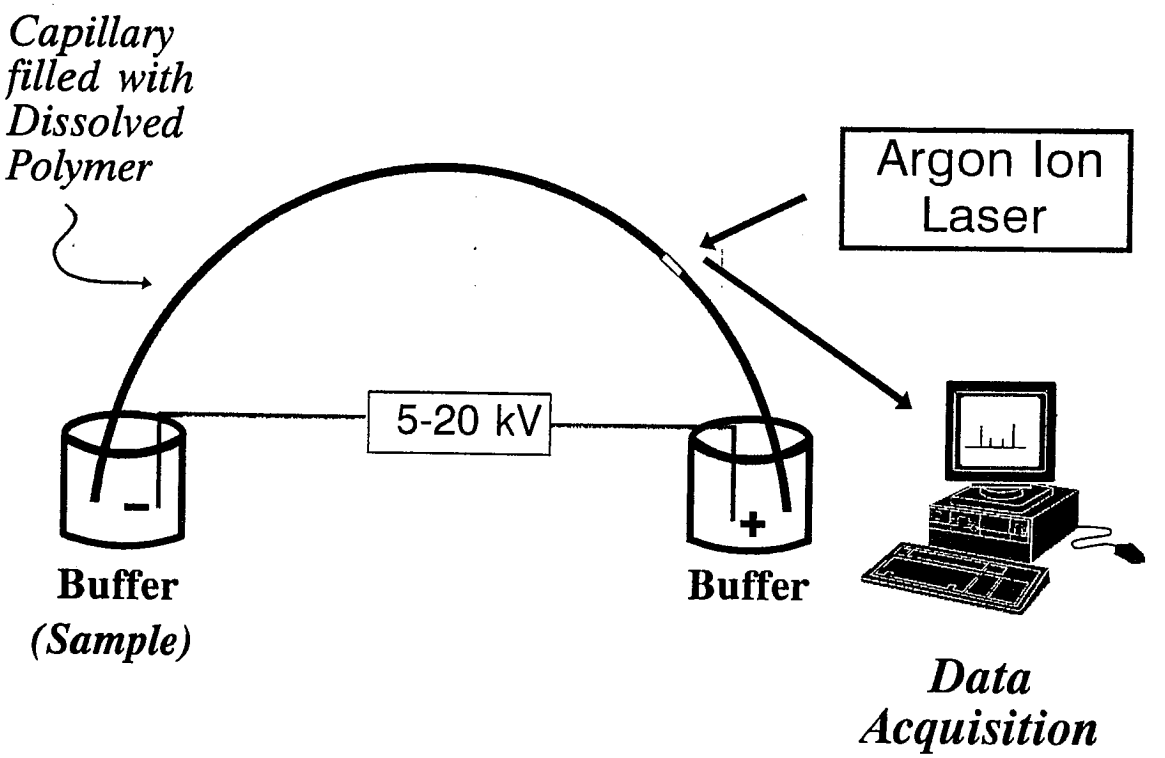


Figure 2-2. Capillary electrophoresis apparatus used for DNA analysis.

Several options in the CE format are available for DNA analysis. The different types of column coatings, injections, separation media, and detection schemes are described below.

A. COLUMN COATINGS

Although DNA separations have been demonstrated in uncoated capillaries [35,45,565] (**2F**), most researchers prefer to use coated capillaries to improve migration time precision [12,16] and reduce electrostatic interaction with the column wall [52,53] (see **APPENDIX 1**). An appropriate capillary wall coating needs to be reproducible in thickness, degree of polymerization, and column-to-column performance [269]. This can often be challenging with narrow capillaries [269]. Efficient coating of a capillary's inner surface has been improved in the past several years by the work of several groups including Hjerten [280] and Schomburg [283,407]. In many cases, gas chromatography (GC) column coating technology has been adapted to the narrower CE capillaries [23,314]. Both hydrophilic coatings, such as polyacrylamide [280] or polyvinyl alcohol [283], and hydrophobic coatings, such as the DB-17 [23,33] or the DB-1 [23,216], have been used.

When replaceable separation mediums are used, the lifetime of the capillary is limited only by the intregrity of the capillary wall coating. Unfortunately, many capillaries last for only a few injections before degradation of the inner wall coating diminishes resolution. This instability has been attributed to base-catalyzed hydrolysis of the siloxane bond (DB-1 and DB-17) at the silica surface [269]. While previous

workers have demonstrated up to 418 runs in a single capillary [75], the procedures described in this work have shown that it is possible to reuse the same capillary for several thousand runs.

B. INJECTION OF DNA FRAGMENTS

1. Hydrodynamic Injection

DNA may be injected onto a CE column by pressure or voltage (**Figure 2-3**). A hydrodynamic (HD) injection involves forcing a plug of the sample onto the end of the capillary by applying a pressure differential (**Figure 2-3a** and **2G**). Hydrodynamic sampling is generally considered the most precise method of injection because it is based solely on the volume loaded [686]. The volume of the sample introduced to the capillary by HD injection (V_{HD}) may be defined by the Poiseuille equation:

$$V_{HD} = \frac{(\Delta P \pi d^4 t)}{(128 \eta L_t)} \qquad (2\text{-}6)$$

where ΔP is the pressure difference across the ends of the capillary for a given unit of time (t), L_t is the capillary length, d is the inner diameter of the capillary, and η is the sample viscosity [686]. Quantity (Q) of sample components injected can then be determined by $Q_i = C_i \times V_{HD}$, where C_i is the concentrations of the sample components (i).

Resolution is often diminished when using pressure injections because a broad sample zone is introduced on the capillary, which can adversely affect separation efficiency. While it is often difficult to inject enough sample material for detection

from dilute samples, HD injections are independent of the sample matrix, which becomes important with PCR samples [86,151].

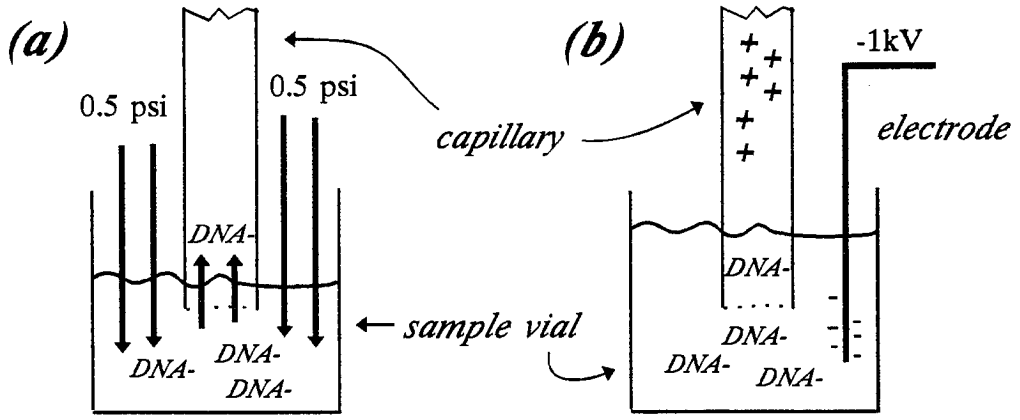


Figure 2-3. A schematic of (a) hydrodynamic and (b) electrokinetic injections. With an HD injection, pressure (0.5 psi) introduces an homogeneous plug of the DNA sample; with EK, an electric potential of 1,000 volts draws the charged species, including DNA, onto the capillary. EK injections are more sensitive but are adversely affected by ionic matrices, like PCR samples.

2. Electrokinetic Injection

Electrokinetic (EK) injection selectively introduces the charged species in the sample into the capillary by the application of a potential difference to the sample [86,372] (**Figure 2-3b**). More sample material can be introduced into the capillary by simply increasing the potential or the time applied. Resolution with EK injection is generally better (than HD) due to the fact that samples are introduced onto the column in a narrow sample zone. However, EK injection is less reproducible and quantitative than HD as the charge-to-mass ratios of the ions in the sample will influence the rate of column loading [86] while ionic strength can influence the quantity of sample

loaded [581]. The equation described by Rose and Jorgenson [14] demonstrates the effect of sample conductivity:

$$Q = \frac{\pi r^2 c_s(\mu_{ep}+\mu_{eo})Et\lambda_b}{\lambda_s} \qquad (2\text{-}7)$$

where Q is the amount of sample material injected, r is the radius of the capillary, c_s is the sample concentration, E is the electric field applied, t is the injection time, λ_s is the sample conductivity, λ_b is the conductivity of the buffer electrolyte, and μ_{ep} and μ_{eo} are the mobility of the sample molecules and the electroosmotic mobility, respectively. Notice that the amount of material injected is inversely proportional to the ionic strength of the sample. For this reason, it is difficult to electrokinetically inject PCR samples, which may have >50mM chloride in the sample matrix. There are several solutions to dealing with an ionic sample matrix, including salt removal and sample stacking (see below).

3. Sample Cleanup Prior to Injection

Salt removal has been described as an important step in analyzing PCR-amplified DNA by capillary electrophoresis [23,24,52,155] (**2H**). Since chloride ions, resulting from the KCl and $MgCl_2$ used in the PCR buffer, have a higher charge-to-mass ratio than the larger DNA ions, they preferentially migrate onto the CE column when EK injection is used [155]. However, another solution exists which avoids an extra sample cleanup step.

4. Sample Stacking

The desire to load more material from the sample vial onto the column has led to an interesting technique known as field amplified injection or sample stacking [111,112,261,440]. Burgi and Chien [440] discovered that the preparation of a sample in a lower concentration of the electrophoresis buffer produces a stacking effect (**Figure 2-4**). Upon application of the electric field with an EK injection, the resistance and field strength in the sample plug increase, which causes the sample ions to migrate rapidly and stack as a sharp band at the boundary between the sample plug and the electrophoresis buffer. This stacking occurs because the effective field strength is lower in the buffer region than in the sample, and thus, the sample ion mobility is reduced in this region [581]. The amount of on-line sample stacking is proportional to the buffer-to-sample ionic strength ratio [581]. Hence, samples prepared and diluted in water should give the highest degree of stacking. A method of sample stacking has also been described which involves injecting a plug of water into the capillary before injecting the sample [48,112,581].

Problems with sample injection from highly ionic matrices (e.g. PCR samples) or detection of low sample concentrations can be reduced with stacking. This work describes the utilization of sample stacking techniques for efficient injection of PCR samples [559]. Briefly, the sample is diluted 50-fold in deionized water, which reduces the ionic strength (relative to the buffer) and enhances sample stacking. However, since the sample concentration is reduced more sensitive detection techniques are required (see below).

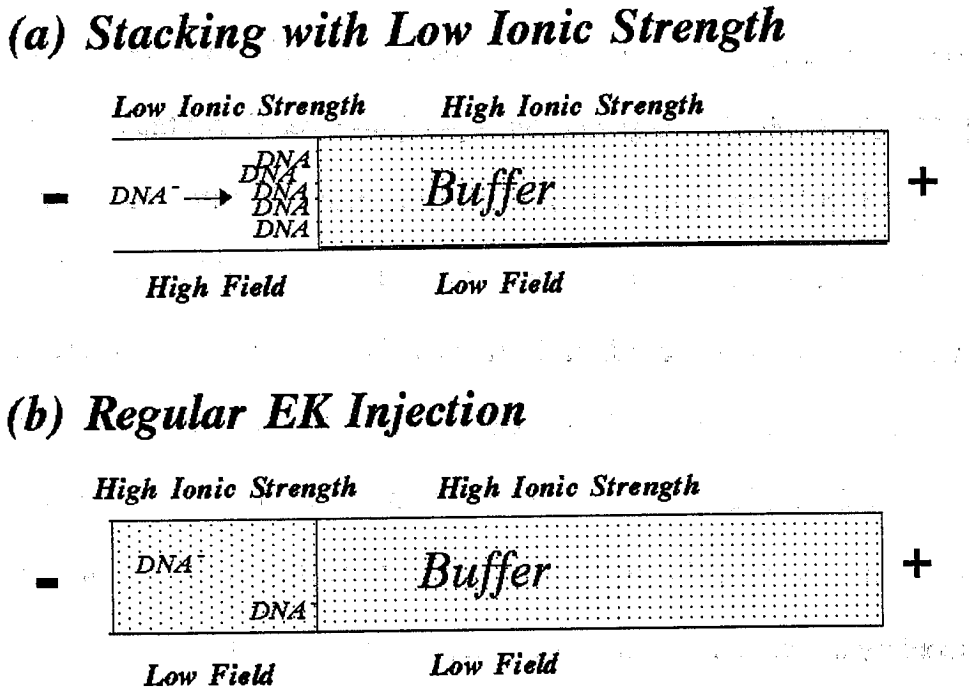


Figure 2-4. Sample stacking results from differences in ionic strength between the sample and the buffer. With low ionic strength sample matrices (a), the DNA experiences a high electric field in the sample region and rushes to the sample-buffer interface where the sample components stack up. From a high ionic strength matrix (b), very little DNA is injected because of the low field experienced by the sample.

C. SEPARATION MEDIA FOR DNA

For each additional nucleotide unit added to a DNA molecule, the charge is increased proportional to the addition in mass. As a result, DNA fragments larger than about 10 bp possess essentially the same electrophoretic mobility [631]. Since DNA possesses this constant mass-to-charge ratio, some form of sieving matrix is needed to separate DNA fragments by molecular size. In traditional gel

electrophoresis, the requirement for a sieving matrix is met with agarose or polyacrylamide gels. In CE, capillaries filled with polyacrylamide gels or a viscous polymer solution are used.

1. Gel-filled Capillaries

Some of the first work with CE separations of DNA fragments involved transferring the agarose and cross-linked polyacrylamide matrices of slab gel electrophoresis into a capillary format [80,108,288]. While resolution of oligonucleotides and double stranded DNA (dsDNA) has been demonstrated by numerous research groups [73,282,675], these gel-filled capillaries face numerous difficulties when used routinely. Poor gel-to-gel reproducibility, bubble formation under electrophoretic conditions, gel matrix collapse under high electric fields, and difficulties in preparing the gels are a few of the problems involved with using crosslinked polyacrylamide gels in a capillary format [133,403]. In addition, the rigid, chemically-crosslinked pore structure retains high molecular weight DNA and is prone to contamination [47]. With each successive run, there is an increasing chance that the presence of impurities will produce anomalous peaks [675].

Most gel-filled capillaries have a limited lifetime of only a few runs, which prohibits their use in routine operations [675]. A further problem is that samples may only be introduced by electrokinetic injections as pressure injections would dislodge the gel from the capillary. As a result, non-crosslinked separation media are being sought for routine application of CE to DNA analysis.

2. Entangled Polymer Networks

In 1989, Zhu *et al.* [533] demonstrated that a dilute, low-viscosity polymer solution could resolve DNA fragments. Instead of forming a permanent chemical gel (e.g., cross-linked polyacrylamide) inside the capillary, concentrated polymer strands can become entangled and form a physical gel (see Chapter 3). This sieving matrix has the advantage of being able to be pumped into and out of the capillary before and after each analysis, which aids column longevity by eliminating many of the problems mentioned above. More importantly, these polymer solutions are inexpensive and easy to prepare.

Promising candidates for soluble polymer sieving media include linear polyacrylamide (LPA) [75], hydroxyethyl cellulose (HEC) [24,35], hydroxypropyl methyl cellulose (HPMC) [23,533], and other cellulose derivatives [69] (see also [675]). These polymer networks exhibit many advantages over the gel-filled capillaries (**TABLE 2-1**) and produce a system which is reproducible, replaceable, and robust. Higher stress (i.e., higher electric fields and faster run times) can be applied to the system without collapse of the matrix. A wide range of DNA fragment sizes may be separated because the polymer chains are more flexible and the resultant "pores" are transient (see **Figure 3-8**). There are some disadvantages though to using entangled polymers. Resolution is often not as good with pumpable polymers. While Karger and coworkers [75] have shown that more viscous buffers can resolve DNA fragments to the point that sequencing is possible (i.e., 1 bp), replacing such viscous media is difficult.

TABLE 2-1. Chemical Gels Compared with Physical Gels for Use in CE/DNA.

	Chemical gels	Physical gels
Cost per column (commercial)	~$100	<$20‡
Replaceable (between runs)	no	yes
Longevity	usually <50 runs	>1000 runs demonstrated
Achievable Resolution	1 bp	usually 2-4 bp
Separation Range	limited because of fixed pore size	wider range of pore sizes

‡The coated capillaries used in these studies may be purchased in 10 m rolls for ~$450. A 30 cm capillary would therefore only cost $14. The soluble polymer separation medium may cost less than 1 cent at the quantities used in this study and can be used for muliple analyses.

D. DETECTION OF DNA FRAGMENTS

The narrow capillaries which improve resolution at the same time adversely affect the sensitivity. In addition, the narrow bore of the capillary limits sample introduction. With only nanoliters of sample being injected, detection must be extremely sensitive. On-line detection of DNA fragments in CE has been demonstrated with UV absorbance and laser-induced fluorescence.

1. UV Absorbance

The nucleobases of DNA absorb strongly at 260 nm allowing the detection of DNA fragments moving through the capillary [675,677]. This inherent absorbance is a major advantage for some studies as no derivatization of the molecule is required,

leaving the structure and the mobility of the DNA unperturbed [675]. However, with the application of narrow capillaries in CE, sensitivity is diminished because the pathlength of detection is reduced (2J). The wide availability and relatively inexpensive design of UV detectors will ensure their continued use, although fluorescent detection is a more sensitive technique.

2. Laser-induced Fluorescence

A more sensitive means of detecting DNA fragments is laser-induced fluorescence (2K). In LIF methods, a laser excites a fluorophore which is attached to the DNA molecule (2L). This fluorescent tag may be attached in a derivatization step either prior to or during the separation (see below). The emission of photons from the excited fluorophore is then detected by a photomultiplier tube (PMT) and converted into the electropherogram. For routine use, LIF is becoming increasingly popular because of its superior sensitivity (2M). Schwartz *et al.* [315] recently published a detailed review of LIF detection and its applications.

The sensitivity of fluorescence detection results in less sample material being consumed for each analysis, allowing some of the sample to be saved for retesting or further characterization (see [417]). Fluorescent labeling may be performed in any one of three ways with PCR products: (a) adding a fluorescent dye on the 5'-end of the forward or reverse oligonucleotide primer, (b) incorporating fluorescently labeled dNTPs into the PCR product, and (c) using a fluorescent intercalating dye to bind to the DNA [51]. Methods (a) and (b) are performed during the PCR process while (c)

is post-PCR.

There are several advantages to using intercalating dyes. Better sensitivity is possible as multiple fluorescent tags can bind each molecule (vs. one tag for fluorescently labeled primers). In addition, on-column derivatization using fluorescent intercalating dyes is less expensive and more efficient than use of fluorescently-tagged PCR primers or dNTPs (2N). The number of commercially available fluorescent intercalators, which may be used for labeling DNA restriction fragments as well as PCR products, is steadily growing (see Chapter 3).

III. Uses of CE in DNA Analysis

A. QUALITATIVE ANALYSIS

Most of the previous work with DNA separations has involved restriction digests, where a plasmid or other DNA molecule is cut into smaller fragments which differ by length and/or sequence (**2P**). The most popular restriction digests include ϕX174 *Hae*III, pBR322 *Hae*III, and pBR322 *Msp*I, where DNA fragments in the size range of 50-1500 bp can be generated. Work with larger DNA fragments is usually done with λ-*Hind*III (125-23,130 bp). These digests are popular because they are relatively inexpensive, can be obtained at high concentration in a purified form, and contain fragments of known size. For these reasons, most researchers use restriction digests to demonstrate the resolution of their CE system.

1. Use of CE for PCR Product Analysis

PCR product analysis by CE has evolved somewhat slower than DNA restriction digest evaluations due to difficulties with injecting amplified DNA from highly ionic matrices, lack of quantitative procedures for sizing DNA, and a shortage of adequate systems to study. Interest in PCR product analysis by CE has increased recently because of perceived benefits to medical diagnostics [23,150,234,235,271, 309,383,470,492,523,529,531,532] and forensic DNA typing [24,27,33,52,149,151, 152,155,247,248,559].

2. Prior Work Involving CE and Forensic DNA Analysis

Several reports have appeared in the literature describing CE separations for DNA typing applications. Srinivasan *et al.* [27,52] separated PCR-amplified alleles from two VNTR loci (apoB and D1S80) and some short mtDNA fragments (130-140 bp). A mixture of two sets of heterozygous alleles (at a 4:1 ratio) were easily resolved and detected in 30 minutes [52]. This group used the dimeric intercalating dyes TOTO-1 and YOYO-1 with a CE-LIF system. Pearce and Watson [149] explored the use of a phosphate buffer (pH 5.7) with HPMC for PCR-amplified apoB alleles. Williams and coworkers [155] at the Armed Forces DNA Identification Laboratory examined several buffer and gel chemistries for the separation of HUMTH01 alleles. Prior to the work described herein, the possibility of STR analysis was addressed in several papers [24,33]. McCord and coworkers examined a soluble polymer buffer [24] and intercalating dye [33] system and demonstrated the resolution needed to resolve several STR (4 bp) or VNTR allelic ladder components, including SE33 and D1S80 [24] and VWA, HUMTH01 (TC11), MBP, and YNZ22 [33]. These separations typically took from 25-35 minutes per run.

None of these reports described procedures for routine application of CE to DNA typing, rather they were feasibility studies. The work herein (Chapter 5) builds upon this previous research and presents methods and techniques for efficient and effective DNA typing by CE.

B. QUANTITATIVE ANALYSIS

The general use of CE as a quantitative tool has been addressed in several reports [12,84,661]. Several factors are important to obtaining precise results including temperature control (±0.2 °C) [12,84], fast sampling rate [12], covering vials to prevent evaporation (2Q) [84], reproducibility of sample introduction [12, 14], and minimizing buffer depletion [661]. The CE instrument used in these studies overcame the first four problems while frequent changes of the buffer vials may reduce buffer depletion. In addition, coated capillaries have shown an order of magnitude better precision than uncoated capillaries as a consequence of reducing analyte adsorption and EOF variability [12]. For quantitating amounts of analyte, a normalization procedure is recommended, where measured peak area is divided by the migration time [12]. This adjusted peak area accounts for variable speed in which analytes travel past the detector [85].

1. Sizing of DNA Fragments

Sizing with external standards (i.e., running a standard and then running the sample) is difficult due to a lack of precision on the part of CE systems. Migration time differences between runs, which can result from minor changes in buffer ionic strength and viscosity, ionic differences in the sample, or slight changes in the capillary wall surface, prevent confident comparisons of migration times between multiple runs (2R,2T). Most CE researchers determine the size of PCR-amplified DNA by mixing the sample with a standard DNA restriction digest [150,398,471] or

by generating a size calibration curve with the DNA standard [282,588,309]. Since the size of the DNA fragments in the digest are known, they can be used to generate a calibration curve from which the "unknown" PCR product(s) size may be estimated.

Unfortunately, when mixing the standard with the sample, the fragments in the DNA digest can often overlap with, or fail to be resolved from, the PCR product of interest. In addition, extreme sequence differences between the "unknown" sample and the "standard" restriction digest can lead to inaccurate sizing due to migration differences resulting from DNA secondary structure [527,646] or the outcome of the digestion process [155]. Thus, an ideal internal sizing standard would be prepared from a PCR process and contain a similar internal sequence to that of the sample.

This work describes the development of a dual internal standard system for comparing multiple runs and sizing DNA fragments independent of external standards (**2U**). The internal standards can also be used to relate the sample to an external standard for accurate and reliable results.

2. Quantitation of PCR Products

Injection variation and inadequate standards have kept quantitation of biological samples by CE from being applied routinely [12]. For lack of better standards, several groups have used restriction digests as internal quantitation standards (**2V**). In this work a single internal standard is used to adjust for variation in sample injection, without introducing multiple DNA fragments which could interfere with the detection of peaks of interest.

Chapter 3

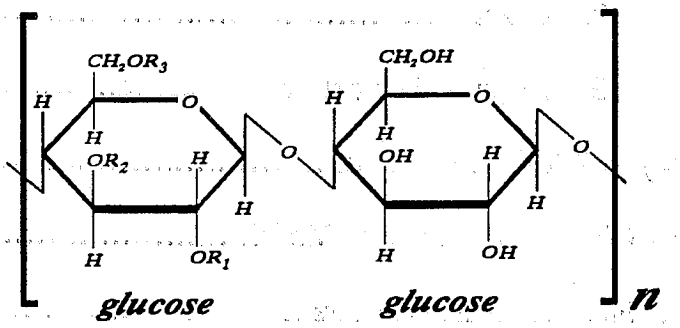
Theory for Engtangled Polymers and DNA Intercalators

Table of Contents

I. Theory of DNA Separations by CE

A. POLYMER SOLUTIONS

An increasing number of researchers are turning to replaceable separation media for DNA separations with CE due to the difficulties stated earlier with gel-filled capillaries. A recent review cited almost 100 references where polymer solutions were used for DNA separations [361] (see also **APPENDIX 1**). The most widely used polymers include linear polyacrylamide, poly(ethylene oxide), and several cellulose derivatives: hydroxyethyl cellulose (HEC), methyl cellulose (MC), and hydroxypropylmethyl cellulose (HPMC) (**Figure 3-1** and **3A**).



Cellulose

Possible Substitutions at R_1, R_2, R_3

MC	$-CH_3$	
HEC	$-CH_2CH_2OH$	$-CH_2CH_2OCH_2CH_2OH$
HPMC	$-CH_3$	$-CH_2\underset{\displaystyle OH}{\underset{\vert}{C}}HCH_3$

Figure 3-1. Structure of cellulose derivatives. Each glucose unit has a three possible sites for derivatization (R_1, R_2, and R_3). Numerous molecular weights for each of these polymers have been used in the literature (3A).

1. Entanglement Threshold

When a polymer is added to a solvent, the individual chains are initially separated from one another, until at higher concentrations they begin to interact. The polymer volume fraction (Φ) at which the polymer chains begin to interact and overlap is called the entanglement or overlap threshold (Φ^*). This volume fraction is defined as $\Phi = C\rho_p$, where C is the polymer concentration and ρ_p is the density of the polymer. Since the density of the polymer is constant, C and Φ may be used interchangeably. At concentrations greater than Φ^*, the polymer chains entangle to form a mesh with transient pores (**Figure 3-2**).

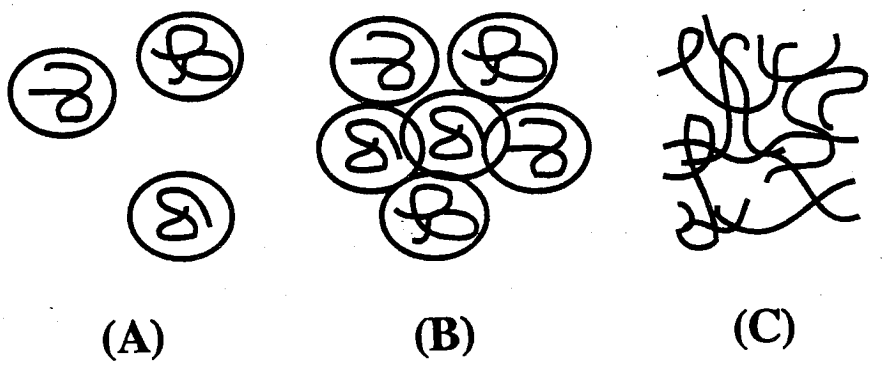


Figure 3-2. A representation of linear polymers in solution. (A) dilute, $\Phi < \Phi^*$; (B) overlap, $\Phi \approx \Phi^*$; (C) semidilute, $\Phi > \Phi^*$. Φ is the volume fraction of the polymer and Φ^* is the entanglement threshold. Adapted from [45]. A circle has been drawn around individual polymer strands to represent their area of influence.

Barron *et al.* [36] found that the entanglement threshold (Φ^*) scales as $N^{-1.2}$ for HEC, rather than the classically predicted $N^{-0.8}$ [566], where N is the number of monomer segments in the polymer chain [36]. This incompatibility is probably related to the fact that HEC is stiff and extended in solution [36], rather than a

randomly coiled polymer as used in the deGennes model [566]. The following relationship was determined empirically to predict the entanglement threshold for any HEC solution:

$$\Phi^* = 3.63\left[\frac{M_n}{M_0}\right]^{-1.2} + 1.18 \times 10^{-4} \qquad \text{(3-1)}$$

where M_n and M_0 are the number-average molecular weight and the average monomer molecular weight, respectively [36]. Assuming a monomer molecular weight of 272 for HEC [36], Eq. (3-1) would predict the 90,000-105,000 MW HEC (N = 331-386) to reach entanglement at concentrations of 0.30-0.36%. The experimentally determined entanglement threshold was found to be 0.37% [36] (3B).

2. Mesh Size

When the polymer strands are entangled, transient pores are formed [356]. For purposes as a molecular sieve in DNA separations, the polymer strands must be well enmeshed with one another (i.e., $C > C^*$) and form a network of "pores" robust enough to resist deformation by the DNA which, influenced by the electric field, pushes against it [70]. But, unlike cross-linked polyacrylamide gels, the pore network is a temporary gel rather than a permanent one [356]. The thermal motion of the polymers ensures that they do not remain as rigid as a cross-linked gel, but rather act as a dynamic sieving system. In fact, Duke and Viovy [356] believe that because a polymer chain is hemmed in by the surrounding molecules in just the same fashion as the DNA, it can consequently diffuse by reptation (see below) in a process called

constraint release [38]. Their theory describes the average lifetime of entanglements for the temporary gel and relates it to the length of the polymer [356]. The longer the polymer, the slower it will diffuse out of place; hence, larger MW polymers will form more stable polymer networks.

The average mesh size (ξ_b) of the polymer network (i.e., the effective "pore" size) may be calculated with an equation derived by Viovy and Duke [38],

$$\xi_b = 2.86\ \xi = 1.43\ R_p \left[\frac{C}{C^*}\right]^{-3/4} \tag{3-2}$$

where R_p is the radius of gyration for the polymer and C^* is the concentration of the polymer where entanglement begins (3C). The appropriate values for R_p and C^* can be estimated from intrinisic viscosity measurements using the equations described by Clark *et al.* [382],

$$R_p \approx \left[\frac{[\eta]\ MW}{2.5\ N_a}\right]^{1/3} \tag{3-3}$$

$$C^* \approx \left[\frac{3\ MW}{4\pi N_a R_p^{\,3}}\right] \approx \frac{0.6}{[\eta]} \tag{3-4}$$

where $[\eta]$, N_a, and MW are the intrinsic viscosity, Avogadro's number, and molecular weight of the polymer, respectively. Furthermore, the intrinsic viscosity may be calculated using the Mark-Houwink-Sakurada equation

$$[\eta] = K*(MW)^a \tag{3-5}$$

where K and a are constants characteristic of a given polymer system at a given

temperature [585]. For HEC dissolved in water, K = 9.53 x 10^{-3} mL/g and a = 0.87 [585].

A representation of the mesh size (ξ_b) is depicted in **Figure 3-3.** The mesh size might be considered analogous to the pore size in cross-linked polyacrylamide gels, which can be adjusted by varying the concentration of cross-linker (**3D**). The concentration of the polymer, independent of polymer length, determines the effective mesh size in entangled solutions [659] (see Eq. 3-2). Thus, two polymer solutions of different molecular weight (e.g., 27,000 MW HEC and 160,000 MW HEC), will form equivalent mesh sizes at the same concentrations, provided the polymers are entangled.

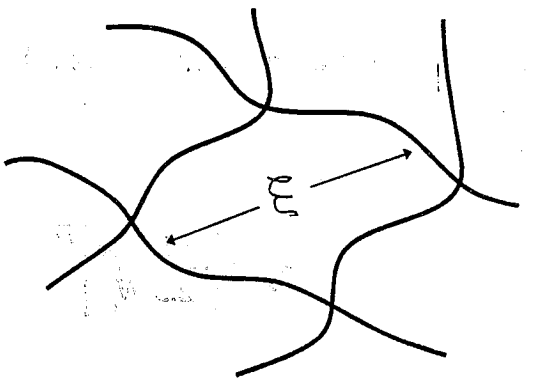


Figure 3-3. The entangled mesh with ξ indicating the mesh size. Each polymer strand has at least two points of entanglement. ξ is always shorter than the length of the polymer.

B. DNA SEPARATION MECHANISMS

While gel electrophoresis has been used to separate biomolecules for almost 30 years, theoretical descriptions for the mechanism have developed more slowly. In the simplest sense, a gel may be considered as a molecular sieve, which retards larger

molecules more than smaller ones. Over the years, two primary mechanisms for DNA separations have been proposed: the Ogston model and reptation. These two theories are complementary as they appear to operate in different size regimes. These mechanisms have been applied to entangled polymer networks as they are thought to form pores similar to agarose gels [34,45]. Recently, however, new theories have been proposed to explain the behavior of DNA in these physical gels, even below the entanglement point [67,356].

1. Ogston Model

The Ogston model regards the DNA molecule as a spherical particle or coil which passes through the pores formed by the gel [673]. Molecules move through the gel in proportion to their ability to find pores which are large enough to permit their passage. **Figure 3-4a** displays a schematic diagram of the Ogston mechanism. Smaller molecules migrate faster because they can pass through a greater number of pores. Thus, the electrophoretic mobility may be determined by multiplying the free solution mobility, μ_o, by the probability that the molecule will find a pore large enough to allow its passage, or

$$\mu = \mu_o P(\xi \geq R_g) \qquad \textbf{(3-6)}$$

where ξ is the radius of the pore in which the coil resides, R_g is the radius of gyration for the DNA molecule, and $P(\xi \geq R_g)$ is the probability that a given pore has a radius greater than or equal to the radius of the migrating particle [585]. After substituting for the probability function, the final equation appears as,

$$\mu = \mu_o \exp(-KC(r+R_g)^2) \qquad (3\text{-}7)$$

where K is a constant of proportionality, C is the concentration of the gel-forming polymer, and r is the thickness of the strands. A plot of log μ verses C, known as a Ferguson plot, should generate a straight line with a slope proportional to R_g^2 (if r « R_g) and a y-intercept of μ_o. The radius of gyration (R_g, nm) for DNA molecules may also be approximated using the equation:

$$R_g(DNA) \cong \sqrt{5.66\ N} \qquad (3\text{-}8)$$

where N is the number of base pairs [38].

For entangled HEC solutions, Grossman and Soane [34] empirically determined that when a DNA fragment's radius of gyration was approximately 1.4 times the mesh size (ξ) the separation mechanism changed. Resolution of DNA fragments rapidly declined as the fragment size became greater than 1.4 times the mesh size. This is due to the inability of the DNA to pass fully through the mesh and suggests a change in mechanism [76].

2. Reptation

When the radius of gyration for the DNA molecules becomes much larger than the mesh size of the sieving medium, the Ogston model predicts that the electrophoretic mobility will slow to zero. However, DNA separations have been demonstrated in cases where $R_g >> \xi$. In these instances, it is thought that the DNA deforms and "snakes" head first (hence the name reptation) through the network of pores [30,32]. **Figure 3-4b** illustrates this mechanism.

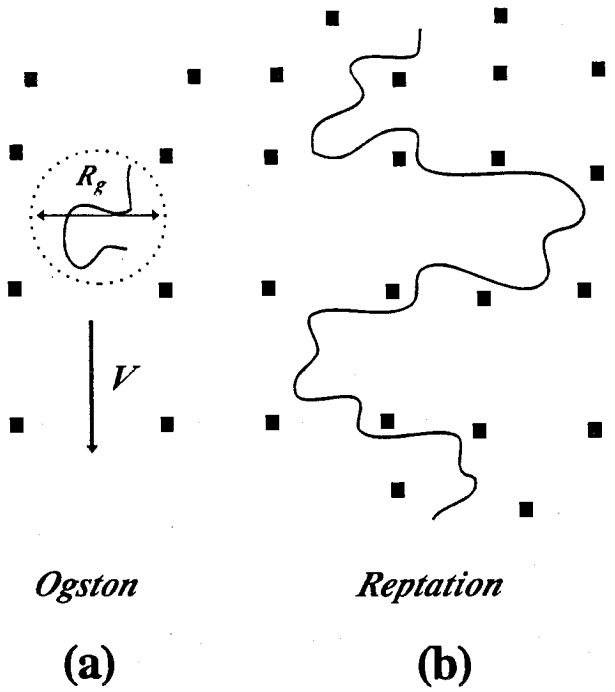


Figure 3-4. Schematic diagram of the (a) Ogston and (b) reptation models. The length and shape of the DNA strand as well as the size and distribution of gel pores affects which mechanism will be followed.

The classical reptation model states that the electrophoretic mobility is inversely proportional to the molecular weight. However, under the influence of high electric fields, the DNA can be oriented parallel to the electric field (**Figure 3-5**) [45].

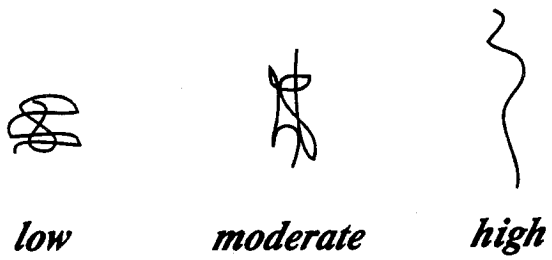


Figure 3-5. A DNA molecule migrating by the reptation mechanism can be elongated by the electric field (E). When E = 0, $R_z \sim N^{0.5}$, whereas for large E, $R_z \sim N^{1.0}$. Adapted from [45].

There is a decreased dependence of mobility on molecular size as the electric field and/or the molecular weight increase. A biased reptation model has been proposed to account for this process [634]. Lumpkin *et al.* [635] derived the expression,

$$\mu \approx \frac{1}{N} + bE^2 \qquad (3\text{-}9)$$

where N is the DNA molecular weight, b is a function of the mesh size of the gel or polymer network as well as the charge and segment length of the migration solute, and E is the electric field [585]. At low electric fields, the first term dominates and the classical reptation model appears. However, with capillary electrophoresis, the effects of DNA stretching in an electric field are important because of the high electric fields which are utilized.

3. Transient Entanglement Coupling Mechanism

The previous discussion has involved DNA mechanisms for gels or entangled polymer solutions which have formed pores. Barron *et al.* [67] have recently shown that DNA separations may be performed in dilute polymer solutions, where no pores are present (i.e., well below entanglement). It is possible that the mobility of large DNA molecules may be retarded by simply interacting with individual strands of the polymer. These polymer strands are pulled along for a short while by the DNA as it moves inside the capillary toward anode. As the interactions are temporary, this theory has been titled transient entanglement coupling.

Video microscopy of DNA conformational dynamics in polymer solutions below the entanglement limit has confirmed the transient entanglement coupling mechanism [444]. Shi *et al.* [444] recently observed that nucleic acids appear to become entangled with the polymer at a single region only. Thus, the formation of a pore network with a polymer solution is not always necessary for DNA separation.

II. Theory of DNA Intercalation

Some polycyclic aromatic molecules, such as ethidium bromide and 9-amino-acridine, can bind to DNA by insertion between the nucleobases of the double helix, in a process known as intercalation (**Figure 3-6**). This type of binding can change the physical and chemical properties of the intercalated DNA as well as the intercalators themselves [25]. Researchers doing DNA separations by CE use intercalators primarily for two reasons: (1) to improve the resolution of similarly sized DNA fragments [135] and (2) as a means of on-column fluorescent derivatization [53] (3E).

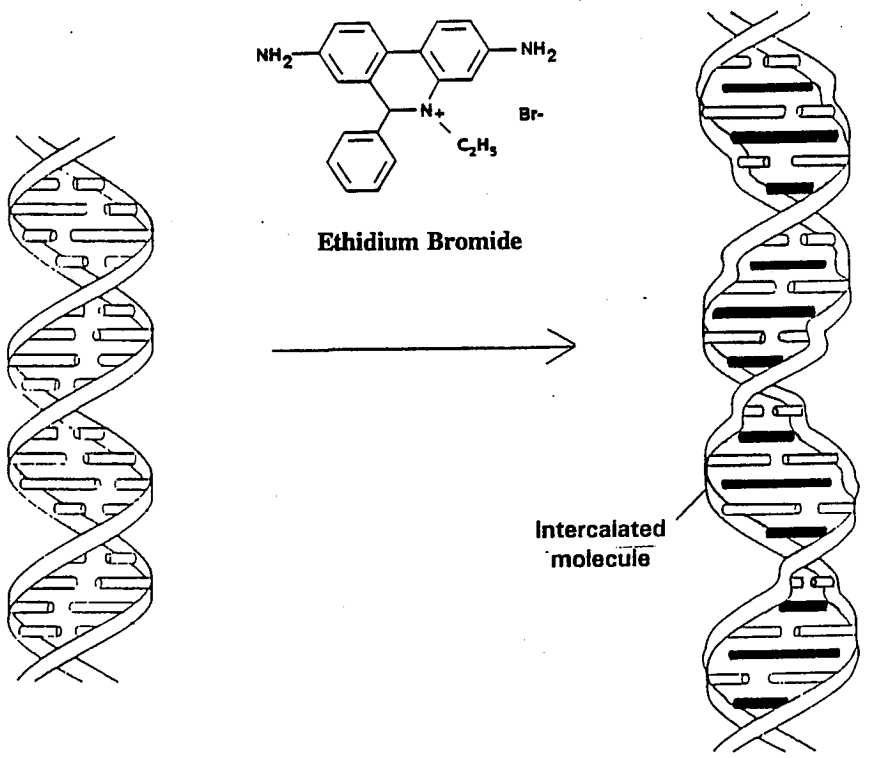


Figure 3-6. An intercalating molecule, such as ethidium bromide, inserts itself between adjacent nucleobases and perturbs the DNA helix by unwinding and lengthening the molecule.

A. CLASSICAL INTERCALATION MODEL

Upon intercalation, the average distance between two stacked base pairs increases from 3.4 Å to ~7-8 Å [32]. The intercalator is sandwiched tightly between the aromatic, heterocyclic base pairs and stabilized electronically in the helix by π-π stacking and dipole-dipole interactions [512]. The double helix unwinds to reduce the stress of separating the bases, and the overall length of the DNA molecule increases [575]. In addition, the overall mass of the intercalator-DNA complex increases, and the charge decreases as the DNA phosphates are neutralized. These effects change the electrophoretic behavior of the DNA.

Guttman and Cooke [135] derived the expression for the electrophoretic velocity of a DNA-intercalator complex as,

$$\nu = \mu_P E \frac{1}{K[L^+]^m} \qquad \textbf{(3-10)}$$

where μ_P is the electrophoretic mobility of the complex, E is the applied electric field, K is the formation constant of the complex, $[L^+]$ is the concentration of the intercalator, and m is the number of intercalator ligands in the complex. This equation shows that an increase in the binding constant and/or in the concentration of the intercalator will decrease the migration velocity of the complex.

Both monomeric and dimeric intercalating dyes exist and can bind to DNA through a mono- or a bis-intercalating process, respectively [28,249]. At saturation, mono-intercalators may bind double-stranded DNA in a one dye per two base pairs stoichiometry while bis-intercalators bind more like one dye per 10 bp [28].

B. FLUORESCENT INTERCALATORS

Upon binding to the DNA, many intercalators experience a large fluorescence enhancement and high quantum yield [28,30]. Lee and coworkers [434] propose that this enhancement of fluorescence upon intercalation may be due to steric hindrance to planarity of the unbound dye. Binding to DNA, constrains the intercalator to a planar conformation and makes it more fluorescent, perhaps through a charge-transfer which may occur between the DNA bases and the intercalator. For example, thiazole orange, which has planar conformations with steric overlap, exhibits a fluorescence enhancement of ~3,000 upon intercalation [434]. Fluorescence quenching is also reduced when planar aromatic intercalators reside in the hydrophobic regions between adjacent bases [675].

Several fluorescent intercalating dyes are commercially available which are useful in CE/DNA applications (**Figure 3-7** and **TABLE 3-1**). These dyes can be efficiently excited by the 488nm or 514nm line of the argon ion laser. Fluorescent derivatization of DNA can be performed by adding the intercalator to the sample [52] and/or the CE electrophoretic buffer [33]. Upon interacting, a DNA-dye complex is formed which strongly fluoresces when excited by the appropriate laser line. Exceptional sensitivity is achieveable when the dye is added to the buffer because the intercalator by itself exhibits very low levels of background fluorescence.

In the first use of fluorescent intercalators with CE separations of DNA, Schwartz and Ulfelder [29] showed a 400-fold improvement in sensitivity using LIF with thiazole orange, when compared to UV detection of the same DNA fragments.

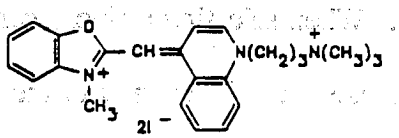
Since then a wide variety of dyes have been examined in CE applications [33,145, 262,678] (**TABLE 3-1** and **Figure 3-7**). When selecting a fluorescent intercalating dye, several factors must be considered, including the affinity of the dye for the DNA, the quantum yield, dye absorption maximum near laser line, and background fluorescence levels [29]. For quantitative measurements, fluorescence emission intensity of the dye-DNA complex should be a linear function of the number of intercalated dye molecules [26,30].

DNA may be pre-labeled with dimeric dyes (prior to electrophoresis) while monomeric dyes, with lower binding constants, must be added to the run buffer in order to maintain a stable complex with DNA during electrophoresis [28,262]. However, intermolecular cross-linking of DNA fragments with dimeric dyes may result in a loss of resolution relative to monomeric dyes [30,145,262]. Thus, monomeric dyes are used primarily in CE separations of DNA because of the higher attainable resolution (**3F**).

TABLE 3-1. Commercially available fluorescent DNA intercalating dyes. From [678]. The structures for the dyes are shown in Figure 3-7.

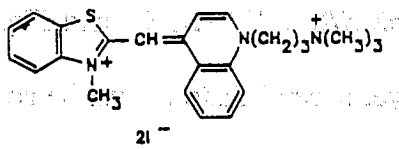
Dye	MW (g/mol)	Ex/Em (nm)	ϵ_{max} ($cm^{-1}M^{-1}$)	Quantum Yield	Binding Constant (K_b)
YO-PRO-1	629	491/509	52,000	0.44	8.2×10^6
TO-PRO-1	645	515/531	62,800	0.25	2.0×10^7
YOYO-1	1271	491/509	98,800	0.52	6.0×10^8
TOTO-1	1303	514/533	117,000	0.34	1.1×10^9
SYBR Green	N.A.‡	497/520	N.A.	N.A.	N.A.

‡N.A. = Information not available until patented.



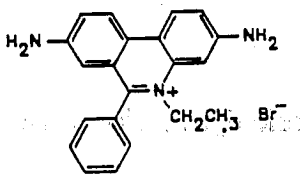
YO-PRO-1

Ex/Em (nm): 491/509



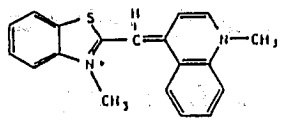
TO-PRO-1

515/531



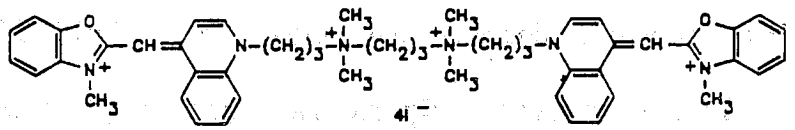
Ethidium Bromide

526/605



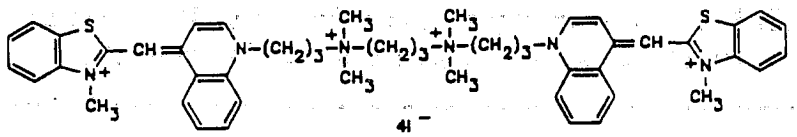
Thiazole Orange

509/525



YOYO-1

491/509



TOTO-1

514/533

Figure 3-7. Fluorescent intercalating dyes commonly used in CE/DNA. Adapted from [678].

Chapter 4

Materials and Methods

Table of Contents

I. Capillary Electrophoresis System

A. INSTRUMENTS

A Beckman P/ACE 2050 capillary electrophoresis instrument with a Laser Module 488 argon ion laser (Beckman Instruments, Fullerton, CA) was the primary CE used in these studies (**Figure 4-1**). A bandpass filter enabled laser-induced fluorescence (LIF) detection at 520 nm. Some work, involving UV analysis at 260 nm, was done on a Spectra-Physics 500 CE (Thermo Separations Products, Fremont, CA).

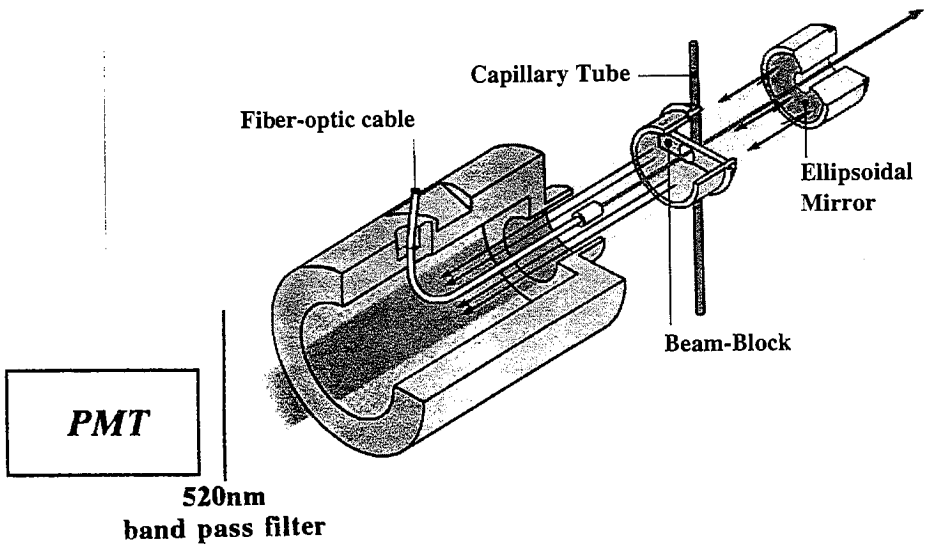


Figure 4-1. Schematic of Beckman CE-LIF System. The fiber-optic cable transmits laser light from the 4 mW laser, which illuminates a section of the capillary. Fluorescence is collected by the ellipsoidal mirror and focused back onto the photomultiplier tube (PMT) after passing through the band pass filter (520 nm). A centered hole in the mirror allows unwanted laser light to pass. The beam block diminishes scattered laser light. Adapted from [677].

B. DATA PROCESSING SOFTWARE

Data points were typically collected at a rate of 10 Hz on Waters Millennium 2010 software version 2.0 (Waters Chromatography, Bedford, MA, USA). For each electropherogram, the migration time of each peak was recorded along with the area and height of the peak. Beckman P/ACE Windows software (version 3.0) was also used when only qualitative information was desired. The control of the autosampler positions was performed with the Windows software.

C. COLUMNS

The most used column in this study was the DB-17 coated capillary (J&W Scientific, Folsom, CA). Product literature from the manufacturer states that the DB-17 is a 50% phenyl, 50% methyl polysiloxane coated capillary (**Figure 4-2**). Wall coating thicknesses of 0.05 or 0.1 μm are available. Uncoated (bare) fused silica capillaries were also obtained from J&W Scientific. Capillaries with internal diameters of 50, 75, or 100 μm and lengths of 27 cm, 37 cm, 47cm, and 57 cm (20, 30, 40, and 50 cm effective length, respectively) were employed. These lengths are available in the Beckman capillary cartridge based upon how many turns around a spool the capillary makes.

An optical window was produced by etching a short section of the polyimide coating with hot fuming sulfuric acid and cleaning it with ethanol as described by Boček and Chrambach [108]. This method protects the integrity of the inner wall coating. When fresh capillaries were prepared, the new columns were hand-flushed

by attaching a syringe to the end of the capillary column and forcing buffer through the length of the capillary to remove entrapped air [24].

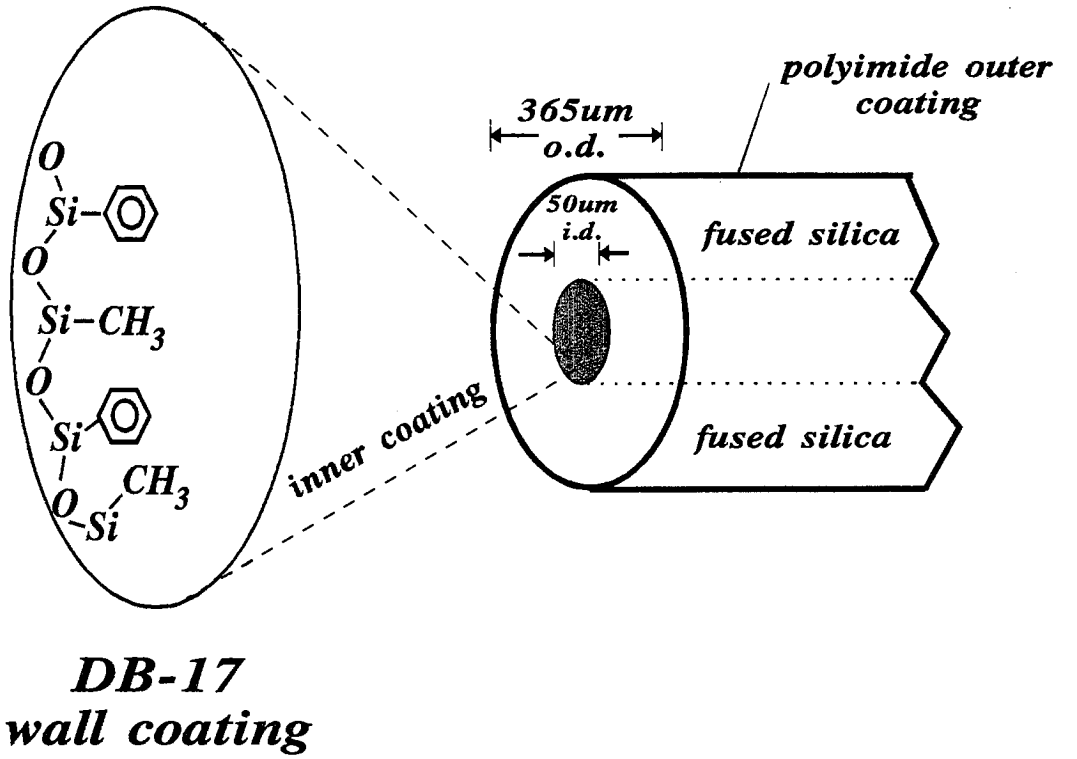


Figure 4-2. DB-17 coated capillary (J&W Scientific). The external polyimide coating makes the fused-silica capillary more robust and flexible. The inner wall coating covers the surface silanol groups and prevents electroosmotic flow.

The capillary, housed inside a sealed cartridge, was typically held at a constant temperature of 25.0 ($\pm$0.1) °C through liquid cooling. However, a few separations were studied at temperatures ranging from 15 °C to 50 °C. Precise temperature control improves run-to-run reproducibility.

Separations were performed with the cathode on the injection side in what is

commonly referred to as reverse polarity. This orientation of the electrodes forces the negatively charged DNA to move through the capillary toward the outlet side and past the on-column detector.

D. ELECTROPHORETIC BUFFER AND ADDITIVES

The run buffer consisted of 100 mM tris[hydroxymethyl]-aminomethane (trizma base), 100 mM boric acid, and 2 mM EDTA (TBE) at a pH of 8.2 with additives of 1% HEC (hydroxyethyl cellulose) and 50 or 500 ng/mL of the fluorescent intercalating dye YO-PRO-1 (Molecular Probes, Eugene, OR). For some studies, the pH of the buffer was adjusted to 8.7 or 8.9 with CsOH [33,35]. The separation buffer was prepared using a modification of procedures published by McCord *et al.* [33].

All chemicals used were of molecular biology grade, when available. The trizma base and boric acid were obtained from Sigma (St. Louis, MO, USA), the EDTA was from Fisher Scientific (Fair Lawn, NJ, USA), and the hydroxyethyl cellulose was from Aldrich (Catalog # 20,863-3, Milwaukee, WI, USA). Several other polymers were examined at various concentrations as part of the resolution studies. These polymers included hydroxypropyl methyl cellulose (100cP and 4000cP from Sigma), hydroxyethyl cellulose (24,000-27,000 MW, 90,000-105,000 MW, and 140,000-160,000 MW from Polysciences, Inc., Warrington, PA, USA), hydroxyethyl cellulose (Fluka medium viscosity--75-125cP), and polyethylene oxide (100,000 MW, 200,000 MW, and 300,000 MW from Polysciences).

1. Hydroxyethyl Cellulose

The HEC was dissolved in the TBE buffer by shaking vigorously overnight at a concentration of 1.0% (w/v). The solution was then filtered through a 500 mL 0.22 μm or 0.45 μm cellulose acetate disposable filter (Corning Inc., Corning, NY, USA). The buffer was usually prepared 500 mL at a time and stored at room temperature.

2. Fluorescent Intercalating Dye YO-PRO-1

To 15 mL of the HEC buffer, 11.9 μL of 1 mM YO-PRO-1 dye (**Figure 4-3**) were added to bring the dye to a concentration of 500 ng/mL. When 50 ng/mL YO-PRO-1 solutions were prepared, 3.2 μL of the 1mM YO-PRO-1 stock solution were added to 40 mL of the HEC buffer.

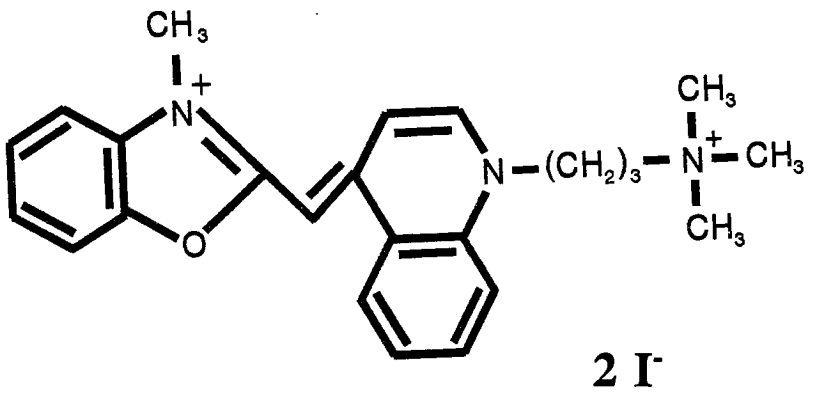


Figure 4-3. Structure of YO-PRO-1. Chemical abstracts registry number and name are 152068-09-2 and Quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-, diiodide.

Solutions containing the light sensitive intercalating dye were prepared daily, although solutions up to a week old were used after being stored at room temperature

with no obvious deterioration. The diluted dye solutions were covered with aluminum foil to prevent contact with light. Occasionally, with UV detection, the intercalating dye ethidium bromide (Sigma) was added to the run buffer at concentrations ranging from 1.27 to 6.35 μM [24]. In buffers used for fluorescence analysis, both YO-PRO-1 and ethidium bromide were added when 100 μm i.d. capillaries were used [33,151]. Several other fluorescent intercalating dyes are available from Molecular Probes (Eugene, OR) and were examined as well, including TOTO-1, YOYO-1, TO-PRO-1, and SYBR™ Green I (**Figure 3-7**).

Laser-induced fluorescence was achieved by the excitation of the intercalated YO-PRO-1 dye-DNA complex with the argon-ion laser 488 nm line and detection of emitted light at 520 nm. The cyanine dye YO-PRO-1, which is a benzothiazolium-4-quinolinium monomer, possesses an absorption maximum of 491 nm which is well matched to the 488 nm line of the argon laser [678]. Upon binding to DNA through an intercalative mechanism, the YO-PRO-1 emits at 509 nm. The fluorescence can be efficiently collected with a 520 nm bandpass filter.

E. COLUMN MAINTENANCE

1. Washing

Prior to each injection, the capillary wall was cleaned with a methanol rinse (1-4 min) and filled with the viscous polymer run buffer (2-6 min) [24]. With proper rinsing, columns have lasted over 1000 runs without a significant loss of resolution. Washes between runs also prevent sample carryover in repetitive runs.

2. Storage

At the end of each work day, the capillary was rinsed with methanol (2-10 min) and subsequently with deionized water (10 min). The column was stored overnight with both ends in water. Each morning, or between uses of different buffers, the capillary was rinsed for 10 min with HPLC-grade methanol and subsequently for 10 min with run buffer to equilibrate the capillary wall and interior.

F. INJECTIONS

1. Electrokinetic

For electrokinetic injection, an electric potential ranging from 1-10 kV was applied to the sample for durations ranging from 1-30 s depending on the sample concentration [151]. The typical injection for PCR-amplified short tandem repeats was 5 s at 1 kV [152,559]. Each figure will contain the injection conditions.

2. Hydrodynamic

All pressure injections were performed at 3.44 MPa (0.5 psi). This particular pressure is set by the Beckman P/ACE instrument. In some circumstances, the hydrodynamic injection of the sample was proceeded by a 10 s injection of water to increase sample stacking [151]. Most pressure injections of PCR-amplified samples were for 45 s, although loading times of up to 90 s were studied.

G. SEPARATION CONDITIONS

Both constant voltage [151,559] and voltage gradient separations [152] were investigated. Separation voltages were optimized to the samples being separated. Ohm's law plots (see **Figure 2-1**) would typically be run with a new buffer or capillary system in order to determine the upper limit for applied voltage.

1. Constant Voltage

Constant voltage runs were typically at 5 kV (185 V/cm for a 27cm capillary), although conditions for individual runs may differ and will be listed with the figures in the text. For PCR product quantitation, separations usually proceeded at 15kV (556 V/cm), as resolution was sacrificed for speed.

2. Voltage Programming

Voltage programming was used to increase the overall speed while at the same time improving the resolution in a particular region. In one approach, with a 50μm i.d. x 37 cm capillary, the voltage gradient involved running at 15 kV (405 V/cm) for three to five minutes and then immediately reducing the applied potential to 5 kV (135 V/cm) [152]. With a 50μm i.d. x 27 cm capillary, the same degree of resolution and speed could be achieved by running at 7.5 kV (278 V/cm) for 5 minutes and then lowering the voltage to 2.5 kV (93 V/cm) [559]. A wide range of gradient separations were examined as part of the resolution studies (see Chapter 5, II).

H. SUMMARY OF SAMPLE ANALYSIS

With the Beckman CE system, a "method" is programmed into the computer which controls the autosampler and correlates each electropherogram to a particular sample (**Figure 4-4**). The samples (usually a volume of 25-50 μL) are pipetted into a vial (e.g. PCR tube) and placed on a spring in a 4 mL wide-mouth vial. After a silicon rubber cap is screwed on top, each of the capped 4 mL vials is loaded into the autosampler. Finally, the names of the samples are typed into the computer to relate the autosampler position to the appropriate sample. In a typical CE analysis, the process would proceed as follows (**Figure 4-4**). The detector parameters and temperature are first set. The column is then rinsed with methanol. This is done by placing the capillary inlet in the methanol vial (#31) and the outlet in a waste vial (#10) and applying pressure to the inlet. In step 4, the column is filled with the viscous polymer buffer (vial #32) as it is loaded into the capillary by pressure displacement at 20 psi. Again, the waste vial (#10) is at the outlet end of the capillary. Prior to injection, the inlet end of the capillary is dipped in deionized water (#33) to prevent contamination of the sample with buffer salts. The sample (#11) is placed at the inlet of the capillary and a voltage applied (electrokinetic) or pressure used (hydrodynamic) to draw the sample into the capillary. Finally, buffer vials (#34 and #1) are placed on both ends of the capillary, and electrophoresis is allowed to proceed under the application of the separation voltage. When the run is complete, the process is repeated again, with additional samples being injected from vials 12, 13, 14, and so forth. The same buffer and rinse vials were used repeatedly

for typically 10-20 samples. As these steps are completely automated, a sample can be injected, separated, and detected in only a few minutes with no operator interaction.

P/ACE 2000 Series Version 3.0 - Beckman Instruments Inc.

Method: C:\PACE\EXAMPLE.MTD 06 Jun 95 11:52

STEP	PROCESS	DURATION	INLET	OUTLET	CONTROL SUMMARY
1	SET DETECTOR				LIF: 488:520 nm Rate: 5 Hz Rise: 1.0 sec Gain: 100
2	SET TEMP				Temp: 25 C Wait until reached
3	RINSE	1.0 min	31	10	Forward: High Pressure
4	RINSE	2.0 min	32	10	Forward: High Pressure
5	WAIT	0.1 min	33	10	
6	INJECT	5.0 sec	11	1	Voltage: 1.00 kV
7	SEPARATE	15.0 min	34	1	Constant Voltage: 5.00 kV Current Limit: 250.0 uA Integrator On

Figure 4-4. An example "method" used in operating the Beckman P/ACE instrument. The operator designates the duration of each process along with the inlet and outlet vial numbers. Vial contents: *inlet:* 31=MeOH, 32=buffer, 33=deionized water, 34=buffer, 11=sample; *outlet:* 1=buffer, 10=waste.

II. DNA Samples

A. INTERNAL STANDARDS

Internal standards were DNA fragments generated separately by BioVentures Inc. (Murfreesboro, TN, USA). The M100, M150, M200, M300, and M400 (100 bp, 150 bp, 200 bp, 300 bp, and 400 bp size fragments, respectively) were developed and used for these studies. The DNA fragments were typically obtained in the concentration range of 10 to 200 ng/μL. Some DNA quantitation work was also done with the QS-200 200 bp DNA fragment from GenSura (Del Mar, CA, USA). According to product literature, the concentration of the GenSura 200 bp DNA fragment was determined to be 100 ng/μL by UV absorbance at 260 nm in an instrument that had been calibrated by the UV absorbance standard SRM 935 from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

For use as internal standards with the CE-LIF methods, dilutions were made to approximately 1 ng/μL with deionized water. From 500 μL stock solutions of 1 ng/μL, multiple analyses could be performed with the same amount of internal standard. Typically, 1 μl of PCR product was added to an internal standard mix at a volume of 22-50 μL and mixed 5-10 times with the pipet [151,152].

B. RESTRICTION DIGESTS AND SIZING STANDARDS

Hae III digested ϕX 174 (Sigma, St. Louis, MO) and pBR322 (Sigma) were diluted to approximately 200 ng/mL using deionized water. K562 cell line DNA was obtained from Life Technologies, Inc. (Gaithersburg, MD) and diluted in 10 mM

Tris-HCl, 1 mM EDTA (TE) buffer [151]. The 20 bp ladder (Superladder-low, Cat. No. SLL-101), containing 50 DNA fragments, each 20 bp apart, in the range of 20-1000 bp, was obtained from GenSura (Del Mar, CA, USA). Separations of large DNA fragments were studied with the λ-*Hind*III digest (Promega, Madison, WI, USA) and large DNA ladders from GenSura (i.e., Superladder-mid1, Cat. No. SLM-101, and Superladder-high, Cat. No. SLH-101).

C. EXTRACTED DNA FOR QUANTITATION AND TYPING

Mitochondrial DNA (mtDNA) samples were extracted from human hair and blood samples as described by Wilson *et al.* [247]. The genomic DNA for the sizing and typing experiments was extracted as described in Comey *et al.* [349]. Briefly, dried blood stains or Q-tip swabs were incubated for 2 hours at 56 °C in stain extraction buffer containing Proteinase K and dithiothreitol (DTT). An organic extraction was then performed with phenol/chloroform/isoamyl alcohol (24:24:1) to remove the proteins. Finally, Tris-EDTA buffer was added and the aqueous layer containing the DNA was recovered by centrifugation.

In one study, anonymous blood samples from 97 unrelated African American blood donors, obtained from the Broward County's Sheriff's Office Crime Laboratory (Ft. Lauderdale, FL), were examined [559]. These samples were used to compare results obtained by CE to those from slab gel electrophoresis. Many of the samples had been previously amplified and typed by FBI laboratory researchers for use in population studies.

D. DNA SAMPLE PROCESSING

After a sample is obtained from an individual in the form of a blood stain or a Q-tip swab of cheek cells from the inside of the mouth, the DNA is extracted and quantitated by hybridization with a human DNA specific probe (see below). Several nanograms of the recovered DNA is amplified via PCR. The PCR product(s) are then typed (in the case of STRs) by either polyacrylamide gel electrophoresis or CE (**Figure 4-5**).

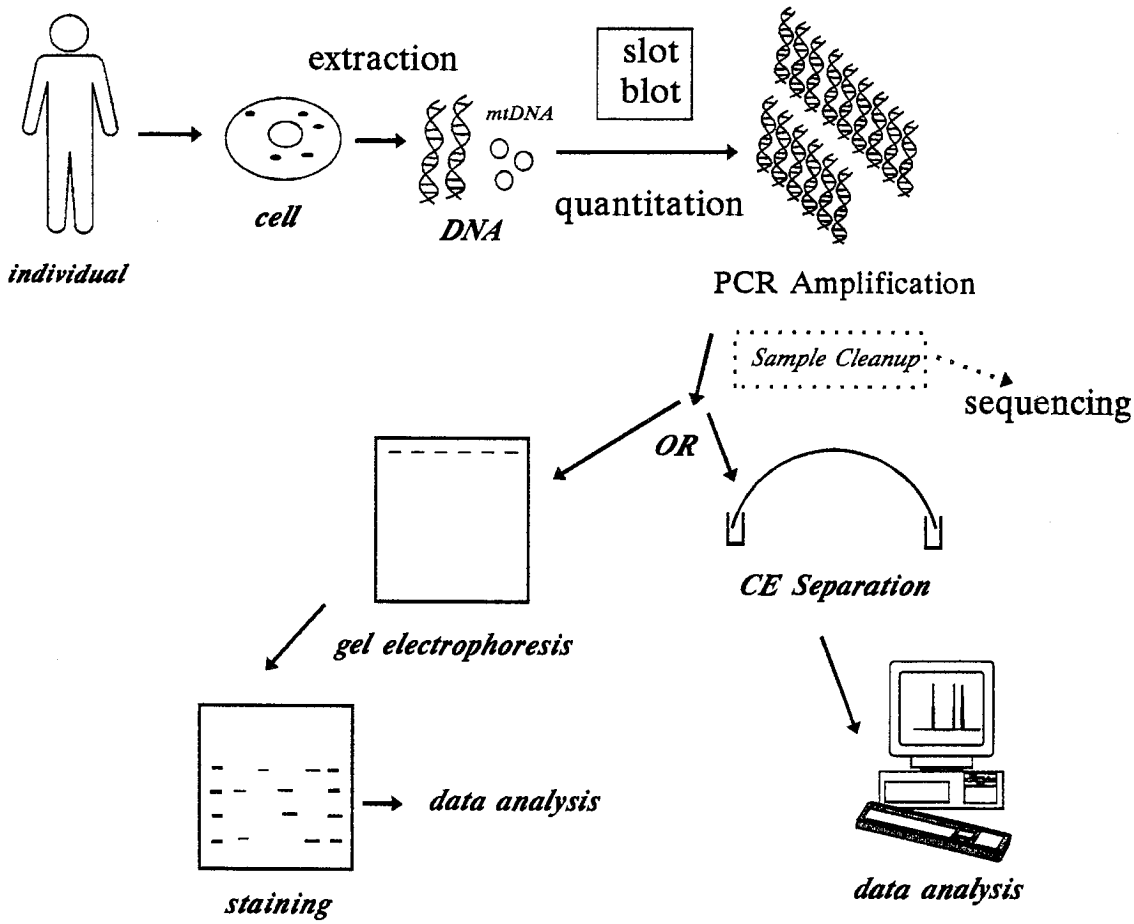


Figure 4-5. The steps for obtaining a DNA genotype (e.g. using an STR) from an individual.

III. DNA Amplifications by PCR

A. MITOCHONDRIAL DNA

Several different amplification schemes were performed on two hypervariable areas of the control region of human mtDNA [151,247]. The primers used are shown below:

HV1		
L15997	5'-CAC CAT TAG CAC CCA AAG CT-3'	**(A1)**
H16395	5'-CAC GGA GGA TGG TGG TCA AG-3'	**(B1)**
HV2		
L047	5'-CTC ACG GGA GCT CTC CAT GC-3'	**(C1)**
H408	5'-CTG TTA AAA GTG CAT ACC GCC A-3'	**(D1)**

The numbers signify the 3' base with respect to the origin of replication as indicated by Anderson *et al.* [505]. The letter L designates the light strand and H the heavy (i.e., GC rich) strand of the mtDNA duplex. Thus, the combination of primers A1 and B1 will generate a PCR product over the HV1 region of 437 bp in length (**Figure 4-6**), while the combination of C1 and D1 will produce a 402 bp long DNA fragment for HV2. The entire control region can be amplified by combining A1 and D1 for a 1021 bp long amplicon. When amplifying mtDNA from hair or bone, the HV1 and HV2 regions are often split into smaller sections in order to increase amplification efficiency [247].

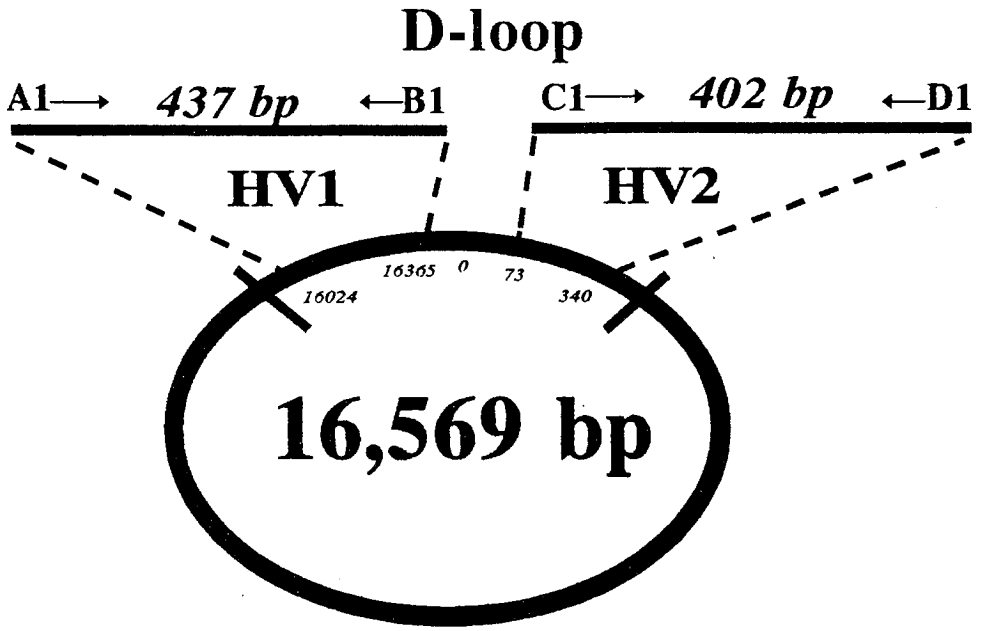


Figure 4-6. The sizes of the PCR products generated from amplifying the D-loop of mitochondrial DNA using the primer pairs shown; A1 and B1 define the HV1 region while C1 and D1 define HV2. The primer sequences and positions are shown on the previous page. The overall size of mtDNA is 16,569 bp.

PCR was performed in 0.2 mL MicroAmp™ Reaction Tubes (Perkin Elmer, Norwalk, CT) with 200 μM each dATP, dCTP, dGTP, and dTTP; 1 μM of each primer (two per reaction; see above); 50 mM KCl, 10 mM Tris-HCl pH=8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, and 4 μg BSA (Sigma); and 5.0 units/reaction of AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT). Amplifications were performed in 25 μL volumes using the Perkin Elmer GeneAmp PCR System 9600 thermal cycler and the following parameters: 1 min 95 °C initial denaturation followed by 36 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s [151,247].

B. THE SHORT TANDEM REPEAT HUMTH01

Eight alleles have been characterized for the STR system located in the first intron of the human tyrosine hydroxylase gene (chromosomal position 11p15.5). The sequence for an allele containing nine repeats is shown in **Figure 4-7.** The HUMTH01 alleles possess a tetranucleotide repeat of AATG and when amplified with the primer pair listed below, generate alleles in the size range of 179 bp (5 repeats) to 203 bp (11 repeats) **(Figure 4-8).**

5'-GTGGGCTGAA AAGCTCCCGA TTATCCAGCC TGGCCCACAC AGTCCCCTGT ACACAGGGCT TCCGAGTGCA GGTCACAGGG AACACAGACT CCATGGTG**AA TGAATGAATG AATGAATGAA TGAATGAATG AATG**AGGGAA ATAAGGGAGG AACAGGCCAA TGGGAATCAC CCCAGAGCCC AGATACCCTT TGAAT-3'

Figure 4-7. Sequence of HUMTH01 allele 9 (195 bp). The tetranucleotide repeats have been displayed in bold while the first and last repeat unit have been underlined for emphasis. The listed sequence is the reverse complement of the Genbank sequence.

Amplifications were performed with the Perkin-Elmer GeneAmp PCR System 9600 thermal cycler using 1.2 units of AmpliTaq enzyme (Perkin-Elmer, Norwalk, CT), dNTPs (200 μM each), 1 μL of 12.5 μM each primer, and 5 μl 10X buffer (Perkin-Elmer, Norwalk, CT) in a volume of 50 μl. PCR primers used for amplifying the HUMTH01 locus, described by Edwards *et al.* [159] are as follows: 5'-ATT CAA AGG GTA TCT GGG CTC TGG-3' and 5'-GTG GGC TGA AAA GCT CCC GCA TAT-3'. Thermocyling temperatures were 95 °C for 45 s, 60 °C for

30 s, and 72 °C for 30 s, for 27 cycles. Approximately 1-5 ng of genomic DNA were used in amplification protocols for the individual typing samples.

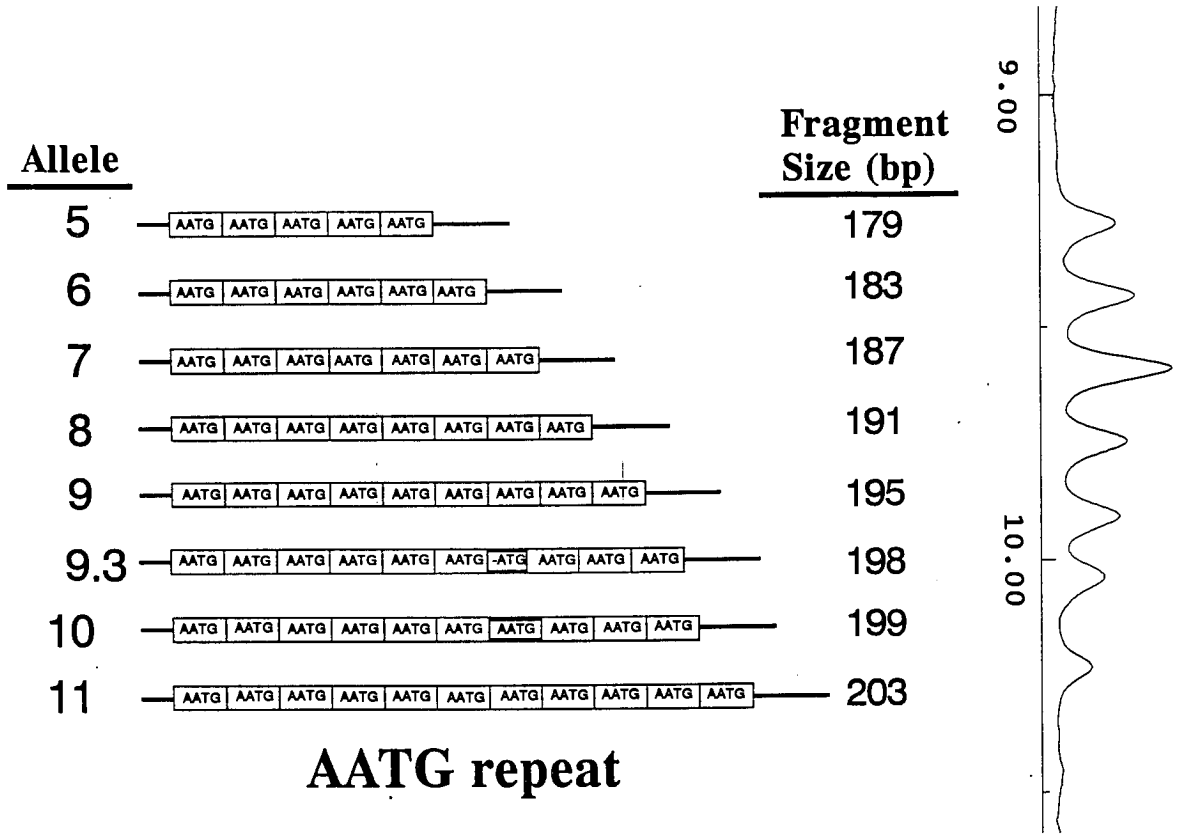


Figure 4-8. The eight characterized HUMTH01 alleles possessing the AATG core repeat sequence. Allele 9.3 differs from allele 10 by a single adenine deletion in the seventh repeat unit. The HUMTH01 allelic ladder used for these studies is displayed vertically; allele 10 is not present. Conditions as in Fig. 4-9.

C. ALLELIC LADDERS

A HUMTH01 allelic ladder was prepared with alleles 5, 6, 7, 8, 9, 9.3, and 11. The stock allelic ladder was built by mixing alleles from individual samples with genotypes (5,9.3), (6,9), (7,8), and (9.3,11). The original HUMTH01 ladder was put together by Greg Parsons of the FBI Laboratory. To produce more of the standard

ladder, the stock was diluted 1:10^6 with deionized water and reamplified [161,645].

The multiplexed allelic ladders (**Figure 4-9**) containing alleles from the STR systems VWA, FES/FPS, and CSF1P0 and the VNTR D1S80 (Roche MP3) were a gift from Dr. Marcia Eisenberg of Laboratory Corporation of America (formerly Roche Biomedical Laboratories, Research Triangle Park, NC).

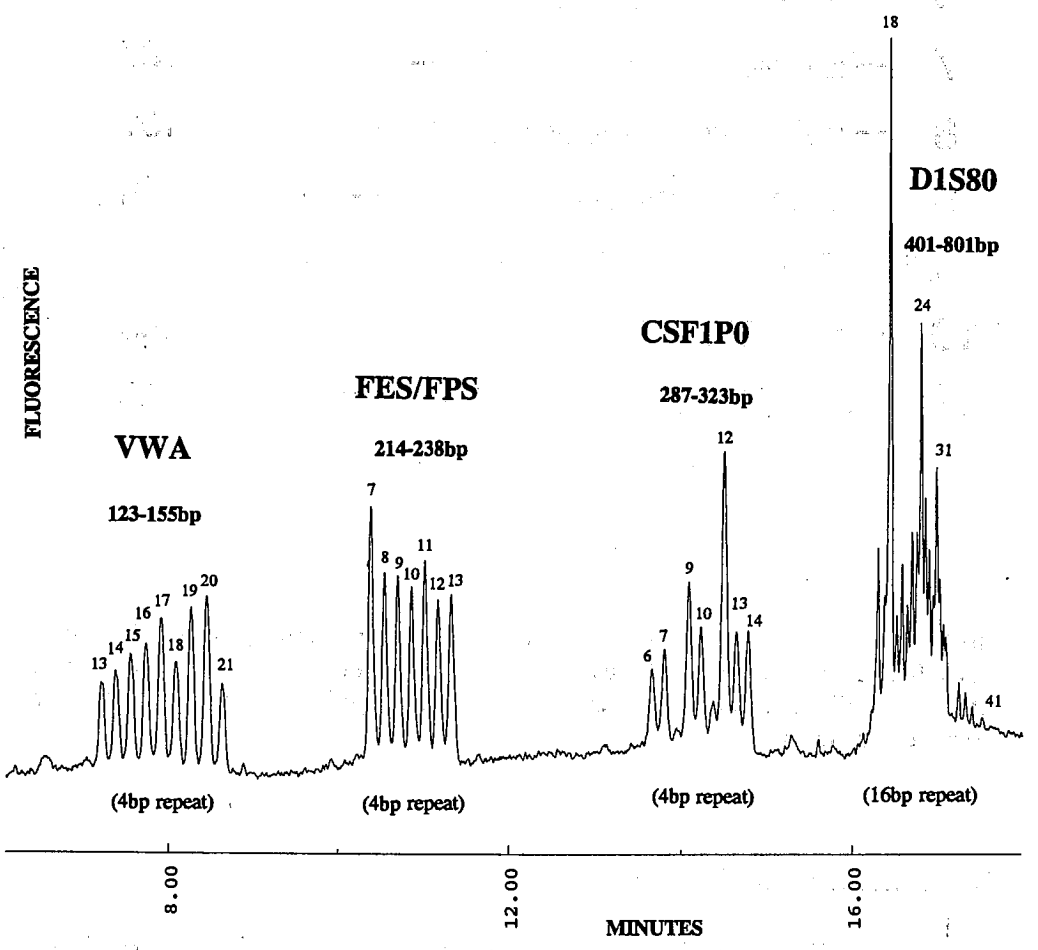


Figure 4-9. Multiplex STR and VNTR allelic ladders for the VWA, FES/FPS, CSF1P0, and D1S80 loci. The numbers above the peaks indicate the number of repeats in each allele. The separation is optimized for the 100-350 bp region with voltage programming so that the D1S80 alleles are not well resolved. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 500 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 5 s @ 1 kV; *Separation:* 0-5 min @ 7.5 kV, 5-16 min @ 1.5 kV, 16-18 min @ 7.5 kV; *Sample:* 1:50 dilution of Roche Multiplex #3 in deionized water.

IV. Post-PCR Sample Preparation and Cleanup

A. PRIMER REMOVAL

In some situations (e.g., cycle sequencing [252,247]), it is desirable to remove the primers following PCR. This may be done by centrifugation, where the primers pass through large pores in a filter, or spin-size exclusion, where the larger PCR product passes through the column and the smaller primers are retained in the spin-column.

Primers were separated from amplified DNA by subjecting the sample in Microcon-100 tubes (Amicon, Beverly, MA, USA) to centrifugation. All sample components less than 100,000 MW (~160 bp) pass through the filter. Two 300 μL washes with deionized water were performed using centrifugation at 3000 x G for 5 min. After adding 30 μL of deionized water to the filter, the tube was inverted and centrifuged at 10,000 x G for 5 min. A retentate of approximately 30 μL was collected.

For the application of CE to quantitating PCR-amplified mitochondrial DNA (see Chapter 5, IV), 1 μL of retentate, which contained the recovered PCR product, was added to 24 μL of deionized water containing an internal standard, typically the 200 bp DNA fragment.

B. FLOAT DIALYSIS

When only salt removal was required, float dialysis was performed. This process was usually necessary when using UV detection to enhance the quantity of

DNA injected onto the CE column.

The amplified DNA was processed by pipeting it onto a 0.025 μm membrane filter (Millipore VSWP) which was floating in a petri dish filled with deionized water as shown in **Figure 4-10** [155,188]. The low (100 bp or 150 bp) and high (300 bp) internal standards (Bioventures Inc., Murfreesboro, TN) were added at this point, for some sizing experiments [152]. Dialysis was typically carried out on the floating filter for 20-30 minutes [24].

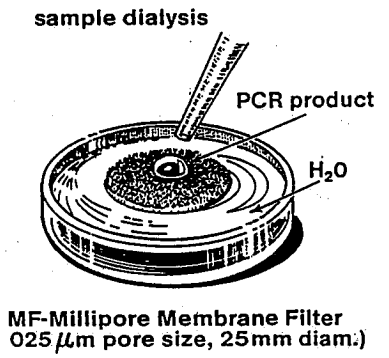


Figure 4-10. Float dialysis for salt removal from a sample prior to electrokinetic injection. A sample is typically pipetted onto the floating membrane for 20-30 minutes. The salts diffuse through the membrane into the water. From [155].

V. Other Methods of PCR Product Quantitation

A. QUANTITATION BY HYBRIDIZATION

The quantitation by hybridization with chemiluminescent detection (i.e., slot blot) procedure was performed as described by Walsh *et al.* [687]. The mtDNA control region from K562 DNA was amplified by PCR and purified by centrifugation with a Microcon-100 tube. These PCR products were then quantitated by fluorescent spectrophotometry (see below). From this resulting DNA, standards were prepared in 10 μL of 10 mM Tris-HCl, 0.1 mM EDTA (TE) buffer by serial dilution to amounts of 40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng, 0.625 ng, and 0.312 ng.

PCR products were assayed after purification by centrifugation through a Microcon-100 tube (see above). Biotinylated oligonucleotides (HV1B = H16255 = Biotin-5'-CTT TGG AGT TGC AGT TGA TG-3' and HV2 = L172 = Biotin-5'-ATT ATT TAT CGC ACC TAC GT-3') at a concentration of 1 pmol/μL were used as the slot blot probes. The serially diluted standards were arranged down each side of the membrane while up to 32 samples can be loaded in the center (4 columns x 8 rows). Either extracted genomic DNA or PCR products may be ascertained depending upon the sequence of the biotinylated probe; the signal intensity is directly related to the number of probes bound to the DNA. After exposure of the membrane to X-ray film for 15 min, a visual comparison was made between the sample and the standards.

B. FLUORESCENT SPECTROPHOTOMETRIC ANALYSIS

A Perkin Elmer 650-40 Fluorescent Spectrophotometer was used following the procedure described by Rye *et al.* [26]. YOYO-1 (the homodimer of YO-PRO-1, Molecular Probes, Eugene, OR) was the fluorescent dye used for quantitation. The YOYO-1 dye was diluted 1:5000 in TE buffer. For each sample, 5 μL of the PCR product were added to 2 mL of the YOYO-TE buffer solution. K562 DNA was diluted to 25 ng/μL and used as a reference. Measurements were usually made in triplicate and averaged.

VI. Data Analysis

A. QUANTITATION OF DNA

The quantity of a PCR product was determined by comparison to an internal standard of known size and concentration [151,247]. Typically a 100 ng/μL 200 bp quantitation standard was diluted to 0.400 ng/μL by placing 2 μL of the 200 bp standard into 498 μL of deionized water. One μL of a PCR sample was examined by adding it to 24 μL of the 0.400 ng/μL diluted 200 bp standard. Following CE separation, the quantity of DNA in the sample was estimated based upon a ratio of the peak areas as shown below,

$$[PCR\ Product] = \frac{25\mu L}{1\mu L} * \frac{Area\ PCR\ product}{Area\ 200bp\ Std} * (0.384\ ng/\mu L) \qquad (4\text{-}1)$$

where (0.384 ng/μL) is the amount of 200 bp DNA in the CE sample (i.e., 24μL/ 25μL). It should be noted that the actual relationship between the internal standard and the sample is also affected by a factor related to the difference in bp size. This factor is due to the difference in the number of fluorophores attached to the DNA molecule.

B. SIZING OF DNA

Two internal standards were typically used to size PCR-amplified DNA [152,559]. The DNA fragments of interest were delimited by the dual internal standards of known molecular size and concentration. The size of unknown peak(s) was then determined by comparison with the internal standards. The same two

internal standards were also added to a reference standard to directly relate the size and quantity of the reference standard to the sample, assuming linearity. Typically the 150 bp and 300 bp PCR products (GC content ~50%) generated by BioVentures were used as the internal standards for sizing HUMTH01 alleles (179-203 bp) and the amelogenin X/Y fragments (212 or 218 bp). The size of an unknown DNA fragment was calculated with the following equation:

$$\textbf{DNA fragment size} = 150 \text{ bp} + [(MT_{DNA} - MT_{150}) * \text{slope}] \qquad (4\text{-}2)$$

where MT is migration time and

$$slope = \frac{(300 - 150)}{(MT_{300} - MT_{150})}$$

These equations may be used as long as the region between the two internal standards, in this case the 150 bp and 300 bp fragments, is linear. For use in typing short tandem repeats, both individual samples and the allelic ladder DNA sizes were calculated in this manner.

C. DETERMINATION OF SAMPLE GENOTYPE

Using the dual internal standard approach, the genotype of a sample was determined by comparing the calculated size in base pairs to that obtained from the standard allelic ladder. Any allele sizing result falling outside 3.3 standard deviations (99.9% confidence limit, as determined by 50 replicate injections) of the calculated bp size obtained from the standard was rejected [559].

Chapter 5

Results and Discussion

Table of Contents

I. Injection from High Ionic Strength Matrices

The goal of this research was to develop capillary electrophoresis procedures for the analysis of PCR products used in human identification DNA typing. Experiments were performed to improve the injection of PCR samples and to understand the factors important to obtaining high resolution with CE. The resolution studies were performed in order to optimize the separation time for PCR product analysis, which will facilitate the routine use of CE. The issues of precision, sensitivity, and stability of the CE system were also examined. From this work, quantitative procedures for DNA fragment sizing and quantitation were developed.

A. HYDRODYNAMIC INJECTION

Experiments were undertaken to improve hydrodynamic injections for the purposes of quantitative analysis of PCR products. One problem with PCR samples has been poor sensitivity upon injection from a high salt matrix. Previous work in the literature suggested that a plug of water prior to the sample injection would be beneficial to enhancing sensitivity and resolution [48]. A series of experiments were performed to develop a two-step injection procedure with a water plug prior to the sample.

The injection of a ϕX174 *Hae*III digest was examined by changing the time of a water injection while holding the sample injection time constant. It was found that up to a 10 s water injection prior to the sample could be performed without loss in resolution. The water injection was then held constant at 10 s while the injection time

of the sample was varied between 30 s and 90 s. The long injection times were used to improve injection precision and the amount of material loaded onto the column. The highest number of theoretical plates for the 234 bp peak of the ϕX174 *Hae*III digest sample was obtained with a 10 s water injection followed by a 45 s sample injection [151]. Resolution loss was observed with injections over 60 s in duration, presumably as a result of sample overload.

The importance of this preceding water plug for long hydrodynamic injections can be seen in **Figure 5-1**. Peak fronting of the larger DNA fragments (603-1353 bp) was reduced when the 10 s water injection preceded the 45 s sample injection (**Figure 5-1a**). When the water plug was removed (**Figure 5-1b**), large peak shoulders became evident for the last four peaks (see the boxed area). This peak fronting may be attributed to the sample stacking process [384]. Without a preceding water plug (**Fig. 5-1b**), some DNA can diffuse or migrate into the sieving buffer and thus partially escape the ongoing stacking at the water-buffer interface. By placing a water plug in front of the sample (**Fig. 5-1a**), a larger zone exists between the sample and the buffer interface which reduces the amount of DNA that can diffuse or migrate into the sieving buffer ahead of the bulk sample, and thus reduces peak fronting. On the other hand, if the water plug is too large, the electric field across the sample zone can become uneven at the start of electrophoresis, resulting in sample dispersion. In fact, peak tailing became evident when the preceding water injection was increased to 30 s.

This hydrodynamic injection scheme (10 s water followed by 45 s HD sample injection) furnished reproducible peak migration times and peak areas and worked

well for the quantitation of PCR products (see below) [151]. When greater sensitivity was desired, electrokinetic injection was performed.

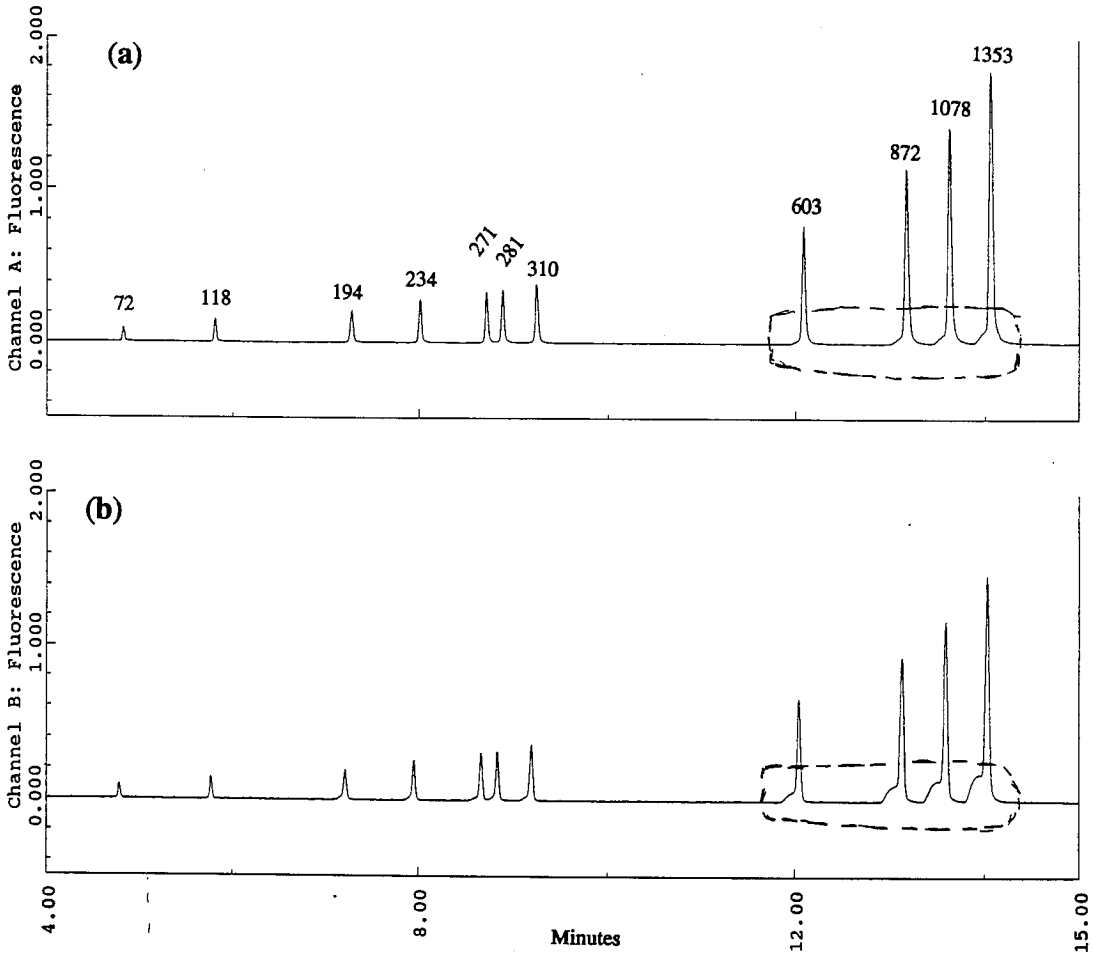


Figure 5-1. The separation of a DNA restriction digest following a hydrodynamic injection; (a) a 10 s water plug preceded a 45 s injection of the sample; (b) no water plug was injected prior to the sample. The larger DNA fragments have severe peak shoulders in (b) (see boxed area). Conditions: *Capillary:* 50 μm i.d. x 37 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.1, 50 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 45 s @ 0.5 psi; *Separation:* 0-4.5 min @ 15 kV, 4.5-15 min @ 5 kV; *Sample:* 588 ng/mL ϕX174 *Hae*III digest diluted in deionized water.

B. ELECTROKINETIC INJECTION

To better understand the role of sample injection in CE an experiment was performed comparing EK and HD injections. The same sample was analyzed using both techniques. For the injection conditions used, a 10-fold improvement in the amount of sample injected was seen with EK (5 s @ 1 kV) over HD (45 s @ 0.5 psi) (**Figure 5-2**). A sample of the HUMTH01 allelic ladder was used in order to observe the effect of the injection method on resolution. Although peak shape appeared to improve slightly with EK (**Figure 5-2b**), high resolution may be obtained with either type of injection as demonstrated by the separation of the HUMTH01 alleles, which differ by only 4 bp. The implication of these results is that as long as stacking is present, hydrodynamic injection can be used as an effective sample introduction technique, thus eliminating the need for dialysis.

A further examination was made to test the effect of electrokinetic injection times and voltages on the sensitivity and resolution of the short tandem repeat HUMTH01 alleles. The use of low voltages for a "long" period of time (e.g., 5-10 s @ 1 kV) produced superior resolution and precision over analyses using high voltages for a short period of time (e.g., 1-2 s @ 10 kV). The superior resolution with lower voltages during EK injection is in agreement with the literature [23]. In general, injections were more reproducible if sampling occurred for longer than 2-3 seconds. Depending on sample concentration and ionic strength, slight variations in injection time or voltage were often required. For example, if sample overloading was seen for 5 s @ 1 kV, the injection time could be lowered to 2 s @ 1 kV to improve the

resolution. On the other hand, if the sample concentration on the column was too low, the injection voltage could be increased (e.g., 5 s @ 2 kV). For most of the PCR samples examined in this work, an electrokinetic injection of 5 s @ 1 kV was most effective following a 1:50 dilution of the sample in deionized water.

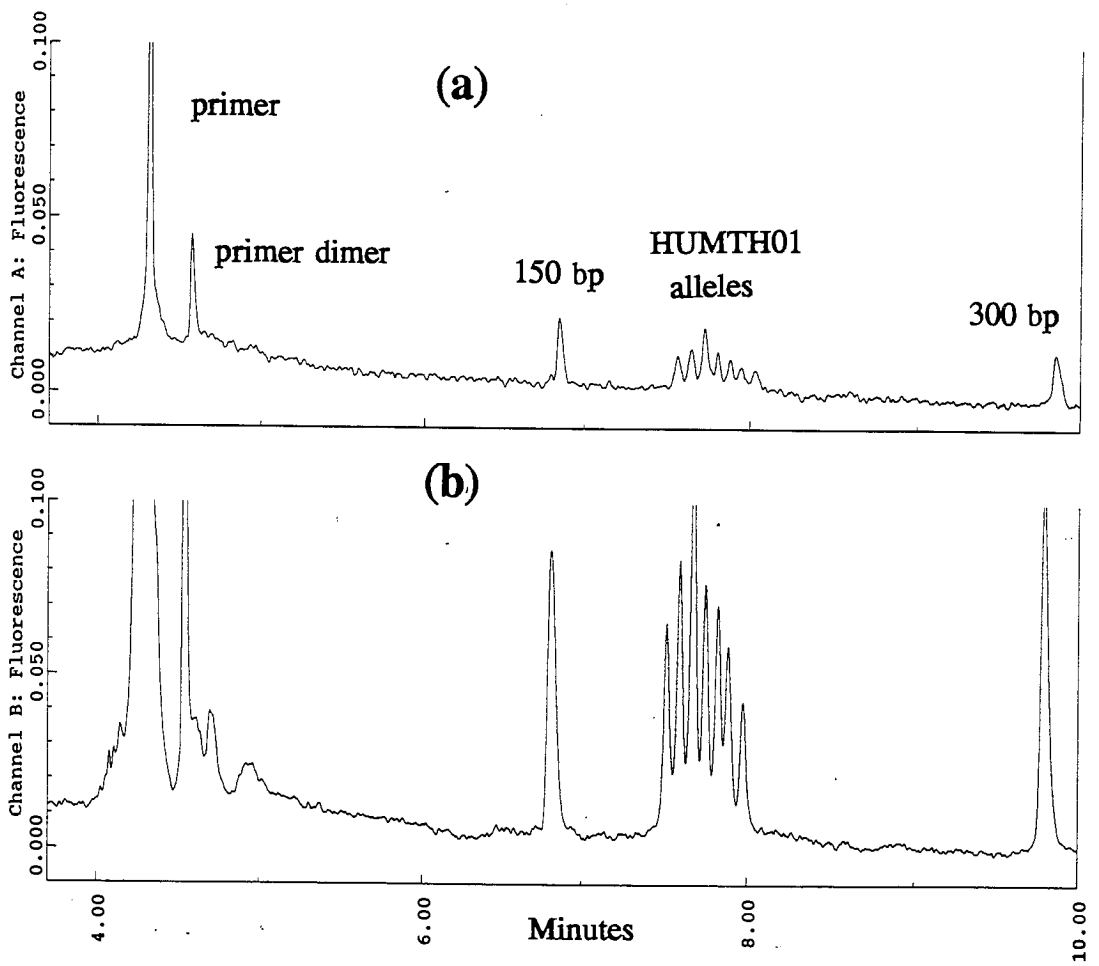


Figure 5-2. A comparison of (a) hydrodynamic and (b) electrokinetic injections for the HUMTH01 allelic ladder. The HUMTH01 alleles differ by 4 bp and range in size from 179-203 bp. The 150 bp and 300 bp fragments have been included as internal markers and are present at a concentration of 2 ng/mL. Conditions as in Fig. 5-1 except injection, where (a) 45 s @ 0.5 psi and (b) 5 s @ 1 kV. *Sample:* HUMTH01 ladder with added 150 bp and 300 bp internal standards (following 30 min float dialysis).

The dilution of samples in water is a beneficial procedure because it eliminates the requirement for sample dialysis and is very amenable to automation. Conceivably, a portion of a PCR sample could be taken directly from the thermal cycler and diluted in water prior to CE separation in a robotic workstation. This would allow fast separations with minimal processing time and reduced labor. The disadvantage of this procedure is that lower sample concentrations are injected and greater sensitivity is required making laser-induced fluorescence a necessity.

II. Factors Affecting DNA Resolution

One goal of this research has been to develop an understanding of the factors affecting resolution in CE. Prior to this work, few systematic studies had been undertaken to understand the effect of various parameters on CE resolution of DNA; and none had been performed on PCR products. For CE to be an effective tool for PCR product analysis, the separation of double-stranded DNA (dsDNA) smaller than ~1000 bp needs to be demonstrated. For example, in the analysis of tetranucleotide STRs used in DNA typing, the resolution of fragments differing in length by 4 bp, in the size range of 100-350 bp, is required. The examination of the factors involved in separating DNA fragments has led to an increased understanding of the CE variables and aided in reducing the separation time while maintaining high resolution.

A. COLUMN COMPONENTS

1. Capillary Inner Diameter

Better precision and resolution were observed with 50 μm i.d. (vs. 100 μm i.d.) capillaries. Precision improves because less ion depletion can occur in the buffer well with narrower capillaries since smaller currents are generated with applied voltage. In addition, heat dissipation is much easier at high electric fields for a 50 μm vs. a 100 μm capillary (see **Figure 2-1**) because the surface area-to-volume ratios are 80 and 40, respectively. Thus, high voltages, and consequently, more rapid separations with less longitudinal diffusion are permitted with the 50 μm i.d. capillary. Another interesting observation was the amount of intercalating dye

required to obtain equivalent resolution. Both ethidium bromide and YO-PRO-1 intercalating dyes were required to obtain 4 bp resolution of STRs using a 100 μm i.d. capillary [33], but equivalent resolution was obtained without ethidium bromide using 50 μm i.d. capillaries.

2. Capillary Length

An examination was made of the effect that column length plays in DNA separations using entangled polymers. The general trend was an improvement in speed with shorter capillaries with a minimal loss in resolution. Good resolution was still maintained with an effective column length of only 7 cm (**Figure 5-3**). The separation of the 271 and 281 bp fragments is often used as a high-resolution indicator. Typically migration times of 15-20 minutes are needed to resolve the 271 and 281 bp doublet with this same separation voltage and a longer capillary (see Fig. 5-1). The separation shown in **Figure 5-3** was equivalent to that obtained on a gel-filled capillary [326], which generally have produced superior resolution when compared to entangled polymers. The minimal role of capillary length on resolution has been substantiated with the high resolution separations that have been performed on glass microchips where separation distances can be <4 cm [471,652]. In fact, there have been a number of studies which tend to confirm the postulate that separation efficiency is more dependent upon injection and other factors than capillary length [236,382,445].

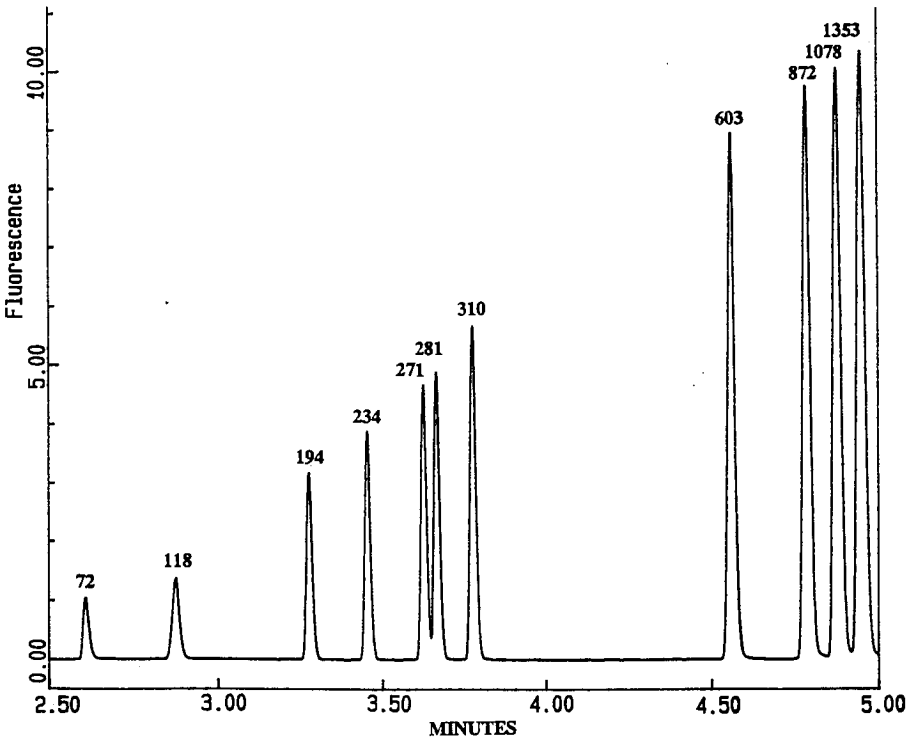


Figure 5-3. The separation of ϕX174 *Hae*III restriction digest with an effective length of 7 cm. With longer capillaries, migration time increases with little benefit to resolution (see Fig. 5-1). Conditions: *Capillary:* 50 μm i.d. x 27 cm (**7 cm to detector**) DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 500 ng/mL YO-PRO-1; *Temperature:* 25 °C; *Injection:* 5 s @ 1 kV (from outlet); *Separation:* 5 kV (6.3 μA); *Sample:* 588 ng/mL ϕX174 *Hae*III digest. Numbers above the peaks indicate the DNA fragment size (bp).

3. Capillary Coating

Several wall coating thicknesses were examined including 0.05, 0.1, and 0.2 μm. No discernable difference could be seen in terms of resolution or stability between the different thicknesses. While the vast majority of the separations described in this work were performed with the DB-17 coating (see **Figure 4-2**), other coatings were briefly examined, including the methyl-coated polysiloxane DB-1 (J&W Scientific) and a C-18 bonded column from Supelco, Inc. (Bellefonte, PA). In agreement with Ulfelder [216], who compared DB-17 and DB-1, no significant changes in resolution or selectivity between these columns and DB-17 were observed.

However, the instability of the Supelco column prevented its use for more than 7-8 runs, presumably because the wall coating degraded.

Our laboratory has been able to operate routinely for over 1000 runs on a single DB-17 capillary, an order of magnitude superior to routine use reported in the literature. This stability most likely results from protection of the capillary's inner surface by the formation of a secondary layer on the capillary wall with the viscous entangled polymer (e.g., 1% HEC) [23,231]. The methanol rinse between each run may also help to activate the hydrophobic surface. In an experiment where the HEC was removed completely from the buffer solution, peak tailing was evident (along with the lack of resolution in DNA fragments, as expected), indicating possible wall interactions with the DNA fragments. Upon replacement of a viscous polymer solution, normal peak shapes were observed. The formation of a dynamic wall coating was further substantiated with some work involving a 20 μm i.d. bare fused silica (uncoated) capillary filled with 1% polymer solution. Instead of coming out in reverse molecular weight, as expected if any EOF was present, the DNA fragments came out in order of increasing size (see **Figure 2-5**). These results suggest that a secondary wall coating from the viscous polymer in the buffer would render the effect of the primary coating (DB-1 or DB-17) insignificant.

B. SAMPLE SEQUENCE EFFECTS

The internal sequence, and hence the conformation, of a DNA fragment can sometimes significantly influence a separation. For this reason, most of the resolution studies described in this work were performed with DNA ladders where fragments contained repeated sequences. A sample with a consistent conformation between fragments should be a more effective indicator of the resolution for a CE system [527]. Short tandem repeats are particularly useful for high resolution studies of a

CE system because of the small, uniform differences between alleles (e.g., 4 bp).

Previous to this work, most DNA separations by CE have involved restriction digests such as ϕX174 *Hae*III and pBR322 *Hae*III (see **APPENDIX 1**). The problems with using these restriction digests may be illustrated from the GC content data compiled in **TABLE 5-1**. For example, consider the 123 and 124 bp fragments in the pBR322 digest. These fragments differ by only 1 bp in overall size, but are not so similar in terms of internal sequence. This fact may be exploited to resolve the two fragments. Note that the 124 bp fragment contains a higher GC content (64.5% vs. 56.1% for 123 bp). Intercalating dyes, which have a stronger affinity for GC base pairs [46], could potentially bind to the 124 bp peak in a selective manner, change its conformation with respect to the 123 bp fragment, and improve the resolution. This fact has been demonstrated and will be discussed later (see **Figure 5-14**). Several groups have even demonstrated the separation of two DNA fragments which were the same length using conformational differences between the fragments [75,135,398]. While these separations are respectable, they do not permit an appropriate, unbiased measure of the resolution when using particular CE parameters. Thus, the use of DNA ladders, with repeated sequences and similar conformations, are more appropriate for resolution studies.

The separation of a 20 bp ladder, which contains 50 fragments from 20 bp to 1,000 bp (each at 20 bp intervals), permits high resolution mapping over the entire PCR product range, without concern about possible conformational differences between adjacent fragments (**Figure 5-4**). A 100 bp ladder has been added to the sample for easier peak identification. The decline in peak signal with increasing DNA size is a result of a decreased efficiency for the enzymatic reaction (i.e., ligation) with longer DNA fragments [75]. This 20 bp ladder, along with various STR and VNTR allelic ladders, was used throughout these resolution studies.

TABLE 5-1. The GC Content of Two Commonly Used Restriction Digests. In the first column, the DNA fragment size is listed. For each fragment, the number of GC (guanine and cytosine nucleotides) in that fragment has been calculated along with the %GC (#GC divided by total fragment size). The sequences were obtained from Sigma. The AT/GC ratio has been suggested as a measure of DNA flexibility [73].

ϕX174 *Hae*III (11 restriction fragments)

bp size	#GC	%GC	AT/GC
72	37	51.39%	0.946
118	59	50.00%	1.000
194	99	51.03%	0.960
234	109	46.58%	1.147
271	124	45.76%	1.185
281	124	44.13%	1.266
310	153	49.35%	1.026
603	258	42.79%	1.337
872	378	43.35%	1.307
1078	471	43.69%	1.289
1353	600	44.35%	1.255

pBR322 *Hae*III (22 restriction fragments)

bp size	#GC	%GC	AT/GC
8	7	87.5%	0.143
11	6	54.5%	0.833
18	9	50.0%	1.000
21	14	66.7%	0.500
51	36	70.6%	0.417
57	35	61.4%	0.629
64	43	67.2%	0.488
80	47	58.8%	0.702
89	54	60.7%	0.648
104	67	64.4%	0.552
123	69	56.1%	0.783
124	80	64.5%	0.550
184	109	59.2%	0.688
192	83	43.2%	1.313
213	131	61.5%	0.626
234	138	59.0%	0.696
267	136	50.9%	0.963
434	252	58.1%	0.722
458	195	42.6%	1.349
504	267	53.0%	0.888
540	302	55.9%	0.788
587	250	42.6%	1.348

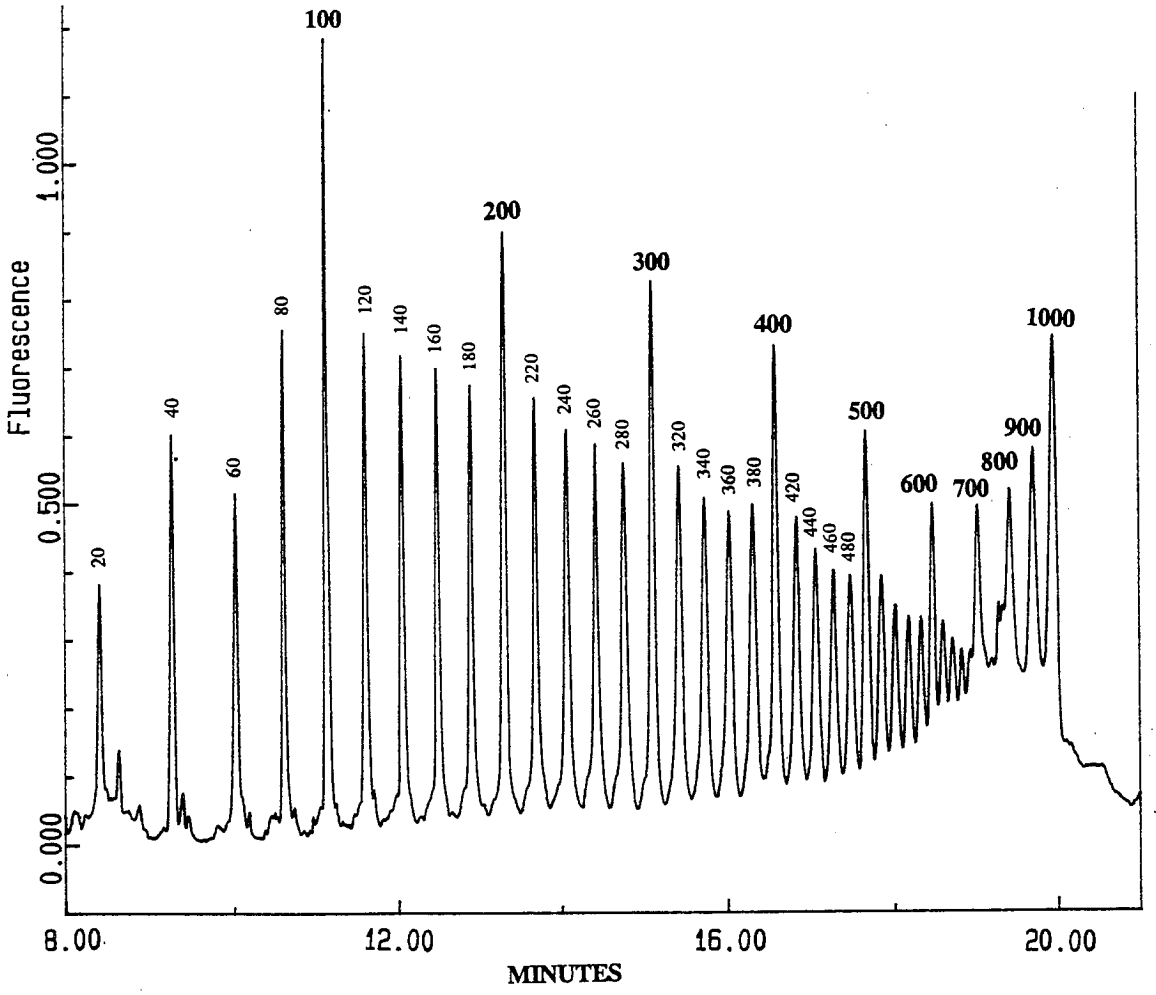


Figure 5-4. GenSura 20 bp ladder with DNA fragments from 20 bp to 1000 bp in 20 bp increments. The declining peak signal with increasing fragment size is a result of the enzymatic reaction used to generate the ladder. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 3% HEC (24,000-27,000 MW), 100 mM TBE, pH 8.2, 500 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 5 s @ 1 kV; *Separation:* CV: 5 kV; *Sample:* 160 ng/mL 20 bp ladder + 40 ng/mL 100 bp ladder (GenSura).

C. INSTRUMENTAL FACTORS

1. Temperature

The separation of the STR (VWA, FES/FPS, and CSF1PO) and VNTR (D1S80) allelic ladders was evaluated over a temperature range of 25-50 °C. From 25-35 °C, temperature appeared to have very little effect on the resolution of the DNA

fragments (**Figure 5-5**). The separation time (solid line) for the 214 bp peak (FES/FPS allele 7) can be reduced by ~1.5 minutes without a noticeable loss in resolution. The resolution (dotted line) was measured between the 214 and 218 bp peaks. As stated earlier, the ability to resolve 4 bp differences is important to typing STRs. For constant voltage separations, the migration times decreased with increased temperature, primarily because the solution viscosity was reduced, which allowed more ion movement and higher currents. At constant current, the migration times remained relatively constant over the temperature range examined here with no significant changes in resolution (data not shown). Other reports of temperature study results are consistent with these findings and interpretation [298,395].

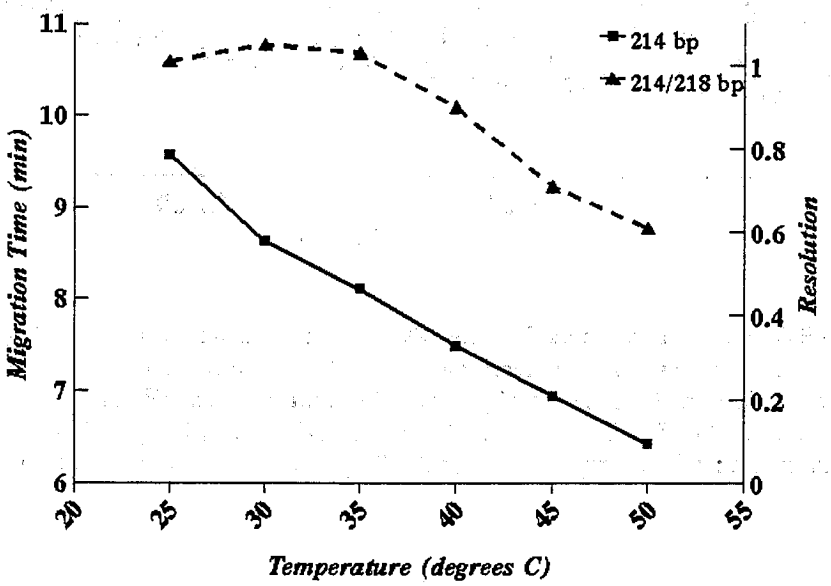


Figure 5-5. Effect of temperature on resolution (dotted line) and migration time (solid line) at constant voltage. Conditions as in Fig. 5-4, except 1% HEC (Aldrich) and temperatures as indicated. The sample is the Roche Multiplex 3; data points are for a 214 bp fragment (FES/FPS allele 7). Resolution was determined using Eq. 2-5.

2. Electric Field Strength

The impact of the electric field on resolution was evaluated with a series of experiments using the STR and VNTR allelic ladders. Voltages ranging from 1 kV (37 V/cm) to 20 kV (741 V/cm) were applied across a 27 cm capillary. Alleles 7 (214 bp) and 8 (218 bp) were chosen as an indicator of the separation because they are 4 bp apart, are in the middle of the STR region (100-350 bp), and represent the general trend of tetranucleotide STRs. Under the column and buffer conditions used in these studies, the resolution in the 100-350 bp region began diminishing above ~200 V/cm (**Figure 5-6**). Data points are not shown above 275 V/cm because the STR alleles, which were 4 bp apart, could not be distinguished. However, an Ohm's law plot with these same conditions was linear out to 450 V/cm.

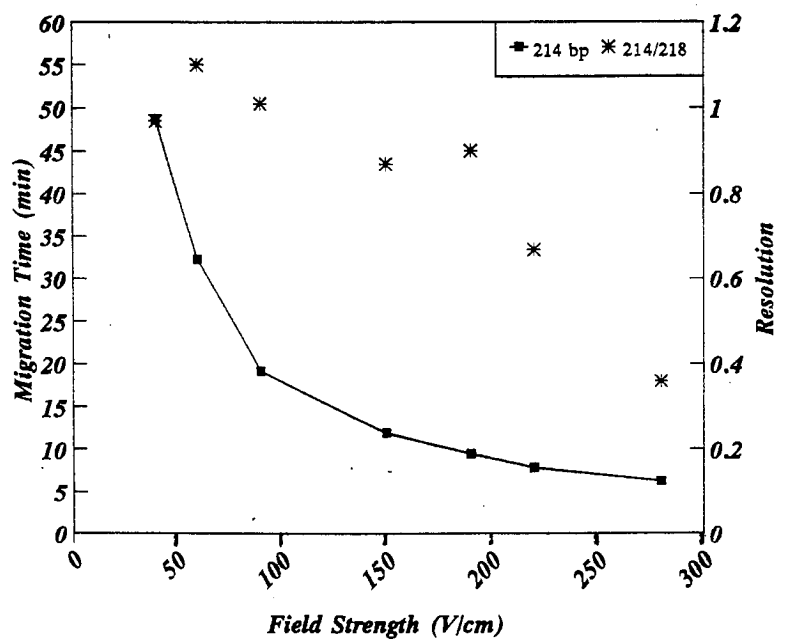


Figure 5-6. Effect of electric field strength on the resolution (asterisks) and migration time (squares with solid line). Resolution between the 214 and 218 bp fragments diminishes above 200 V/cm (calculated using Eq. 2-5). However, an Ohm's law plot under these conditions does not show deviation until 450 V/cm. Conditions as in Fig. 5-5 except at a constant temperature of 25 °C and field strengths as indicated.

Notice that the resolution does not improve significantly by going to lower field strengths, primarily because more longitudinal diffusion may occur (**Figure 5-6**). Moreover, migration times markedly increase below 100 V/cm. Thus, the optimum balance for the STR region of interest (100-350 bp), in terms of migration time and resolution, was 150-185 V/cm, or 4-5 kV applied across a 27 cm capillary.

For longer PCR products, such as the larger alleles in the VNTR D1S80, the voltage may need to be even lower to enhance resolution (**Figure 5-7a**). The dramatic dropoff in resolution at higher field strengths is primarily due to alignment of the fragments in the electric field [127]. At higher voltages, the change from the Ogston regime to the biased reptation regime (see Section F below) can occur earlier with substantial loss in resolution (**Figure 5-7c**). A close examination shows this phenomenon; at 93 V/cm (**5-7a**), all DNA fragments are fully resolved; at 185 V/cm (**5-7b**), the 36 (721 bp) and 37 (737 bp) repeat peaks have begun to comigrate and are not being resolved as well from their neighbors as at the lower field strength; finally, when the applied voltage is increased to 370 V/cm (**5-7c**), no fragments larger than 500 bp (allele 22) can be separated. Alignment of the DNA fragments in the electric field occurs earlier with higher voltages, which leads to a rapid loss in resolution. These results highlight the fact that as DNA fragments become larger it is more difficult to separate them when using high voltages [78,127].

An examination of the literature (see **APPENDIX 1**) revealed that there has consistently been an optimum range for the applied voltages used in DNA separations (**Figure 5-8**). A majority of these separations were performed in the 151-200 V/cm range at the expense of some resolving power (compared to 101-150 V/cm), but with greater speed. The detailed study carried out as part of this research shows why this range is generally preferable despite Ohm's law measurements that might have suggested higher voltages.

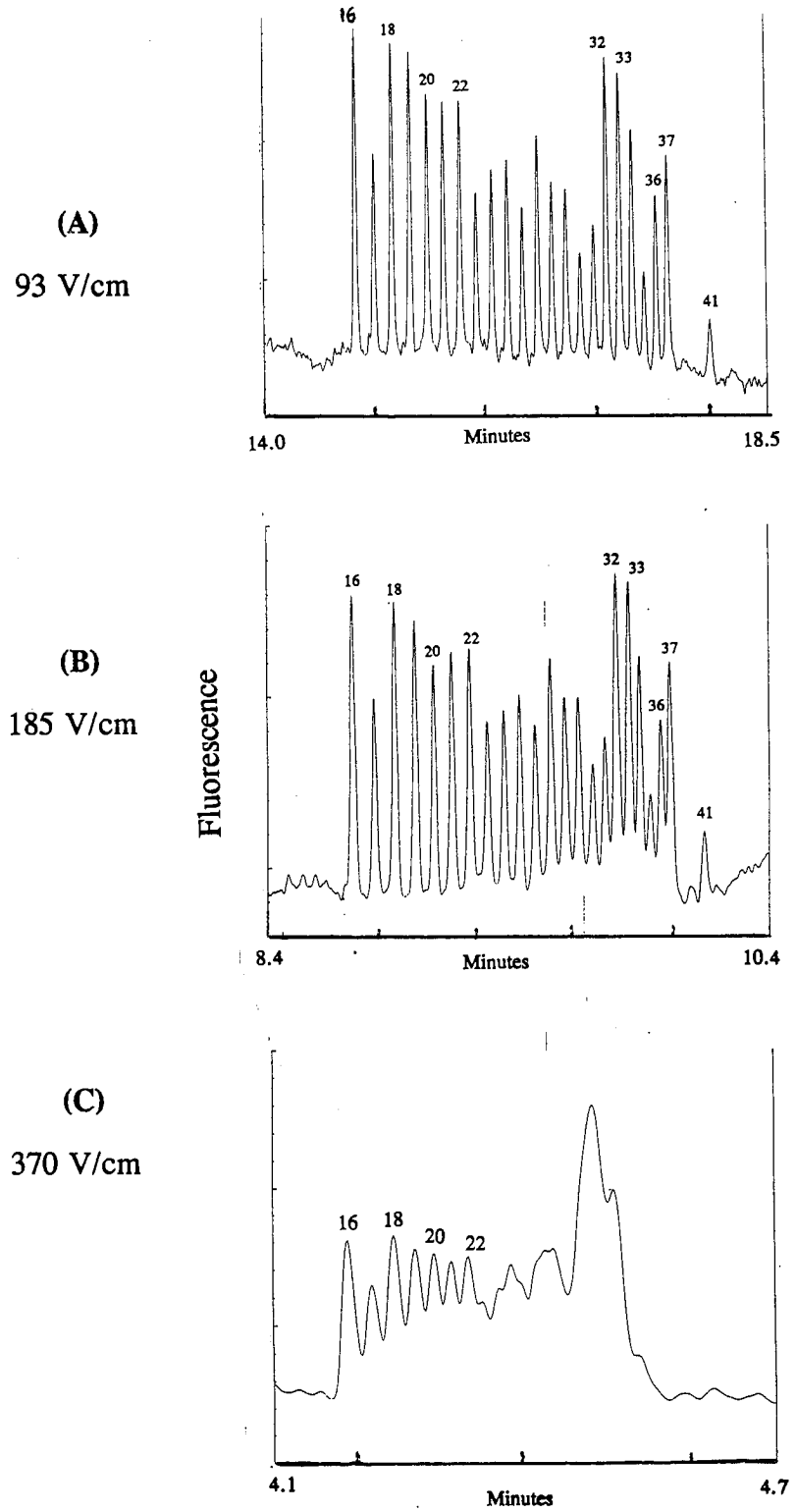


Figure 5-7. The onset of biased reptation begins with lower DNA size when higher field strengths are used. Time scales differ. D1S80 alleles at (A) 93 V/cm, (B) 185 V/cm, and (C) 370 V/cm. DNA fragments larger than ~500 bp (allele 22) fail to be resolved above 300 V/cm. Conditions as in Fig. 5-3 except 0.3% HPMC (4000 cP), and voltages as indicated. Sample was the FBI D1S80 allelic ladder (1:50 with H_2O).

Based upon the observations made in **Fig. 5-6 and 5-7**, the stretching of the DNA appears to be the primary cause of resolution loss. Furthermore, the Ohm's law point of deviation is not a good indicator of the optimum voltage when looking at DNA. Optimum field strengths are DNA length dependent. For small PCR products, 150-200 V/cm was found to be the optimum field while resolution of larger PCR products improved with electric fields below 100 V/cm. For DNA separations, the optimum voltage may be well below the determined Ohm's law point of deviation because of the ability of DNA to be stretched in the electric field.

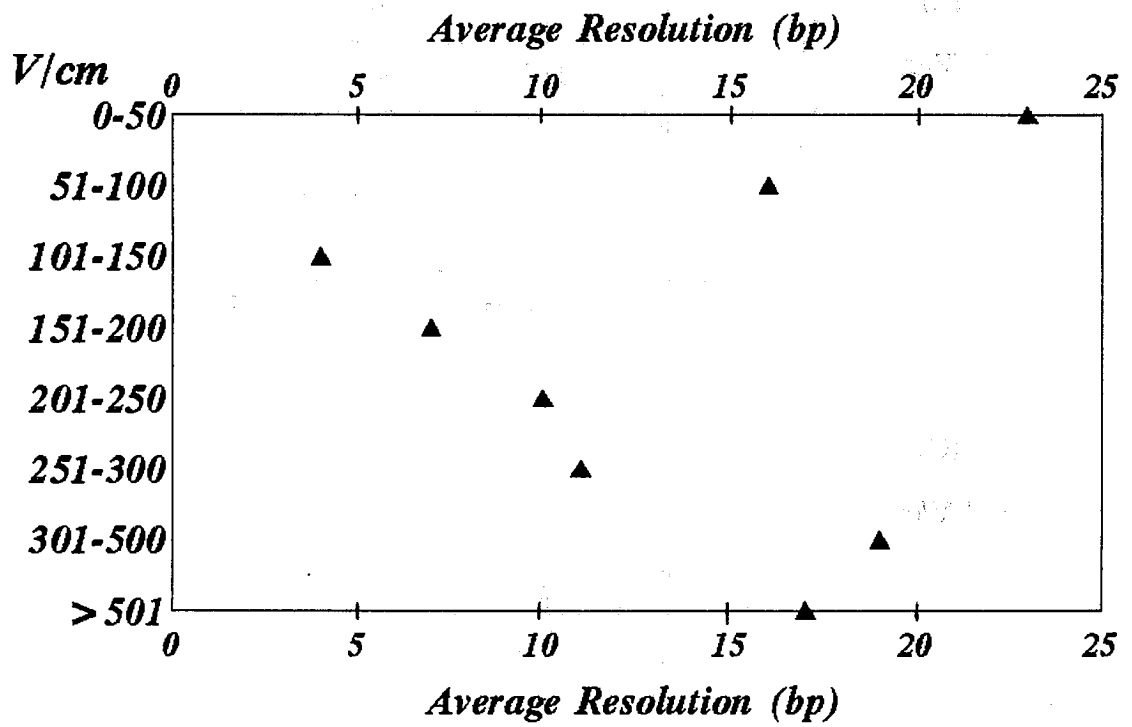


Figure 5-8. Resolution using different field strengths from CE/DNA literature. Information was obtained from examination of the data in APPENDIX 1. The average resolution was a combined summary from estimates of results displayed at each field strength (triangles). Most separations in the literature are demonstrated with ϕX174 *Hae*III digest; these estimates therefore come from the ability to split the 271 and 281 bp peaks.

3. Voltage Programming

As lower voltages were found useful in improving resolution, but at the expense of longer run times, voltage gradients were examined in an effort to reduce the overall separation time. The application of a high electric field (e.g., 400 V/cm) at the beginning of a run was found to move the DNA quickly through the column, in effect, shortening the length of the column. Shortly before the DNA fragments would normally pass the detector window, the potential difference across the capillary was reduced to a lower voltage (e.g., 150 V/cm) in a single step to improve the resolution in that particular region. This single-step voltage gradient increased the peak capacity (by spreading the separation region) and reduced the overall separation time.

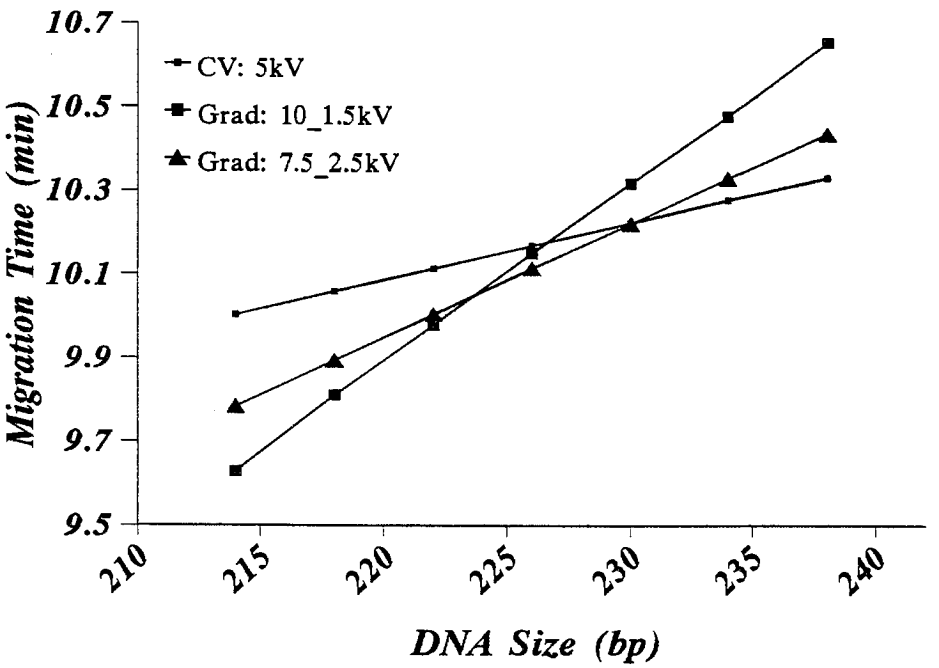


Figure 5-9. The separation of STR alleles, which differ in size by 4 bp, was improved with voltage programming. The slope is an indicator of the separation spread. Conditions as in Fig. 5-6 except *Separation:* (**CV**, dots): 0-15 min @ 5 kV; (**Grad 10_1.5kV**, squares): 0-4 min @ 10 kV, 4-24 min @ 1.5 kV; (**Grad 7.5_2.5kV**, triangles): 0-5 min @ 7.5 kV, 5-20 min @ 2.5 kV.

Several voltage gradients were applied to expand various regions of an electropherogram. In **Figure 5-9**, two single-step voltage gradients are compared to a constant voltage run for the separation of FES/FPS alleles (PCR products ranging from 214-238 bp). While the overall migration times are very similar, the optimum separation (as indicated by the steepness of the slope) existed when the lowest field strength was applied in the STR region (squares). Voltage programming was also used to improve the speed and resolution of HUMTH01 alleles (**Figure 5-10** and **TABLE 5-2**). A similar resolution was obtained between the gradient separation (5 kV in the HUMTH01 region) and the constant voltage run (CV: 5 kV), but with faster times and at slower data collection rates (bp/sec) (**TABLE 5-2**). Data collection rates and storage can become important if the idea of using multiple capillaries in parallel is to be employed as a means of creating greater sample throughput [99].

TABLE 5-2. Comparison of Separations for HUMTH01 Alleles. Theoretical plates and resolution were calculated using Eq. 2-4 and 2-5, respectively. The precision was determined after sizing the alleles using the dual internal standard approach [559]. The results of 7 alleles from the HUMTH01 ladder were averaged over 9 replicate injections.

	CV: 10kV	CV: 5kV	Gradient‡
Separation Time	8.3-8.6 min	17.2-17.8 min	7.0-7.6 min
Theoretical Plates	660,000	1,500,000	225,000
Resolution	0.65	1.26	1.16
Precision (n=63)	0.95 bp	0.35 bp*	0.57 bp
bp/sec	1.8	0.80	0.83

‡Gradient: 0-4.5 min @ 15 kV, 4.5-10 min @ 5 kV
*Only 8 runs rather than 9 were performed so (n=56).

Notice also that the number of theoretical plates is drastically reduced when using a gradient, as compared to the constant voltage methods, yet the peak separation

does not decrease (**TABLE 5-2**). This observation highlights the difference between resolution and efficiency. Peak widths are increased as a result of longitudinal diffusion in the lower voltage portion of the separation. However, the resolution of the peaks is much improved. Thus, the use of theoretical plates as an indicator of a CE system's performance is not always appropriate for DNA separations, although references to plate counts are common in the literature [146,251,382].

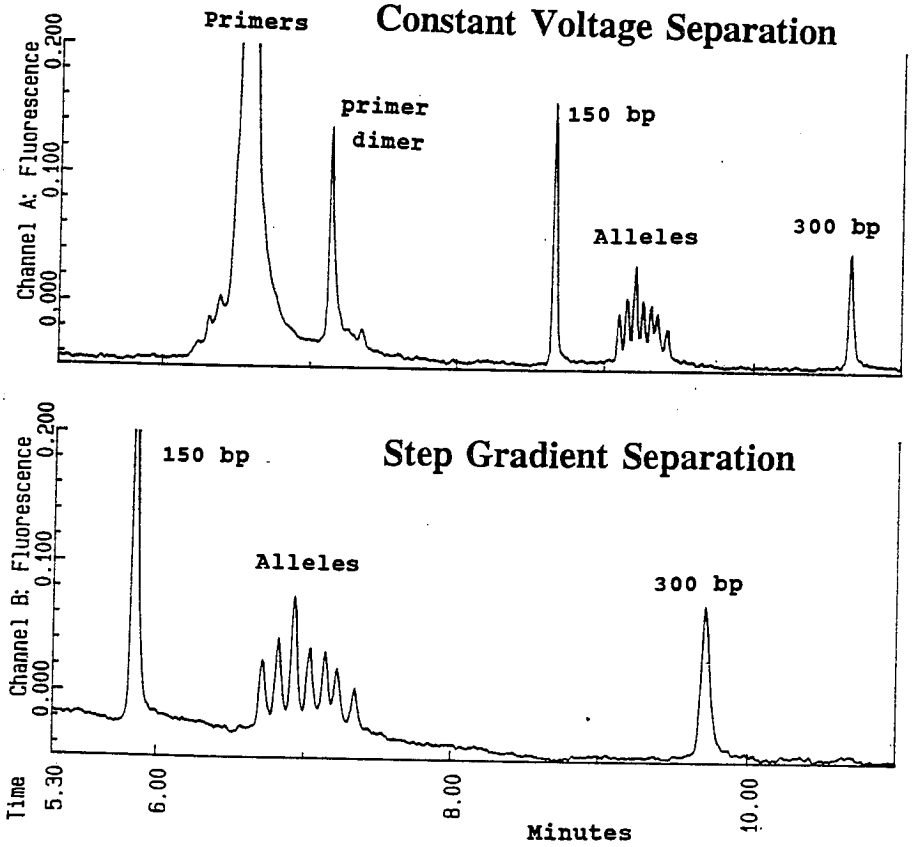


Figure 5-10. A comparison of separation speed for the HUMTH01 alleles between a constant voltage and a step gradient separation [152]. The time scales are the same. In the lower frame, the primers and primer dimer migrate faster than 5 minutes and are not seen. Alleles represented include 5 (179 bp), 6 (183 bp), 7 (187 bp), 8 (191 bp), 9 (195 bp), 9.3 (198 bp), and 11 (203 bp). Conditions: *Capillary:* 50 μm i.d. x 37 cm (30 cm to detector) DB-17 coated; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 50 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 5 s @ 1 kV; *Separation:* **Constant Voltage** = 10 kV; **Step Gradient** = 0-5 min 15 kV, 5-11 min 5 kV.

D. BUFFER COMPONENTS

1. Entangled Polymer Solution

Type of Polymer

Three soluble polymers were examined as separation media for DNA separations: hydroxyethyl cellulose (HEC), hydroxypropyl methyl cellulose (HPMC), and polyethylene oxide (PEO). Several molecular weights were examined for each type polymer at a variety of concentrations. Of the three polymers studied, HEC and HPMC gave superior resolution for STRs and VNTRs over PEO at similar molecular weight (~100,000 MW) and concentrations (0.3-1.0%). Most of the separations discussed below were performed with HEC because HEC went into solution much easier than HPMC, which could have an impact on routine use. In addition, DNA separations with HEC have been studied in the past, which allowed a direct comparison with results obtained by others [34,36,45].

Concentration of Polymer

The separation of a 100 bp DNA ladder was examined in polymer networks formed from various molecular weights and concentrations of HEC. This ladder was chosen because the 4 bp resolution needed to separate STRs alleles was not achievable with many of the examined polymer concentrations. Interestingly, the migration times of the 100 bp ladder increased as a direct function of the polymer concentration *regardless* of the polymer length (**Figure 5-11**). Notice that with equivalent concentrations of HEC (0.5%), almost identical migration times were observed for the DNA fragments (see solid diamonds and open hour-glasses). HEC "C" was the 140,000-160,000 MW HEC while the HEC "E" was the 90,000-105,000 MW HEC. In addition, 1% solutions of HEC "A" (Aldrich, unknown MW, 86-113 cP for a 2% solution) and HEC "B" (Fluka, 75-115 cP for a 2% solution) produced similar

separations of the DNA ladder. These are interesting results because solution viscosity (i.e., molecular weight, see Eq. 3-5) has been reported to be an important parameter in the separation process [69]. The viscosities of the two HEC polymers discussed above ("C" and "E") were quite different for 0.5% solutions yet similar separations were observed.

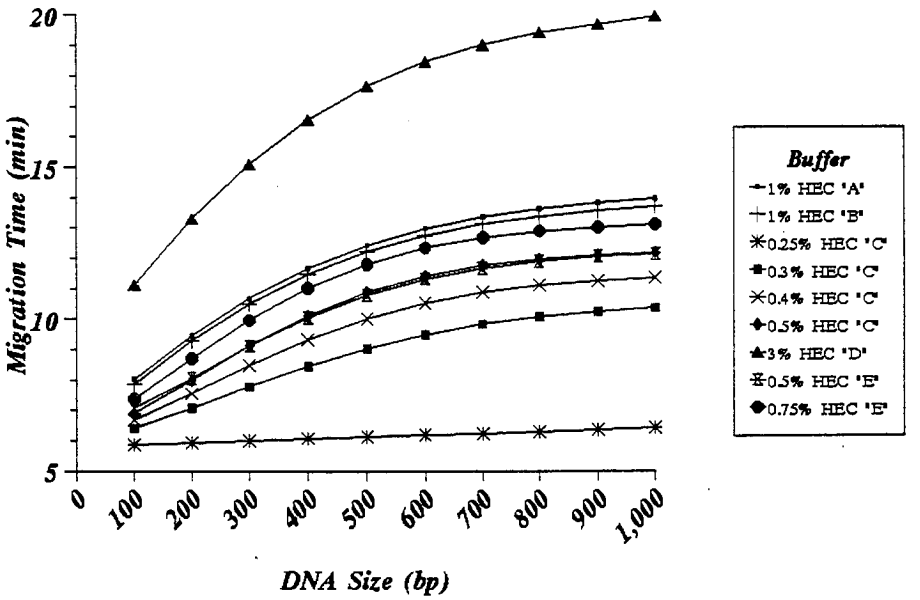


Figure 5-11. The migration times for a 100 bp ladder in entangled solutions of HEC over a range of polymer concentrations and molecular weights. HEC "A" = Aldrich, HEC "B" = Fluka medium viscosity, HEC "C" = 140,000-160,000 MW (Polysciences), HEC "D" = 24,000-27,000 MW (Polysciences), HEC "E" = 90,000-105,000 MW (Polysciences). Conditions as in Fig. 5-4 except where noted for the types and concentrations of HEC.

These data suggest that mesh size, which is determined primarily by the polymer concentration (**Figure 5-12**, see Eq. 3-2), is the dominant factor in the separation process. If polymer strand length (i.e., molecular weight) does not play a major role in the "pore" formation process, then using shorter strands should decrease the viscosity, allowing a smaller mesh size to be formed at manageable concentrations in order to produce a better separation of small DNA fragments (100-1000 bp) [67].

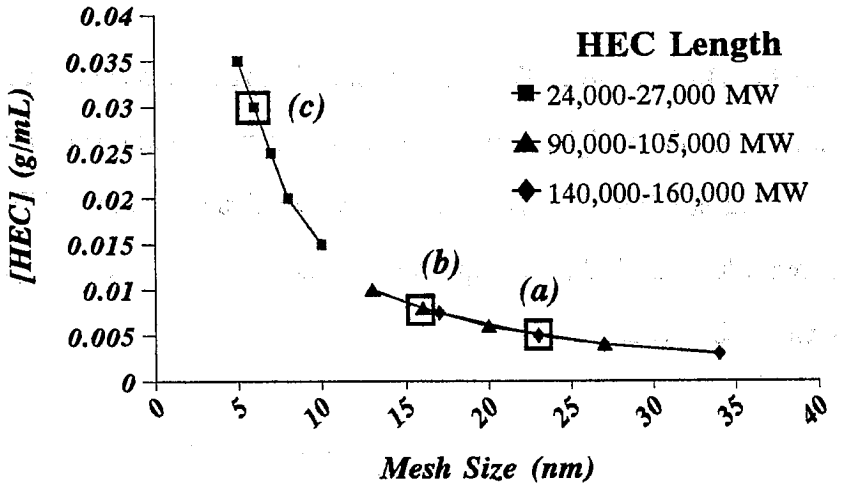


Figure 5-12. The calculated mesh size of several HEC polymers differing in molecular weight. The mesh size was determined using Eq. 3-4. Notice that mesh size is solely a function of polymer concentration *not* molecular weight. Results from the three boxed areas are displayed in Fig. 5-13.

A comparison of different points along the HEC mesh size curve shown in **Figure 5-12** demonstrated that mesh size is important to resolution (**Figure 5-13**). When the polymer network became too tightly enmeshed, the separation of larger PCR products (e.g. D1S80 alleles) decreased dramatically. With a 0.5% HEC solution involving the 140,000-160,000 MW polymer, the HEC formed an average mesh size of approximately 23 nm (**TABLE 5-3**). This allowed the larger D1S80 alleles to be resolved (**Figure 5-13a**), although not as well as if the concentration was lowered further to produce a more open polymer network. For example, at 0.3% HEC (140,000-160,000 MW), where the calculated mesh size is 34 nm (**TABLE 5-3**), the largest D1S80 alleles were baseline resolved (data not shown). Resolution of the D1S80 alleles diminished as the mesh size was reduced with a higher concentration of HEC (**Fig. 5-13b&c**). With 3% HEC (24,000-27,000 MW) (**5-13c**), the calculated mesh size was 6 nm (**TABLE 5-3**). Notice that migration times increased with higher HEC concentrations because the DNA molecules have a difficult time passing through the tigher mesh. More importantly, resolution decreased for the 400-800 bp PCR products and even the STRs below 200 bp in

length.

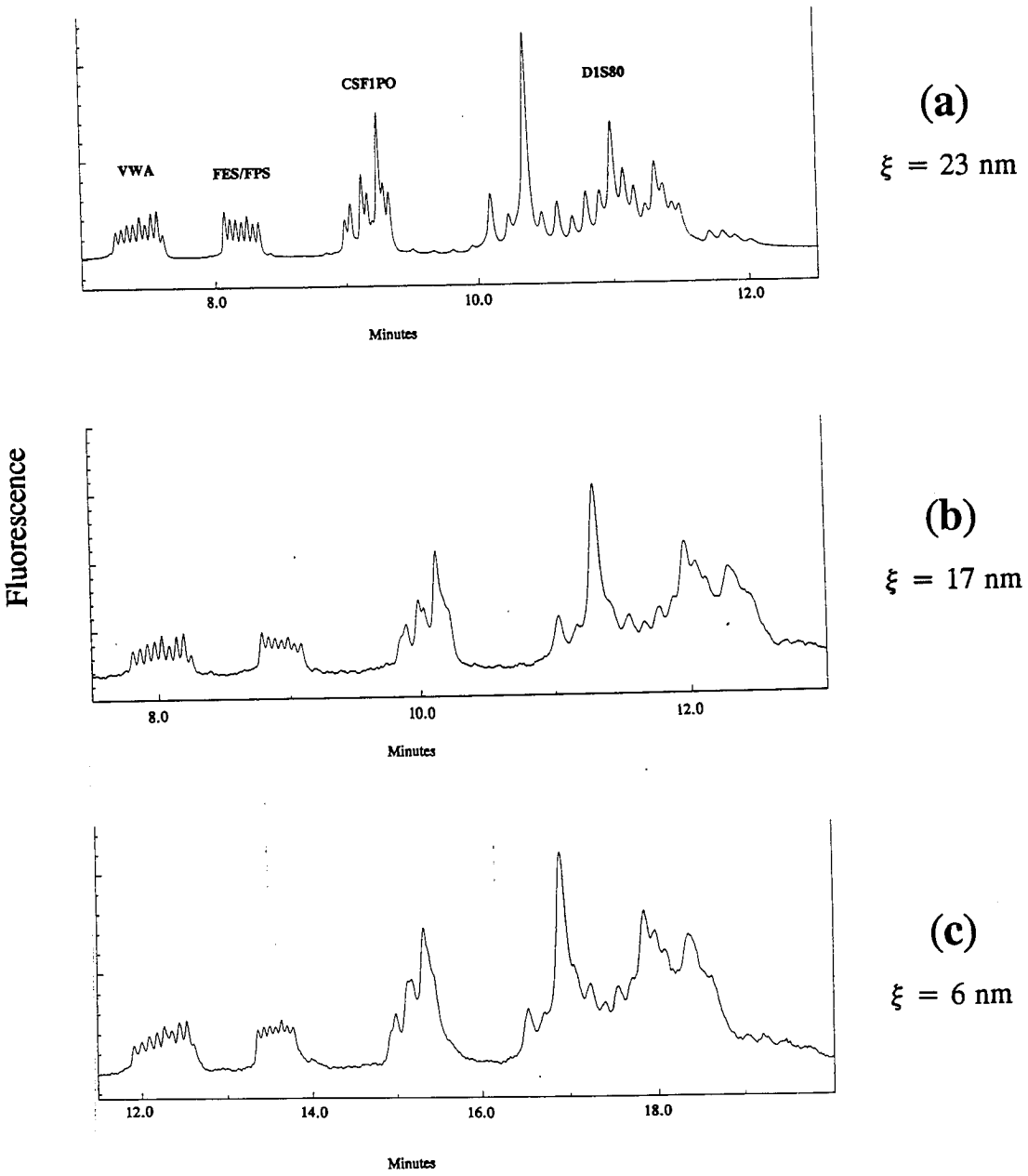


Figure 5-13. The separation of STRs and D1S80 alleles at different polymer concentrations and molecular weights. Time scales differ. The calculated mesh sizes have been included and correspond to the boxed points in **Fig. 5-12.** Conditions as in Fig. 5-11 except polymer concentration and MW as noted. (a) 0.5% HEC (140,000-160,000 MW), (b) 0.75% HEC (90,000-105,000 MW), (c) 3% HEC (24,000-27,000 MW). Sample as in Fig. 4-9.

TABLE 5-3. Average Mesh Sizes for Several Molecular Weights and Concentrations of HEC. Calculations were performed using Eq. 3-4. Φ^* from [36].

Molecular Weight	Φ^*	Concentration	Mesh Size (nm)
24,000-27,000	1.8%	3.0%	6
24,000-27,000	1.8%	2.0%	8
95,000-105,000	0.37%	0.75%	17
140,000-160,000	0.21%	0.5%	23
140,000-160,000	0.21%	0.3%	34

Figure 5-13 suggests that an optimum resolution window can be adjusted by changing the concentration of the polymer. As a general trend, more dilute polymer solutions result in improved separations for larger DNA fragments. This observation may be explained by considering the relationship between a DNA molecule's size and the mesh size of the polymer network. When the radius of gyration for a DNA fragment becomes larger than the average mesh size, the DNA fragments begin to reptate, and resolution rapidly diminishes. A value of 1.4 times the polymer network mesh size has been reported (i.e., when $R_g \geq 1.4\xi$, then the separation mechanism switches from Ogston to reptation) [34]. Following this idea, two possibilities exist for the beginning of reptation and the subsequent loss in resolution: (1) the electric field can stretch the DNA which reduces the cross-sectional area available for interaction with the polymer network [76] (see **Figure 3-5** and **Figure 5-7**) or (2) the mesh size can be reduced by increasing the polymer concentration (see **Figure 5-13**). Both of these events have been observed and described in this work.

Therefore, in order to resolve D1S80 alleles (400-800 bp) in CE, the electric field must be reduced or the mesh size increased to shift the onset of biased reptation to a higher DNA fragment size. Since the former parameter can lead to excessive run times, it should not be considered for routine use. In the future, the voltage

stretching effects might be overcome by shorter column lengths [471] or pulsed field techniques [144]. For the present, increasing the average mesh size holds more promise.

In order to form the largest possible mesh size, it would be desirable to work as close to the entanglement threshold as possible. However, operating near the entanglement thresholds determined by Barron *et al.* [36] does not produce adequate resolution for the PCR products examined below 1000 bp. The optimum mesh size appeared to be somewhat higher than that formed at the entanglement threshold. Several factors may be responsible for this apparent discrepancy. The entanglement threshold is only a determination of where the polymer strands begin to interact. The transient "pores" may be very short-lived near the onset of entanglement. Furthermore, the force exerted by the electrically driven DNA molecules could push the polymer strands apart. However, increasing the polymer concentration should result in "hemming in" neighboring polymer strands with an ensuing longer-lived mesh structure. This idea is in keeping with the constraint release model of Viovy and Duke [38]. A longer polymer requires more time to diffuse or reptate out of position, and thus the transient pores around the DNA molecule would remain in place longer and produce superior resolution.

To summarize, the concentration of the polymer is important to form the appropriate mesh size, but the length is important as well to allow a larger mesh to be formed and to maintain that form. Thus, larger molecular weight polymers worked better in this regard. An analogous result was obtained by Heiger *et al.* [282], who found that a better separation of larger DNA fragments was obtainable with an increase in the pore size for cross-linked polyacrylamide. In polyacrylamide gel-filled capillaries, increasing the pore size may be achieved by reducing the amount of crosslinking agent for a given percentage of polyacrylamide.

Polymer Length (Molecular Weight)

The general influence of polymer length on a DNA separation was considered by comparing the effects of 100,000 MW PEO, ~90,000 MW HPMC (4000cP), and 90,000-105,000 MW HEC. With 0.5% solutions of the HPMC and the HEC, STR alleles (differing by 4 bp) and D1S80 alleles (differing by 16 bp), could be separated. However, with a 1% solution of the PEO, very poor resolution was observed. The STR alleles and D1S80 were unresolved blobs. These dramatic differences may be accounted for by considering the flexibility of the polymer chain itself. The lack of hydrophilic side chains for polyethylene oxide, unlike those found with HEC (see **Figure 3-1**), would render PEO much more flexible in solution than HEC. Rather than forming stiff polymer strands in solution, PEO would adopt more of a random coil formation and not form an equivalent mesh (either in size or structure) to the stiffer HEC. Thus, when the polymers are extremely flexible, larger molecular weights will be required to form an equivalent mesh to shorter, stiffer polymers. This idea has been confirmed recently. With extra long PEO (2.5% solutions of 8,000,000 MW), Chang and Yeung [676] showed excellent resolution of DNA fragments, similar to what can be achieved with 0.5% HEC (140,000-160,000 MW) solutions. Their results suggest that a longer PEO polymer at a higher concentration is required to form an adequate sieving matrix, perhaps because the strands coil back on themselves.

2. Fluorescent Intercalating Dye

Type of Dye

The binding preference to particular DNA sequences was examined for several commercially available fluorescent intercalating dyes. As fragments can possess varying GC contents, the restriction digest pBR322 *Hae*III was used as a test sample for sequence dependent binding of intercalators (see **TABLE 5-1**). Important differences in the separations of the pBR322 digest were observed with equivalent amounts of YO-PRO-1 and TO-PRO-1 (**Figure 5-14**). Interestingly, the two fluorescent dyes differ in structure at only one site (i.e., a sulfur atom for TO-PRO-1 and an oxygen atom for YO-PRO-1). The 123 and 124 bp peaks could not be split with YO-PRO-1 (**5-14 upper trace**) while they were well resolved with TO-PRO-1 (**5-14 lower trace**). Moreover, the 434 and 458 bp peaks were split with YO-PRO-1 but *not* resolved with TO-PRO-1. The spacing of the largest DNA fragments (540 and 587 bp) is also unusual with TO-PRO-1. The GC contents of the pertinent peaks have been included as an explanation for the behavior of TO-PRO-1-DNA interactions (**Figure 5-14**). The TO-PRO-1 dye appears to have selected affinity for certain DNA fragments, which upon excess binding shifts the intercalator-DNA complex to slower mobilities. The separation of two peaks can improve (in the case of the 123/124 bp), if the larger fragment has the higher GC content, or comigration can occur, (434/458bp) if the smaller fragment in a doublet has greater affinity for the intercalator. With STRs, where internal sequences are more uniform, no discernable differences were seen.

For sizing and quantitation purposes, it is desirable to have peak migration time change in a reproducible manner with the DNA size. Those dyes which bind universally (based upon the total number of intercalating sites) rather than discriminately (preferring a particular sequence) will be useful in relating the size or

quantity of a known internal standard to the size or quantity of an unknown DNA fragment. YO-PRO-1 behaves more like a universal binder. While several researchers have used intercalators to improve a DNA separation [53,135,145,234], quantitative interpretation of results with internal sequence description has not been reported.

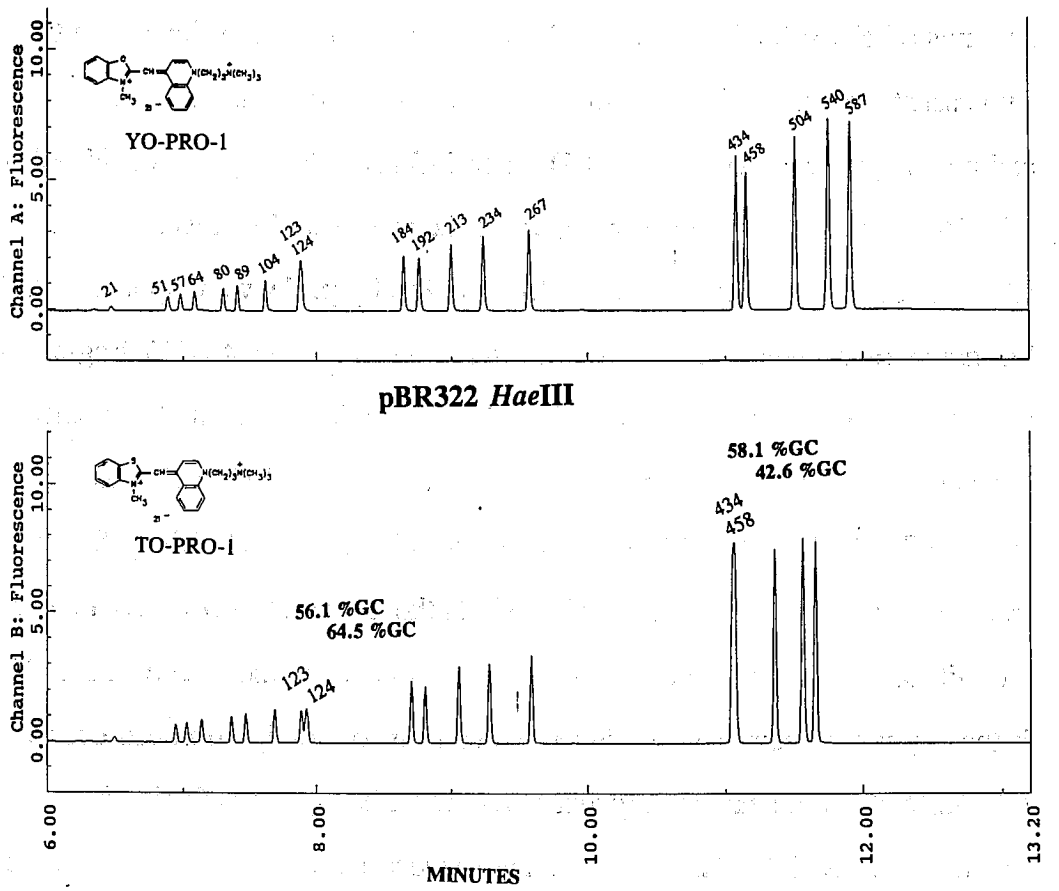


Figure 5-14. Sequence dependent intercalation of a restriction digest shown with a comparison of YO-PRO-1 (upper trace) and TO-PRO-1 (lower trace). When YO-PRO-1 bound to the DNA fragments, peak migration was according to size. On the other hand, TO-PRO-1 bound with greater affinity to the fragments with a higher GC content (124 and 434 bp) and slowed their migration. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 50 ng/mL YO-PRO-1 or TO-PRO-1; *Temp.:* 25 °C; *Injection:* EK: 2 s @ 1 kV; *Separation:* CV: 5 kV (6.3 μA); *Sample:* 612 ng/mL pBR322 *Hae*III.

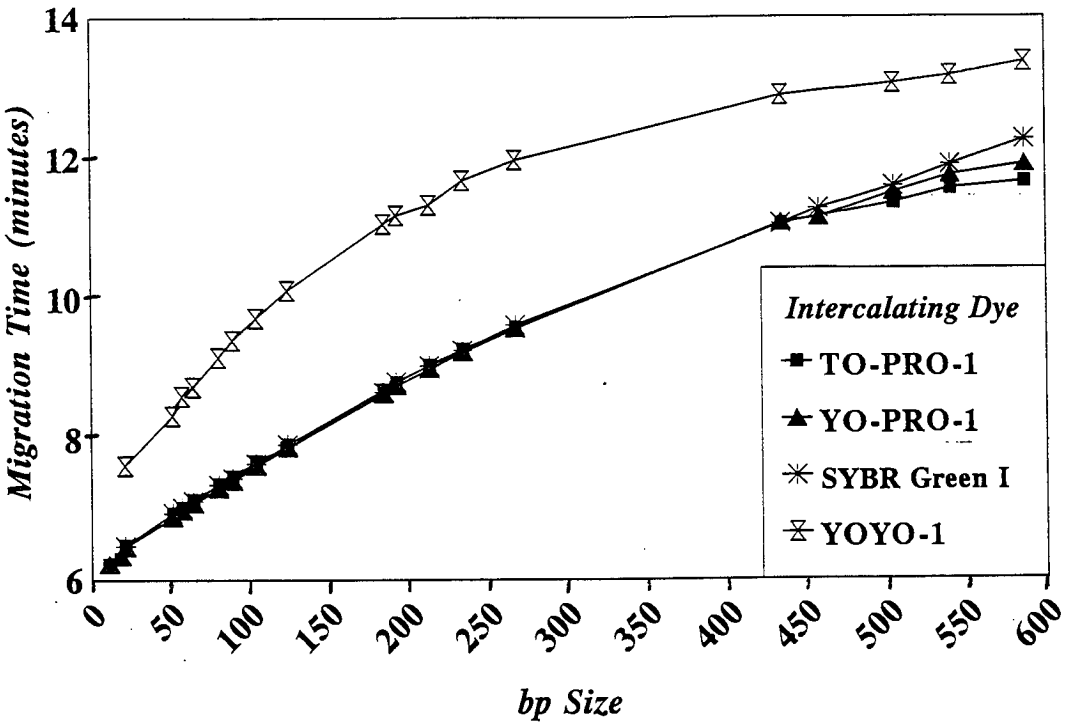


Figure 5-15. The effect of several intercalating dyes upon the migration time of a pBR322 *Hae*III digest. Conditions as in Figure. 5-14, with 50 ng/mL of YO-PRO-1, TO-PRO-1, and YOYO-1. SYBR Green I was diluted 1:10^6 from a stock solution (10,000X). YO-PRO-1, TO-PRO-1, and SYBR Green I are mono-intercalators while YOYO-1 is a dimer and a bis-intercalator [678]. See Fig. 3-7 for dye structures.

Fluorescent intercalators can impact the migration time of the pBR322 restriction digest differently (**Figure 5-15**). YO-PRO-1, TO-PRO-1, and SYBR™ Green I behaved in a similar manner as they are monomeric intercalators. SYBR™ Green showed better linearity between migration time and DNA size, and at the concentration used was at least 10 times more sensitive than YO-PRO-1. A higher binding constant and a greater quantum yield may account for the increased sensitivity with SYBR™ Green [Dr. Vicki Singer, personal communication].

The dimeric YOYO-1 migrates more slowly than YO-PRO-1 and the other mono-intercalators when complexed to DNA (**Figure 5-15**). YOYO-1 is larger and

more highly charged than YO-PRO-1 (see **Figure 3-7** and **TABLE 3-1**). Hence, the electrophoretic mobility of the intercalator-DNA complex will decrease with increased mass and phosphate neutralization. The binding constant of YOYO-1 is stronger than YO-PRO-1, which inversely affects migration time (see Eq. 3-10). Resolution of smaller fragments (21-123 bp) was improved as demonstrated by the steeper slope (**Figure 5-15**). However, unlike the mono-intercalators, the YOYO-1 dimer does not appear to possess a linear relationship between migration time and DNA size, in the 100-350 bp region. Therefore, the dual internal standard sizing procedure for STRs (see Section III.A.3) could not be performed effectively with YOYO-1. In addition, resolution was diminished with the larger DNA fragments (434-587 bp) when complexed with either YOYO-1 and TOTO-1 (data not shown). There is further evidence in the literature indicating that mono-intercalators do produce better resolution than bis-intercalators [145,262].

Concentration of Dye

The effects of the YO-PRO-1 dye on resolution, sensitivity, migration time, and separation efficiency were examined over a concentration range of 50 ng/mL (0.08 μM) to 1500 ng/mL (2.4 μM) (**Figure 5-16**). Separation of HUMTH01 alleles improved with more intercalator (**5-16C**) but with a concomitant increase in migration time (**5-16A**). While peak signal increased with higher amounts of dye (**5-16B**), sensitivity (i.e., signal-to-noise) diminished above 500 ng/mL because the baseline noise increased due to the larger levels of intercalator in the buffer. A substantial improvement in migration time precision also resulted from increasing the dye concentration (see Section III.B.1). A concentration of 500 ng/mL was chosen for sizing experiments because of improved precision and higher resolution for STRs (but with some loss in separation speed). On the other hand, 50 ng/mL YO-PRO-1 was

found more effective for quantitation purposes where speed and sensitivity (e.g., lower background noise) were more desirable.

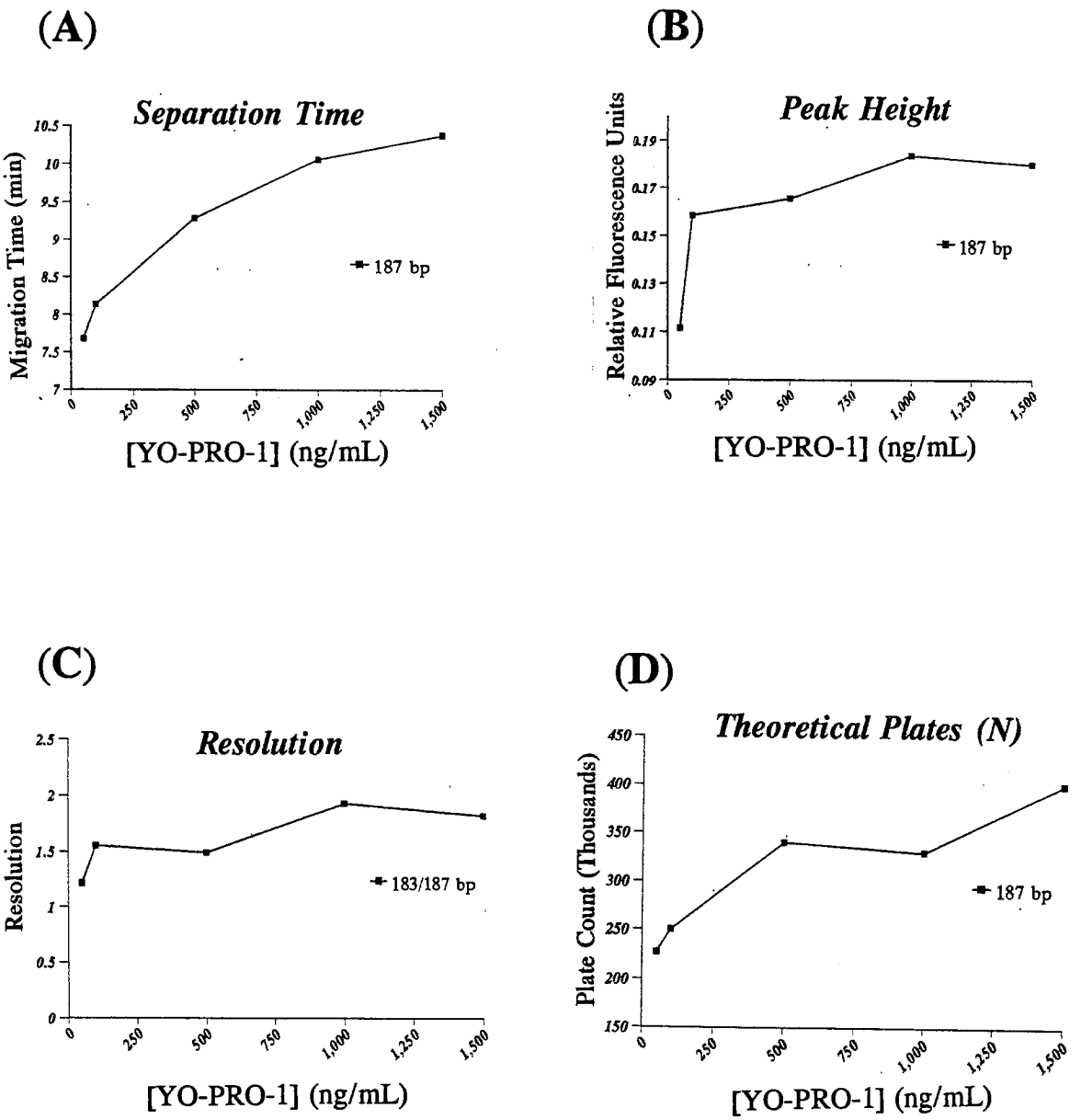


Figure 5-16. The effects of increasing YO-PRO-1 concentration from 50-1500 ng/mL on the separation of HUMTH01 alleles. Measurements were made with alleles 6 (183 bp) and 7 (187 bp). (A) separation time, (B) peak height, (C) resolution, and (D) theoretical plates. Conditions as in Fig. 5-10 except YO-PRO-1 concentrations as indicated and gradient separation was 0-4.5 min @ 15 kV, 4.5-15 min @ 5 kV.

E. GENERAL SUMMARY OF RESOLUTION FACTORS

In a plot of migration time *vs.* DNA size for the 20 bp ladder, two major "regions" may be seen (**Figure 5-17**). The region below 400 bp fragment size is fairly linear, while above 400 bp, the relationship of migration time to DNA size becomes logrithmatic. The PCR-amplified short tandem repeats examined in this study fell in the first region while D1S80 alleles extended over much of the second region. Under the CE conditions used in these studies, resolution generally decreased over the entire range from 20-1000 bp.

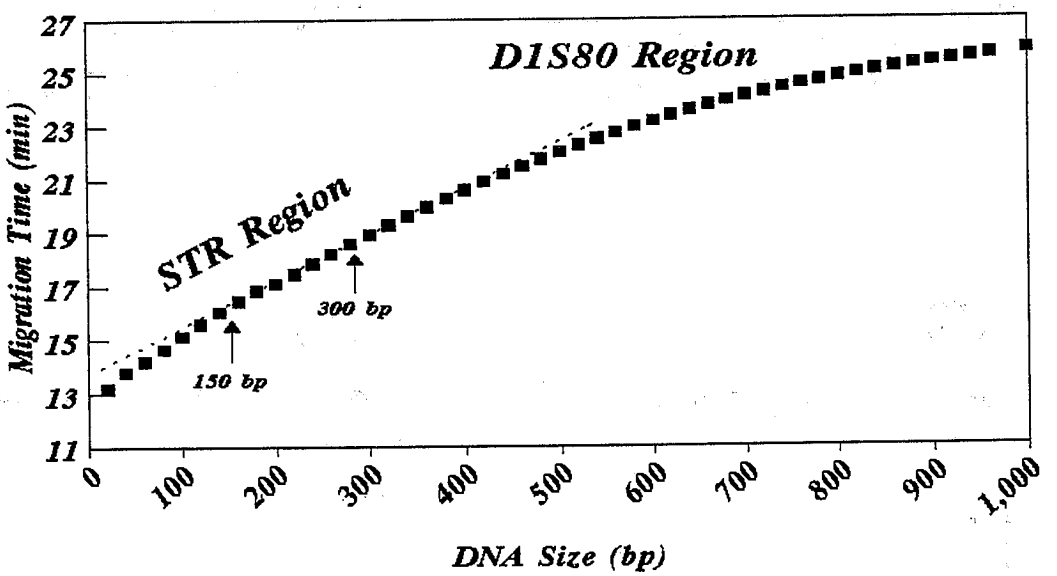


Figure 5-17. The relationship between migration time and DNA size is no longer linear after ~400 bp. The STR and D1S80 regions represent where most of the resolution studies were conducted. Conditions as in Fig. 5-10 except a constant voltage separation of 5 kV (135 V/cm). Sample was the GenSura 20 bp ladder.

Methods for improving resolution vary between the two regions. For example, a tighter mesh is desirable for PCR products less than 400 bp (STR alleles) and can be accomplished by increasing the concentration of the soluble polymer.

Furthermore, higher concentrations of the intercalating dye were beneficial to resolving STRs. On the other hand, the separation of larger PCR products (400-1000 bp) can be improved with larger mesh sizes and lower field strengths. The larger mesh size can be obtained with long, stiff polymers, such as 140,000-160,000 MW HEC. The resolution of large DNA fragments decreased rapidly with high electric fields probably because of alignment to the applied field. Consequently, the separation voltage should be optimized in terms of speed and resolution for the region of interest. In addition, voltage programming, higher temperatures, and shorter, narrower columns can be used to reduce separation times without substantially diminishing resolution.

F. COMPARISON OF RESULTS TO DNA SEPARATION THEORIES

Results from separating the 20 bp ladder were plotted in terms of DNA size *vs.* the natural log of the mobility for each peak. The linear region of this semilog plot is often used to indicate adherence to the Ogston sieving model [76]. When the Ogston model holds, the natural log of mobility should be proportional to the radius of gyration for the DNA molecule (Eq. 3-7) and hence the bp size of the DNA (Eq. 3-8). Upon switching from Ogston to reptation, the relationship is no longer linear. Using a 20 bp DNA ladder as a high resolution tool, apparent deviation from Ogston was observed at 320 bp (**Figure 5-18**). Thus, the Ogston model appears to fit data from the soluble polymers examined in this study, at least for the smaller DNA fragments and STRs.

By way of comparison, Grossman and Soane [34,45], using a similar HEC system, found that for DNA fragments larger than 310 bp (in the ϕX174 *Hae*III), agreement to the Ogston model degraded. However, due to the nature of their DNA sample, they did not have any fragments between 310 bp and 603 bp, which

prevented a better pinpointing of deviation from Ogston sieving. The same group showed that the onset of the reptation regime could be predicted to occur when R_g (DNA) $\approx 1.4\xi$ [34]. The calculated R_g for a 320 bp fragment is 42.6 nm (using Eq. 3-10) or 1.8 times the calculated average mesh size for this particular concentration of HEC (i.e., 23 nm). The discrepancy probably arises from the use of intercalating dyes in this study, which can alter the flexibility of the DNA molecule.

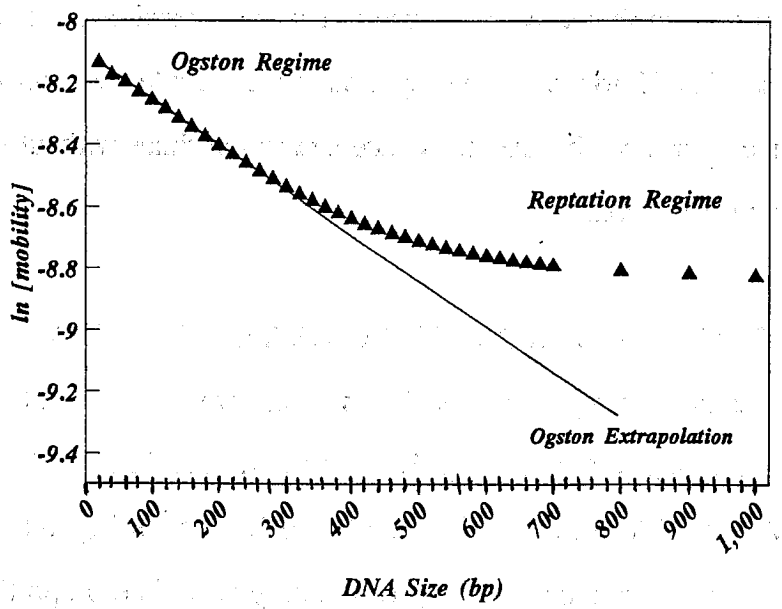


Figure 5-18. Ogston regime can be shown with a semilog plot of fragment size vs. ln (mobility). Conditions as in Fig. 5-4 except the polymer was 0.5% HEC 140,000-160,000 MW. *Sample:* 160 ng/mL 20 bp ladder with 40 ng/mL 100 bp ladder (GenSura).

The transition from a sieving to a reptating mechanism occurs much earlier in CE than conventional slab gel-electrophoresis. This discrepancy may be attributed to the higher field strengths used in CE and to the smaller pore sizes in entangled polymer solutions (when compared with agarose gels [585]). For example, Chiari *et al.* [202] found the transition from the Ogston to the reptation regimes occurred at 200 bp for a 10% solution of linear polyacrylamide, whereas this limit had been set at approximately 2000 bp in slab-gel electrophoresis.

III. Precise and Accurate Sizing of DNA Fragments

A. USE OF STANDARDS FOR SIZING IN CE

1. External Standards

The sequential nature of sample processing in CE analyses normally requires the use of an external standard run previous to the unknown. The reliability of an external standard is limited to the precision of the CE system. In other words, if conditions vary from run-to-run, relating the mobility of a previously processed external standard to the mobility of the unknown DNA fragment, can lead to inaccurate sizing of the unknown. For the purposes of sizing PCR products (specifically STR alleles), with an external standard, a study was made of migration time stability. When the buffer was not changed over the course of 10 runs, peak migration times changed by approximately 3 seconds (**Figure 5-19**).

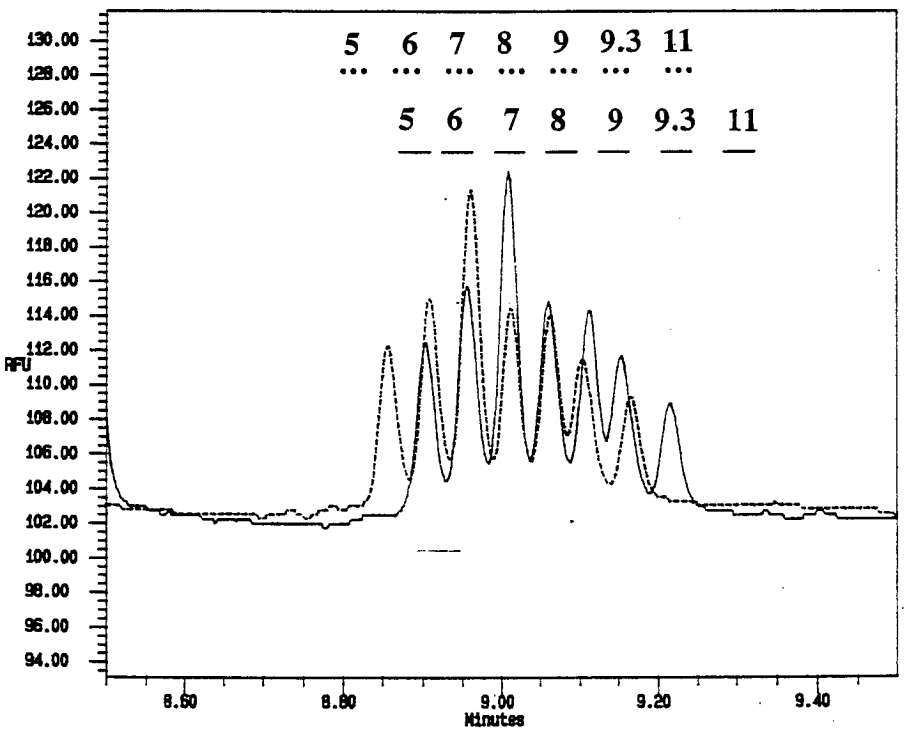


Figure 5-19. Peak drift over 10 runs as demonstrated with two HUMTH01 allelic ladders; 1st run (___), 10th run (...). The absolute migration time drift was ~3 seconds. The alleles are 4 bp apart and are designated by the numbers above them. Conditions as in Fig. 5-23.

For some applications, this seemingly minor peak drift would not be significant, but for allele identification in STR typing it could be disasterous. When peaks are passing the detector at a rate of 1.25 bp/second, a 3 second change in peak migration time can result in an apparent change of 4 bp, or an entire allele in the case of tetranucleotide STRs. Failure to correct for peak drift when sizing with external standards (i.e., an allelic ladder) could lead to mistyping the STR alleles. Frequent changes of the buffer vials were found to diminish absolute migration time drift between runs, presumably because the intercalating dye was depleted from the outlet buffer vial when the vials were not changed (see below). The use of single or dual internal standards were also found to greatly improve precision.

2. Single Internal Standard

Adjustments for migration time drift may be performed with a single internal standard. Sodium fluorescein (10^{-7} M) was originally tested as a reference marker. However, the fluorescein was found to exhibit too much run-to-run variation both in terms of peak area and migration time, possibly because its separation mechanism was different than that of DNA. Instead, individual DNA fragments in the size range of 100-400 bp have been used to adjust for peak drift between runs [151,152].

An individual internal standard may be added to an external reference as well as an unknown to provide a relative relationship between components in the two samples. Using a 150 bp internal standard to relate the sizes of sample alleles to a mixture of possible alleles, discrimination of closely spaced DNA peaks was demonstrated (**TABLE 5-4**). In the first column, the migration time (MT) ratios of the HUMTH01 allelic ladder to the 150 bp marker are shown (A1-A7). The MT ratio for A1 corresponds to HUMTH01 allele 5, A2 to allele 6, and so forth. Sample typing was performed by determining the sample's MT ratio and comparing it to the

nearest MT ratio. For example, sample #2 had one allele with a MT ratio of 1.127 which was closest to the ladder A3 (1.124) (**TABLE 5-4**). Repeating the analysis of the allelic ladder, after several samples have been processed, allowed a check of the system's stability.

TABLE 5-4. Results from Using a Single Internal Standard Sizing Method. The number shown is the migration time (MT) ratio of the PCR-amplified HUMTH01 allele to a 150 bp DNA fragment. Conditions as in Fig. 5-10 gradient separation.

Possible Alleles	Sample #1	Sample #2	Sample #3	Alleles after 12 runs
A1 = 1.098				A1 = 1.098
A2 = 1.110	1.113		1.114	A2 = 1.109
A3 = 1.124		1.127		A3 = 1.121
A4 = 1.136	1.137			A4 = 1.132
A5 = 1.148			1.147	A5 = 1.143
A6 = 1.158				A6 = 1.152
A7 = 1.173				A7 = 1.165
	---	---	---	
	A2, A4	A3	A2, A5	

With a single internal standard, non-uniform peak drift can possibly lead to misidentification of peaks which are further in size from the internal standard. For example, in **TABLE 5-4**, the ratio of the 150 bp internal standard to A1 (179 bp) remains constant at 1.098 while for A7 (203 bp) the ratio changes from 1.173 to 1.165 over 12 runs. The use of an arbitrary ratio for allele determination has an additional disadvantage in that it does not provide an indicator of the actual DNA size. These problems may be overcome with the use of multiple internal standards.

3. Dual Internal Standards

If the relationship between migration time and DNA size is linear in the region

of interest, only two internal standards are needed to define the "calibration curve" (**Figure 5-20**) [559]. The use of dual internal standards can improve the precision by adjusting for any minor variations in conditions between runs. The relationship between migration time and DNA size is linear in the 150 to 300 bp region (**Figure 5-21**). However, this dual internal approach is not effective for large DNA fragments (e.g., D1S80 region) because the relationship between migration time and DNA size becomes non-linear (see **Figure 5-17**).

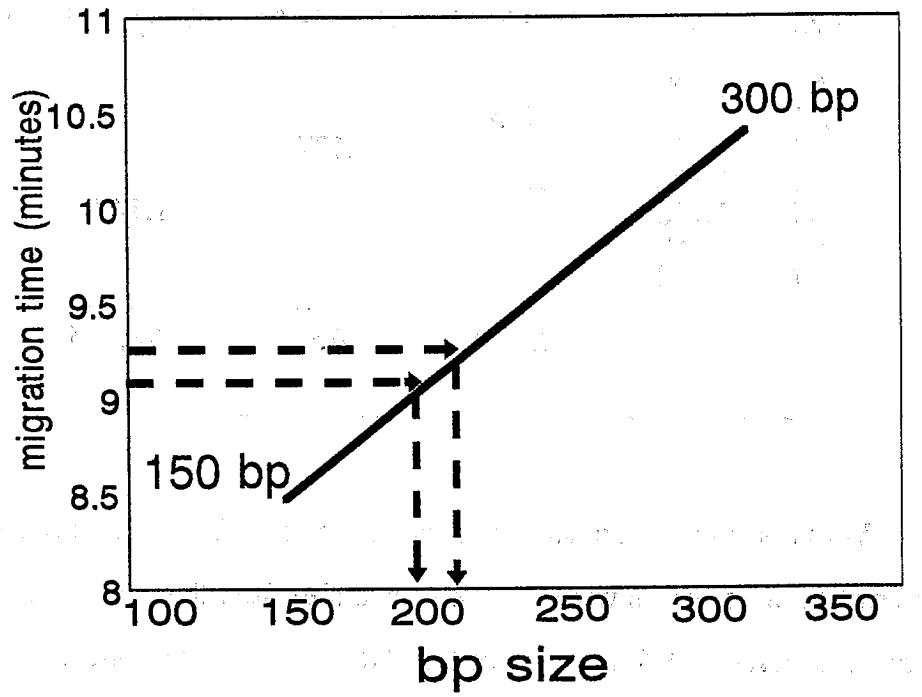


Figure 5-20. The concept of sizing using dual internal standards. Individual peak migration times for alleles are turned into base pair sizes on a line formed from the 150 bp and 300 bp internal standards. This region has been shown to be linear (Fig. 5-21).

Sizing in the non-linear region may be overcome by fitting more calibration points to the logrithmatic portion of the curve. This becomes more difficult mathematically as more peak migration times have to be processed. A more elegant

solution lies with multiple wavelength detection [431,541]. Rather than relying on the relationship between migration time and DNA size, spectrally distinct fluorescent dyes can be used for the internal sizing standard and the unknown DNA. Multiple channel detection would then permit spectral rather than spatial resolution [431,541]. An allelic ladder or DNA sizing ladder tagged with one color can be directly compared to unknown PCR products labeled with a second color [431].

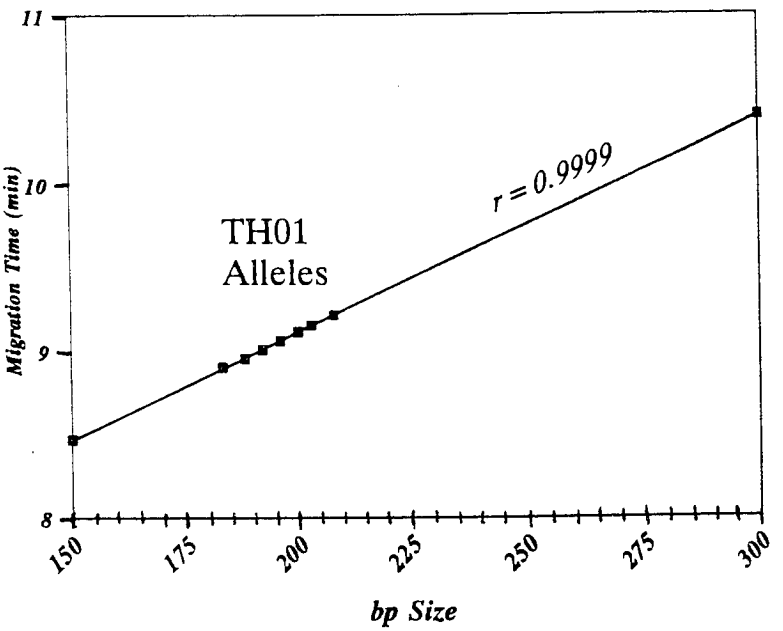


Figure 5-21. The relationship of migration time to bp size is linear from 150 bp to 300 bp (correlation coefficient = 0.9999). The standard deviation about the regression line for the HUMTH01 region is 0.3 bp. Conditions as in Fig. 5-19.

B. SIZING OF SHORT TANDEM REPEATS BY CE

The dual internal standard system described above has been successfully applied to typing PCR-amplified short tandem repeats [152,559]. Briefly, the method involved generating a two point regression to define the linear region between the 150 bp and 300 bp internal standards. Next, the base pair sizes of the alleles are

calculated by interpolation of the allele migration times on the linear regression. Finally, the calculated bp sizes of the alleles are compared to the calculated bp sizes of the allelic ladder run at the start of the set of analyses. Usually 10 samples were run in a set. The closest allele was chosen to designate the type of the sample. This approach is consistent with the recent recommendations of Smith [474] for accurate sizing of STRs using automated fluorescence detection with internal lane standards [319,473,541].

1. Precision

High run-to-run precision was desired in order to reliably relate the sizes of an external allelic ladder standard to the individual samples. Several variables were examined in order to determine their effect upon the precision of the CE system (**TABLE 5-5**). Among those variables examined, dye concentration appeared to be the most important factor. This variation may be due to the effect that the intercalator has on migration time. The cationic intercalator neutralizes sites on the anionic DNA molecule and thus decreases the effect of the electric field on the molecule. At lower dye concentrations, the DNA molecules may not reach saturation with the YO-PRO-1 dye. In addition, small differences in dye concentration likely produce migration time variations. Since a log relationship exists between dye concentration and migration time (**Figure 5-16A**), the shifts in peak migration time will be more pronounced at lower dye concentrations, and thus peak sizing will be less precise.

The best precision was obtained with the removal of the methanol wash between the runs, most likely because disturbance to the capillary wall was reduced (**TABLE 5-5**). However, this slight improvement in precision was offset by a loss in the system's robustness. A degradation in column performance over time was

observed when a methanol rinse was not performed between each run. Slightly higher standard deviations were also observed with a single step gradient separation. This is due to the fact that changing the voltage during the run was not as reproducible as keeping the voltage constant [152].

TABLE 5-5. Variables Affecting Precision in CE Sizing. Ten replicate injections of the HUMTH01 allelic ladder. All seven alleles have been sized using the dual internal standard approach. The pooled standard deviations for the seven sized alleles are listed below. Conditions as in Figure 5-23, except where noted with the condition varied.

Condition Varied	Standard Deviation
Normal (500 ng/mL YO-PRO-1)	0.14 bp
No methanol wash between runs	0.09 bp
4 min methanol wash between runs	0.13 bp
Constant Current (6.7 μA)	0.13 bp
50 ng/mL YO-PRO-1	0.28 bp
100 ng/mL YO-PRO-1	0.14 bp
Gradient Separation 0-5.5 min @ 7.5 kV, 5.5-11 min @ 2.5 kV	0.18 bp

In order to determine the precision of the CE system with an intercalating dye concentration of 500 ng/mL, the HUMTH01 allelic ladder was analyzed 50 times and sized with the 150 and 300 bp internal standards. The combined results of 350

calculated allele sizes (i.e., 7 alleles in the ladder times 50 runs) are shown in **Figure 5-22.** Gausian distributions were observed with an average precision of 0.2 bp or 0.1% R.S.D. (1 σ) for these 50 injections. This result was obtained by calculating the standard deviation from averaging the sizes of each individual allele. The standard deviations about each allele were then averaged to yield the 0.2 bp value.

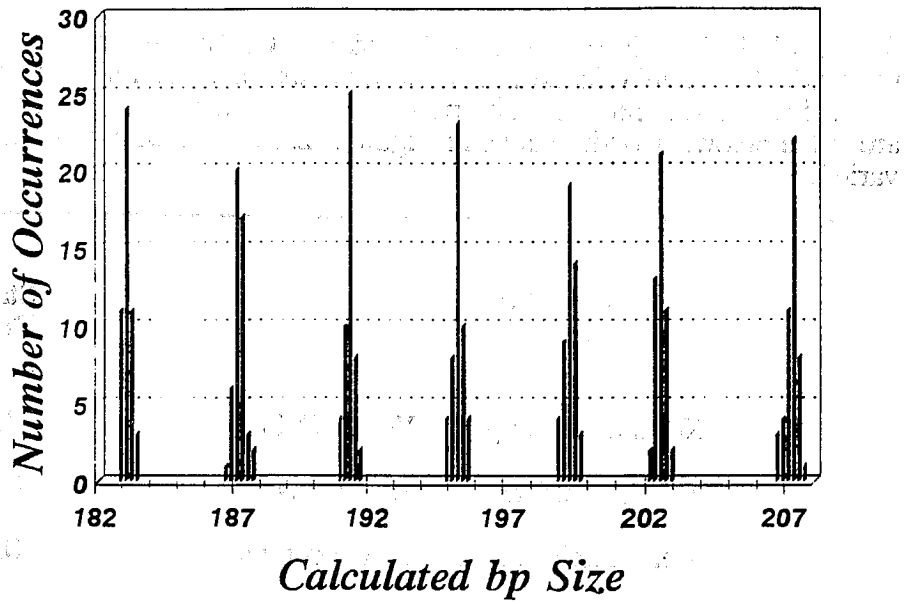


Figure 5-22. Histogram of the calculated allele sizes from 50 replicate injections of the HUMTH01 allelic ladder. Each bar represents a 0.2 bp bin. Conditions as in Figure 5-23. From [559].

The overall drift was less than 0.25 bp for eight injections with an average standard deviation of 0.1 bp (**TABLE 5-6**). The pooled standard deviation for 100 samples increased to 0.3 bp [559]. By way of comparison, Fregeau and Fourney [417] obtained $\pm$0.2-0.5 bp within a gel and $\pm$0.5-1.5 bp between gels using automated fluorescence detection with internal lane standards.

TABLE 5-6. Eight replicate injections of the HUMTH01 allelic ladder. The size of each allele was calculated using the 150 bp and 300 bp dual internal standard approach. The calculated allele sizes are shown to two decimal places for purposes of precision testing only and do not indicate a "partial" base pair. The Δbp value indicates the difference between adjacent alleles while Δ indicates the total change in allele size over the 8 runs. Conditions as in Figure 5-23.

Alleles (bp)

Inj.	5	6	7	8	9	9.3	11
1	182.18	186.35	190.34	194.33	198.32	201.60	206.47
Δbp	+0.20	+0.09	+0.25	+0.23	+0.21	+0.19	0
2	182.38	186.44	190.59	194.56	198.53	201.79	206.47
Δbp	-0.02	+0.05	-0.06	+0.11	+0.09	+0.09	+0.07
3	182.36	186.49	190.53	194.67	198.62	201.88	206.54
Δbp	-0.03	-0.11	-0.19	-0.19	-0.18	-0.18	+0.01
4	182.33	186.38	190.34	194.48	198.44	201.70	206.55
Δbp	-0.01	+0.07	+0.23	+0.05	+0.13	+0.11	+0.10
5	182.32	186.45	190.57	194.53	198.57	201.81	206.65
Δbp	+0.06	-0.12	-0.10	-0.10	-0.09	-0.08	-0.08
6	182.38	186.33	190.47	194.43	198.48	201.73	206.57
Δbp	-0.09	0	0	0	-0.09	-0.09	-0.27
7	182.29	186.33	190.47	194.43	198.39	201.64	206.30
Δbp	+0.12	+0.05	+0.05	+0.05	+0.23	+0.15	+0.25
8	182.41	186.38	190.52	194.48	198.62	201.79	206.55

	5	6	7	8	9	9.3	11
Δ	+0.23	+0.03	+0.18	+0.15	+0.30	+0.19	+0.08
ave.	182.33	186.39	190.48	194.49	198.50	201.74	206.51
std dev	0.072	0.060	0.095	0.101	0.109	0.093	0.103

2. Accuracy in Typing

For the purposes of this discussion, accuracy in typing may be defined as confidently identifying an allele through correlation to the allelic ladder. Over the course of three days, 100 population samples, which had been PCR-amplified using HUMTH01 primers, were examined using this CE method (**Figure 5-23**). Examples of separations for several population samples are shown in **Figure 5-24.** The separation buffer vials were not changed between samples for the entire 100 samples in an effort to see what effect buffer depletion might have on the system. The samples were examined in sets of ten with an allelic ladder being run before and after each set. The results of one of the sets is shown in **TABLE 5-7.**

High run-to-run precision was observed when the 150 and 300 bp internal standards were used to correct for migration time drift. Because the buffer vials were not changed over the course of the 120 runs, migration times changed over the course of the 120 runs (100 samples + 20 standard ladders). For example, the migration time for the 150 bp fragment changed from 8.47 min (1st run) to 8.29 min (120th run). During these same 120 runs, the calculated size for allele 5 (as determined by Eq. 4-2) dropped from 183.47 bp to 182.06 bp, or an overall change of 1.41 bp. These changes in calculated sizes probably resulted from the steady depletion of intercalating dye from the outlet buffer vial over the course of multiple runs without buffer replenishment. (Loss of dye in the outlet buffer vial would account for the decreasing migration times because the DNA would not be neutralized as much and would thus migrate faster.) While the overall calculated sizes varied, within a single set the calculated allele sizes for individual samples never varied by more than 0.5 bp from the standard ladder alleles run at the beginning of the set (**TABLE 5-7**). In fact, as **TABLE 5-7** is the tenth set of samples (i.e, runs #109-120 on the same buffer vials), these samples should show the most variation. However, the beginning and

ending ladders in this set vary from each other by less than 0.2 bp (e.g., beginning allele 5 = 182.26 and ending allele 5 = 182.06). These results are significant because under the rigors of routine operation, where the buffer vials may not be changed as often as high precision research, the correct genotype can still be determined if internal standards are used.

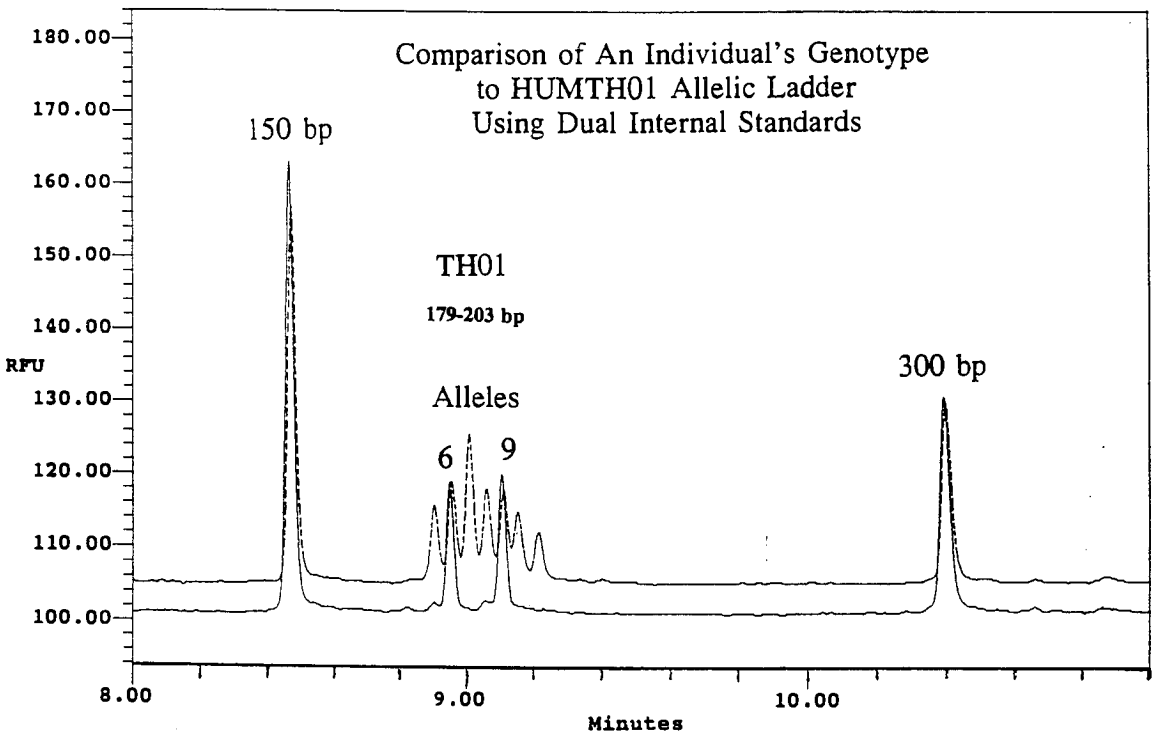


Figure 5-23. Comparison of an individual's genotype to the HUMTH01 allelic ladder. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 500 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 5 s @ 1 kV; *Separation:* CV: 5 kV (185 V/cm); *Sample:* 1:50 dilution of the PCR-amplified DNA with deionized water + 150 bp and 300 bp internal standards.

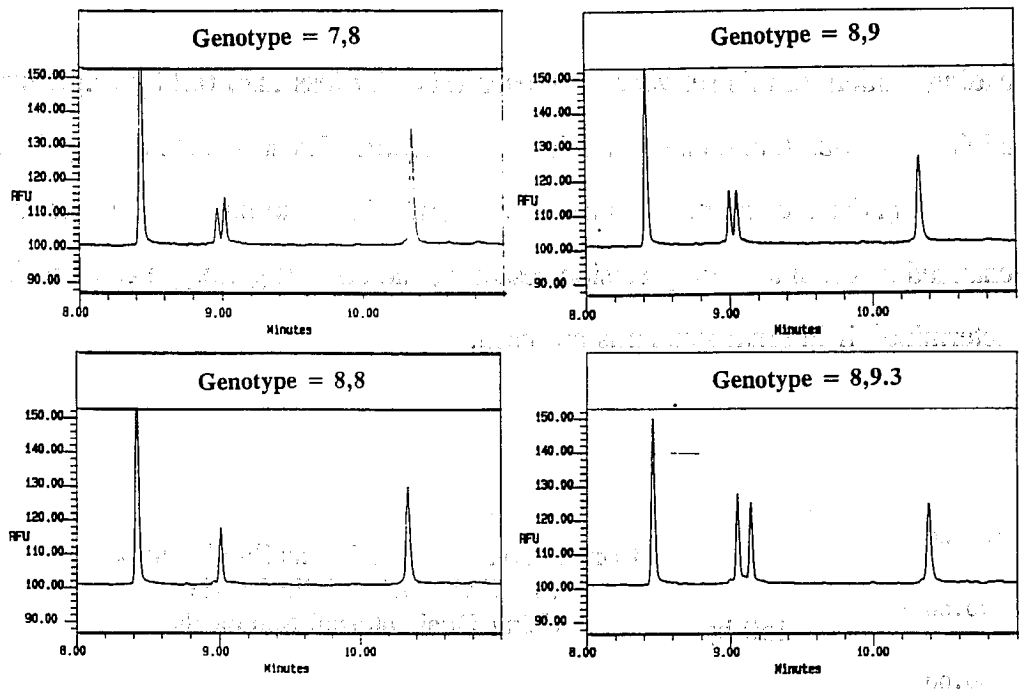


Figure 5-24. Four population samples containing a common allele 8 from the STR system HUMTH01. The alleles are bracketed by the taller 150 bp and 300 bp internal standards. Conditions as in Fig. 5-23.

TABLE 5-7. A set of ten population samples with an allelic ladder standard run before and after the samples to calibrate the CE system. The numbers across the top were the calculated sizes of each allele in the ladder and were used as a reference to type the individual samples, based upon their estimated sizes. All numbers are in base pairs. Conditions as in Figure 5-23. From [559].

Alleles (bp)						
5	**6**	**7**	**8**	**9**	**9.3**	**11**
182.26	186.37	190.39	194.32	198.35	201.51	206.22
		190.85				
				198.70	201.84	
					201.75	
	186.46			198.64		
		190.65	194.57			
	186.65				201.90	
		190.65			201.73	
			194.74	198.66		
		190.54	194.47			
		190.64		198.60		
182.06	186.10	190.23	194.27	198.14	201.40	206.05

In all of these studies, genotyping was performed by comparing the calculated size (in bp) for an individual sample to that obtained from the previously run allelic ladder (**Figure 5-23**). For example, the second sample shown in **TABLE 5-7** contains two alleles which were sized as 198.70 bp and 201.84 bp. The first allele is closest in size to allele 9 (calculated size = 198.35 bp) in the allelic ladder, and the second allele is closest in size to allele 9.3 (calculated size = 201.51). Any result falling outside 3.3 standard deviations (i.e., can be called with 99.9% certainty) of the calculated bp size obtained for the standard alleles was rejected. As the pooled standard deviation is less than 0.3 bp (determined with 100 samples), a sample allele should fall within 1 bp of a given standard allele to be properly identified. In a blind study between CE and conventional gel analysis, the results between the two methods were found to be in complete agreement [559].

The CE system described here was unable to separate a sample containing the two alleles 9.3 and 10. Alleles 9.3 and 10, which differ by only 1 bp (see **Figure 4-8**), could be distinguished individually but not together. However, since the system can accurately and precisely determine peak times, a "7,9.3" genotype could be distinguished from a "7,10" genotype [559]. With five injections of each sample, the average size for the 9.3 allele was 202.63 ($\pm$0.08) bp while the 10 allele was sized at 203.37 ($\pm$0.05) bp. In the same analysis set, the 9.3 allele contained in the allelic ladder was sized at 202.61 bp. The match criteria for this set of injections would be $\pm$0.26 bp {($\pm$0.08bp) x 3.3σ}. For calling a 9.3 allele, the calculated size must be 202.37 to 202.89 bp (i.e., $\pm$0.26 bp around the 202.61 bp allelic ladder 9.3). Thus, the individual sample with the "7,9.3" type met the match criteria for allele 9.3 while the "7,10" one did not.

The feasibility of typing D1S80 alleles with CE has also been demonstrated (**Figure 5-25**). Using only the 300 bp peak to adjust for migration time variation

(i.e., due to the non-linear nature of the D1S80 region, the dual internal standard approach is not appropriate), five samples were correctly typed for D1S80. As part of the same set of samples, the amelogenin (sex-typing marker) alleles, which differ by only 6 bp, were resolved and accurately sized with the dual internal standard procedure (**Figure 5-25**).

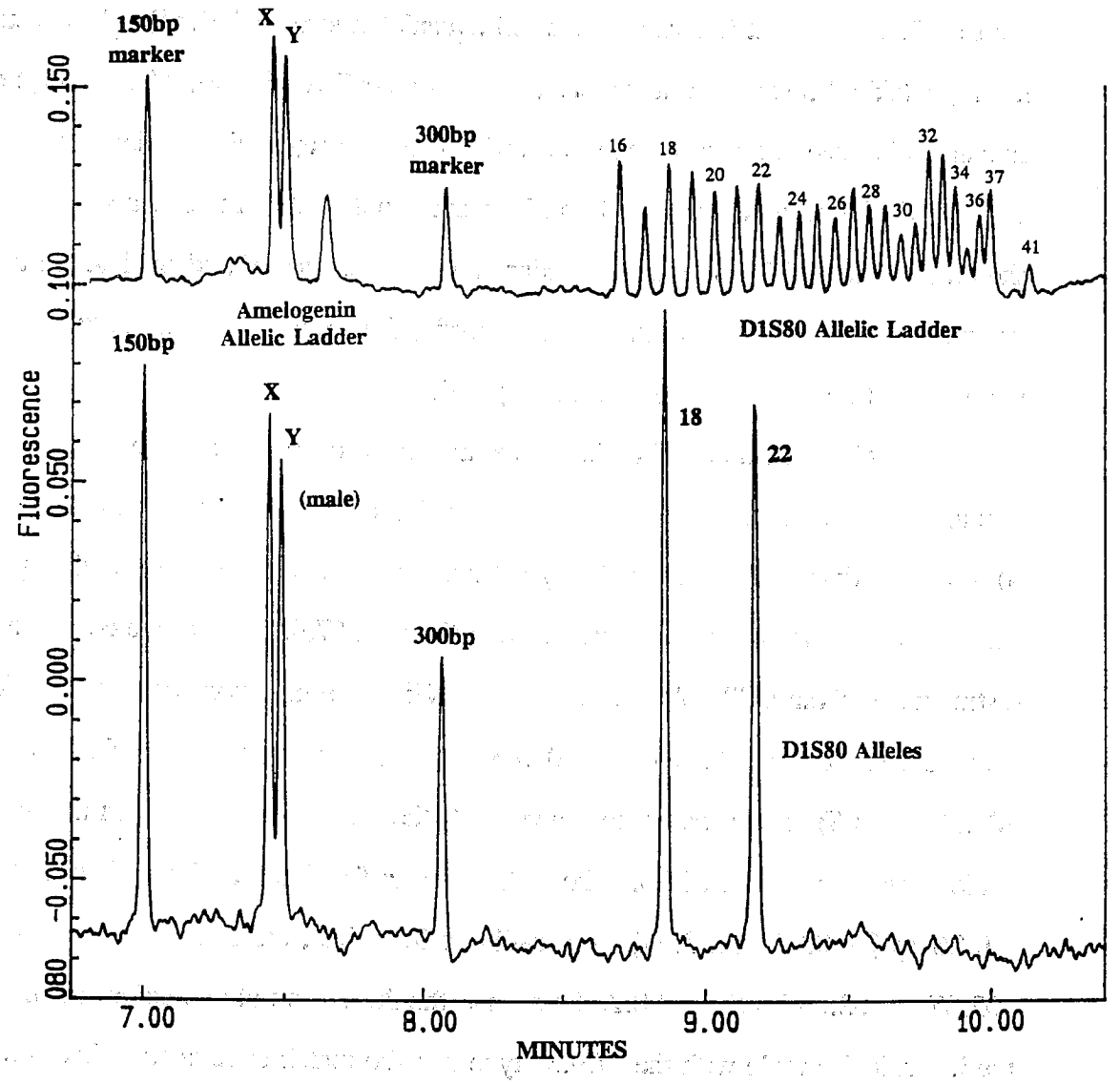


Figure 5-25. Determination of sex and D1S80 type in 20 minutes. The sizes for the amelogenin alleles were determined using the dual internal standard system (150 and 300 bp markers) while the D1S80 type was assessed using the single internal standard method with the 300 bp marker. Conditions as in Fig. 5-23 except 0.3% HPMC (4000cP) and EK: 5 s @ 2 kV.

3. Accuracy in Sizing

Accuracy in sizing refers to the ability to determine the actual length of the DNA fragment (i.e., equivalent to the result obtained by sequencing). Sizing of DNA fragments can vary depending on intercalation dye binding and the magnitude of the electric field, both of which affect the DNA conformation (**TABLE 5-8**). Higher fields or dye concentrations lead to a larger spread between the 150 bp standard and HUMTH01 alleles and yield an estimate of a larger allele "size." While absolute sizes may differ between conditions, run-to-run precision using the same conditions was still excellent. When using constant conditions, precision of ≤ 0.2 bp has been routinely demonstrated (see above). At higher voltages, resolution is not as good and so sizing may be less accurate (e.g., "CV:10kV" run in **TABLE 5-8**).

The HUMTH01 alleles were recently sequenced and determined to be in the size range of 179 (allele 5) to 203 bp (allele 11) [161]. However, the calculated sizes for the alleles (**TABLE 5-8**) usually range from 3-4 bp larger than the actual sizes, and so, do not represent the actual length of the alleles. This is likely due to sequence or length variations between the internal standards and the samples of interest [161,319]. Although the GC contents of HUMTH01 alleles (50.3%) and the internal standards ($\sim 50\%$) are approximately equal, AT-rich domains may differ, which would lead to anomalous migration [646] when compared to the 150 bp and 300 bp DNA fragments. Other researchers have also determined the size of HUMTH01 alleles to be slightly larger than the actual sequenced size. In one of the first papers regarding the HUMTH01 locus, the alleles were sized in the range of 183-207 bp by Edwards *et al.* [160]. Fregeau and Fourney [417] noted that the repeat nature of STRs may influence the structure of the DNA product and alter their migration upon electrophoresis relative to non-repeat DNA markers. This may account for the discrepancies between the predicted and observed lengths. Thus, an

allelic ladder may be desirable for calibration, particularly for interlaboratory comparisons [268,480].

TABLE 5-8. Effects of Voltage and Dye Concentration on Sizing HUMTH01 Alleles (both affect conformation).

	Allele 5‡	SD (bp)	Allele Spacing*
50 ng/mL YO-PRO-1			
CV: 5kV	177.69 bp (n=8)	0.35	4.0 bp
CV: 10kV	186.00 bp (n=9)	0.95	3.8 bp
Gradient1 0-5.2min @ 15kV, 5.2-10min @ 5kV	182.01 bp (n=9)	0.49	3.9 bp
Gradient2 0-4.5min @ 15kV, 4.5-10min @ 5kV	184.84 bp (n=9)	0.57	3.5 bp
CV: 5kV 27cm x 50μm DB-17	181.98 bp (n=10)	0.28	4.0 bp
500 ng/mL YO-PRO-1			
CV: 5kV	182.34 bp (n=8)	0.10	4.0 bp
CV: 5kV	183.34 bp (n=10)	0.14	4.0 bp
Gradient 0-5.5min @ 7.5kV, 5.5-11min @ 2.5kV	183.88 bp (n=10)	0.18	4.1 bp

‡Average of alleles sized with dual internal standard method. Sequenced size: 179 bp [161].
*Average between alleles 5-6, 6-7, 7-8, & 8-9. Should be 4 bp [161].

The reliability of the dual internal standard system introduced for the STR HUMTH01 has also been demonstrated with sex typing. Using same 150 bp and 300 bp internal standards, but with a different buffer system, the X and Y alleles from the amelogenin locus were accurately typed in several samples (see **Figure 5-25**). The X

allele was sized at 212.08 (±0.56) bp (n=7) while the Y allele was sized at 218.00 (±0.53) bp (n=7). The actual sizes of these alleles, as determined by sequence analysis, are 212 bp and 218 bp, respectively [681]. Obtaining the exact sizes in the case of amelogenin alleles may be a result of similar DNA secondary structure between the alleles and the internal standards.

To summarize, absolute migration times cannot be used for accurate sizing. Changing capillaries or buffers can produce migration time differences. In one instance, switching columns resulted in a 0.2 min (equivalent to 16 bp) decrease in absolute migration time for the 150 bp peak (all other conditions were held constant). However, the relative migration times between the internal standards and the samples remained constant and high precision between runs was obtained. It should also be pointed out that the "actual size" obtained with this method does not matter for correct genotyping as long as the allelic ladder is used for a reference standard.

IV. Quantitation of PCR Products

A. ANALYTICAL LIMITS OF CE METHOD

The issues of sensitivity, linear range, and precision were studied in order to develop a rapid quantitation system for PCR products. The PCR products examined in these studies were produced from the mitochondrial DNA D-loop region.

1. Sensitivity

Before discussing the minimum detectable quantity of DNA determined in these studies, it is important to point out several things. First, the quantity of DNA measured on the column is dependent upon the type and duration of the injection. As discussed earlier (see **Figure 5-2**), EK injections can load more DNA onto the column than HD injections. However, pressure injections, which introduce a uniform plug of sample material, were found more reproducible and effective for quantitation purposes. Second, the quantity of DNA measured by CE-LIF methods is related to the number of fluorophores detected, not the actual DNA. Peak heights and areas increase incrementally as DNA size increases. This results from the fact that the number of fluorophore binding sites increases in direct proportion to the DNA size with intercalating dyes. This phenomenon may be seen with a restriction digest (see **Figure 5-1**). Equivalent numbers of each fragment are present, but larger fragments have a greater number of bound fluorophores. In fact, a linear relationship exists between both peak area (squares) and peak height (triangles) and DNA fragment length for equimolar fragments of a restriction digest (**Figure 5-26**). Thus, when noting the limit-of-detection for a CE system, the length of the DNA must be considered because longer DNA fragments will bind more fluorophores and produce a greater signal.

The limit-of-detection for the rapid mtDNA quantitation procedure described

below was found by diluting a previously quantitated PCR product. A 402 bp PCR product could be seen from samples with levels of DNA as low as 400 pg in a 25 μL volume (signal-to-noise = 3).

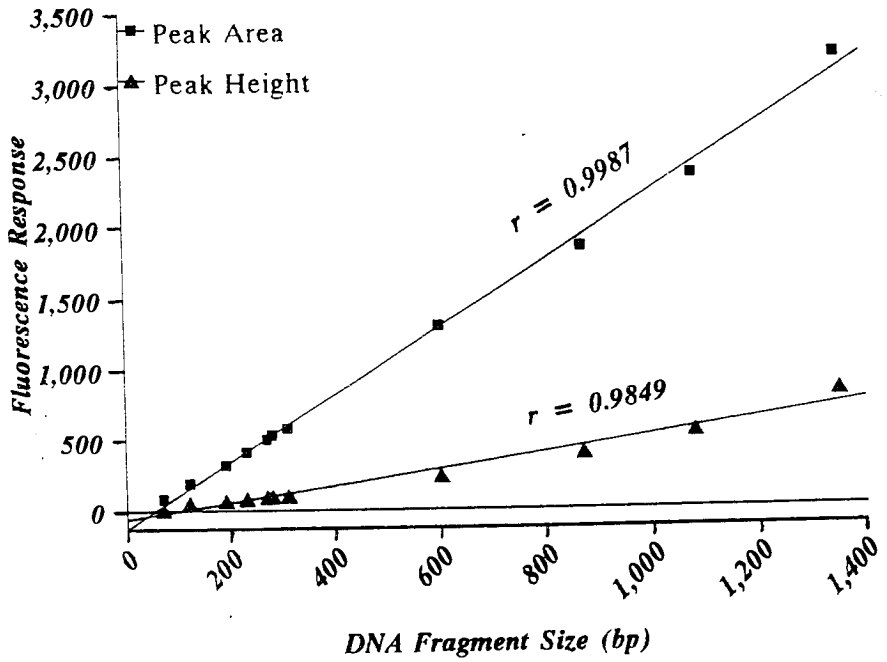


Figure 5-26. Plot of fluorescence response *vs.* DNA fragment size for ϕX174 *Hae*III. Both peak area (squares) and peak height (triangles) showed a linear increase with DNA fragment size illustrating the increasing number of bound fluorophores. The DNA fragments of the ϕX174 *Hae*III digest were at equal concentrations. Conditions: *Capillary:* 100 μm i.d. x 57 cm DB-17; *Buffer:* 1% HEC, 100 mM TBE, pH 8.7 w/ CsOH, 50 ng/mL YO-PRO-1, 1.27 μM EB; *Temp.:* 20 °C; *Injection:* 10 s @ 5 kV; *Separation:* CV: 15 kV (42 μA); *Sample:* 400 ng/mL ϕX174 *Hae*III digest.

2. Linear Range

A serial dilution of a 402 bp PCR product (mtDNA HV2 region) was used to examine the linear range of response between DNA quantity and the detected fluorescence. Both the peak area and peak height response were linear (r = 0.9999) over a range of 1.5 to 200 ng (**Figure 5-27**).

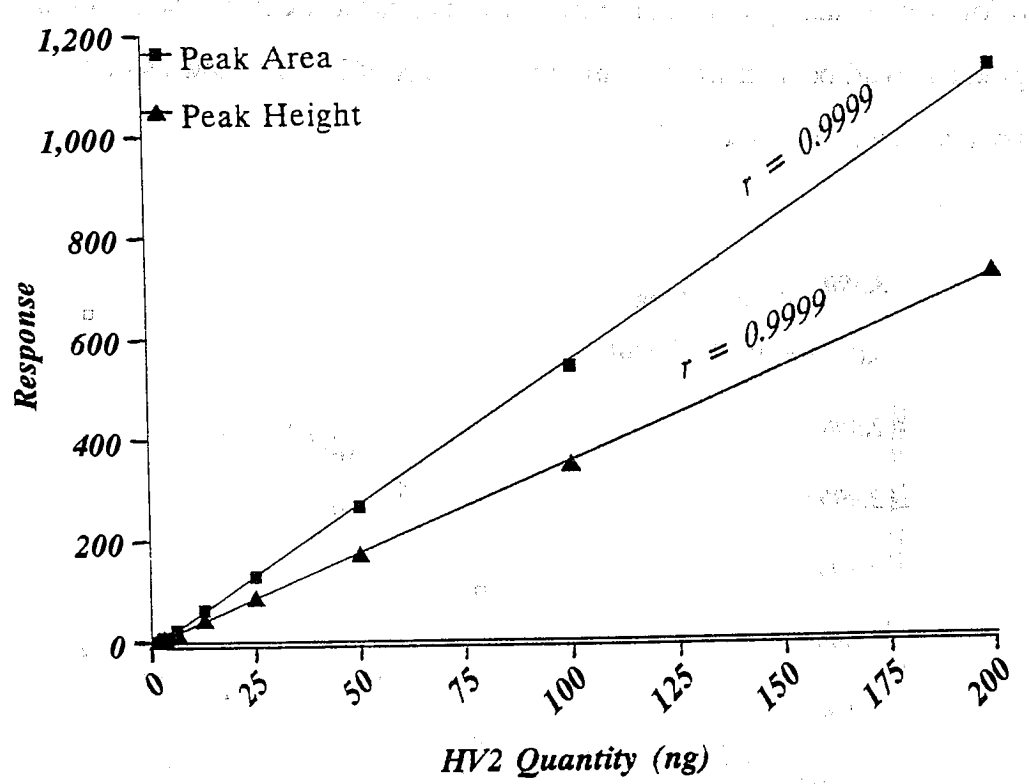


Figure 5-27. Serial dilution of a 402 bp PCR product (mtDNA HV2). Both the peak area (squares) and peak height (triangles) were linear (r=0.9999, n=8) over a range of 1.5-200 ng. Measurements were made from 25 μL samples. Conditions as in Figure 5-30.

3. Peak Area Precision

Peak precision was studied by preparing eight samples of a 402 bp PCR product and sequentially analyzing them. The peak areas and heights were numerically integrated by the computer program (Millenium software). The peak area precision was found to be 4.15% R.S.D. (n=8) while the peak height precision was slightly higher at 5.34% R.S.D. (n=8). A gradual loss in signal (dotted lines) was observed for both peak area and peak height over the course of the eight runs (**Figure 5-28**). This phenomenon may be attributed to gradual loss of the intercalating dye and other buffer ions from the outlet buffer vial. With lower levels of fluorescent intercalating dye available in the buffer, less dye will bind to the DNA and result in a

lower fluorescent signal (as well as the faster migration times discussed earlier). In an experiment where the outlet buffer vial was changed between every run rather than every 10 runs, the peak area precision improved from 8% to 3% R.S.D [151]. However, in a routine operation, the inferior precision may be tolerated to avoid the frequent changes in buffer vials.

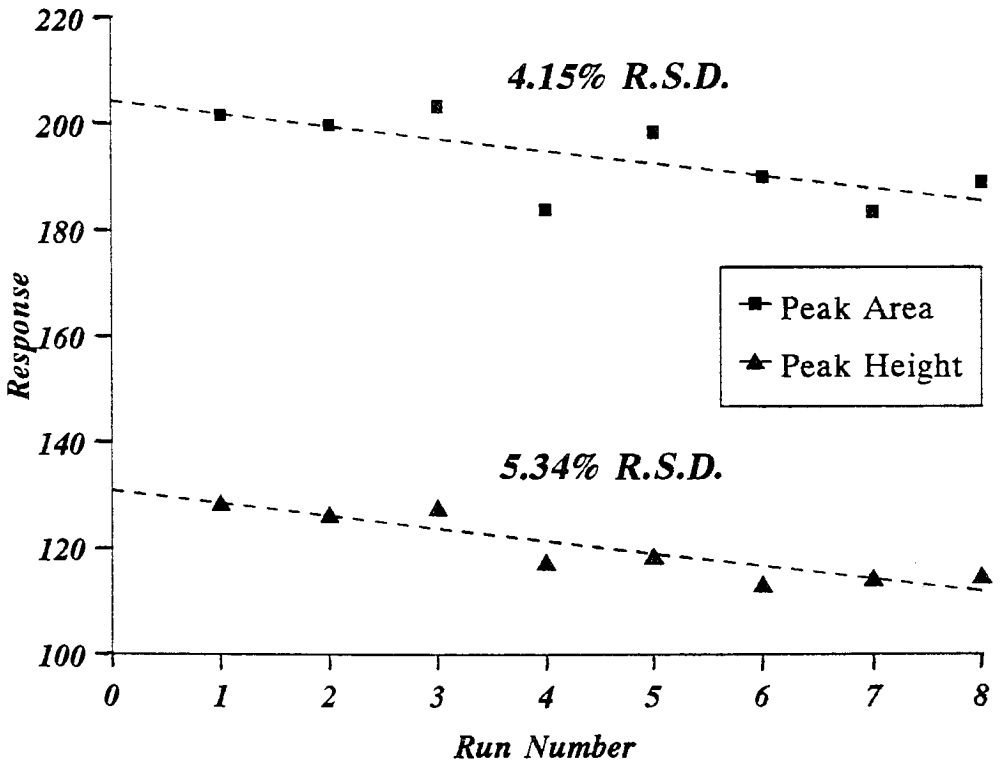


Figure 5-28. The fluorescent signal response from 8 vials each containing 20 ng/μL of a 402 bp PCR product (mtDNA HV2). The peak area precision was 4.15% R.S.D.; the peak height precision was 5.34% R.S.D. The downward trend (dotted line) has been attributed to YO-PRO-1 depletion from the outlet buffer vial [151]. Conditions as in Fig. 5-30.

4. Peak Signal is Related to Time in the Detector Window

Experiments were conducted to determine the importance of accounting for the length of time a DNA fragment spends in front of the detector. An increase in peak response was observed when DNA fragments moved slower through the capillary

(i.e., spent more time in front of the detector) (**Figure 5-29**).

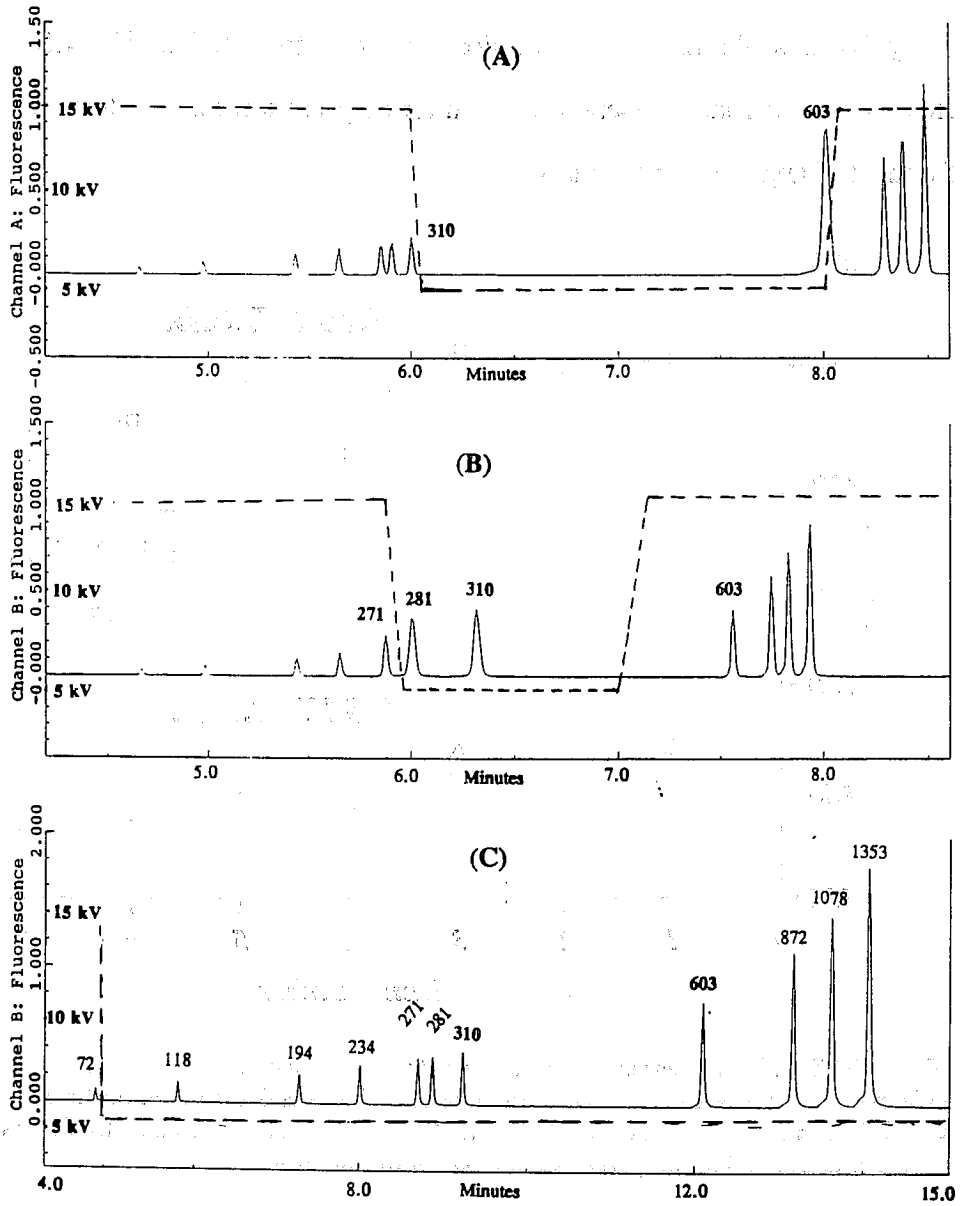


Figure 5-29. A comparison of the changes in peak response depending on the speed at which the DNA fragment passes the detection point. Voltage programming (dotted line) was used to alter the time which the DNA spent in front of the detector. Conditions: *Capillary:* 50 μm i.d. x 37 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.1, 50 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 10 s water, 45 s sample @ 0.5psi; *Separation:* (A) 0-6 min @ 15 kV, 6-8 min @ 5 kV, 8-10 min @ 15 kV; (B) 0-5.75 min @ 15 kV, 5.75-7 min @ 5 kV, 7-10 min @ 15 kV; (C) 0-4.5 min @ 15 kV, 4.5-15 min @ 5 kV; *Sample:* 588 ng/mL ϕX174 *Hae*III digest.

Using voltage programming, the peak sizes of a restriction digest were observed to grow larger than expected with a lower voltage in that region (**Figure 5-29**). The dotted lines show the changes in applied voltage. When the electric field strength was lower, the DNA moved slower and produced abnormally high peak signals because they were in the detector window for a longer period of time. For example, compare the peak size for the 603 bp fragment in **Figure 5-29A**, where the DNA was moving slower from a lower applied voltage, to the results in **Figure 5-29B**, where the DNA is moving much faster. The 603 bp peak is much larger in **Fig. 5-29A** because it is moving past the detector slower. A more dramatic change in peak signal was observed with the 281 and 310 bp peaks in the same figures. **Figure 5-29C** has been included to demonstrate the expected peak signal size for each of the DNA fragments. These results indicate that for peak reponse is influenced by the time spent in the detection window, which is related to how fast the DNA is travelling through the capillary.

The consideration of how fast DNA moves through the capillary becomes important when trying to relate the quantities of two fragments which may differ significantly in size. Depending on their length, DNA fragments move through the sieving buffer at different rates. Larger DNA has a greater frictional capacity to interact with the entangled polymer solution, and as such, moves slower. As a peak's intensity is related to the time spent in the detector window, adjustment must be made for longer DNA fragments. The time which a DNA molecule spends in front of the detector is related to its length and the mechanism by which it is separating. Thus, when using internal standards to quantitate PCR products (see below), it is desirable to have a DNA fragment which is similar in size so that it will pass the detector at a similar rate as the unknown DNA.

5. Use of Internal Standards

Absolute peak areas can fluctuate day-to-day because of differences in laser intensity, capillary cartidge mirrors, capillary light scatter, and variations in buffer and dye concentration. In addition, changes in buffer viscosity can affect the amount of sample introduced onto the column. Thus, it is important to use an internal standard of known concentration to adjust for these variations. Good reproducibility was achievable when the quantities of PCR products were compared to an internal standard (**TABLE 5-9**). The adjusted area was calculated by dividing the peak area by the migration time for each peak. The area ratio was obtained through dividing the PCR product adjusted area by the 200 bp adjusted area. The relationship between the 200 bp internal standard and the PCR product remained constant, as evidenced by the area and MT ratio, even when measured under different conditions. Thus, internal standards are useful in obtaining consistent results.

TABLE 5-9. Reproducible Results May Be Obtained with an Internal Standard, even if different columns and injections are used. MT = migration time

	Adjusted Area	MT (min)	Area Ratio	MT Ratio
mtDNA Sample*				
Product	7228	14.983	0.668	1.205
200 bp	10828	12.433		
Same Sample repeated 1 wk later‡				
Product	12262	14.233	0.668	1.210
200 bp	18356	11.767		

* 50 μm i.d. x 57 cm DB-17; EK: 10 s @ 5 kV; CV: 20 kV (16 μA)
‡ 100 μm i.d. x 57 cm DB-17; EK: 15 s @ 5 kV; CV: 20 kV (67 μA)

B. PCR-AMPLIFIED MITOCHONDRIAL DNA

Once the conditions for reproducibility and reliability of PCR product quantitation by CE-LIF were established and compared to traditional techniques (see below) [151], they were applied to the quantitation of PCR-amplified mitochondrial DNA fragments [247]. As noted in a recent report, the quality of the DNA sequencing is highly dependent upon the quantity and cleanliness of the DNA template in the sequencing reaction [439]. The amplitude of any peak within a sequencing chromatogram is a function of three parameters: (1) the sequence and concentration of the template, (2) the primer used for sequencing, and (3) the nature of the enzyme and the resulting reaction [247]. If contaminating PCR products are present in the sequencing reaction, they can generate a mixture of signals at positions throughout the chromatogram where the sequence of the contaminant differs from the template of interest. In addition, failure to remove unreacted PCR primers following the PCR reaction may result in mispriming of the DNA template during cycle sequencing [247]. Both of these scenarios increase the noise in a sequence chromatogram, which makes it more difficult to unambiguously determine the sequence. Thus, it is desirable to have a pre-sequencing method which can detect any contaminating DNA fragments and the presence of excess PCR primers and quantitate the PCR products of interest. A capillary electrophoresis method has been developed to meet these needs.

Due to the presence of co-extracted inhibitors and unknown template number for PCR, mitochondrial DNA (especially that obtained from hairs) does not always amplify with the same efficiency [247]. As it is desirable to have 20-50 ng of template DNA for cycle sequencing [247], CE can provide an accurate assessment of the amount of PCR product present for the sequencing reaction. In addition to being automated, only 1 μL of the limited PCR product is consumed in the analysis because of the sensitivity of CE-LIF. Prior to development of this method, a mini

polyacrylamide gel with silver staining was run to assess amplification, and fluorescent spectrophotometry [26] was used to determine PCR product quantity. Not only was this two-step process time-consuming, but it also expended ~10 μL of a 25 μL PCR product. In addition, the fluorescent spectrophotometry method would often give falsely high readings when excess PCR primers were present. If the PCR product needed to be sequenced several times or if the amplification was poor (e.g., only a few ng/μL), most of the PCR product would be consumed by the previous methods.

1. Analysis by Other Quantitation Techniques

A direct comparison of CE-LIF results with those obtained by other methods demonstrated the quantitative potential of CE (**TABLE 5-10**). The hybridization and spectrophotometry methods are described in Chapter 4. In samples 1, 2, 5, 6, 8, 9, and 12, the CE results compared favorably with the fluorescent spectrophotometer. Fluorometric measurements may be higher than CE results in most of the samples because the primers have not been adequately removed. Since analyses of PCR products based on fluorescence or UV absorption measure total DNA content in the sample, they cannot discriminate between amplified product and excess primers. Consequently, without purification to remove the single-stranded DNA primers, accurate conclusions as to the quantity of amplified product may not be made. The results for samples 7, 10, 13, and 14 in **TABLE 5-10** illustrate this limitation due to primer contamination. At low levels of PCR product, the hybridization and CE results are comparable, while the fluorometric readings are higher. In fact, in all of these samples, the slot blot and CE approaches show similar results. As the hybridization method is specific for the target DNA, a corresponding CE result demonstrates the reliability of the technique.

TABLE 5-10. Results from DNA Quantitation Methods. From [151]. HV1A, HV1B, and HV2 PCR amplified mtDNA samples were quantitated by CE-LIF, fluorescent spectrophotometer, and hybridization (slot blot). MDQ = minimum detectable quantity ($\sim$ 1 ng/μL for this CE-LIF method). All results are listed in ng/μL.

Sample Description	CE Results[a]	Spec[b]	Hybridization[c]
1. HV1A	30	29.0	ND[d]
2. HV1A	15	17.9	ND
3. HV1A Control[e]	below MDQ	2.98	ND
4. HV1A Control	below MDQ	5.49	ND
5. HV1B	56	55.1	100
6. HV1B	26	32.5	25
7. HV1B	1.1	5.70	0.6
8. HV1B	2.2	4.25	1.3
9. HV1B Control	5.5	4.99	2.5
10. HV1B Control	below MDQ	12.6	0.6
11. HV2	3.0	6.97	5
12. HV2	21	23.8	20
13. HV2 Control	5.0	18.8	5
14. HV2 Control	below MDQ	8.60	0.6

[a]CE conditions as in Fig. 5-27, except 5 s @ 5 kV.
[b]Fluorescent spectrophotometer conditions as listed in experimental. Measurements were made in triplicate and averaged.
[c]Hybridization procedure as described in experimental.
[d]ND = not determined. No slot blot HV1A probes were available at time of study.
[e]Controls are PCRs run without template DNA.

2. CE-LIF Analysis

A 200 bp internal standard was added to each sample for quantitation purposes. The ratio of the adjusted product peak area to the adjusted internal standard peak area was used to calculate the quantity of the product (see Eq. 4-1). In addition, the length of the PCR product can be determined by comparing the peak migration time to sizing standards (containing the same internal standard) run under similar conditions.

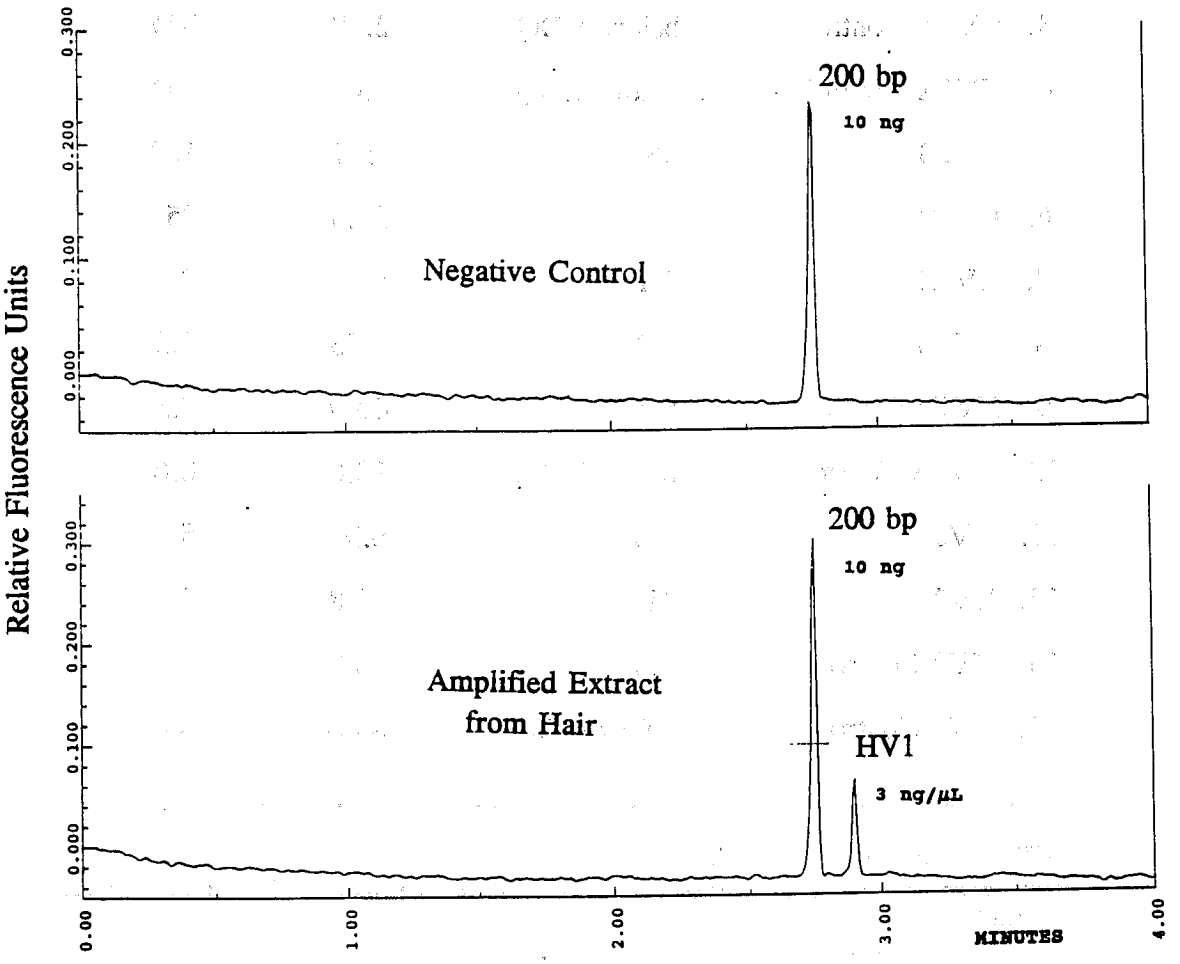


Figure 5-30. Rapid mitochondrial DNA quantitation. The negative control contained no template DNA. The quantity of the HV2 PCR product (402 bp) was determined by Eq. 4-1 through comparison of its peak area to the peak area of the 200 bp internal standard. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 1% HEC (Aldrich), 100 mM TBE, pH 8.2, 50 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* HD: 45 s @ 0.5 psi; *Separation:* 15 kV (~20 μA); *Sample:* 1 μL PCR product + 24 μL 200 bp (0.4 ng/μL) in water.

Separation time has been reduced to less than four minutes by utilizing higher field strengths (**Figure 5-30**). Typically, unreacted and unremoved PCR primers will appear at approximately 2 minutes with the 200 bp standard and either HV1 (437 bp) and/or HV2 (402 bp) at around 3 minutes. When the entire D-loop (1021 bp) is amplified, it may be seen at 3.6 minutes (**Figure 5-31**). It should be noted that resolution of the system is still adequate to resolve HV1 (437 bp) and HV2 (402 bp) should multiplex amplification of these regions be performed (**Figure 5-31**).

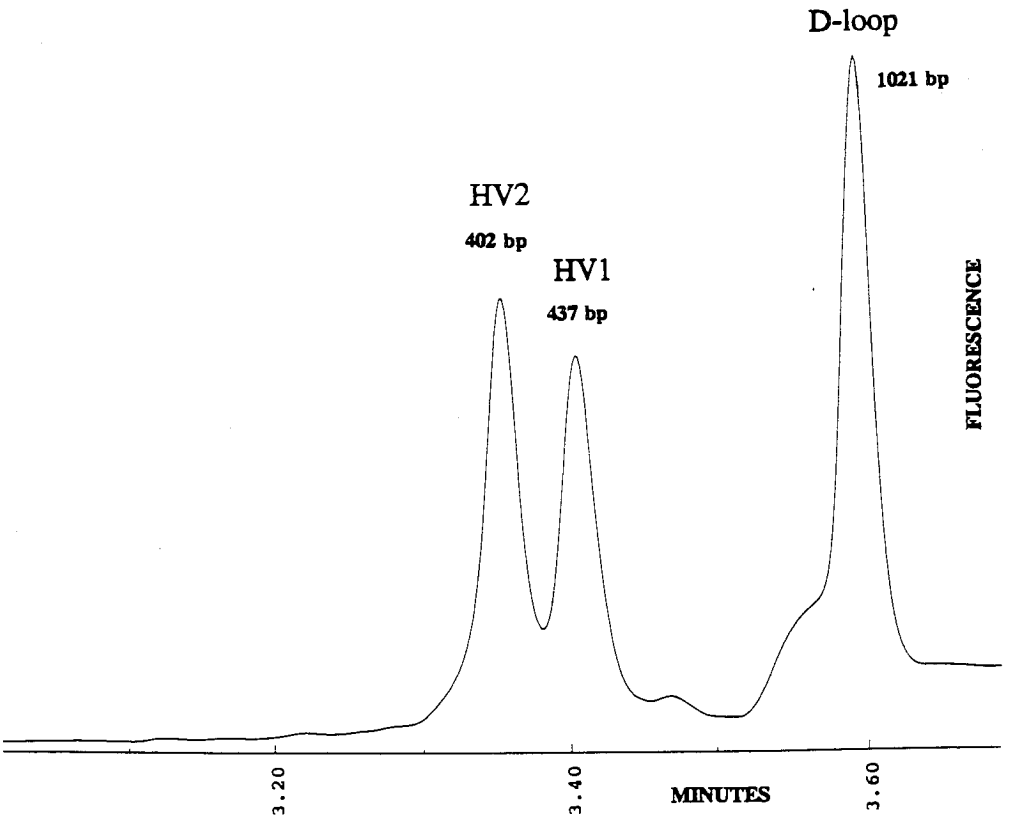


Figure 5-31. PCR products from the multiplex amplification of the mtDNA HV1 and HV2 regions can be resolved. The peak shoulder on the front of the D-loop peak was a result of not using a 10 s water plug prior to sample injection. Conditions as in Fig. 5-30.

This CE method has been used to analyze several thousand PCR-amplified mtDNA samples and is being prepared by the Hairs and Fibers Unit of the FBI Laboratory for use in mitochondrial DNA casework.

3. Application to Problems with mtDNA

Melanin Inhibition

One of the difficulties of amplifying mitochondrial DNA from hair is that *Taq* polymerase inhibitors (such as melanin) can be co-extracted with the DNA [535]. These inhibitors potentially reduce the efficiency of the amplification by blocking the active site of the polymerase [535]. In one experiment, where a grey hair (i.e., negligible amounts of melanin) and a dark hair (i.e., melanin containing) from the same individual were extracted and amplified side-by-side, the CE quantitation method showed a 5-fold better yield for mtDNA from the grey hair as compared to the black one. Without the separation power of CE to show that no contaminanting DNA was present (e.g., spectrophotometry can only give an overall reading and hybridization methods cannot detect non-specific fragments), assessment of the relative quanititative yield of the PCR reactions could not be easily performed. To overcome melanin inhibition, extra *Taq* polymerase can be added to enhance amplification of dark hairs [Mark Wilson, personal communication].

Loss of DNA During Post-Amplification Purification

The removal of primers following PCR can be important to future steps in sequence analysis of the amplicons. However, in some cases, it appeared that portions of the PCR product were also being removed. By adding internal standards to PCR products before and after the post-amplification filtration cleanup procedure, the loss of DNA during the centrifugation was examined. In one experiment,

approximately 45% of the PCR product was lost during the filtration process (**Figure 5-32**). The DNA of interest most likely adsorbed to the filter as the concentrated filtrate (i.e., the material which came through the filter) showed only primer peaks [151]. Based upon these results, measures have been taken to more efficiently recover amplified DNA from the centrifugation filter. Traditional fluorometry and quantitation by hybridization could not separate and quantitate the individual components of a sample and were therefore unable to perform this type of analysis. The ability to determine this result highlights one advantage of using CE as a diagnostic tool.

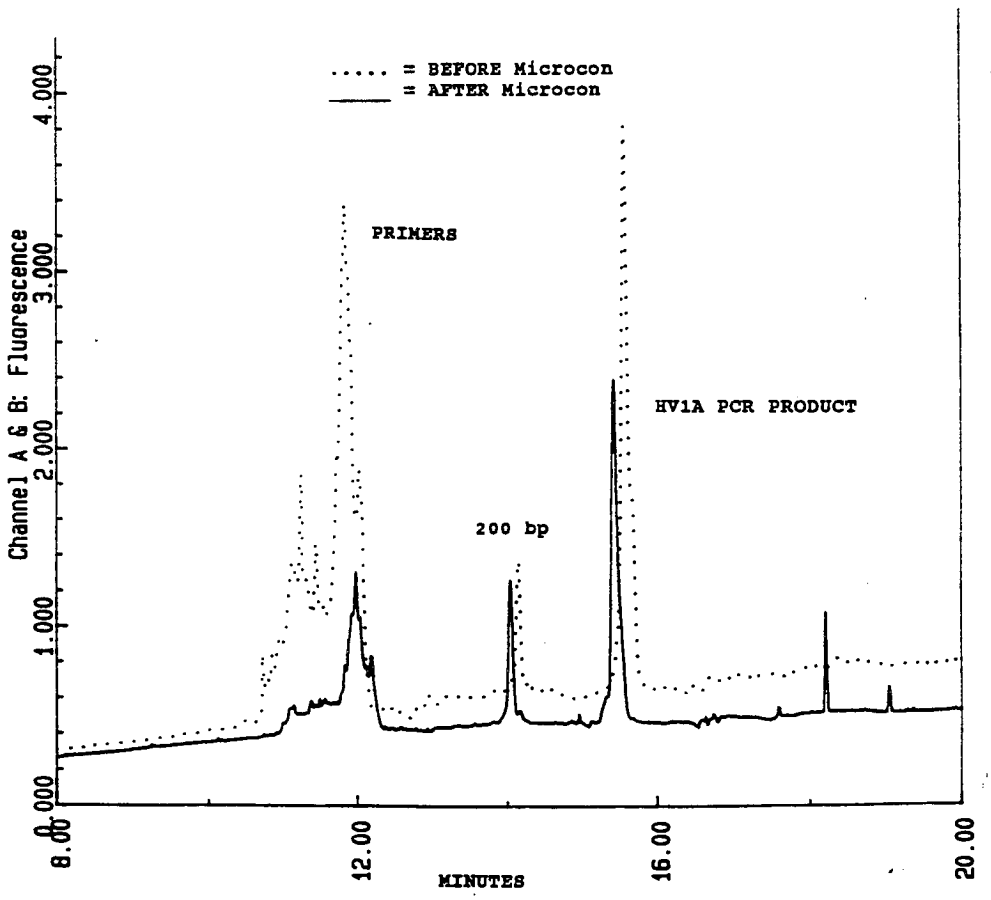


Figure 5-32. PCR product is lost in the primer cleanup step. Electropherogram of 1 μL of HV1A PCR product. The ratio of the product peaks indicates that 45% of the DNA is lost in the cleanup step. BEFORE = 1 μL PCR product prior to filtration added to 34 μL of 200 bp standard. AFTER = 1 μL Microcon 100 retentate added to 34 μL of 200 bp standard. Conditions as in Fig. 5-27.26

V. Summary

A. FACTORS INFLUENCING RESOLUTION

High resolution of DNA fragments ranging in size from 100-1000 bp depends upon the relationship of the DNA size to the mesh size in the polymer network. Smaller PCR products (e.g., STRs, 100-350 bp) were well separated with 1% HEC (Aldrich; ~40,000 MW) while the best resolution for larger PCR products (e.g., D1S80, 350-1000 bp) was obtained with 0.3% HEC (140,000-160,000 MW). Low concentrations of high molecular weight polymers were able to form a wider mesh than high concentration of shorter polymers, which most likely slowed the onset of reptation and the subsequent loss in resolution. Lower voltages also improved the resolution of large PCR products by reducing the amount of DNA stretching and alignment in the electric field. Voltage programming, where a high field strength was applied for several minutes followed by a lower field around the region of interest, was found useful for improving the speed of the separation without reducing resolution [152].

The type and concentration of an intercalating dye can influence a separation. For the STR separations examined in this study, 500 ng/mL of the mono-intercalator YO-PRO-1 gave the optimum results [559]. A short (20 cm effective length) and narrow (50 μm i.d.) capillary with a hydrophobic coating yielded excellent results for hundreds, and sometimes even thousands, of separations.

B. IMPROVEMENTS IN INJECTION PROCEDURES

Hydrodynamic [151] and electrokinetic [559] injection procedures were developed for rapid and sensitive PCR product analysis by CE. Effective sample stacking was demonstrated by lowering the sample's ionic strength with a 50-fold dilution in water. When combined with sensitive LIF detection, time consuming pre-

injection cleanup steps, which were required prior to this work, were eliminated.

C. PCR PRODUCT SIZING

The use of dual internal standards for PCR product sizing was introduced [152,559]. Good agreement between CE methods and traditional gel electrophoresis techniques for STR typing of the HUMTH01 locus was demonstrated with over 100 samples analyses [152,559]. A standard deviation of 0.14 bp (n=70) was obtained with the proper CE conditions [559].

D. PCR PRODUCT QUANTITATION

A method for the rapid quantitation of PCR products was developed to evaluate PCR-amplified mitochondrial DNA prior to cycle sequencing [247]. Results obtained from the CE method were comparable to fluorescent spectrophotometry and quantitation by hybridization but with the advantages of being able to detect the presence of primers and contaminating DNA fragments [151]. With a 402 bp PCR product and 50 ng/mL of the intercalating dye YO-PRO-1, fluorescence was found to be linear from 200 ng/mL down to ~400 pg/mL. A run-to-run peak area precision of 3% R.S.D. was obtained with an internal standard and hydrodynamic injection [151]. Important factors to consider for obtaining reliable results include precise pipetting of samples and internal standards, sample mixing with the pipets, and regular replenishment of the buffer vials.

E. SYSTEM STABILITY

Good stability has been demonstrated with the methods developed as part of this work. Routine application of these CE procedures is now possible. Several reasons exist for the improved stability. First, lower field strengths, which are

permitted with short columns, preserve the column coating because of lower Joule heating (see [157]). Narrower capillaries also help in this regard. The high polymer concentration increases the viscosity (which lowers any EOF--see [192]) and limits interaction of positively charged buffer components (e.g., intercalators like ethidium bromide or YO-PRO-1) with the capillary wall (see [52,53]). Less DNA is also injected when using LIF detection, which can reduce the amount of material available for interacting with the capillary surface.

CHAPTER FOOTNOTES

Chapter 1

(1A) For a genetic marker to be useful in forensic identification, several characteristics should be met. These marker attributes include (1) a well established pattern of inheritance, (2) a polymorphic character with a high degree of heterozygosity, (3) an independent inheritance from the other markers used, (4) a low mutation rate, (5) well established population data consisting of allele, phenotype and/or genotype frequencies, (6) analysis techniques which are simple, rapid, reproducible, and of reasonable cost, (7) reliable allele detection, and (8) small sample requirements [347].

(1B) In the original Jeffreys method [212], a single probe labeled multiple VNTR loci under low stringency conditions and generated a complex pattern resembling the bar codes used by supermarkets. This process is commonly referred to as "DNA fingerprinting." However, in forensic samples, where there is the possibility of mixed samples, a multiple locus system could generate patterns which would be difficult to fully interpret. Thus, most RFLP typing performed today in forensic laboratories involves single locus probes, where only one (a homozygote) or two (a heterozygote) alleles are seen [343].

(1C) The RFLP process can take several weeks to complete. First, a sample of blood or some other biological material is collected. The DNA is extracted from the cells by breaking open the cell walls and removing the protein packaging (histones) of the DNA. Next, a restriction enzyme is added to cut the long, extracted DNA fragments into smaller pieces. The most commonly used restriction enzyme in the United States is *Hae*III which recognizes the sequence GG/CC [207]. Every time "GGCC" appears in the genome, two DNA fragments will be produced--one ending in "GG" and the other beginning in "CC" (the reverse would be true on the other strand). The chopped up DNA fragments are then run on an agarose gel in order to separate them based upon size.

Next, a Southern blot is performed. Here, the bands of DNA are transferred from the gel to a nylon membrane. By using a highly alkaline solution, the DNA strands become single stranded. One of the strands is affixed to the membrane by crosslinking the DNA onto the membrane with UV light (or using a positively-charged membrane). A radioactive or chemiluminescent probe, which contains a VNTR sequence, is allowed to hybridize to the DNA attached to the nylon membrane. Stringent binding can occur at the proper hybridization temperature and ionic strength, allowing the probe to find its complementary sequence with complete fidelity. Excess probe is removed with several stringency washes. Finally, the location of the probe is noted by placing the membrane in contact with x-ray film. The resulting samples in the autoradiogram should contain two bands representing an allele from one's mother and another allele from one's father (one band if both alleles are the same size). Human identity testing is primarily done for forensic and paternity purposes (**Figure 1-4**).

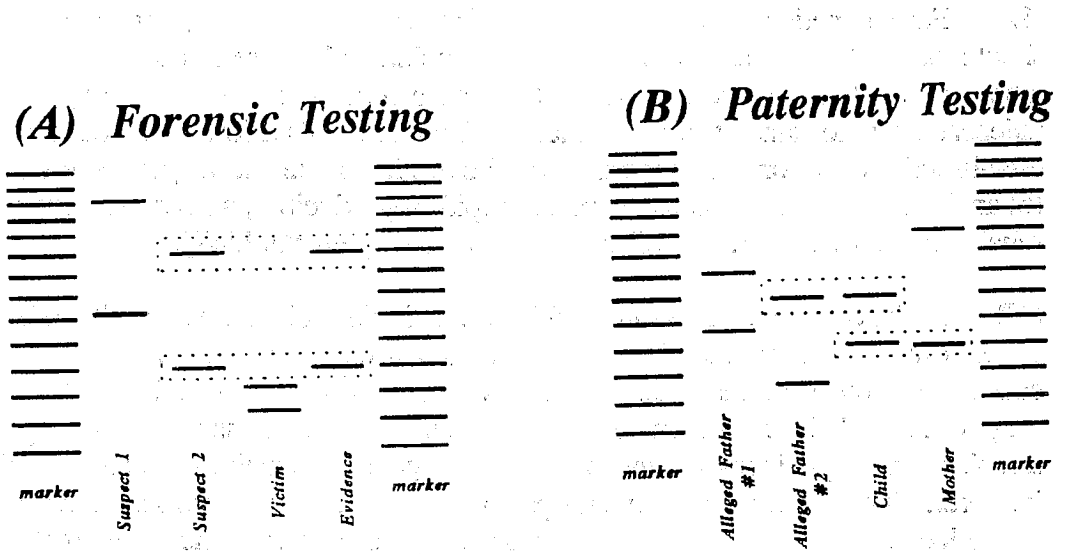


Figure 1-4. The use of RFLP profiling in (A) a forensic case and (B) a paternity case.

(1D) The temperature cycling for PCR proceeds as follows. The DNA template is first heated to approximately 95 °C for 30 s or more to completely separate the two strands of the DNA molecule. Next, the temperature of the PCR reaction mixture is lowered to approximately 60 °C where the two primers (synthesized oligonucleotides 20-30 bp in length) bind to their complementary sequences. The annealing temperature used here is only an example and depends on the template sequence. These primers are synthesized in order to bracket a preselected segment of the DNA template. With the proper choice of annealing temperature, the binding of the primers can be stringent enough to prevent any single base mismatches. The first primer matches part of the upper or "Watson" strand at one end of the segment, while the second primer matches the lower or "Crick" strand at the other end. Finally, the temperature of the solution is raised to 72 °C, the optimum temperature for synthesis of DNA by the *Taq* polymerase, and the area between the two primers is filled in with the complementary nucleotides (dNTPs) by the polymerase. Since the result of this process is a doubling of the number of strands of targeted DNA, repeated cycles of heating and cooling lead to a chain reaction. Each new PCR product becomes the template for the next cycle.

(1E) More advanced methods of PCR product detection are discussed by Lazar [322] and include automated gel-based fluorescence detection [163,541], CE [152,559], HPLC [118,124], and sequence-specific oligonucleotide probes [425,427].

(1F) The variety of STR lengths which can exist within a population is thought to originate from strand slippage during DNA replication [257,547,644]. **Figure 1-5** illustrates how a few alleles produce the multiple genotypes which are useful in distinguishing between individuals.

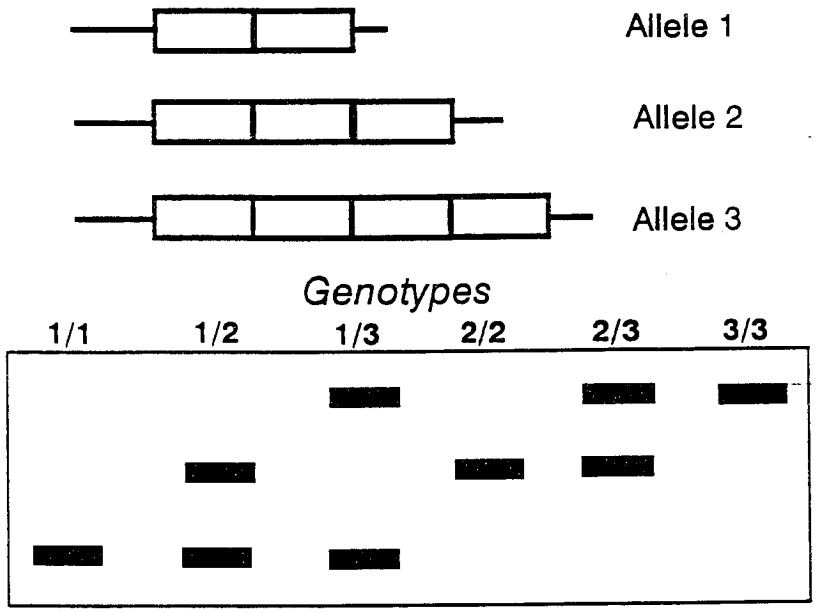


Figure 1-5. Variation in the number of tandem repeats. If n is the number of alleles, then n + (n-1) + (n-2) + ... equals the number of possible genotypes.

(1G) By way of example, 29 alleles have been identified for the D1S80 (pMCT118) locus, with a heterozygosity of 0.95 [683]. These D1S80 alleles differ from each other by a 16 bp repeat [259]. D1S80 alleles, which are named for the number of repeat units they contain, usually range between 14 and 41, although a few alleles have been reported with more than 50 repeat units [683]. The D1S80 VNTR system has been extensively tested and is now commercially available [683].

(1H) While plenty of STR systems exist which have a simple repeat unit, many STRs exhibit complex repeat patterns. The human beta-actin related pseudogene H-beta-Ac-psi-2 locus (ACTBP2, also known as SE33), for example, is generally a tetranucleotide repeat, but allele size differences of 1, 2, or 3 bp also exist [475]. While this makes the system much more polymorphic, and thus, more worthwhile in a forensic context, extremely accurate sizing is required. The situation can be further complicated if allelic variants with the same overall size, but different internal sequence, are possible [268,325,475]. Different separation techniques may have different effects on DNA mobility, which would make it difficult to compare results across different laboratories.

For this reason, the DNA Commission of the International Society of Forensic Haemogenetics [480] has recommended that all alleles in an STR allelic ladder be sequenced in order to establish the sequence of the repeat unit(s), the number of repeats present, and the actual size of the allelic fragment (see [161]).

(1J) Multiplexing can save time and money, but difficulties may arise when co-amplifying several loci. Tully *et al.* [164] found that the primers for one locus could complex with those of other loci and thus completely inhibit amplification. Finding the optimum PCR conditions, particularly the annealing temperature and the primer concentrations, can be challenging and time-consuming. For example, in evaluating the multiplex amplification of VWA, TH01, F13A1, and FES/FPS, Kimpton *et al.* [344] examined the effects of the concentration of the buffer, the individual primers, the dNTPs, the *Taq* polymerase, and the template DNA. The number of amplification cycles and the denaturing and annealing temperatures were also studied.

(1K) Another approach to analyzing multiple sites on the genome while conserving limited DNA has been demonstrated by Lorente *et al.* [448,478]. These studies involved recovering the original genomic DNA template following each PCR amplification and reusing it for the next reaction in what is termed sequential multiplex amplification (SMA). Using SMA, less than 5 ng of template genomic DNA was needed to type five different loci [478].

(1L) The first commercialized and most widely used PCR-based test of forensic DNA samples is the AmpliType™ HLA-DQα Forensic DNA Amplification and Typing Kit developed by Cetus Corporation (Emeryville, CA). A reverse dot blot system (**Figure 1-6**) is used with allele-specific oligonucleotide probes to detect each of the DQα alleles. Six alleles, known as 1.1, 1.2, 1.3, 2, 3, and 4, can been distinguished for the 242 bp region (239 bp for alleles 2 and 4) in the second exon of the DQα gene [425]. Twenty-one genotypes are possible with various combinations of these alleles.

(1M) For the reverse dot blot hybridization scheme seen in **Figure 1-6**, biotin-labeled primers are used in the DNA amplification. During hybridization of the PCR product to the oligonucleotide probe which is attached to the membrane, a streptavidin/ horseradish peroxidase complex is added. The streptavidin binds to the biotin, which is present on the 5'-end of every amplified DNA molecule. Horseradish peroxidase (HRP) catalyzes the oxidation of the colorless soluble substrate 3,3',5,5'-tetramethyl-benzidine (dye) into a blue precipitate. Thus, hydridization is detected by the formation of a colored dot [425].

This reverse dot blot system has been validated for forensic casework [425] and has sufficient sensitivity to type HLA-DQA1 alleles from cigarette butts [239]. More recently, other sequence variable loci have been coamplified and typed with the DQα system [286,682].

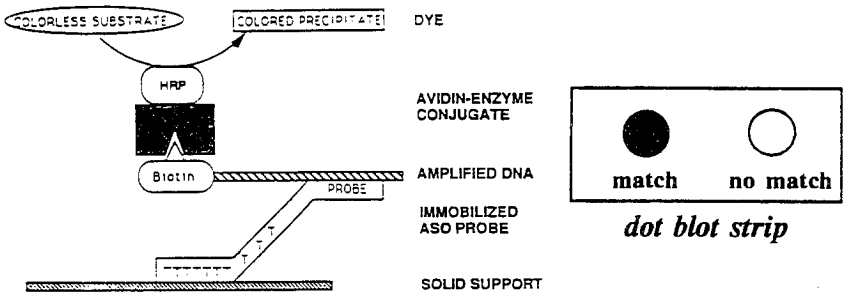


Figure 1-6. Reverse dot blot detection with allele-specific oligonucleotide (ASO) probes.

(1N) Simultaneously amplification and detection of multiple sequence polymorphisms may be performed with the AmpliType™ PM PCR Amplification and Typing Kit [682]. The genetic loci amplified with the polymarker kit include HLA DQα, Low Density Lipoprotein Receptor (LDLR), Glycophorin A (GYPA), Hemoglobin G Gammaglobin (HBGG), D7S8, and Group Specific Component (GC) [286,682]. These markers are also typed by reverse dot blot technology. The amplified PCR products from the last five loci are hybridized to a nylon membrane that contains 13 immobilized oligonucleotide probes. The HLA DQα locus is typed on a separate probe strip. The chromosomal location, size of the PCR products, and number of alleles for each locus is shown in **TABLE 1-4** [88].

TABLE 1-4. AmpliType™ PM Loci. From Ref. [682].

Locus	Chromosomal Location	PCR Product Size	Number of Alleles
HLA DQα	6	242/239 bp	6
LDLR	19	214 bp	2
GYPA	4	190 bp	2
HBGG	11	172 bp	3
D7S8	7	151 bp	2
GC	4	138 bp	3

Product literature lists the combined power of discrimination for U.S. Caucasians at 0.9997 [682]. Population studies and forensic validation of the PM loci were recently performed by Budowle *et al.* [286]. The AmpliType™ PM PCR Amplification and Typing Kit is currently being used by the FBI Laboratory in forensic cases.

(1P) Mitochondrial DNA has several advantages in a forensic context. First, the small, circular nature of mtDNA makes it more robust and resistant to degradation. Second, the high copy number means that small amounts of recovered biological evidence are more likely to be successfully typed. Most mammalian cells contain hundreds of mitochondria and thousands of mtDNAs. Third, all copies of an individual's mtDNA sequence are identical, since they only come from one's mother, making mtDNA easier to sequence for forensic purposes. (In order to unambiguously sequence a nuclear DNA gene, the two alleles must first be separated [190].) However, disadvantages also exist for using mitochondrial DNA in human identification. Since genetic recombination does not occur with each generation, not as much variation exists in mtDNA. However, mutation rates are 10-50 times higher than nuclear DNA, though, which can add variability in a population. Barring mutation, siblings and maternal relatives have an identical mtDNA sequence.

(1Q) The sensivitity of PCR necessitates constant vigilance on the part of a laboratory to ensure that contamination does not affect DNA typing results.

Chapter 2

(2A)
TABLE 2-2. Comparison of CE and Slab Gel Electrophoresis.

	CE	Slab Gels
Automation Capability	yes	some
Speed	minutes	hours
Quantitative	yes (real-time)	extra step
Waste Generated	nL-μL	mL-L
Sample Processing	sequential	simultaneous
Multiple Samples Run	can be parallel (arrays)	multiple lanes
Sample Consumption	few nL	1-5 μL
Cost	\$25,000-65,000[‡]	\$2,000-5,000
Preparative Technique?	no	yes
Established Technique?	still evolving	yes

[‡]UV detection (\$25,000); LIF detection (\$65,000)

(2B) The high surface area-to-volume ratio of capillaries gives them good heat dissipation characteristics. It is this feature which permits voltages to be applied in CE that are an order of magnitude higher than those permitted in slab gel electrophoresis.

(2C) The problem with pressure driven flow, as used in systems such as HPLC, is that parabolic flow profiles are produced yielding more disperse analyte zones. Band broadening is reduced in CZE (relative to HPLC) as the bulk flow past the detector is driven by electroosmosis, which has a relatively flat flow profile. Separations with efficiencies of one to two orders of magnitude higher than HPLC are not uncommon in CE [251].

(2D) An analysis proceeds with first filling the capillary with the electrolytic buffer. Some instruments use high pressure on the inlet side while others use a vacuum suction on the outlet side. High pressures are important to load viscous buffers used in DNA separations. Next, the inlet buffer vial is replaced by the sample vial and injection of the sample takes place either under pressure or the application of an electric field [372]. Typically only a few nanoliters of sample are placed in the capillary. The separation proceeds once the inlet buffer vial is replaced on the capillary and the high electric field is applied. Finally, the analytes are inspected as they move past an on-column detector.

(2E) Commercial CE instruments, with autosamplers, offer fully automated injection, separation, and detection. Real-time data analysis of peak areas, peak heights, and peak migration times in the electropherogram (E-gram) are available with sophisticated

computer programs. Much of the commercially available software was developed in large part from previous HPLC experience.

(2F) The movement of DNA through a column is dependent upon the condition of the inner wall of the capillary and the extent of EOF (**Figure 2-5**). With uncoated capillaries and large EOF, counter-migration CE occurs because the DNA "swims upstream" against the EOF.

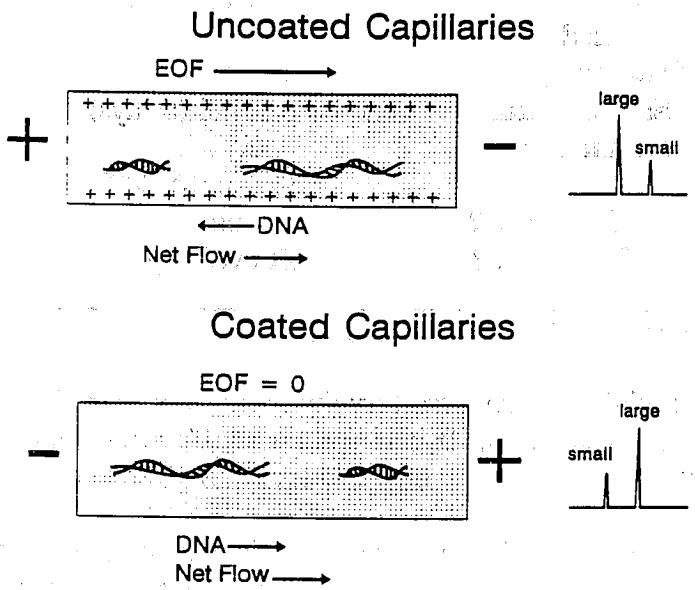


Figure 2-5. The order of DNA eluting from the capillary is dependent upon the column inner wall coating. Depending on the extent of electroosmotic flow, DNA fragments elute by size either (A) large → small (uncoated) or (B) small → large (coated).

(2G) HD injections can be performed by several means including (a) the application of pressure at the inlet side which pushes the sample into the column, (b) the application of a vacuum at the outlet side which pulls the sample into the column, or (c) raising the inlet side which forces the sample into the capillary through siphoning due to gravity.

(2H) Several methods have been developed to reduce the salt content of a PCR sample prior to CE injection (**TABLE 2-3**). While most of these sample cleanup procedures work nicely, they require an extra step, which reduces the capability for automation [155].

TABLE 2-3. PCR Sample Purification Methods.

Type of Clean-up	Method of Salt Removal
Float Dialysis	salts diffuse through a membrane into a Petri dish of deionized water
Ultracentrifugation	salt are spun through a filter; DNA is recovered by inverting the filter
Spin-Size Exclusion	DNA comes through the filter pores; salts are retained by the filter
Ethanol Precipitation	DNA is recovered as a pellet; salts are washed away with water

(2J) This is the result of Beer's law, in which absorbance is equal to the products of the sample absorbivity (ϵ), the pathlength of detection (b), and the sample concentration (c). As ϵ is fixed by the chemical nature of the sample, in order to improve the sensitivity in CE, either more sample must be injected onto the column or the pathlength can be increased [6].

(2K) Fluorescence has been recognized since the early 1980s as important to sensitive detection with CE. Jorgenson and Lukacs [4] demonstrated good sensitivity with fluorescamine-derivatized peptides. Early work with fluorescent detection involved arc lamps [4], which are difficult to focus onto a narrow capillary. Richard Zare (*Science* **1985**, *230*, 813-814) is attributed with the introduction on-column laser-induced fluorescence (LIF) in 1985.

(2L) Direct detection of DNA has also been demonstrated based on the native fluorescence of the DNA molecule in its natural state, with low-UV lasers as an excitation source [354].

(2M) While superior sensitivity is available with LIF, several difficulties prevent its widespread use. Limited excitation wavelengths are available with lasers meaning that fluorophores must be found which can be efficiently excited with a particular laser. In addition, the expense of some lasers prohibit their use in most laboratories. The most commonly used lasers in connection with CE include the argon ion laser (488 nm excitation) [29] and the green HeNe laser (543 nm) [522] although several UV lasers

have been used [53,409]. See Schwartz *et al.* [315] for a complete list of lasers used in CE.

(2N) Using fluorescently tagged primers allows the detection of overlapping fragment sizes, which can be useful in multiplexed STR amplification and detection [163,473].

(2P) Growth in the field of dsDNA separations by CE has been exponential over the past few years (**Figure 2-6**). Over 100 papers have been published describing CE as a qualitative and a quantitative tool for DNA analysis (**APPENDIX 1**).

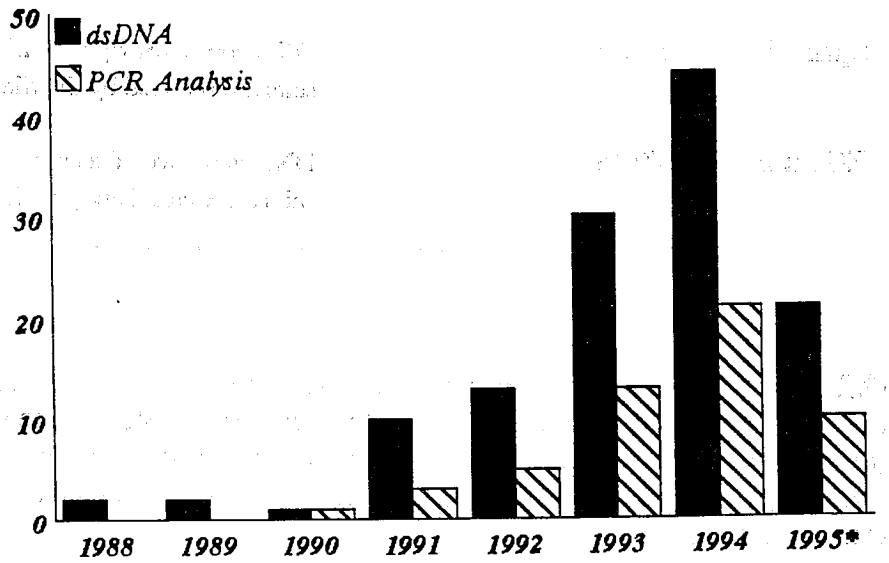


Figure 2-6. Papers in the literature describing dsDNA separations (dark bars) using CE. PCR product analysis by CE (lined bars) is also on the rise. Results are compiled from the information in APPENDIX 1. * = papers are tabulated through May 1995.

(2Q) An evaporation rate of 0.05 nL/s has been reported for sample volumes of 5μL, which corresponds to more than 4 μL per day [84]. In the Beckman P/ACE instrument, microvials are covered with silicone rubber caps to prevent evaporation (Beckman Technical Bulletin 101B).

(2R) Arakawa *et al.* [309] did use an external calibration curve from ϕX174 *Hae*III to size PCR products. However, because they report a migration time precision of only 0.47-2.77% for ϕX174 *Hae*III, they don't have the ability to accurately relate the size of the external standard to the actual sample. In a later report, this same group improved their precision to 0.55% RSD (within-run, n=10) and 0.71% RSD (day-to-day, n=6) for ϕX174 *Hae*III peak migration times [383].

(**2T**) Del Principe and coworkers [467] observed that migration time diminished by 15 ($\pm$1) second from run-to-run (over 7 runs). Since the drift was constant and predictable, they calculated the DNA fragment sizes taking the migration time variation into account. The original calibration curve was formed with a log (bp) vs. migration time plot of a pBR322 *Eco*RI standard. This technique can be risky if unforeseen changes occur between runs and requires careful calculation of adjustment factors.

(**2U**) The use of internal standards for DNA fragment sizing was first shown to improve migration time reproducibility by Guttman *et al.* [251]. Using the migration of a 40-mer relative to that of a 50-mer, they improved the column-to-column precision (n=11) from 4.8% R.S.D. to only 0.7%. R.S.D. [251]. Using a 24-mer internal marker, the ability to accurately size to within 99% has been shown [400]. The internal marker is run first with a 100 bp ladder external calibration standard followed by the sample with the same internal marker to relate the sample to the standard.

(**2V**) Using ϕX-174 DNA-*Hinc*II digest as an internal standard, Nathakarnkitkool *et al.* [35] analyzed a 361-bp segment of an androgen receptor mRNA transcript in less than 20 min with a peak area relative standard deviation (RSD) of 3.06% (n=6). Schwartz and coworkers [23] obtained peak area precisions (n=7) for the 11 DNA fragments in ϕX 174 *Hae* III digest ranging from 1.91-8.43% RSD.

Chapter 3

(3A) Thirteen different types of polymers have reportedly been used as DNA separation media [675]. These polymers are available from numerous sources in several different molecular weights (MW) and can be dissolved in aqueous buffers over a wide range of concentrations. **TABLE 3-2** illustrates the number of different polymers which have been used in the literature with HEC, MC, and HPMC. A consensus has yet to be reached as to which polymers (type, MW, or concentration) are better to use for a particular separation, partly because the results between different laboratories involve dissimilar polymers, DNA samples, or CE conditions.

(3B) Experimental determination of Φ^* may be performed by plotting the log of the specific viscosity, η_{sp}, as a function of the polymer volume fraction [45]. For noninteracting polymer molecules, i.e., $\Phi < \Phi^*$, dilute solution theories predict that the slope of such a plot should be approximately 1.0. When the polymer chains begin to interact, the slope should increase. Thus, the point at which the slope deviates from ~ 1.0 is indicative of chain interaction, or in other words, Φ^*.

(3C) Using dynamic light-scattering studies, Grossman *et al.* [307] found the empirical relationship between polymer concentration (C, g/mL) and the mesh-size of the entangled polymer network (ξ) to be,

$$\xi(\text{Å}) = 6.0\ C^{-0.68}$$

for their HEC solution.

(3D) While the dynamic nature of the system makes it difficult to model, the flexibility of the polymer network can be beneficial when trying to separate a wide range of DNA fragment sizes. DNA fragments, ranging in sizes of over three orders of magnitude, may be separated in a relatively short period of time (**Figure 3-8**). This separation was performed with a 0.4% HPMC ($\sim$90,000 MW) solution. The viscosity of the polymer solution was only slightly greater than water. Conventional gel electrophoresis, with rigid chemical pores, would probably require multiple gel compositions to accomplish the same separation. Tightly cross-linked gels with small pores would be needed for the smaller fragments ($<$587 bp) while more loosely formed gels with larger pores (i.e., agarose) would be required for the larger DNA (2027 $\rightarrow$ 23,130 bp).

TABLE 3-2. Cellulose Derivatives Used in CE/DNA. Information was compiled from APPENDIX 1. Only one reference per polymer has been displayed for clarity. The most widely used include HEC (86-113cP), MC (4000cP), and HPMC (4000cP).

Polymer	Source	Molecular Weight	Viscosity (2%sol,25°C)	Reference
Hydroxyethyl Cellulose (HEC)				
	Aldrich	Not Known	86-113cP	[559]
	Serva	Not Specified	300mPa.s	[35]
	Fluka	"Cellosize-WP-40"	75-125mPa.s	[39]
	?	191,800 MW	Not specified	[34]
	Polysciences	24,000-27,000 MW	Not specified	[36]
	Polysciences	90,000-105,000 MW	Not specified	[36]
	Polysciences	140,000-160,000 MW	Not specified	[36]
	Aqualon	~35,900 MW	Not specified	[36]
	Aqualon	~63,800 MW	Not specified	[36]
	Aqualon	~306,000 MW	Not specified	[36]
	Aqualon	~438,800 MW	Not specified	[36]
Methyl Cellulose (MC)				
	Sigma	100,000 MW	4000cP	[27]
	Sigma	20,000 MW	15cP	[382]
	Aldrich	Not specified	4000cP	[387]
	Fluka	Not specified	high	[16]
	Polysciences	86,000 MW	Not specified	[47]
MC-4000	ShinEtsu	Not specified	4290cP	[69]
MC-8000	ShinEtsu	Not specified	7980cP	[69]
Hydroxypropylmethyl Cellulose (HPMC)				
	Sigma	~90,000 MW	4000cP	[23]
	Sigma	~26,000 MW	100cP	[23]
	Sigma?	Not specified	50cP	[149]
	Aldrich	Not specified	4000cP	[145]
	Serva	Not specified	15000cP	[269]
HPMC-4000a	ShinEtsu	Not specified	4550cP	[69]
HPMC-4000b	ShinEtsu	Not specified	4390cP	[69]
HPMC-15000	ShinEtsu	Not specified	15900cP	[69]

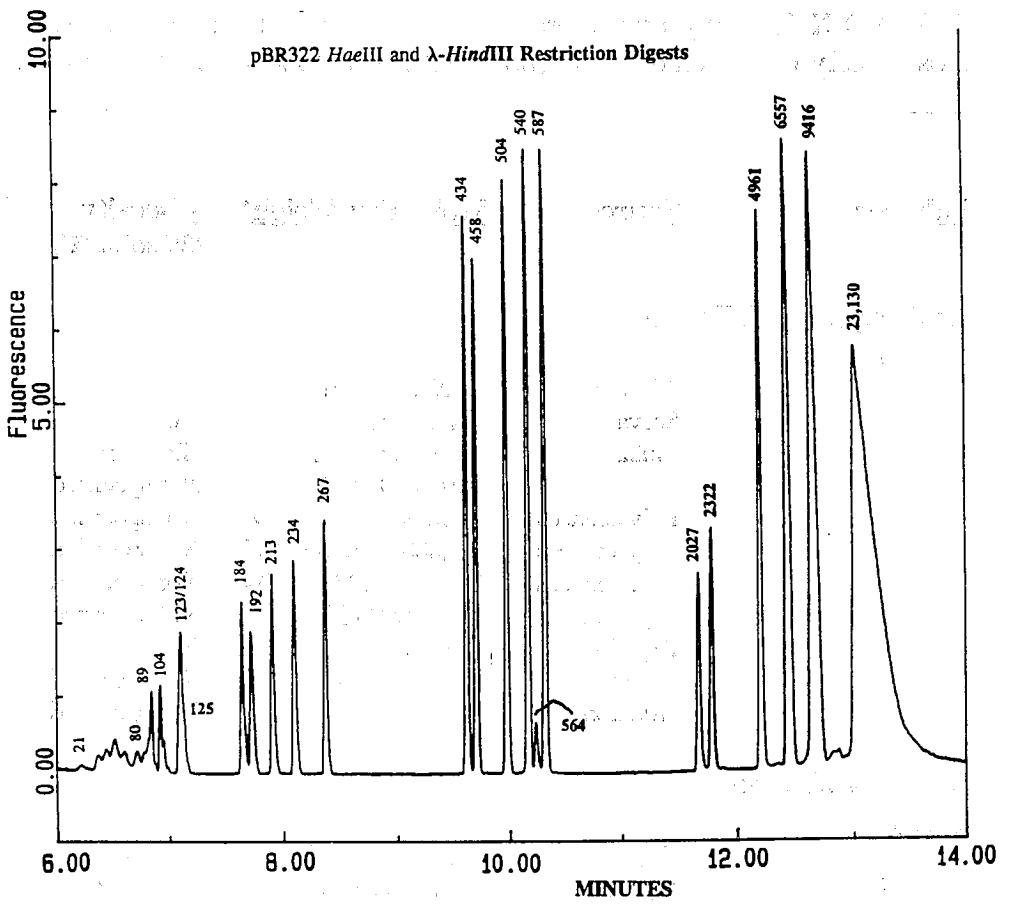


Figure 3-8. The efficient separation of DNA fragments ranging in size from 21 bp to 23,130 bp demonstrating the flexible nature of the mesh formed with soluble polymers. Conditions: *Capillary:* 50 μm i.d. x 27 cm DB-17; *Buffer:* 0.4% HPMC (4000cP), 100 mM TBE, pH 8.2, 500 ng/mL YO-PRO-1; *Temp.:* 25 °C; *Injection:* 5 s @ 1 kV; *Separation:* 5 kV; *Sample:* 204 ng/mL pBR322*Hae*III mixed with 200 ng/mL λ-*Hind*III.

(3E) From the data compiled in **APPENDIX 1**, there has appeared in the CE literature 52 references where intercalating agents were used to improve sensitivity and/or resolution of DNA fragments. The most commonly used intercalators include ethidium bromide (used 28 times) and thiazole orange (used 9 times).

(3F) Srinivasan *et al.* [52] speculate that TOTO-1 in the run buffer binds to free capillary surface silanols and creates cationic charges that may interact with DNA fragments and result in band broadening. Clark and Sepaniak [53] found that micromolar levels of the dimer POPO-3 produced unresolved fragment profiles while ethidium bromide, a monomer, could be used at levels up to 12.5 μM without deleterious effects.

ABBREVIATIONS

bp	base pair
CE	capillary electrophoresis
CV	constant voltage
CZE	capillary zone electrophoresis
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
EK	electrokinetic
EOF	electroosmotic flow
EB	ethidium bromide
HD	hydrodynamic
HEC	hydroxyethyl cellulose
HPCE	high performance capillary electrophoresis
HPMC	hydroxypropyl methyl cellulose
i.d.	internal diameter
LIF	laser-induced fluorescence
LPA	linear polyacrylamide
MC	methyl cellulose
mtDNA	mitochondrial DNA
MW	molecular weight
N	number of theoretical plates

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEO	poly(ethylene oxide)
PMT	photomultiplier tube
psi	pounds per square inch
R	resolution
r	radius
RFLP	restriction fragment length polymorphism
R.S.D.	relative standard deviation
STR	short tandem repeat
TBE	Tris-borate-EDTA
t_m or MT	migration time
μ_e	electrophoretic mobility
VNTR	variable number of tandem repeats

APPENDIX 1

dsDNA Separations in the CE Literature

An Examination of the Conditions and Results

Legend:

__ = no information provided for that parameter

Reference:
Reference number refers to listing in the literature survey; the primary researcher was also included

Sample:
DNA restriction digest or PCR samples and their sizes

Conditions:
CE instrument, column length (length to detector) x column inner diameter, column coating, temp.
Buffer type, pH, separation medium (e.g., 1% HEC), additives (e.g., 500 ng/mL YO-PRO-1)
Injection type (EK or HD): time @ kV or psi; Separation (CV, gradient, pulsed): applied voltage

Detection:
UV or LIF with wavelength(s)

Separation:
Electrode polarity and the time required to elute the last (or major) peaks

Resolution:
Average resolution under the conditions listed. Typically involved evaluation of the ability to split the 271 and 281 bp peaks in the ϕX174 *Hae*III digest.

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[11] Ulfelder	φX174 *Hae*III	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 (0.5μm) 28 °C 89mM Tris-borate, 2mM EDTA, pH 8.6, 0.5% LPA?, 5mM EB EK:10s @ 35 V/cm (2kV); CV: 175 V/cm (10kV) 27cm x 50 μm i.d. and 10kV (~4 min); 50mM NaCl improves resolution	LIF 488:530	⊖→⊕ ~4 min (Fig. 6c) 35 min	271/281 split ~5-7bp (Fig. 7b)
[16] Strege	1-kbp DNA ladder λ DNA-*Hind*III 125-23,130 bp	Beckman P/ACE 2000 CE, 50cm(43) x 75 μm i.d. PAA coated FS 30 °C 50mM Tris-borate, 2.5mM EDTA, pH 8.0, 0.5% methyl cellulose (Fluka-high viscosity); HD: 5s @ 0.5psi; CV: 10kV	UV 254nm	⊖→⊕ 20 min	506/517 split <11 bp
[22] Brownlee	φX174 *Hae*III SV40 RF DNA	Homebuilt CE, 20cm(_) x 100 μm i.d. 3%T,5%C PAA gel-filled _°C 9mM Tris-borate, 0.2mM EDTA, pH 8.0, 10 μg/mL EB HD: ??; CV: 10kV (10μA); 20mM CTAB with 4M urea added to some	fluor 250:585	⊖→⊕ 8 min	not good
[23] Ulfelder	φX174 *Hae*III 115 (HIV) & 242 bp PCR products	Beckman P/ACE 2000 CE, 27cm(20) or 57cm(50) x 100 μm i.d. DB-17 89mM Tris-borate, 2mM EDTA, pH 8.5, 10μM EB, 0.5% HPMC (4000cP) EK:10s @ 2kV; CV: 10kV 25 °C	UV 260nm	⊖→⊕ 35 min	271/281 split 5 bp
[24] McCord	pBR322 *Hae*III D1S80 & SE33 allelic ladders	SP1000 CE, 70cm(60) x 100 μm i.d. DB-17 (0.05μm) 25 °C 100mM Tris-borate, 0.1mM EDTA pH 8.7 w/CsOH, 0.5%HEC (Aldrich), 1.27μM EB; EK: 10s @ 5kV; CC: 38 μA (-12kV)	UV 260nm	⊖→⊕ 30 min	123/124 split 16bp D1S80 4bp SE33
[27] Srinivasan	φX174 *Hae*III D1S80 alleles	Beckman P/ACE 2100 CE, 47cm(40) x 100 μm i.d. DB-17 30 °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% methyl cellulose (Sigma) EK:5s @ 10kV; CV: 10kV TOTO-1 & YOYO-1 used	LIF 488:530	⊖→⊕ 40 min	271/281 split 3-4 bp ?
[29] Ulfelder	φX174 *Hae*III 242, 368, 900 bp PCR products	Beckman P/ACE 2050 CE, 47cm(40) x 100 μm i.d. DB-17 25 °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (Sigma), 0.1 μg/mL Thiazole Orange EK:10s @ 35 V/cm (1.6kV); CV: 175 V/cm (8.2kV)	UV 260nm LIF 488:530	⊖→⊕ 25 min	271/281 split ~5 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[33] McCord	TC11, VWA, & MBP allelic ladders; pBR322 *Hae*III	P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 (0.1μm) 25 °C 100mM Tris-borate, 0.1mM EDTA pH 8.7 w/CsOH, 1%HEC (Aldrich), 50 ng/mL YO-PRO-1, 1.27 μM EB; EK: 10s @ 5kV; CC: 38 μA (13kV)	LIF 488:520	⊖→⊕ 30 min	195/198 (TC11 9/9.3) 3 bp
[34] Grossman & Soane	φX174 *Hae*III	Homebuilt CE, 50cm(35) x 50 μm i.d. fused silica 30 °C 89mM Tris-borate, 5mM EDTA, 0.25% (0.1-0.5% tested) HEC (?) HD: vac 2-3s; CV: 15kV (300 V/cm)	UV 260nm	⊕→⊖ 20 min	271/281 not split >30bp
[35] Oefner	pBR322 *Hae*III, etc. φX174 *Hae*III, etc. 361 bp PCR product	Homebuilt CE, numerous conditions were tested including electric field strength, HEC concentration, pH, ionic strength, various cations, ethidium bromide, sample concentration, column coating, etc.	UV 260nm	⊕→⊖ ⊖→⊕ 20 min	1-2bp w/ 5μM EB
[36] Barron & Soane	φX174 *Hae*III	Homebuilt CE, 50cm(35) x 51 μm i.d. fused-silica 30 °C 89mM Tris-borate, 5mM EDTA, pH 8.15, HEC (several MW & % used) HD: vac ~3 nL sample; CV: 11kV (220 V/cm)	UV 260nm	⊕→⊖ 20 min	271/281 split 10 bp (Fig. 10)
[37] McCord	TC11, VWA, MBP, SE33, D1S80 allelic ladders	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 25 °C 100mM Tris-borate, 0.1mM EDTA, pH 8.7 w/ CsOH, 1% HEC (Aldrich) 50 ng/mL YO-PRO-1, 1.27 μM EB, EK:10s @ 5kV; CC: 38μA (13kV)	LIF 488:520	⊖→⊕ 30 min	195/198 (TC11 9/9.3) 3 bp
[39] Schomburg	φX174 *Hae*III	Homebuilt CE, __cm(44) x 75 μm i.d. fused-silica coated with PAA, PVA 20 mM Na-phosphate, pH 7, 1% PAA or 1% HEC, 20 °C EK: 4s @ 95 V/cm; CV: 223 V/cm (23 μA)	UV 260nm	⊖→⊕ 20 min	271/281 baseline (Fig. 4a)
[45] Grossman & Soane	φX174 *Hae*III	Homebuilt CE, 50cm(35) x 50μm i.d. fused silica 30 °C 89mM Tris-borate, 5mM EDTA, 0.1-0.5% HEC (191,800 MW) HD:2-3s; CV: 15kV ($\Phi^* = 0.39\%$ $\xi \sim 264$ Å)	UV 260nm	⊕→⊖ 20 min	271/281 not split > 30 bp
[46] Oefner	pBR322 *Msp*I	ABI 270A or P/ACE 2100, 72cm(50) x 50μm & 67cm x 75μm fused silica 10mM tris-borate, 0.1mM EDTA, pH 8.7, 25mM NaCl, 0.5% HEC, 1.27μM EB; HD: vac, 5s; CV: 15kV (16μA); 35 °C; alkali cations...	UV 260nm	⊕→⊖ ⊖→⊕ 20 min	180/190bp split 10 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[47] MacCrehan	1kb ladder pBR322 & ϕX174 DNA Marker V	Instrument?, 100cm(80) x 75 μm i.d. polyacrylamide coated fused silica 100mM Tris-borate, 2mM EDTA, pH 8; 0.2,0.4,0.6% MC (MW 86,000) ____; CV: 30kV; __°C	UV 260nm	$\ominus\rightarrow\oplus$ 20 min	184/192 split 8 bp
[48] Ulfelder	ϕX174 *Hae*III 220,330,520,530 bp PCR-RFLP products	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 (0.1μm) 25 °C 89mM Tris-borate, 2 mM EDTA, pH 8.5, 0.5% HPMC (Sigma-4000cP) EK:10s @ 175 V/cm (10kV) after 1mm H_2O plug; CV: 10kV 10μM EB	UV 260nm	$\ominus\rightarrow\oplus$ 30 min	271/281 split <5 bp
[52] Srinivasan	ϕX174 *Hae*III apoB, D1S80 alleles (PCR products)	Beckman P/ACE 2100 CE, 57cm(50) x 50 μm i.d. DB-17 25 °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% MC (Sigma), __ng/mL TOTO; EK:5s @ 10kV; CV: 10kV 3%T,3%C gel-filled capillaries also used w/ & w/o urea buffers	LIF 488:530	$\ominus\rightarrow\oplus$ 45-60 min	271/281 split 10 bp
[53] Sepaniak	ϕX174 *Hae*III	Homebuilt CE, 50cm(40) x 50 μm i.d. DB-1 (0.05μm) __°C 100mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% MC (Sigma), 2.5 μM EB EK:10s @ 4kV; CV: 15kV POPO-3 was also studied	LIF 325:570 543:570	$\ominus\rightarrow\oplus$ 15 min	271/281 split <5 bp
[67] Barron & Soane	ϕX174 *Hae*III λ-*Hind*III 72 - 23,130bp	same conditions as [36] except CV: 265 V/cm and below Φ^* thorough examination of MW 24,000-27,000 and MW 90,000-105,000 HEC	UV 260nm	$\oplus\rightarrow\ominus$ 15-25 min	271/281 split with 0.15% MW 105,000
[68] Novotny	pBR322 *Hae*III	ISCO Model 3850 CE, 65cm(45) x 50 μm i.d. PA coated fused silica 100mM Tris-100mM glycylglycine, pH 8.2, 0.2-1% HEC (MW 105,000) EK:10-15s @ 5kV; CV: 19.5kV; __°C; 1% HEC (MW 27,000)	UV 260nm	$\ominus\rightarrow\oplus$ 20 min	184/192 baseline 8 bp
[69] Baba	ϕX174 *Hae*III ϕX174 *Hinc*II 1 kbp ladder	Waters Quanta 4000 CE, 50cm(42.5) x 100 μm i.d. PA coated fused silica 50mM Tris-borate, 2.5mM EDTA, pH 8.3, 0.7% MC-4000 (MC-4000, MC-8000, HPC-11000, HPMC-4000a&b, HPMC-15000 were tested) EK:10s @ 10kV; CV: 10-20kV (200-400 V/cm, 18-22 μA); 24-27 °C	UV 254nm	$\ominus\rightarrow\oplus$ 20 min	271/281 Not ~25 bp
[73] Paulus	ϕX174 *Hae*III 100 bp ladder pBR322 *Msp*I pBR328 *Bgl*I+*Hinf*I	Homebuilt CE, 40cm(20) x 75 μm i.d. 6%T,0%C gel-filled 22 °C 100mM Tris-borate, 2mM EDTA, 7M urea, pH __ EK:__kv; CV: 200 V/cm (8kV, 7.5μA) *see Table 1 (p.29) for DNA fragment sizes*	UV 260nm	$\ominus\rightarrow\oplus$ 30 min	238/242 split 4 bp (Fig.2)

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[75] Karger	φX174 *Hae*III pBR322 *Msp*I pBR322 *Hae*III λ-*Hind*III 1-kbp ladder	Homebuilt CE, 40cm(20) x 75 μm i.d. hydrophilic coated FS 30 °C 100mM Tris-borate, 2mM EDTA, pH 8.4, 3% LPA (1.5-6% tested) EK:4s @ 75 V/cm (3kV); CV: 300 V/cm (12kV) *90 sec φX174 separation:* 3%T LPA, 40cm(20) x 25 μm i.d., CV: 700 V/cm (28kV, 19μA), 500 ng/mL EB	UV 260nm	⊖→⊕ 10 min 2-75 min	123/124 w/EB → 1bp
[99] Mathies	φX174 *Hae*III λ-*Hind*III 70-10,000bp	Homebuilt Array CE, 50cm(25) x 100 μm i.d. DB-17 22 °C 45mM Tris-borate, 1mM EDTA, pH 8.3, 1% HEC (MW 105,000) EK:10s @ 35 V/cm (1.75kV); CV: 125 V/cm (6.25 kV) 1 μM EB	LIF 488:590	⊖→⊕ 20 min 8x	271/281 split < 5 bp
[108] Chrambach	φX174 *Hae*III	Beckman P/ACE 2001 CE, 27cm(20) x 150 μm i.d. PA coated FS 40 °C 89mM Tris-borate, 2.5mM EDTA, pH __, 1.7% SeaPlaque AGAROSE EK:16s @ 1kV; CV: 5kV (185 V/cm)	UV 260nm	⊖→⊕ 12 min	271/281 Not ~30 bp
[122] Baba	1-kbp ladder 75-12,216 bp CGE vs. CE (HPLC also)	ABI 270A CE, 50cm(30) x 100 μm i.d. PA gel-filled 30 °C 50mM Tris-borate, 2.5mM EDTA, pH 8.0 EK:1s @ 5kV; CV: 10kV (200 V/cm, 9-11 μA) Waters Quanta 4000 CE, 50cm(42) x 100 μm i.d. PA coated FS __°C 50mM Tris-borate, 2.5mM EDTA, pH 8.0, 0.5% MC (Sigma, 4000cP) EK:10s @ 5kV; CV: 10kV (200 V/cm, 20 μA)	UV 260nm UV 254nm	⊖→⊕ 25 min ⊖→⊕ 20 min	506/517 baseline <10 bp 506/517 split ~15 bp
[135] Guttman	φX174 *Hae*III pBR322 *Msp*I 123-bp ladder	Beckman P/ACE 2000 CE, 47cm(40) x __μm i.d. PA gel-filled 25 °C 100mM Tris-borate, 2mM EDTA, pH 8.5, 1 μg/mL EB EK:5s @ 0.5psi; CV: 250 V/cm (11.8kV)	UV 254nm	⊖→⊕ 25 min	271/281 split 5 bp (Fig.2) 160/160 split
[144] Morris	φX174 *Hae*III	Homebuilt CE, 20cm(15.5) x 75 μm i.d. 9%T LPA gel-filled __ °C 90mM Tris-borate, 2mM EDTA, pH 8.2, 2 μg/mL propidium iodide (PI) EK:1s @ 6kV; PULSED FIELD: 325-400 V/cm dc, ac modulation 140%	LIF 543:610 HeNe laser	⊖→⊕ 25 min	271/281 baseline 3-4 bp
[145] Morris	φX174 *Hae*III	Homebuilt CE, 33cm(28.5) x 75 μm i.d. PA coated FS __ °C 89mM Tris-borate, 2mM EDTA, pH 8.2, 0.3-0.5% HPMC (Aldrich) EK:4s @ 4kV; CV: 180 V/cm (6kV) 3μg/mL EB or 5μg/mL PI	LIF 543:620 HeNe laser	⊖→⊕ 20 min	271/281 baseline 3-4 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[146] Yeung	ϕX174 *Hae*III	SP 1000 CE, 43cm(35) x 50 μm i.d. DB-1 10-40 °C 100mM Tris-borate, 2 mM EDTA, pH 8.2, 0.5% methyl cellulose 4000cp EK:1s @ 200 V/cm (8.6kV); CV: 400-700 V/cm (17-30kV)	UV 260nm	⊖→⊕ 3-9 min	271/281 split < 5 bp
[147] Guttman	ϕX174 *Hae*III pBR322 *Msp*I	Beckman P/ACE 2100 CE, 47cm(40) x 100 μm i.d. LPA gel-filled 20 °C 100mM Tris-borate, 2mM EDTA, pH 8.35 HD:5s @ 0.5psi; GRADIENT: 400-100 V/cm in 20 min	UV 254nm	⊖→⊕ 10 min	271/281 split 10 bp 160/160 split
[149] Watson	ϕX174 *Hae*III pBR322 *Hae*III ApoB & 500 bp PCR product	BioRad HPE 100 CE, 50cm(47.3) x 50 μm i.d. coated capillary? __ °C 100mM Phosphate, pH 5.7, 1.25% HPMC (50cP) EK:6s @ 160 V/cm (8kV); CV: 160 V/cm (8kV)	UV 260nm	⊖→⊕ 35 min	184/192 split 8 bp 271/281 Not
[150] Andrieu	ϕX174 *Hae*III 142,394,442 bp PCR products	Beckman P/ACE ?? CE, 37cm(30) x 100 μm i.d. LPA gel-filled __ °C Beckman LIFluor dsDNA 1000 kit containing thiazole orange EK:90s @ 5kV; CV: 7.4kV	LIF 488:530	⊖→⊕ 24 min	271/281 split 12 bp
[151] Butler/ McCord	100,200,400bp PCR products Quantitation	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 20 °C 100mM Tris-borate, 1mM EDTA, pH 8.7 w/ CsOH, 1% HEC (86-113cP), 1.27μM EB, 50 ng/mL YO-PRO-1, EK and HD...; CV: 15kV	LIF 488:520	⊖→⊕ 20 min	<10 bp
[152] Butler/ McCord	TH01 ladder and alleles (PCR products)	Beckman P/ACE 2050 CE, 37cm(30) x 50 μm i.d. DB-17 25 °C 100mM Tris-borate, 2mM EDTA, pH 8.2, 1% HEC (86-113cP), 50 ng/mL YO-PRO-1, EK:5s @ 1kV; Gradient: 0-5min 15kV, 5-10min 5kV	LIF 488:520	⊖→⊕ 10 min	195/198 split 3 bp
[153] Williams	100bp DNA ladder 123bp DNA ladder ϕX174 *Hae*III	ABI 270A-HT CE, 50cm(30) x 100 μm i.d. DB-17 30 °C 100mM phosphate, pH 8.0 OR 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5-0.75% HEC, 10 μM EB; EK:3s @ 100 V/cm; CV:100 V/cm (5kV)	UV 260nm	⊖→⊕ 40 min	271/281 split 10 bp
[154] Williams	SE33 ladder ϕX174 *Hinf*I 134 bp PCR product	Dionex CES1A CE, 48cm(40) x 75 μm i.d. μPAGE3™ (3%C, 3%T) amb μPAGE buffer (J&W Scientific), 10 μM EB EK:_____; CV: 210 V/cm (10kV)	UV 260nm	⊖→⊕ 60 min	240/244 split in SE33 4 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[155] Williams	φX174 *Hae*III φX174 *Hinf*I HUMTH01 ladder Marker XI-100bp TH01 PCR products mtDNA 136 bp	*CGE:*Dionex CES1A CE, __cm(40) x 75 μm i.d. μPAGE-3 (3%T,3%C) μPAGE buffer with 10 μM EB; EK:5s @ 7kV; CV: 210 V/cm __°C *DSE:*ABI 270A-HT CE, 70cm(50) x __ μm i.d. coated? FS or fused silica Dionex Nucleophor sieving buffer OR ABI DNA fragment analysis buffer with 20% urea and 10μM EB OR 100mM Phosphate, pH 8.0, 0.5% HEC, 10 μM EB with DB-17 EK:5s @ 7kV; CV: 210 V/cm 30 °C (see paper for the rest)	UV 260nm	⊖→⊕ 50 min (Fig.5) 22 min (Fig.18)	183/191 TH01 alleles baseline 4 bp
[157] Martin	φX174 *Hae*III 880 bp PCR product (sized at 790bp)	Beckman P/ACE 2100 CE, 57cm(50) x 100 μm i.d. DB-1 (0.1μm) 25 °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (H-7509, Sigma) EK:15s @ 10kV (after HD:11s water plug); CV: 10kV (175 V/cm)	UV 254nm	⊖→⊕ 20 min	271/281 split 10-12 bp
[192] Singhal	φX174 *Hae*III pGEM-3 DNA/ *Hinf*I,*Rsa*I,*Sln*I	Homebuilt CE, 80cm(50) x 75 μm i.d. fused silica 20 °C 50mM Tris-borate, 2mM EDTA, 7M urea, pH 8.85, 0.25-0.75% HEC, TBAP,etc. tested; HD: 20s (~8 nL); CV: 250 V/cm (20kV) (Aldrich)	UV 260nm Z-flow cell	⊕→⊖ 25 min	281/310 split 30 bp
[199] Gambari	pBR322 *Hae*III 299 bp PCR product	Beckman P/ACE 2050 CE, 47cm(40) x 100 μm i.d. fused silica 20 °C 100mM Tris-borate, pH 8.3, 0.5% HPMC, 10 μg/mL EB EK:40s @ 4kV; CV: 12kV (~10μA)	UV 254nm	⊖→⊕ 20 min	184/192 split ~10 bp
[201] Izumi	φX174 *Hae*III 832 bp PCR product	JASCO CE-800 CE, 50cm(20) x 100 μm i.d. DB-WAX __°C 70mM Tris-HCl, 14mM EDTA, pH 8.2, 0.25% glucomannan EK:1s @ 5kV; CV: 100 V/cm = 5kV (~40μA)	UV 260nm	⊖→⊕ 30 min	271/281 barely split 15 bp
[202] Righetti	1-kbp ladder pBR322 *Hae*III λ-*Hind*III	Waters Quanta 4000 CE, 40cm(__) x 100 μm i.d. LPA coated FS 24 °C 100mM Tris-borate, 2mM EDTA, pH 8.2, 4%T LPA (4,6,8,10% tested) EK:3s @ 4kV; CV: 4kV (100 V/cm, 10.5 μA)	UV 254nm	⊖→⊕ 45 min	184/192 split 8 bp
[216] Ulfelder	φX174 *Hae*III pBR322 *Msp*I 115 & 242 bp PCR product	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 (0.1μm) 25 °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (Sigma-4000cP) EK:10s @ 35 V/cm; CV: 175 or 350 V/cm 10μM EB *fast separation:* changed to 37cm capillary, 350 V/cm, 1.25μM EB	UV 260nm	⊖→⊕ 34 min 8 min (Fig.5)	271/281 split <10 bp 160/160 split

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[218] Baba	1-kbp ladder ϕX174 *Hae*III pBR322 *Hae*III CGE vs. CE (HPLC also)	*CGE:* ABI 270A CE, 50cm(30) x 100 μm i.d. PA gel-filled 25 °C 100mM Tris-borate, 2mM EDTA, pH 8.3, (3%T,0.5%C) EK:5s @ 5kV; CV: 13kV (260 V/cm, 15-25 μA) *CE:* Waters Quanta 4000 CE, 50cm(42) x 100 μm i.d. LPA coated FS 50mM Tris-borate, 2.5mM EDTA, pH 8.0, 0.5 or 0.7% MC EK:10s @ 5kV or 10kV; CV: 10kV (200 V/cm, 18-20 μA) 23-25 °C	UV 260nm UV 254nm	⊖→⊕ 15 min ⊖→⊕ 25 min	271/281 split 10 bp 271/281 Not ~30 bp
[229] Mensink	pBR322 *Hae*III 372 bp PCR product SSCP Analysis	Bio-Rad BioFocus 3000 CE, 31cm(__) x 75? μm i.d. PA coated FS 40mM Tris-acetate, 2mM EDTA, pH 8.3, 4% LPA (4%T,0%C) EK:10 or 20s @ 167 V/cm; CV: 250 V/cm (7.75kV) 25 °C	UV 260nm	⊖→⊕ 25 min	184/192 split ~10 bp
[230] Dovichi	ϕX174 *Hae*III 100 bp ladder 123 bp ladder λ-*Hind*III M13mp18 *Taq*I	Homebuilt CE, __cm(30) x 32 μm i.d. PA coated FS __ °C 89mM Tris-borate, 2mM EDTA, pH 8.2 (1xTBE), 0.4% HPMC (4000cP) EK:5s @ 100 V/cm; CV: 200 V/cm 100mM NaCl POPO-3, YOYO-3, and YOYO-1 were tested; bp:dye ratio examined 4-8% LPA compared with 0.2-0.8% HPMC	LIF 543:580 612:640 488:535	⊖→⊕ 15-60 min (Fig.4)	271/281 split HPMC ~15bp LPA ~ <5bp
[232] Righetti	pBR322 *Hae*III 1-kbp ladder λ-*Hind*III/*Eco*RI	Waters Quanta 4000 CE, 40cm(31) x 100 μm i.d. AAEE coated FS 24 °C 100mM Tris-borate, 2mM EDTA, pH 8.2, 10%T,0%C poly(AAEE) EK:3s @ 4kV; CV: 4kV (100 V/cm, 10.5 μA)	UV 254nm	⊖→⊕ 60 min	184/192 baseline ~4 bp
[234] Righetti	VNTR alleles 111 & 115 bp PCR products	Waters Quanta 4000E CE, 37cm(__) x 100 μm i.d. PA coated FS __ °C 100mM Tris-borate, 2mM EDTA, pH 8.3, 10 μM EB, 6%T LPA EK:10s @ 140 V/cm (5.2kV); CV: 100 V/cm (3.7kV)	UV 254nm	⊖→⊕ 30 min	111/115 split 4 bp
[235] Righetti	Triplet repeat 480 & 540 bp PCR products	Waters Quanta 4000 CE, 40cm(31) x 100 μm i.d. AAEE coated FS 22 °C 100mM Tris-borate, 2mM EDTA, pH 8.3, 8%T poly(AAEE) EK:4s @ 4kV; CV: 4kV (100 V/cm, 10 μA)	UV 254nm	⊖→⊕ 45 min	480/540 well resolved ~20 bp
[237] Bocek	1-kbp ladder ϕX174 *Hae*III 0.05-12.2 kbp	Beckman P/ACE 2001 CE, 27cm(20) x 150 μm i.d. PA coated FS 40 °C 89mM Tris-borate, 2.5mM EDTA, pH 8.3, 1.7% SeaPlaque AGAROSE EK:16s @ 1kV; CV: 185 V/cm (5kV)	UV 260nm	⊖→⊕ 12 min	271/281 Not 30 bp 506/517 split

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[247] Butler	400 & 436 bp PCR Products **Quantitation**	Beckman P/ACE 2050 CE, 27cm(20) x 50 μm i.d. DB-17 25 °C 100mM Tris-borate, 2mM EDTA, pH 8.2, 1% HEC (86-113cP, Aldrich) HD:45s @ 0.5psi; CV: 15kV (~22kV) 50 ng/mL YO-PRO-1	LIF 488:520	⊖→⊕ 4 min	~30 bp
[248] Butler/ McCord	PCR products TH01 alleles ~400 bp mtDNA **Quantitation**	Beckman P/ACE 2050 CE, 37cm(30) or 27cm(20) x 50μm i.d. DB-17 100mM Tris-borate, 2mM EDTA, pH 8.1, 1% HEC (86-113cP, Aldrich) HD:45s @ 0.5psi; CV: 15kV (22μA) or GRAD: 0-5.2min @ 15kV, 5.2-10min @ 5kV; 50 ng/mL YO-PRO-1; 25 °C	LIF 488:520	⊖→⊕ 3 min 10 min	3 min ~30bp 10 min 3bp
[250] Yeung	φX174 *Hae*III	Homebuilt 100 Capillary Array CE, 50cm(35) x 75 μm i.d. DB-1 __°C 100mM Tris-borate, 2mM EDTA, pH 8.2, 0.5% MC (Sigma-4000cP) EK:10s @ 100 V/cm (5kV); CV: 50 V/cm (2.5kV) 0.8μM TOTO-1	LIF 514:CCD	⊖→⊕ 65 min 100X	271/281 Not 30 bp
[262] Mathies	φX174 *Hae*III	Homebuilt Array CE, 50cm(25) x 100 μm i.d. DB-WAX __°C 40mM Tris-acetate, 1mM EDTA, pH 8.0, 0.75% HEC (MW 105,000) EK:10s @ 125 V/cm (6.25kV); CV: 125 V/cm (6.25kV) EB,TOTO,YOYO,TOTAB,9AA tested	LIF 488: 515-545 >590	⊖→⊕ 30 min	271/281 split 4-5 bp
[267] Ulfelder	PCR products 53,71,97,163 bp **Quantitation**	Beckman P/ACE 2200 CE, 47cm(40)/37cm(30) x 100μm i.d. PAA coated Beckman TBE buffer (4% LPA??); EnhanCE™ (TO) HD:10s @ 0.5psi or EK:10s @ 40 V/cm; CV: 200 V/cm 20 °C **compared CE to slab gels...**	UV 254nm LIF 488:530	⊖→⊕ 18 min	53/72 split 5-10 bp
[269] Yeung	φX174 *Hae*III λ-*Hind*III pBR322 *Hae*III pBR328 *Bgl*I/*Hin*fI	*CGE:* Homebuilt CE, 40cm(25) x 75 μm i.d. 30% HydroLink gel-filled 1mM borate, pH 9.1, 400 ng/mL EB; EK:1s @ 1kV; CV: 2kV __°C *CE:* Homebuilt CE, 60cm(50) x 50 μm i.d. DB-1 __°C 1mM borate, pH 8.5, 500 ng/mL EB, 0.5% HPMC (4000cP) and (15000cP-Serva); EK:1s @ 10kV; CV: 15kV	LIF 488:blue	⊖→⊕ 50 min (Fig.4) 25 min (Fig.5)	271/281 split 10 bp 184/192 split 8 bp
[271] Righetti	127 & 135 bp PCR products	Waters Quanta 4000E CE, 37cm(__) x 100 μm i.d. PA coated FS __°C 100mM Tris-borate, 2mM EDTA, pH 8.3, 10 μM EB, 6%T LPA EK:6-10s @ 165 V/cm (6.1kV); CV: 165 V/cm (6.1kV)	UV 254nm	⊖→⊕ 30 min	127/135 split 8 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[274] Colburn	1-kbp ladder ϕX174 *Hae*III	ABI 270A CE, 41cm(21) x 50 μm i.d. fused silica __°C SepraGene™ 500 Buffer from ABI (tris-borate system) HD:3s w/ vacuum; CV: 350 V/cm (14.3kV)	UV 260nm	$\oplus \rightarrow \ominus$ 13 min	271/281 Not 15-20 bp
[281] Karger	ϕX174 *Hae*III λ-*Hind*III ϕX174 *Hinc*II	Homebuilt CE, 50cm(25) x 75 μm i.d. fused silica 27°C 100mM Tris-borate, 2.5mM EDTA, pH 8.1, 7M urea, 0.1% SDS *(MECC)* HD:3-4 nL w/ siphoning; CV: 15 kV (300 V/cm, 18 μA)	UV 260nm	$\oplus \rightarrow \ominus$ 25 min	341/345?? 4 bp
[282] Karger	ϕX174 *Hae*III 1-kb ladder 500 bp PCR product	Homebuilt CE, 40cm(30) x 75 μm i.d. PA coated __°C 100mM Tris-borate, 2mM EDTA, pH 8.3, 3%T, 0.5%C PA gel-filled EK:0.5s @ 10kV; CV: 250 V/cm (10kV, 12.5μA) pulsed fields also	UV 260nm	$\ominus \rightarrow \oplus$ 20 min	271/281 split $<$10 bp (Fig. 1)
[290] Morris	ϕX174 *Hae*III	Homebuilt CE, 25cm(20) x 75 μm i.d. 3.5%T, 3.3%C gel-filled 22°C (rt) 90mM Tris-borate, 2mM EDTA, pH 8.3, 125 ng/mL EB EK:5s @ 3kV; PULSED FIELD: 180 V/cm dc (17 μA) at 78 Hz ac with 120% ac depth of modulation	LIF 325:580 HeCd laser	$\ominus \rightarrow \oplus$ 20 min	271/281 split 10 bp
[298] Guttman	ϕX174 *Hae*III	Beckman P/ACE 2000 CE, 27cm(20) x 100 μm i.d. DB-17 20-50°C 100mM Tris-borate, 2mM EDTA, pH 8.5, __%T LPA EK:2s @ 45mW; CV: 400 V/cm (10.8kV) OR CC: 20 μA	UV 254nm	$\ominus \rightarrow \oplus$ 10 min	271/281 split $<$10 bp
[302] Karger	123 bp ladder	Homebuilt CE, 40cm(30) x 75 μm i.d. polyvinylmethylsiloxanediol coated 100mM Tris-borate, pH 8.4, 3%T LPA __°C EK:2s @ 8kV; CV: 300 V/cm (12kV, 15 μA)	UV 260nm	$\ominus \rightarrow \oplus$ 15 min	1 x 10^6 plates ~10 bp ??
[309] Tsuji	ϕX174 *Hae*III 245 & 359 bp PCR products PCR-RFLP	ABI 270A CE OR Beckman P/ACE 2000 CE, 50cm(30) OR 37cm(30) x 100 μm i.d.; (100 ng/mL thiazole orange & 2mM EDTA in LIF buffer) 100mM Tris-borate, pH 8.3, 3%T,0.5%C PA gel-filled EK:2s @ 5kV; CV: 10kV 30°C	UV 260nm LIF 488:530	$\ominus \rightarrow \oplus$ 20 min	271/281 split (LIF) 10 bp
[317] Novotny	λDNA 8.3-48.5 kb	Homebuilt CE, 20-40cm(10-20) x 75 μm i.d. LPA coated __°C 45mM Tris-borate, 1mM EDTA, pH 8.1, 0.6% LPA (5-6,000,000 MW) EK:30s @ 25 V/cm; Pulsed: 25 V/cm sine-wave, 12 Hz	UV 260nm	$\ominus \rightarrow \oplus$ 45 min	good for kilobase DNA

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[326] Guttman	ϕX174 *Hae*III	Beckman P/ACE 2000 CE, 27cm(20 or 7) x 100 μm i.d. PA gel-filled FS 100mM Tris-borate, 2mM EDTA, pH 8.4, 50 °C EK:3s @ 3kV; CV: 600 V/cm (16.2kV)	UV 254nm	⊖→⊕ 6 min	271/281 baseline 10 bp
[328] Mitchelson	ϕX174 *Hae*III 126 bp PCR product SSCP Analysis	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 22 °C 90mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (4000cP), 4.8% glycerol, 3μM EB; EK:10s @ 175 V/cm; CV: 228 V/cm (13kV)	UV 260nm	⊖→⊕ 30 min	271/281 split 10 bp
[331] Williams	PCR products TH01 alleles 100 bp ladder	Dionex CES1A CE, 40cm (__) x __μm i.d. μPAGE3™ buffer, 3%C, 3%T PAA gel-filled μPAGE3™ EK: "nL volumes"; CV: 210 V/cm	UV 260nm	⊖→⊕ __ min	179/183 split 4 bp
[332] Tornar	ϕX174 *Hae*III PCR products D1S80 alleles	Beckman P/ACE 2100 CE, 47cm(40) x 100 μm i.d. DB-17 20 °C 100mM Tris-borate, pH 8.7 w/ CsOH, 1% HEC (Aldrich?), 500 ng/mL EB and 13 μg/mL YO-PRO-1; EK:___; CV: 9kV	LIF 488:520	⊖→⊕ 20 min	271/281 split <10 bp 16 bp D1S80
[354] Yeung	ϕX174 *Hae*III	Homebuilt CE, 60cm(22) x 75 μm i.d. 3%T LPA gel-filled 50mM Tris-borate, 1mM EDTA, pH 8.3 EK:3s @ 3.5kV; CV: 6kV (8 μA)	LIF 275:___ 248:___	⊖→⊕ 45 min	271/281 Not 40 bp
[359] Williams	100 bp ladder (XI) ϕX174 *Hinf*I 134 bp PCR product	Dionex CES1 CE, __cm(40) x __μm i.d. 3%T,3%C μPAGE gel-filled μPAGE Tris-borate and urea buffer (J&W Scientific), 10 μM EB EK:9s @ 7kV; CV: 225 V/cm	UV 260nm	⊖→⊕ 60 min	413/417 split 4 bp
[382] Sepaniak	ϕX174 *Hae*III pBR322 *Hinf*I pBR328 *Hinf*I	Homebuilt CE, 36cm(30) x 25,50,or 75 μm i.d. PA coated FS __ °C 45mM or 90mM Tris-borate-EDTA, pH __, 2.5μM EB, __% MC (20,000 or 100,000 MW); EK:20s @ 20 V/cm; CV:up to 1000 V/cm	LIF 325:570 543:570	⊖→⊕ 2-5 min	271/281 split 10 bp
[383] Tsuji	ϕX174 *Hae*III ϕX174 *Hinc*II pBR322 *Hapa*II 175 and 202 bp PCR product	Beckman P/ACE 2000 CE, 37cm(30) x 100 μm i.d. 3%T,0.5%C gel-filled 100mM Tris-borate, 2mM EDTA, pH 8.3, 100 ng/mL thiazole orange EK:2s @ 3.7kV; CV: 7.4kV (200 V/cm) 30 °C	LIF 488:530	⊖→⊕ 20 min	291/297 split 6 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[384] Guttman	φX174 *Hae*III 97 bp PCR product	Beckman P/ACE 2100 CE, 37cm(30) x 100 μm i.d. LPA gel-filled 20 °C Beckman eCAP™ dsDNA 1000 kit, various injection schemes explored HD:10s tris-acetate, 20s sample @ 0.5psi; CV: 7.4kV (200 V/cm)	UV254nm LIF 488:530	⊖→⊕ 20 min	271/281 split ~5 bp
[387] Morris	1-kb DNA ladder λ-*Hind*III	Homebuilt CE, 33cm(28) x 75 μm i.d. PA coated __ °C 89mM Tris-borate, 2mM EDTA OR 100mM Tris-50mM Acetic acid, 5.5mM EDTA, 0.01%-0.4% MC (4000cP), 3μg/mL EB EK:4s @ 2kV; CV: 180 V/cm (5.9kV) or pulsed fields	LIF 543:620	⊖→⊕ 24 min	506/517 split ~12-15 bp
[393] Yeung	φX174 *Hae*III	Homebuilt CE, 40cm(40) x 50 μm i.d. DB-1 (0.2μm) __ °C Phosphoric acid, pH 2.8 w/ NaOH, 0.5% MC (4000cP, Sigma) EK:5s @ 500 V/cm (20kV); CV: 20kV ? (500 V/cm)	LIF 275:fluor sheath flow	⊖→⊕ 20 min	271/281 ? > 20 bp
[396] Dionex Corp.	φX174 *Hae*III pBR322 *Msp*I	Dionex CESI CE, 50 cm(__) x 100 μm i.d. NucleoPhor coated __ °C Dionex NucleoPhor SB1.5kB Sieving Buffer, No EB EK:20s @ 20kV; CV: 10kV (200 V/cm)	UV 260nm	⊖→⊕ 30 min	271/281 split ~3 bp 147/147 split
[397] Dionex Corp.	φX174 *Hae*III pBR322 *Msp*I	Dionex CESI CE, 50cm(__) x 100 μm i.d. NucleoPhor coated __ °C Dionex NucleoPhor SB1.5kB Sieving Buffer, No EB EK:20s @ 20kV; CV: 10kV (200 V/cm)	UV 260nm	⊖→⊕ 30 min	271/281 split ~3 bp
[398] Dionex Corp.	φX174 *Hae*III pBR322 *Msp*I	Dionex CESI CE, 50cm(__) x 100 μm i.d. NucleoPhor coated __ °C Dionex NucleoPhor SB 1.5kb buffer EK:4s @ 1kV; CV: 12.5kV (250 V/cm)	UV 260nm	⊖→⊕ 22 min	271/281 split 5 bp 147/147 split
[399] Dionex Corp.	φX174 *Hae*III pBR322 *Msp*I ~400,650,1000bp PCR products	Dionex CESI CE, 20cm(__) x 100 μm i.d. NucleoPhor coated __ °C Dionex NucleoPhor SB 1.5kb buffer EK:5s @ 5kV; CV: 15kV (300 V/cm)	UV 260nm	⊖→⊕ 18 min	147/147 split 180/190 split ~10 bp
[400] ABI	100 bp ladder (XI) 314bp PCR product	ABI 270A-HT CE, 60cm(40) x 100 μm i.d. ? bare fused silica 30 °C ABI buffer (contains polyamines from wall coating) EK:80s @ 5kV; CV: 13 kV	UV 260nm	⊖→⊕ 18 min	300/314 bp ~15 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[431] Mathies	TH01 ladder & alleles ϕX174 *Hinc*II	Homebuilt Array CE, 50cm(25) x 75 μm i.d. PA coated FS 22 °C 45mM Tris-borate, 1mM EDTA, pH 8.3, 0.8% HEC (MW 438,000) EK:3s @ 80 V/cm (4kV); CV: 80 V/cm (4kV) 1μM 9-AA or 1μM TO	LIF 488:520 :590	$\ominus \rightarrow \oplus$ 18 min	191/195 split (8)/(9) 4 bp
[441] Ulfelder	ϕX174 *Hae*III 1-kbp ladder 97bp PCR product	Beckman P/ACE 2100 CE, 37cm(30) x 100 μm i.d. coated 20 °C eCAP™ dsDNA 1000 kit or LIFluor dsDNA 1000 kit w/ thiazole orange HD:10s @ 0.5psi; CV: 7.4kV (200 V/cm)	LIF 488:530 UV254nm	$\ominus \rightarrow \oplus$ 20 min	506/517 split 271/281 split 10 bp
[443] Morris	megabase DNA	Homebuilt CE, 22cm(18) x 75 μm i.d. LPA coated __ °C 45mM Tris-borate, 1mM EDTA, pH __, 3 μg/mL EB, 0.00375% HEC (438,000 MW) and 0.0020% PEO (8,000,000 MW); EK:15s @ 1kV; Pulsed: 100 V/cm + 14 Hz square wave, 250% modulation	LIF	$\ominus \rightarrow \oplus$ 13 min	great for megabase DNA
[445] Sepaniak	ϕX174 *Hae*III	Homebuilt CE, 36cm(15 or 30) x 50 μm i.d. LPA coated FS __ °C 45mM Tris-borate, 1mM EDTA, pH 8.5, 0.25% MC (MW 100,000) mixed concentrations of MC also used (size-selective gradients) EK:20s @ 18 or 9 V/cm; CV: 500 V/cm (18kV) 2.5μM EB	LIF 543:570	$\ominus \rightarrow \oplus$ 2-5 min	271/281 split 10 bp
[459] Aubert	ϕX174 *Hae*III Marker VI D1S80 alleles YNZ22 ladder	Beckman P/ACE 2100 CE, 57cm(50) x 50 μm i.d. DB-1 25 (20-35) °C 89mM Tris-borate, 2mM EDTA, pH 8.3, 0.5% HPMC, 10μM EB EK:5s @ 10kV; CV: 25kV →other PCR products examined including dystrophin multiplex...	UV 260nm	$\ominus \rightarrow \oplus$ < 10 min	220/234 barely ~20 bp
[467] Del Principe	pBR322 *Eco*RI 220,520, & 740bp PCR products	Bio-Rad HPE 100 CE, 50cm(__) x 50 μm i.d. BioRad coated __ °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (Sigma) EK:8-15s @ 180 V/cm (9kV); CV: 160 V/cm (8kV)	UV 260nm	$\ominus \rightarrow \oplus$ 20 min	PCR-RFLP ~30bp ??
[468] Bocek	123 bp ladder ϕX174 *Hae*III pBR322 *Msp*I	Homebuilt CE, 410cm(300) x 75 μm i.d. LPA coated 50 (10-70) °C 89mM Tris-borate, 2.5mM EDTA, pH __, 1% agarose (SeaPrep & AcrylAide) HD:20s @ 10cm; CV: 2kV	UV 260nm	$\ominus \rightarrow \oplus$ 45 min	271/281 Not ~30 bp
[470] Righetti	95 & 98bp PCR products	Waters Quanta 4000E CE, 37cm(30) x 100 μm i.d. PA coated __ °C 100mM Tris-borate, 2mM EDTA, pH 8.3, 6%T, 0%C LPA EK:6-10s @ 140 V/cm (5.2kV); CV: 140 V/cm (5.2kV) or 100 V/cm	UV 254nm	$\ominus \rightarrow \oplus$ 40 min	95/98 split ~3 bp No EB!

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[471] Mathies	ϕX174 *Hae*III DQα (242 bp) PCR product	Homebuilt CHIP, 4.5cm(__) x 50 μm i.d. LPA coated channels __°C 40mM Tris-acetate, 1mM EDTA, pH 8.2, 0.75% HEC (MW 438,000), 1μM Thiazole orange or 0.1μM TO6 EK:5s @ 180 V/cm; CV: 180 V/cm	LIF 488:530	$\ominus\rightarrow\oplus$ 2-7 min	271/281 split 10 bp
[472] Mitchelson	ϕX174 *Hae*III	Beckman P/ACE 5500 CE, 57cm(50) x 100 μm i.d. DB-17 22°C 90mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (4000cP, Sigma) 0-7.5% glycerol EK:15s @ 175 V/cm (10kV); CV: 228 V/cm (13kV)	UV 260nm	$\ominus\rightarrow\oplus$ 10-35min	271/281 Not ~20 bp
[492] Sunzeri	ϕX174 *Hae*III 115 (HIV) & 242 bp PCR products	Microphoretic Systems Model 1200 CE, 50cm(__) x 100 μm i.d. DB-17 or DB-WAX; ABI SepraGene 500 Buffer w/ 100 μg/mL Hoechst 33258 dye EK:15s @ 6kV; CV: 7.7kV __°C	UV 260nm FL 457nm	$\ominus\rightarrow\oplus$ 30 min	271/281 Not 40 bp
[494] Mensink	312 & 362 bp PCR products Quantitation	BioRad BioFocus 3000 CE, 31cm(__) x 75 μm i.d. PA-coated 25°C 40mM Tris-acetate, 2mM EDTA, pH 8.3, 4% LPA EK:10s @ 280 V/cm (8.7kV); CV: 425 V/cm (13.2kV)	UV 260nm	$\ominus\rightarrow\oplus$ 9 min	312/362 split < 50 bp
[521] Lundberg	236/257 & 401/426 bp PCR products Quantitation	Beckman P/ACE 2100 CE, 37cm(30) x 100 μm i.d. DB-17 (0.1μm) 25°C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC (4000cP), 8.6μM EB; EK:10s @ 2kV; GRAD: 0-1 min @ 2kV, 1-21 min @ 6.5kV	UV 260nm	$\ominus\rightarrow\oplus$ 20 min	401/426 split < 25 bp
[522] Liu	ϕX174 *Hae*III pBR322 *Hae*III 307bp PCR product	Beckman P/ACE 2100 CE, 47cm(40) x 75 μm i.d. coated capillary 22°C Beckman eCAP™ dsDNA-1000 gel, 10μM EB EK:20s @ 3kV or HD:20s @ 0.5psi; CV: 625 V/cm (29.4kV)	LIF 543:600 HeNe laser	$\ominus\rightarrow\oplus$ 11 min	271/281 split 10 bp 123/124 split
[523] Gambari	pBR322 *Hae*III ~300 bp (+ probe) PCR products	Beckman P/ACE 2050 CE, 47cm(40) x 100 μm i.d. coated ? 20°C 100mM Tris-borate, pH 8.3, 0.5% HPMC, 10 μg/mL EB EK:20s @ 4kV; CV: 12kV (~10μA)	UV 254nm	$\ominus\rightarrow\oplus$ 20 min	184/192 split 8 bp
[527] Wenz	pBR322 *Msp*I ϕX174 *Hae*III 271,281,310 bp PCR products	ABI 270A-HT CE, 70cm(50) x 75 μm i.d. uncoated FS 30 (10-60)°C ABI DNA Fragment Analysis Reagent, 3-4% polymer EK:5s @ 140 V/cm (9.8kV); CV: 157-357 V/cm (11-25kV) *Fast Method:* 50cm(30) x 75 μm i.d.; 3% polymer; CV: 240 V/cm; 26°C	UV 260nm	$\ominus\rightarrow\oplus$ 12 min 25 min	271/281 split <10 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[529] Gambari	pBR322 *Hae*III 289 bp PCR product hybridization	Beckman P/ACE 2050 CE, 47cm(40) x 100 μm i.d. FS deactivated 25 °C 100mM Tris-borate, EDTA??, pH 8.3, 0.5% HPMC (source?), 7.5 μg/mL EB; EK:90s @ 4kV; CV: 12kV (~10μA)	UV 254nm	⊖→⊕ 22 min	184/192 split ~10 bp
[530] Hjerten	BioRad RF digest 42-1746 bp	Bio-Rad BioFocus 3000 CE, 24cm(19.3) x 75 μm i.d. LPA coated FS 40mM Tris-acetate, 2mM EDTA, pH 7.5, 1% SDS, 1% methoxylated agarose; EK:20s @ 2kV; CV: 2kV 18 °C	UV 210nm?	⊖→⊕ 40 min	222/249 split 15-20 bp
[531] Righetti	PCR products 116,...,284 bp PCR-RFLP	Waters Quanta 4000E CE, 37cm(30) x 100 μm i.d. LPA coated __ °C 100mM Tris-borate, 2mM EDTA, pH 8.3, 6% LPA, 10 μM EB EK:6-10s @ 6.1kV; CV: 165 V/cm (6.1kV)	UV 254nm	⊖→⊕ 40 min	167/176 split 9 bp
[532] Sunzeri	115bp & 242bp PCR products ϕX174 *Hae*III multiplex PCR	Microphoretic Systems Model 1200 CE, 52cm(__) x 100 μm i.d. DB-17 ABI SepraGene 500 Buffer; also tried SDS buffer and CTAB buffer with urea; EK:__s @ 6kV; CV: 7.3kV (140 V/cm) __ °C	UV ___nm	⊖→⊕ 30 min	271/281 not ~30 bp
[533] Zhu	BioRad DNA digest 88,...,1746 bp 123 bp ladder	Bio-Rad HPC 100 CE, 50cm(__) x 50 μm i.d. coated FS __ °C 89mM Tris-borate, 2mM EDTA, pH 8.0, 7M urea, 0.5% HPMC-4000cP EK:8s @ 8kV; CV: 8kV	UV 260nm	⊖→⊕ 25 min	222/249 split 27 bp
[534] Karger	ϕX174 *Hae*III	Homebuilt CE, 40cm(20) x 75 μm i.d. LPA coated __ °C 100mM Tris-borate, 2mM EDTA, pH 8.3, 3%T LPA EK: _____; CV: 300 V/cm (12 kV, 16 μA) studied coiled columns	UV 260nm	⊖→⊕ 9 min	271/281 split ~5 bp (Fig. 7)
[557] Dovichi	ϕX174 *Hae*III	Homebuilt CE, 28.6cm(__) x 32 μm i.d. ___coated __ °C 89mM Tris-borate, 2mM EDTA, pH 8.3, 100mM NaCl, 0.4% HPMC or 5%T LPA; EK:____; CV: 100 V/cm (2.9kV) or 200 V/cm (5.8kV)	LIF 594:___	⊖→⊕ 22 min	271/281 Not ~40 bp
[558] Mitchelson	ϕX174 *Hae*III PCR products for SSCP analysis	Beckman P/ACE 5050 CE, 57cm(50) x 100 μm i.d. DB-17 22 °C 90mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC-4000cP, 3 μM EB, 4.8% glycerol; EK:13s @ 175 V/cm (10kV); CV: 228 V/cm (13kV)	UV 260nm	⊖→⊕ 20 min	??? (like [472])

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[559] Butler/ McCord	TH01 allelic ladder and samples with 150 & 300bp PCR products	Beckman P/ACE 2050 CE, 27cm(20) x 50 μm i.d. DB-17 25 °C 100mM Tris-borate, 2mM EDTA, pH 8.2, 1% HEC (86-113cP), 500 ng/mL YO-PRO-1; EK:5s @ 1kV; CV: 5kV (~6.7μA)	LIF 488:520	⊖→⊕ 10 min	195/198 split 3 bp <0.3bp SD
[563] Mitchell	φX174 *Hae*III 128 bp & 157 bp PCR products **Quantitation**	ABI 270A-HT CE, 44cm(__) x 50 μm i.d. bonded phase, glass capillary 100mM glycylglycine, pH 8.0, DNA fragment analysis reagent (LPA?) EK:5s @ 5kV; CV: 12kV 30 °C	UV 260nm	⊖→⊕ 24 min	271/281 not ~30 bp
[564] Fasco	φX174 *Hae*III multiplex competitive PCR products	Beckman P/ACE 2200 CE, 44cm(37) x 100 μm i.d. Beckman coated __ °C 89mM Tris-borate, 2mM EDTA, pH 8.5, 0.5% HPMC-4000cP (?), 67 nM YOYO-1; **HD:12s @ 20psi (YOYO-1)** HD:12-18s @ 0.5psi (sample) CV: 200 V/cm (9.4kV)	LIF 488:___	⊖→⊕ 30 min	271/281 split < 10 bp
[565] Barron	λ-*Hind*III φX174 *Hae*III	Homebuilt CE, 50cm(35) x 51μm i.d. **uncoated and PAA coated** 30 °C 89mM Tris-borate, 5mM EDTA, pH 8.15, 0.15% (below C*) HEC 90,000-105,000 MW; HD: ~3nL; CV: 265 V/cm (8.5μA)	UV 260nm	⊕→⊖ 24 min	271/281 split ~15 bp (Fig. 1b)
[577] Righetti	PCR products 59, 295, 354 bp pBR322 *Hae*III	Quanta 4000E CE, 37cm(__) x 100μm i.d. AAEE coated __ °C 89mM Tris-borate, 2mM EDTA, pH 8.3, 6% LPA EK:10-20s @ 100 V/cm; CV: 100 V/cm	UV 254nm	⊖→⊕ 40 min	10-20 bp ??
[586] Morris	λ-*Hind*III 1kb ladder	Homebuilt CE, 20cm(15) x 75μm i.d. _____coated __ °C 89mM Tris-borate, 2mM EDTA, pH 8.3, 7.5% LPA, 2 μg/mL PI EK:2.5s @ 2kV; **Pulsed field**: 0-6min, -250 V/cm DC; 6-20min, 350 V/cm AC; 20-22min, 250 V/cm; 22-30min, 350 V/cm; AC: 5-100Hz random frequency square waves	LIF ___nm	⊖→⊕ 25 min	506/516 ~10 bp
[587] Brunk	PCR-amplified SSU rRNA; PCR-RFLP of 800bp	Beckman P/ACE 2050 CE, 57cm(50) x 100 μm i.d. DB-17 __ °C 89mM Tris-borate, 2mM EDTA, pH 8.3, 0.5% HPMC-4000cP EK:10s @ 35 V/cm (2kV); CV: 175 V/cm (10kV)	LIF 488:___	⊖→⊕ 25 min	?
[588] Karger	φX174 *Hae*III **plasmid digestion**	Homebuilt CE, 60cm(30) x 50 μm i.d. LPA coated __ °C 180mM Tris-borate, 4mM EDTA, pH 8, 6%T LPA EK:10s @ 300 V/cm (18kV); CV: 300 V/cm (18kV)	UV 260nm	⊖→⊕ 14 min	271/281 split 10 bp

Reference	Sample	Conditions: Capillary, Buffer, Additives, Voltage	Detection	Speed	Resolution
[639] Oto	$(CA)_n$ repeats PCR products	Bio-Rad BioFocus 3000 CE, 36cm(__) x 50 μm i.d. PA coated 30 °C Bio-Rad PCR Product Analysis Buffer™ HD:1s @ 414 kPa; CV: 222 V/cm (8kV)	UV 260nm	⊖→⊕ 11 min to 300bp	CA repeats ??
[640] Righetti	pBR322 *Hae*III	Waters Quanta 4000E CE, 37cm(__) x 100 μm i.d. AAEE coated __°C 89mM Tris-borate, 2mM EDTA, pH 8.3, 6% short-chain PAA EK:6s @ 100 V/cm (3.7kV); CV: 100 V/cm (3.7kV)	UV 254nm	⊖→⊕ 60+ min	184/192 split 8 bp
[646] Karger	ϕX174 *Hae*III pBR322 *Msp*I pBR322*Hae*III 100 bp ladder 123 bp ladder 1 kbp ladder	Homebuilt CE, 50cm(40) x 75μm i.d. PAA coated __°C 89mM Tris-borate, 1mM EDTA, pH __, 6% LPA EK:5s @ 100 V/cm; CV: 300 V/cm (15kV) Dyes examined: EB, TO, POPO-3, EthD, TOTO-3 **examined sequence dependent migration behavior**	UV 260nm	⊖→⊕ 37 min	271/281 split <5 bp ??
[647] Bocek	ϕX174 *Hae*III restiction digests of bacteriophages	Homebuilt CE, 61cm(50) x 100μm i.d. LPA coated 30 °C 89mM Tris-borate, 2.5mM EDTA, 2% SeaPrep agarose EK:8s @ 10kV; CV: 10kV (164 V/cm)	UV 260nm	⊖→⊕ 45 min	271/281 split ~10 bp
[648] Oto	73 & 325 bp PCR products	BioRad BioFocus 3000 CE, 36cm(__) x 50μm i.d. LPA coated 30 °C 267mM Tris-borate, 1mM EDTA, pH 8.3, BioRad polymer additives HD:__s @ 60psi/sec; CV: 222 V/cm	UV 260nm	⊖→⊕ 10 min	??
[649] Bocek	ϕX174 *Hae*III	BioRad BioFocus 3000 CE, 54cm(50) x 100μm i.d. LPA coated 30 °C 89mM Tris-borate, 2.5mM EDTA, 2% SeaPrep agarose HD:__s @ 20psi or EK:8s @ 7kV; CV: 10kV (295 V/cm)	UV 260nm	⊖→⊕ 38 min	271/281 split 10 bp
[650] Evans	ϕX174 *Hae*III thymocytes ladder fragments	Beckman P/ACE 2050 CE, 57cm(50) x 75μm i.d. uncoated 25 °C 50mM KH_2PO_4, 0.5mM EDTA, pH 5.0, 400 nM YO-PRO-1, 0.5% HEC (Aldrich); HD:90s @ 0.5psi; CV: 10kV (175 V/cm)	LIF 488:520	⊖→⊕ 30 min	271/281 split 10 bp
[652] Mathies	ϕX174 *Hae*III TH01 alleles	Capillary Array Microchip, 1-3.5cm x 50μm channels 0.75% HEC (105,000 MW), 1.0 μM TO (9%T LPA, 7M urea for TH01) EK:__s @ __kV; CV: up to 250 V/cm	LIF 488:530	⊖→⊕ 2-5 min >20s	271/281 split 10 bp 9.3/10 split

APPENDIX 2

TROUBLESHOOTING GUIDE

Beckman P/ACE 2050 CE

I. Most Common Problems

A. No Current (when voltage is applied)

1. Capillary is plugged - attach syringe to the inlet and push water through; if water droplet does not form at the outlet then remove a small portion of both capillary ends and repeat syringe purge

2. Capillary is broken - replace capillary

B. Poor Resolution

1. Bad capillary - replace capillary

2. Wall adsorption - rinse capillary with methanol and buffer (10 min each) and repeat run; if no significant improvement in resolution, replace capillary

C. No Peaks Detected

1. Laser is not turned on (Error 33) - turn it on!

2. Fiber optic connection is dirty - clean out any dust or other particles

3. Intercalating dye was not placed in the buffer - prepare new run buffer

D. Vial Sticking (Error 11 or 12) - Silcon rubber vial tops have become sticky from the buildup of HEC buffer - rinse both the piercing levers and the vial tops

E. Error Messages

1. Error 18 - voltage leak; make sure the lid is down and the interlock button is pushed in

2. Error 11 - inlet tray position error; vials are getting caught and need to be cleaned; "f50" to set autosampler tray to the home position

3. Error 33 - low excitation light; turn on the laser (or replace)

4. Error 20 - low coolant; add 10-15 mL of coolant

II. Instrument Maintenance

A. Overnight/Long Term Storage

1. Rinse the capillary with water and place both ends in water vials

2. Turn off the laser

B. Daily Cleaning

1. Vial caps - rinse with water

C. Weekly Cleaning - spring-loaded piercing lever which the vials caps touch

1. Remove cover, detector, capillary catridge, and catridge protector.

2. Scrub polymer residue from the spring-loaded plastic piece and rinse well with water.

3. Replace all of the pieces in reverse order.

III. Other Considerations

A. Buffer Vials - the buffer must have no bubbles at the top of the vial as these can be injected into the capillary and prevent the flow of current; bubbles may be removed from the viscous polymer buffer with a transfer pipet

B. Humidity - during the summer months (in Virginia) a dehumidifier was found necessary; operating above 65-70% humidity is not recommended in order to avoid a corona discharge (i.e., arcing between electrodes and water condensation)

APPENDIX 3

PREPARING CE FOR CASEWORK AND THE COURTROOM

To the best of my knowledge, capillary electrophoresis has not been introduced to the courtroom. However, forensic research using CE has been conducted for analysis of illicit drugs [58,293,296,503,574,578,664], gun shot residue [137,295], explosive residue [136,138,485], and now PCR-amplified DNA [152,559]. These forensic applications of CE have been reviewed [56,248]. Ion analysis methods developed by Hargadon and McCord [138] have been used to confirm explosive residue analyzed by ion chromatography, although the cases were settled out of court.

It is not anticipated that there will be a court admissibility issue with CE due to the fact that it is an electrophoretic process and is similar to the separation process utilized in RFLP protocols. However, several issues need to be considered as CE is validated for DNA typing in forensic casework. It should be recognized that the high volume of samples which can be handled at a single time with CE requires the constant vigilance of the operator. Methods and checks need to be developed so that samples will not be switched, either in preparation, in loading the autosampler, in filling out the sample spreadsheet on the computer, or in analyzing the data. When dozens of samples are being rapidly processed in a routine setting, a careless mistake may not be caught using the present procedures. These sample identification problems may be diminished in several ways: (a) the injection could be performed from a microtiter plate format, in combination with robotic sample preparation and thermal cycling, which would minimize user interaction; (b) a barcode reader could

be used to scan a unique label on each sample, as it is lifted up to be injected, with the sample i.d. being electronically recorded with the electropherogram and the final results; (c) computer software could be implemented for automatic DNA fragment sizing (based upon internal standards) and genotype designation (based upon appropriate allelic ladders); and (d) quality assurance/quality control samples could (and should) be blindly tested on a regular basis.

Data storage will quickly become an issue when routine processing begins. For example, a 15-minute run, with data points collected at a rate of 5 Hz, generates approximately 100,000 bytes of information (on P/ACE windows software). While this does not seem significant for an individual sample, for routine analysis it quickly adds up to almost half of a megabyte of information generated every hour. At higher data collection rates (e.g., 10-20 Hz), such as those required for rapid separations (e.g., under 5 minutes), the computer storage needed will be even greater. File maintenance protocols will have to be designed for regular backup and hard drive purging.

Changing buffer vials after every run, to avoid buffer depletion effects, is not an efficient method for routine analysis and reduces the number of samples which can be loaded into the autosampler. If it becomes desirable to change buffer vials often (e.g., every run), the design of smaller buffer vials (less than the 4 mL currently used in the Beckman) may be desirable.

As not all human identity testing laboratories will have access to or the need for a CE, universal consistency of typing will need to be demonstrated against

procedures and protocols used in other laboratories. A preliminary validation has been performed with a direct comparison of CE results to silver-stained polyacrylamide gels [559].

A calibration procedure should be performed to ensure that everything is working fine. For example, a restriction digest could be run prior to the PCR samples to confirm that the capillary is providing adequate resolution and the proper sensitivity. The frequency of this calibration should be at least once a day, or every 30-40 runs when numerous samples are being processed. In addition, the stable threshold of each parameter should be determined, i.e., where and under what conditions does the system break down. Issues to consider include intercalating dye storage time, capillary degradation, uniform buffer preparation, and injection artifacts.

Thresholds should be set as to what constitutes a match between alleles and the allelic ladder. If peaks do not match exactly (and they almost never will because of slight variations between runs), how should this result be reported? What should the match criteria be and how should they be determined? This work used 50 runs to define the system standard deviation with a match window existing over $\pm 3.3\sigma$ (99.9% confidence). Should more separations be used to define the precision window? Should a floating bin approach be taken with only sizing the allele fragments as some are doing for the GENESCANNER (Ron Fourney, TWGDAM talk, June 1995)? Or should allele designations always be made against an allelic ladder, as recommended by Smith [474]?

Lastly, evaluation and implementation of new technologies (e.g., microchips)

and/or CE systems must be ongoing. Separation matrices which rival or exceed those currently used may become available at some future time and should be considered. Commercially available materials should also be evaluated in order to give uniformity across laboratories. The biotechnology field is evolving rapidly with techniques which can revolutionize DNA typing procedures. This work is by no means the final solution to DNA typing but rather a foundation for the future.

The Direction of the CE/DNA Field

CE has yet to gain a substantial foothold in the molecular biology or the DNA typing communities, primarily because of the lack of quantitative CE methods, such as those described in this work, and poor sample throughput. Sample processing speed may be increased in several ways with CE: (1) more rapid single sequential runs [152,382], (2) multiple wavelength analysis, where several samples and/or standards are simultaneously injected and separated but resolved spectrally with different color fluorophores [431], and (3) multiple parallel capillaries where 8 [99] or even 100 [250] samples are separated simultaneously. The future will probably include some combination of all three methods. A commercial capillary array system with multiple color detection is currently being developed. Miniaturization will also aid in speed and throughput. Several groups are designing miniature CE systems on microchips which will allow rapid separations on a nanoscale [143,471,652,656]. It is anticipated that work in all of these areas will be valuable in rapid DNA typing, in many cases building upon the work described here.

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VITA

John Marshall Butler was born in Pomona, California, on April 1, 1969. He is the oldest of Doug and Marsha Butler's seven children. An early interest in puzzle solving led John to investigating careers in science and law. At age 15, he became interested in forensic science and pursued the fields of biology and chemistry with that in mind. In 1990, a friend introduced him to forensic DNA typing with Joseph Wambaugh's book *The Blooding*, which describes the use of genetic fingerprinting to solve a murder/rape case in England. These early interests have led to this work.

John graduated first in his class at Maryville High School (Maryville, Missouri) in 1987. He attended a year of college at Brigham Young University (Provo, Utah) before serving two years as a missionary for the Church of Jesus Christ of Latter-day Saints in the Boston, Massachusetts area. After another two years at BYU, John began graduate studies at the University of Virginia (Charlottesville, Virginia) in the analytical chemistry. Under the direction of Professor Ralph Allen and the tutelage of Dr. Bruce McCord, John has worked for the past two years in the FBI Laboratory's Forensic Science Research Unit located at the FBI Academy (Quantico, Virginia). The development of new techniques for rapid DNA typing, which are described in this work, is a fulfillment of an early quest.

In 1994, John married Terilynne Wright of Charlottesville, Virginia.

John has accepted a postdoctoral position in the DNA Technologies Group at the National Institute of Standards and Technology (Gaitherburg, Maryland) and will continue developing techniques for rapid DNA analysis.