Review

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Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis

DNA typing with short tandem repeat (STR) markers is now widely used for a variety of applications including human identification. Capillary electrophoresis (CE) instruments, such as the ABI Prism 310 and ABI 3100 Genetic Analyzers, are the method of choice for many laboratories performing STR analysis. This review discusses issues surrounding sample preparation, injection, separation, detection, and interpretation of STR results using CE systems. Requirements for accurate typing of STR alleles are considered in the context of what future analysis platforms will need to increase sample throughput and ease of use.

Keywords: Capillary electrophoresis / DNA typing / Forensic science / Review / Short tandem repeat
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CE and CEC

Contents

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1 Introduction

1.1 General aspects

The law enforcement community has greatly benefited from recent developments in the area of DNA testing. Forensic laboratories may now match minuscule amounts of biological evidence from a crime scene to the perpetrator and can reliably exclude falsely accused individuals. In the past two decades, numerous advances in DNA testing technologies have occurred, most notably among them the development of polymerase chain reaction (PCR)-based typing methods [1–2].

Today, the forensic DNA typing community has standardized on the use of short tandem repeat (STR) markers [1–4]. In November 1997, the Federal Bureau of Investigation (FBI) selected 13 STR markers to serve as the core of its Combined DNA Index System (CODIS) [5]. These markers are CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 (Table 1). Multiplex PCR amplification of all or a subset of these STR markers is possible with a variety of commercial STR kits using spectrally resolvable fluorescent dyes (Table 2). The availability of commercial STR kits has greatly simplified the use of STRs in recent years and aided the

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Table 1. Information on 13 STR markers used in the FBI's CODIS DNA database and other STR markers contained in commercial kits

Locus name	Chromosomal location	Repeat motif	GenBank accession	Allele range[a)]	Number of alleles seen[b)]
CSF1PO	5q33.1 c-fms proto-oncogene, 6th intron	TAGA	X14720	6–16	15
FGA	4q31.3 α-Fibrinogen, 3rd intron	CTTT	M64982	15–51.2	69
TH01	11p15.5 Tyrosine hydroxylase, 1st intron	TCAT	D00269	3–14	20
TPOX	2p25.3 Thyroid peroxidase, 10th intron	GAAT	M68651	6–13	10
VWA	12p13.31 von Willebrand factor, 40th intron	[TCTG][TCTA]	M25858	10–24	28
D3S1358	3q21.31	[TCTG][TCTA]	NT_005997	9–20	20
D5S818	5q23.2	AGAT	G08446	7–16	10
D7S820	7q21.11	GATA	G08616	6–15	22
D8S1179	8q24.13	[TCTA][TCTG]	G08710	8–19	13
D13S317	13q31.1	TATC	G09017	5–15	14
D16S539	16q24.1	GATA	G07925	5–15	10
D18S51	18q21.33	AGAA	L18333	7–27	43
D21S11	21q21.1	Complex [TCTA][TCTG]	AP000433	24–38	70
Other STRs included in kits from Applied Biosystems or Promega					
Penta D	21q22.3	AAAGA	AP001752	2.2–17	14 alleles
Penta E	15q26.2	AAAGA	AC027004	5–24	21 alleles
D2S1338	2q35	[TGCC][TTCC]	G08202	15–28	14 alleles
D19S433	19q12	AAGG	G08036	9–17.2	15 alleles
SE33	6q15	AAAG	V00481	4.2–37	>50 alleles

a) Numbers in this column refer to the number of repeat units present in the alleles.
b) See Appendix 1 in [1]

development of large and effective DNA databases [6]. A report by the National Commission on the Future of DNA Evidence [7] concludes that STR typing will likely be the primary means of forensic DNA analysis for the next 5–10 years because of the need for consistency in national and international DNA databases. STR markers offer a number of advantages over previously used methods for DNA typing including the ability to obtain results from degraded DNA samples and extremely small amounts of DNA [1]. The process is fairly rapid and results may routinely be obtained in less than one working day.

Figure 1 illustrates how an STR marker within a DNA template is targeted with a forward and reverse PCR primer that anneal on either side of the repeat region. One of the primers is labeled on the 5'-end with a fluorescent dye that enables detection of the resulting PCR product following amplification. The position of the primers defines the overall PCR product size as does the number of repeats present in the STR region. PCR products are separated by size and dye color using electrophoresis followed by laser-induced fluorescence with multiwavelength detection. An internal standard, containing DNA fragments of known size and labeled with a different dye color, is typically coelectrophoresed with each sample to calibrate sizes from run to run. The collected data in the form of multicolored electropherograms are analyzed by software that automatically determines STR allele sizes based on a standard curve produced from the internal size standard. STR genotyping is performed by comparing the allele sizes in each sample to the sizes of alleles present in an allelic ladder, which contains common alleles that have been previously sequenced [8]. On a capillary electrophoresis (CE) system, the allelic ladder is

Table 2. Commonly used STR kits for analysis on ABI Prism 310 Genetic Analyzer

STR kit name	Source	Dye color	STR markers amplified in kit (shown in order of increasing PCR product size)
AmpFlSTR® Profiler Plus™	Applied Biosystems	B	D3S1358, VWA, FGA
		G	Amelogenin, D8S1179, D21S11, D18S51
		Y	D5S818, D13S317, D7S820
AmpFlSTR COfiler™	Applied Biosystems	B	D3S1358, D16S539
		G	Amelogenin, TH01, TPOX, CSF1PO
		Y	D7S820
AmpFlSTR SGM Plus™	Applied Biosystems	B	D3S1358, VWA, D16S539, D2S1338
		G	Amelogenin, D8S1179, D21S11, D18S51
		Y	D19S433, TH01, FGA
AmpFlSTR Identifiler™ (5-dyes)	Applied Biosystems	B	D8S1179, D21S11, D7S820, CSF1PO
		G	D3S1358, TH01, D13S317, D16S539, D2S1338
		Y	D19S433, VWA, TPOX, D18S51,
		R	Amelogenin, D5S818, FGA
AmpFlSTR SEfiler™ (5-dyes)	Applied Biosystems	B	D3S1358, VWA, D16S539, D2S1338
		G	Amelogenin, D8S1179, SE33
		Y	D19S433, TH01, FGA
		R	D21S11, D18S51
PowerPlex® 1.2	Promega	B	D5S818, D13S317, D7S820, D16S539
		Y	VWA, TH01, Amelogenin, TPOX, CSF1PO
PowerPlex 16	Promega	B	D3S1358, TH01, D21S11, D18S51, Penta E
		G	D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D
		Y	Amelogenin, VWA, D8S1179, TPOX, FGA
PowerPlex ES	Promega	B	D3S1358, TH01, D21S11, D18S51
		G	SE33 (ACTBP2)
		Y	Amelogenin, VWA, D8S1179, FGA
PowerPlex Y	Promega	B	DYS391, DYS389I, DYS439, DYS389II
		G	DYS438, DYS437, DYS19, DYS392
		Y	DYS393, DYS390, DYS385 a/b
Y-PLEX™ 6	ReliaGene Technologies	B	DYS393, DYS19, DYS389II
		Y	DYS390, DYS391, DYS385 a/b
Y-PLEX 5	ReliaGene Technologies	B	DYS389I, DYS389II
		G	DYS439
		Y	DYS438, DYS392
Y-PLEX 12	ReliaGene Technologies	B	DYS392, DYS390, DYS385 a/b
		G	DYS393, DYS389I, DYS391, DYS389II
		Y	Amelogenin, DYS19, DYS439, DYS438

An internal size standard is typically run in the fourth or fifth dye position. Dye colors, blue (B), green (G), yellow (Y), or red (R). See [78] for more information on the Y-STR loci and kits.

run along with the internal size standard in one injection, and sample alleles with the same internal size standard are run in subsequent injections on the capillary in a sequential fashion [9].

In order to accurately genotype STR markers using multicolor fluorescence detection, a separation and detection technique must exhibit the following characteristics: (i) Methods for reliable sizing over a 75–500 bp size range; (ii) high run-to-run precision between processed samples to permit comparison of allelic ladders to sequentially processed STR samples; (iii) effective color separations of different dye sets used to avoid bleed through between four or five different colors; (iv) resolution of at least 1 bp to approximately 350 bp to permit reliable detection of microvariant alleles.

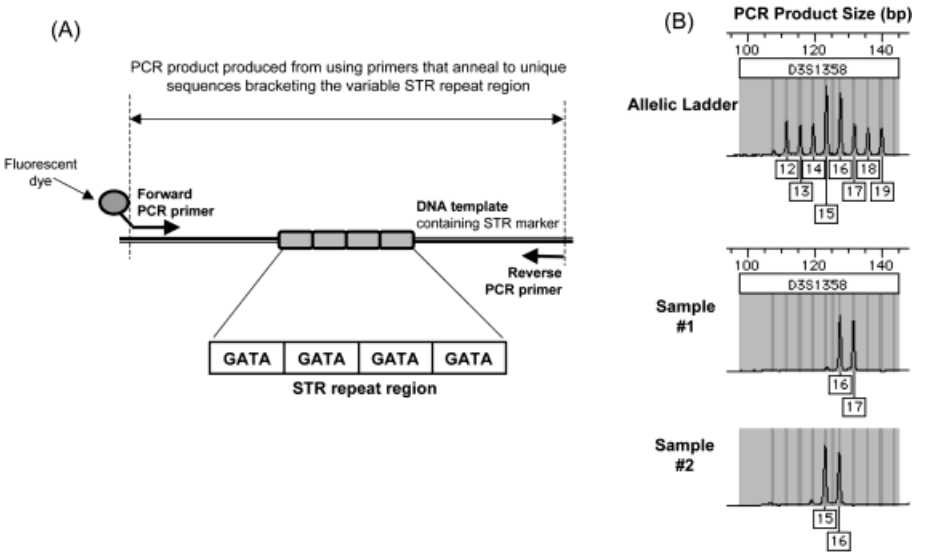


Figure 1. (A) Schematic of PCR primer positions for amplification of a STR DNA marker. The single-headed arrows represent the primer positions. The double-headed arrows illustrate the overall PCR product size using a particular set of primers. The PCR product size is measured and converted back to the number of repeat units present in the sample for genotyping purposes. A fluorescent dye is present on one of the primers in order to label the PCR product with a specific color. (B) Allelic ladder for the STR marker D3S1358 shown above two heterozygous DNA samples exhibiting different genotypes. Values below the peaks indicate the number of tandem repeats present in the measured allele.

Early work with STR markers used polyacrylamide gels [3, 4, 10]. However, CE, where the DNA molecules are separated in a narrow glass tube, has become increasingly popular for STR typing because it eliminates the need to pour gels and to load the DNA samples onto the gel. CE offers greater automation at the injection and detection phases of DNA analysis. In addition, CE consumes only a small portion of the actual sample so that it can be retested if needed. This article will review the use of CE for DNA analysis and its application to STR typing. The primary focus will be on the chemistry, hardware, and software used with the ABI Prism 310 Genetic Analyzer from Applied Biosystems as it is the most widely used instrument today for STR analysis. Higher throughput approaches for STR typing will also be discussed including the 16-capillary ABI 3100 Genetic Analyzer.

1.2 Early work with CE

Since the first description of electrophoresis in small diameter tubes [11, 12], CE has been identified as a powerful analytical technique capable to replace slab gel-based electrophoresis of nucleic acids. In CE the separation takes place in a capillary with an internal diameter of 50–100 μm). The narrow capillary enables the application of high electric fields, and thus faster run times, without overheating problems associated with the high voltages used. In addition, the capillary can be easily manipulated for automated injections. CE has been shown to be a versatile technique and has been used for a variety of forensic applications including analysis of gunshot residues, explosive residues, and drugs as well as DNA typing [13]. Since 1996, CE results have been admissible in courts of law [14].

Early work with CE and STR typing used instruments having UV detection [15] or laser-induced fluorescence detection of a single color [16]. In these cases, dual internal size standards had to bracket the allelic ladder or amplified alleles in order to accurately type the STR alleles [17]. The advent of the ABI Prism 310 Genetic Analyzer in July 1995 with its multicolor fluorescence detection capabilities opened a whole new world to STR typing. The ability to examine more than one wavelength simultaneously during electrophoresis permits a higher density of genetic information to be obtained. CE systems have played a vital role in other applications such as sequencing the human genome [18]. Thousands of CE instruments are in use around the world now for DNA sequencing and genotyping. A search of the PubMed database in October 2003 located more than 1300 references with keywords of DNA and CE.

The ABI 310 Genetic Analyzer instrument is probably the most widely used platform for STR testing today. DNA samples are processed in a serial fashion at a rate of approximately one sample per 30 min on this single-capillary instrument. The multi-capillary ABI 3100 became available in the spring of 2001 and has become

the instrument of choice for many laboratories needing an increased level of throughput. The steps for processing DNA samples through size and color separations are illustrated in Fig. 2. Issues impacting sample injection, separation, detection, and interpretation for reliable STR typing are addressed below (Fig. 3).

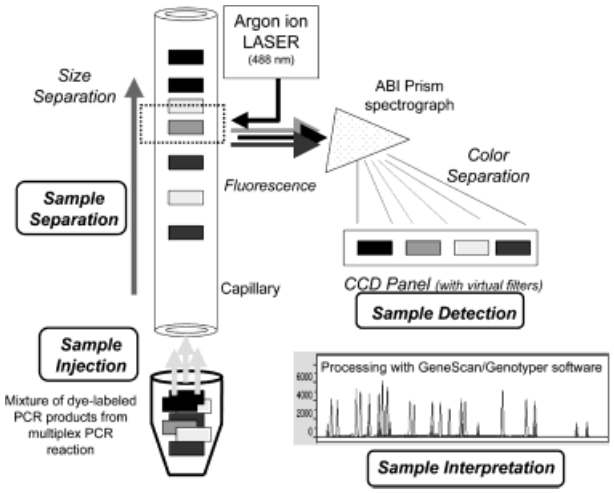


Figure 2. Schematic illustration of the separation and detection of STR alleles with an ABI Prism 310 Genetic Analyzer.

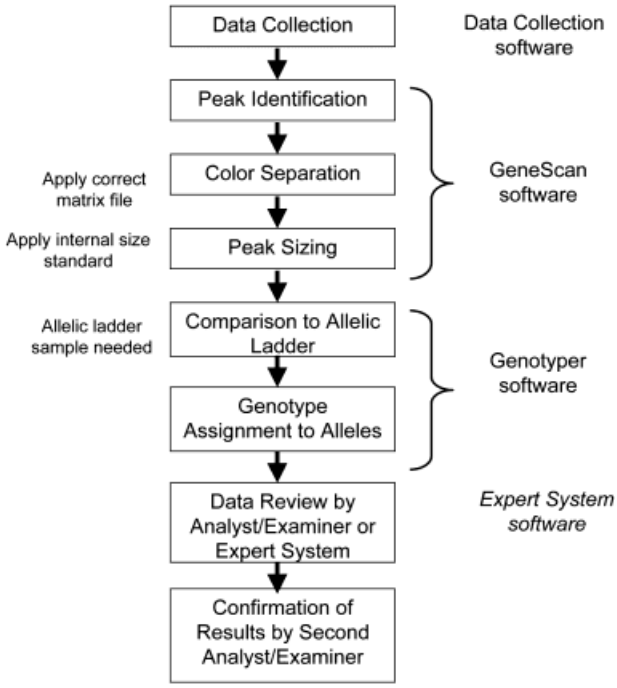


Figure 3. Sample interpretation and genotyping process for STR allele determination (see [1]). Software packages for DNA fragment analysis and STR genotyping perform much of the actual analysis, but extensive review of the data by trained analysts/examiners is often required.

2 Sample preparation and injection

A major advantage of CE is that samples can be loaded onto the separation medium in an automated fashion from a sample plate. Traditional gel electrophoresis techniques require careful manual loading of samples prior to initiating electrophoresis although some methods for comb loading with robotic spotting have been described [19]. Samples for CE separation are usually prepared by diluting a small portion of the PCR product into water or deionized formamide. Another significant advantage for CE in the context of forensic analysis is that only a small portion of the actual sample is examined each time. It may be reinjected additional times if needed for retesting purposes.

Most CE systems utilize electrokinetic injection, where a voltage is applied for a defined time, to move charged molecules from the sample into the capillary. As DNA is negatively charged, a positive voltage is applied to draw the DNA molecules into the capillary. Electrokinetic injections produce narrow injection zones, but are highly sensitive to the sample matrix. In general, the quantity of DNA injected onto a CE column ($[Q_{inj}]$) is a function of the electric field (E), the injection time (t), the true concentration of DNA in the sample ($[DNA_{sample}]$), the area of the capillary opening (πr^2), and the ionic strength of the sample (λ_{sample}) *versus* the buffer (λ_{buffer}). This can be described by the following equation [20]:

$$[DNA_{inj}] = Et(\pi r^2)(\mu_{ep} + \mu_{eof})[DNA_{sample}](\lambda_{buffer}/\lambda_{sample}) \quad (1)$$

where r is the radius of the capillary, μ_{ep} is the mobility of the sample molecules, and μ_{eof} is the electroosmostic mobility, which is hopefully negligible in a coated capillary.

However, this equation assumes no interfering ions are present. The addition of ions such as Cl^- from the PCR reaction mixture will compete with DNA and reduce the total amount of DNA injected onto the capillary because the sample conductivity (λ_{sample}) will be higher. As Cl^- ions are smaller than DNA molecules, they will have a higher charge/mass ratio and subsequently a higher sample mobility (μ_{ep}). Likewise, smaller DNA molecules, such as remaining PCR primers, will travel more quickly into the capillary opening from the sample solution than the larger PCR products.

To reduce this sample bias problem with electrokinetic injection, PCR samples can be purified by means of dialysis [16, 21], spin columns [15, 22, 23] or ethanol precipitation [24]. The dialysis step appears to be the most effective for removing excess salt, while the spin columns are more effective at removing primer peaks, enzyme and deoxy nucleotide triphosphates (dNTPs). However, early in the development of DNA testing with CE, it was demon-

strated that a simple dilution of the sample in water or deionized formamide can be an effective method for sample preparation because the sample ionic strength is reduced relative to the buffer ionic strength [17].

Since formamide is a strong denaturant, it is commonly used in the preparation of single-stranded DNA samples for CE. Merely placing a sample in formamide is sufficient to denature it. However, rapid heating to 95°C and snap-cooling on ice is commonly performed to ensure that the denaturation process has occurred. Use of high-quality formamide with a low conductivity is important. Formamide produces ionic decomposition products including formic acid, which is negatively charged at a neutral pH and will be preferentially injected into the capillary. The formamide by-products can cause problems in both sensitivity and resolution [25]. The quality of formamide can be easily measured using a portable conductivity meter and should be 80 μS or less to obtain the best results. Many laboratories buy ultrapure formamide and freeze aliquots immediately to ensure sample quality. Water has also been successfully used in the preparation of STR samples for CE analysis instead of formamide [17, 26]. Use of deionized water can eliminate the health hazard and the cost of formamide as well as problems with disposal. While studies have shown that water gives fully concordant results with formamide, long-term sample stability suffers because DNA molecules will renature in water after a few days.

A useful method for keeping the sample zone narrow and improving the amount of analyte placed onto the column during an injection involves a process commonly called sample stacking [27, 28]. Stacking, also called field-amplified injection, occurs when the ionic strength of the sample zone is lower than that of the buffer. This is in effect what is happening when a sample is diluted in deionized water or formamide. As the current through the system is constant, the lack of charge carriers in the sample zone produces a strong electric field that ends abruptly at the interface between the sample zone and the buffer inside the capillary. DNA molecules mobilized by this field move rapidly towards the capillary as the injection voltage is applied and "stack" in a narrow zone at the interface. Stacking allows a large sample zone to be loaded onto the capillary with a minimum of band broadening. Stacking also aids in producing efficient separations. With sharp injection zones, shorter capillaries and less gel media is required to effect a separation. The key to producing a good stacking interaction is to produce a zone of low conductivity immediately in front of the sample. This is facilitated in many CE systems by dipping the capillary in water just prior to sample injection. Other methods can also be utilized such as on-line sample dialysis or buffer neutralization with NaOH [29], but these are more difficult to implement. In forensic analyses these methods are typically not employed since sufficient sample stacking occurs through the dilution of the amplified sample.

3 Sample separation

Besides the width of the sample injection zone, there are several other components that impact DNA separations within CE systems: the polymer used for enabling the separation, the capillary, the electrophoresis buffer, and the field strength [30]. STR allelic ladders are useful tools for monitoring system resolution (see Fig. 4).

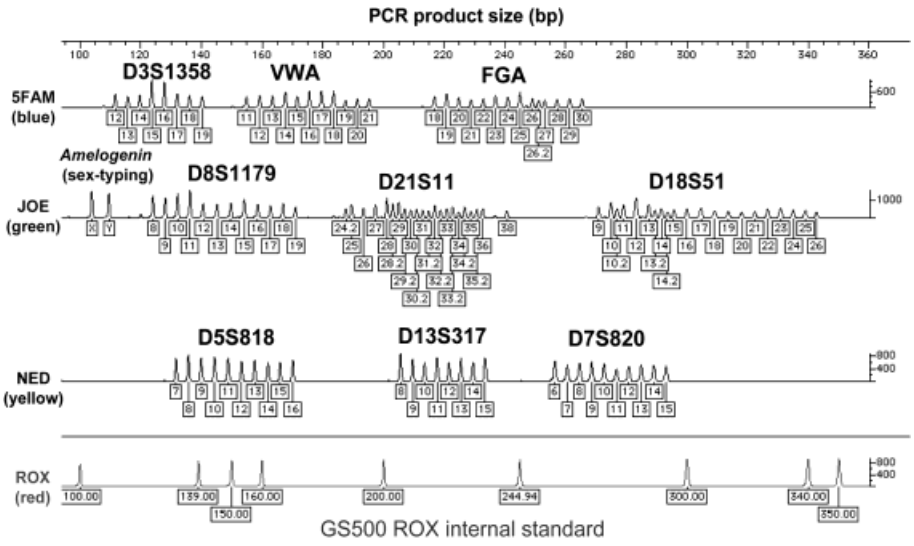


Figure 4. Allelic ladders present in the Profiler Plus STR kit from Applied Biosystems. Note the clean color separation (*i.e.*, no pull-up between dye colors).

3.1 The polymer separation matrix

There are several different types of sieving media utilized in electrophoretic separations, depending on the physical characteristics of the media. Chemical gels such as the common polyacrylamide gels used in denaturing slab-gel electrophoresis are rigid cross-linked materials whose porous structure is linked together by strong covalent bonds. Agarose produces physical gels. This material's shape is the result of weaker intermolecular forces produced *via* entanglement of the various strands of different agarose molecules. Entangled polymers are the third type of sieving media. Similar to physical gels, these materials are also characterized by intermolecular interactions. However, such substances are not true gels, as they cannot hold their shape unless placed in some container such as a capillary. Entangled polymers are characterized by a rapid increase in viscosity as the polymer concentration reaches a certain threshold value. The viscosity of these materials is also dependent on the polymer's molecular weight. All of the above types of materials have been used in CE separations, and thus there is nothing especially novel about the CE method of electrophoresis other than the convenience of containing the gel in a capillary and the enhanced heat dissipation which results from the small cross sectional area of the capillary.

Early attempts to apply CE to the size separation of biomolecules were based on gel-filled capillaries (*e.g.*, cross-linked polyacrylamide or agarose) [31]. However, gel-filled capillaries presented several disadvantages: air bubble formation during the filling of the capillary as well as in the process of shrinkage of the gel during polymerization, limited their applications. Moreover gels, in particular acrylamide, suffer from degradation by hydrolysis, particularly at the alkaline pH commonly used to separate biopolymers. This degradation leads to short lifetimes for gel-filled capillaries. Currently, gel-filled capillaries play a minor role in DNA separation applications [32].

Capillary cross-linked gel systems have been replaced with entangled polymer solutions such as linear (uncross-linked) polyacrylamide [33]. The idea of using polymer solutions to separate biopolymers is not new, as it was proposed years ago by Bode [34, 35]. However, it only became popular in combination with CE, because the very efficient anticonvective and heat dissipation properties of thin capillaries permit separation in fluids without loss of resolution. Grossman and Soane [36, 37] demonstrated that by using a dilute, low-viscosity polymer solution as the separation medium, high-resolution separations of DNA mixtures could be achieved. Barron *et al.* [38] found that dilute solutions of hydroxyethylcellulose well below the entanglement threshold have the ability to separate large DNA fragments from 2000 to 23 000 bp. However, in a systematic study with small double-stranded DNA, the entangled polymer solutions gave superior separations over dilute solutions [39]. Therefore, for many high-resolution applications, such as DNA sequencing and genotyping, the properties of an entangled polymer network are needed.

Even though a great number of polymers exist which could potentially be used as a separation matrix for biological molecules, not all of them are suitable for standard CE systems. Especially in the new multicapillary devices, a low viscosity is needed to keep the technical sophistication low. Therefore, the ideal polymer should have at least the same separation properties as classical gels, combined with a low viscosity that would allow easy replacement. These conditions have been achieved with the performance optimized polymers, POP™-4 and POP™-6, from Applied Biosystems [40]. POP-4 is commonly used for DNA fragment analysis including STR typing while the POP-6 polymer, which is the same polydimethylacrylamide polymer present at a higher concentration, is capable of higher resolution to meet the single-base resolution needs of DNA sequencing.

3.2 The buffer

The buffer that is used to dissolve the polymer in CE systems is important as it stabilizes and solubilizes the DNA, provides charge carriers for the electrophoretic current, and can enhance injection. If the buffer concentration and concomitant conductivity are too high, then the column will overheat resulting in a loss of resolution. In the process of electrophoresis, the composition of the anode and the cathode buffers may change due to electrolysis and migration of buffer ions. Thus, to avoid problems with poor size calibration of the system over time, it is a good policy to periodically replace the CE buffers with fresh solution.

The Genetic Analyzer buffer commonly used with the ABI 310 is 100 mM TAPS and 1 mM EDTA, adjusted to pH 8.0 with NaOH [43]. TAPS is short for *N*-tris-(hydroxymethyl) methyl-3-aminopropane-sulfonic acid. TAPS is used instead of Tris-borate-EDTA (TBE) since TBE is temperature and pH-sensitive. As analysis temperature is increased with TBE, the pH decreases at a rate of 0.02 pH units with every 1°C. As pH decreases so does the fluorescence emission of many dyes [46].

The forensic community primarily uses the ABI 310 for the analysis of STRs. Under the analysis parameters typically employed for STR analysis, the amplified DNA fragments must remain denatured. To accomplish this DNA denaturation, the capillary column run temperature is set

to a higher than room temperature, and buffer additives such as formamide, urea, and 2-pyrrolidinone are added to keep the DNA from reannealing [43]. Even under strong denaturing conditions, DNA molecules can sometimes assume various conformations due to intramolecular attractions and capillary run temperatures of 60°C are commonly employed to help reduce secondary structure in DNA [43]. Thus, high concentrations of urea and elevated temperatures are used to keep the various STR alleles uniformly denatured, since the mobility of DNA fragments can be affected by its conformation. Even with these measures, the operator must take care to maintain their system at a stable ambient temperature, as temperature variations can have profound effects on allele migration [47]. Many laboratories assess an internal standard peak (such as the 250 peak in the ABI GS500 internal standard, see Figs. 4 and 5), which is particularly sensitive to temperature variation to demonstrate that their CE systems are stable and well calibrated [47]. CE analysis of DNA fragments at elevated pH conditions, where the DNA molecule is predominately denatured, suggests that DNA secondary structure is responsible for the variations observed in DNA size determinations with fluctuating temperatures [48–50]. By carefully controlling the run conditions, *i.e.*, pH, buffer, denaturants, and temperature, variations within and between runs can be minimized and overall run precision improved. Run-to-run precision can also be enhanced using a global Southern sizing algorithm rather than the traditional local Southern sizing [47, 51].

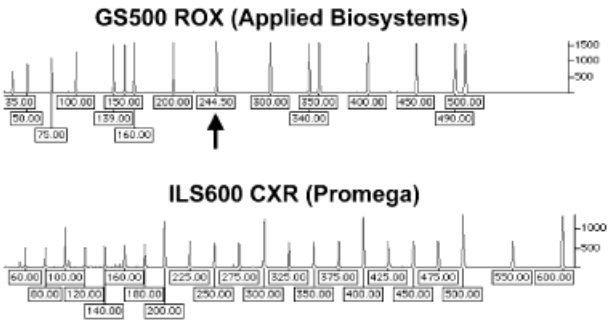


Figure 5. Two different internal size standards commonly used with STR typing. The ~245 bp peak (arrow) in the GS500 ROX standard is not included in the software calculations.

3.3 The capillary

The capillary column is central to the separation capabilities of CE. In uncoated capillary columns, residual charges on the silica surface induce a flow of the bulk solution toward the negative electrode. This process known as electroosmotic flow (EOF) creates problems for reproducible DNA separations because the velocity of the DNA molecules can change from run to run. Capillary and microchip channel walls, which contain charged silanol groups, are chemically modified [41] or dynamically coated [42, 43] to prevent EOF in DNA separations.

One method to accomplish EOF suppression in a fused-silica channel or capillary is to mask the charged sites on the wall by adsorption of neutral linear polymers that provide a viscous layer on the capillary surface [40, 42]. The commercially available poly-dimethylacrylamide POP-4 and POP-6 are successfully used in DNA genotyping by CE because they provide a sieving matrix for the separation of single-stranded DNA and, at the same time, suppress the EOF [43]. POP-4 consists of 4% linear dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone [43, 44]. For STR analysis, the run temperature is typically set at 60°C to further help keep the DNA strand denatured.

When using the ABI 310 Genetic Analyzer, an operator simply loads a batch of samples and leaves the instrument unattended. If a capillary failure occurs, all the subsequent analysis will be ruined. Thus, it is important to understand the potential issues involved in the breakdown of a capillary or series of analyses. Often, the causes of a capillary failure are unknown but they can result in loss of valuable time and effort. As capillary failures occur, migration times can shift or peaks can broaden (Fig. 6). Determining at which point the failure occurred is critical, as separations may be affected several runs prior to the perceived failure. To avoid this problem, it is common practice to dispose of capillaries before their useful lifetime has expired.

Failure to obtain successful results with CE may also occur due to capillary wall effects, which are the results of adsorption of sample and buffer components on the capillary surface. The theory of gel-based separations in CE generally ignores the capillary wall as a contributor to the separation, but under certain conditions the wall can play a major role in the quality of the separation [45]. One effect, which could lead to this type of behavior, is EOF. Under normal conditions this phenomena does not occur because the viscous polymer solution masks charged sites on the wall and resists the bulk flow. However, with continued operation, the buildup of contaminants gradually over the course of many separations can produce active sites along the wall. These sites produce a charge double layer along the capillary wall, which can induce bulk flow, destroying the reproducibility of the migration times and making the resultant data unreadable. Another potential problem with the buildup of active sites on a capillary wall is the adsorption of the DNA molecules resulting in loss of resolution as sample bands become diffuse.

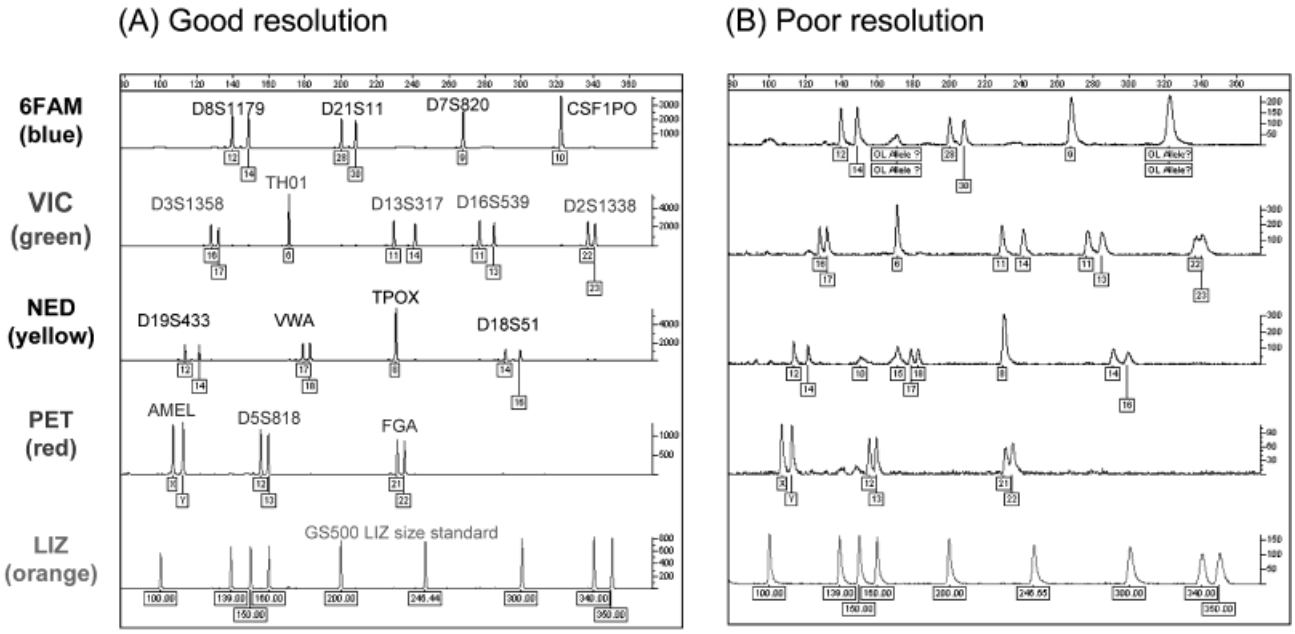
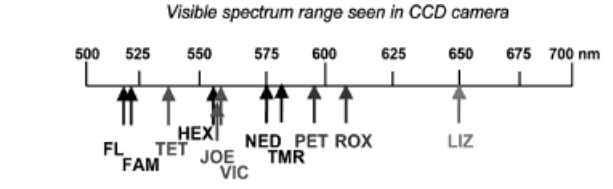


Figure 6. Comparison of same sample with (A) good resolution and (B) poor resolution due to a bad capillary. The STR kit used was the AmpFlSTR® Identifiler™ (5-dyes).

Manufacturers of capillaries often suggest replacing a capillary at around 100 injections to avoid problems with resolution failure. Capillary lifetimes can be improved by rinsing the capillary with consecutive washes of water, tetrahydrofuran, hydrochloric acid, and polymer solution [40]. Unfortunately, the ABI 310 instrument does not permit an on-the-instrument wash so the capillary must first be removed to conduct the rinsing procedure. With good sample preparation, many forensic laboratories see capillary lifetimes extend far past the 100 injections recommended by the manufacturer. Through effective monitoring of sample resolution [30] columns can be replaced when resolution declines. As the capillary column washing step is a manual procedure with the ABI 310, most forensic laboratories view capillary life spans of two to three hundred in number as acceptable, and hence columns with a large number of injections are viewed as expendable items.

4 Sample detection

Multiwavelength detection has expanded the capabilities of DNA analysis beyond a single-dye color and permitted greater multiplexing for STR markers. The key to the utilization of this technology is to covalently bind a different dye onto the 5'-(nonreactive) end of each primer or set of primers [52]. These dyes have a number of interesting properties. They are all excited by a single argon-ion laser tuned to 488 nm, yet fluoresce in different regions of the spectra. A multiwavelength analyzer, such as a charged-coupled device (CCD) camera, can then be used to determine which dye is present, based on the emission of each fragment as it passes the detector window. This technique permits the analysis of fragments of DNA that overlap in size as long as they are labeled with different dyes, which fluoresce at different wavelengths. The ABI 310 Genetic Analyzer uses virtual filters to collect the light striking the CCD camera at particular wavelength intervals. Figure 7 illustrates the fluorescence emission spec-



	Blue	Green	Yellow	Red	Orange	Used with These Kits
Filter A	FL	JOE	TMR	CXR		PowerPlex 16
Filter C	6FAM	TET	HEX	ROX		in-house assays
Filter F	5FAM	JOE	NED	ROX		Profiler Plus
Filter G5	6FAM	VIC	NED	PET	LIZ	Identifiler

Figure 7. Schematic of fluorescent dye emission maxima and virtual filters used in ABI 310 with various STR kits.

tra of the different dyes used to label the DNA and the position of several common virtual filters used in the ABI 310. The correct filter needs to be selected to match the fluorescent dye combinations in use in order to maximize sensitivity.

Note that in spite of the difference in emission wavelengths of the various dyes, there is still some overlap between them. To eliminate this spectral overlap between the dyes, a computer algorithm known as a matrix calculation is utilized to deconvolute the overlapping dyes and produce peaks that can be attributed to one fluorescent dye. Users of this technology must be careful to properly prepare and evaluate the matrix to calibrate their instruments to prevent what is known as "cross-talk", "bleed through", or "pull-up" between the different spectral channels. This "pull-up" problem is easily recognized as it results in the production of small peaks of a different color that occur at exactly the same size as a major peak in a different color. In addition, several artifacts peaks may also occur in some electropherograms such as residual dye "blobs" and spikes [53].

5 Sample interpretation

5.1 Software used

There are three software programs used to process data from the ABI 310 and produce STR genotypes: ABI 310 data collection software, GeneScan®, and Genotyper® (see Fig. 3). These programs were originally written for Macintosh computers but more recently have been adapted to run on Microsoft Windows NT. Applied Biosystems also has developed another program called GeneMapper® that combines the functions of GeneScan and Genotyper. The 310 data collection software [54] performs three primary functions: control of electrophoresis run conditions, control of which wavelengths of light will be examined on the CCD camera through the use of "virtual filters", and enables sample sheets and injection lists to be created whereby the sample name and processing order are specified. The user inputs the name of each sample and which dye colors are present in a sample sheet. The injection list controls the order in which each sample is injected onto the capillary as well as the time and voltage for the electrokinetic injection and electrophoresis voltage and run temperature. The virtual filter is also designated in the injection list depending on the dyes present in the sample being analyzed (Fig. 7). The output from the data collection program is "raw data" that comes in the form of relative fluorescence units on the *y*-axis and number of data points collected on the *x*-axis. The GeneScan and Genotyper programs are necessary to convert the raw data into the appropriately colored peak and to generate STR genotyping information.

GeneScan software [55] also performs three primary functions. It calls peaks based on threshold values specified by the user; it separates the peaks into the appropriate dye color based on a matrix file; and it sizes the STR allele peaks based on an internal size standard labeled with a different colored dye that is run in every sample. Typically, the internal standard is labeled with the red dye ROX while the STR alleles are labeled with blue, green, and yellow dyes (see Table 2). Different internal size standards may be used (Fig. 5). It is important to be consistent in the use of an internal size standard because all STR allele peaks are measured relative to this internal size standard. The default sizing algorithm, and one most commonly used, with the GeneScan program is the local Southern method [47, 56, 57]. The local Southern method measures the size of an unknown peak relative to its position from two peaks in the internal standard that are larger than the unknown peak and two that are smaller than the unknown peak. GeneScan software contains six different screens that may be used as part of data analysis and evaluation: processed data (color-separated), size standard curve, electrophoresis history, sample information, raw data (no color separation), and an analysis log file.

The Genotyper software program [58] takes GeneScan data and converts the sized peaks into genotype calls. Genotyping is performed by comparison of allele sizes in an allelic ladder to the sample alleles. The manufacturer of a particular STR kit normally provides Genotyper macros in order to make the allele calls from the allelic ladders. These macros can be designed to filter out stutter peaks (see [59]) that may interfere with sample interpretation.

5.2 Assessing resolution of DNA separations

Determining the resolution of an electropherogram allows the analyst to evaluate the performance of the CE system [30]. These resolution measurements can be useful in evaluating casework data, or assessing system modifications that may alter electrophoretic conditions. In the review of casework, or in the appraisal of variations made upon the system, resolution measurements can be applied as part of the evaluation process in conjunction with other assessments to judge system performance.

Before forensic laboratories report casework data, electopherograms and supporting data must undergo considerable review. Most laboratories conduct at least a

qualitative resolution assessment of an electropherogram through a visual inspection of peak shape, breadth and separation. Peaks that are poorly shaped, overly broad, merged or lack appropriate baseline separation indicate deteriorated system performance. For example, Fig. 6 compares a good and poor resolution DNA separation with the same STR sample. Such visual inspections offer an excellent qualitative gauge of the system.

Resolution measurements can be conducted if a nonsubjective approach is desired to evaluate casework electropherograms. For casework analysis this may take the form of evaluating the resolution of the allelic ladders typically bracketing casework samples or by evaluating the samples themselves. The allelic ladder typically contains multiple peaks that span the breadth of the electrophoretic run and are consistently applied from run to run. These factors make the allelic ladder an excellent sample to assess the performance of the system. Assessing individual sample resolution may be approached by evaluation the sample peaks or through the assessment of an internal marker.

Due to the vagaries of crime scene samples, much variation would be expected in the resultant sample peaks found in these electropherograms. However, most laboratories include in the preparation of each sample for CE an internal lane standard (ILS) for determining sample peak base sizes. When the amplified sample and internal lane standard are co-injected, the variations of sample-to-sample injections may be evaluated and appropriate sizing conducted along with an assessment of the samples resolution based upon the ILS.

6 Applications of forensic DNA testing

With the analytical aspects of forensic DNA typing considered using CE systems, we can examine the two primary applications of this technology – forensic casework and DNA databasing. Each application has issues and challenges.

6.1 Forensic casework

As with any technology that is applied to forensic casework, the use of CE to determine DNA profiles must be rigorously evaluated through a comprehensive validation program [23, 60]. The DNA Advisory Board through the publication of DNA standards has established the basis for this validation that forensic laboratories are obliged to follow [1, 2]. These validation experiments reveal the operational parameters that are employed in the assessment of peaks detected during CE analysis. The forensic community primarily uses CE for STR analysis although it is used to a lesser extent in mitochondrial DNA sequencing [61].

For those involved with STR analysis, many parameters must be determined that are typically based upon the STR system employed. Commercial kits are available which allow the user to amplify many STR loci simultaneously (Table 2). The analysis of this amplified product may be done in one or two electrophoretic runs depending upon the kit. The evaluation of the peaks derived from this amplification is to some extent kit-dependent, where the amplification product yields fragments interpreted as a "colored" peak by the CE. The assessment of these peaks must take into consideration a number of factors inherent in the amplification such as peak imbalance, stochastic effects, stutter and *n*-1 peaks [1]. The analysis of these parameters must be done with an understanding of the limitations of the CE unit. For example, there is a linear fluorescent range for the instrument that should be well understood to be able to calculate meaningful heterozygote peak ratios. These ratios are important in the determination of alleles in a possible mixture and must be calculated within the operational range of the instrument. Likewise it is important to understand the sensitivity of the system to allow the analyst to develop a threshold fluorescence value above which peaks would be assigned as an allele.

In addition, a properly assigned matrix is critical to the evaluation of observed peaks. As discussed in a previous section, the fluorescent dyes employed in STR analysis have some spectral overlap and with a poorly assigned matrix, peaks of one color will be observed and misinterpreted as a peak of another color. This "pull-up" may yield peaks that could be mistaken as true alleles and hence it is important to review peaks to determine if they are detected in more than one wavelength. Such electropherograms that show a considerable "pull-up" may be revalued with a new matrix.

As discussed previously, artifacts such as spikes and dye "blobs" may be observed in an electrophoretic run. These artifacts may yield peaks in the allelic range and could be initially interpreted as an allele. The experienced operator should be able to review the peak shape and possible multifluorescent attributes of these artifacts to identify these as such and not as true allelic peaks. Through a good understanding of the CE system, appropriate DNA profile determinations can be obtained. The analysis of validation samples is an important mechanism, which provides operators with the opportunity to examine the system and to learn the criteria necessary to make appropriate interpretations especially for challenging samples containing mixtures or degraded DNA profiles [1, 62].

6.2 DNA databasing

DNA databasing has become a useful forensic tool and as more samples are added to the database the probability of a case-to-case match or case to convicted offender match increases. One problem facing most forensic laboratories in the United States concerning the database is the backlog of convicted offender samples waiting to be processed and entered into the database. Most US laboratories do not have the staff or instrumentation necessary to process the volume of samples collected, and hence these laboratories typically out source their samples to commercial laboratories. Many of these laboratories have developed highly automated systems to handle this demand. Some of the CE systems employed for this high-throughput typing is detailed below.

Another problem encountered by forensic laboratories engaged in databasing is the need to perform a second reading of the electropherograms prior to loading the profiles into the database. Typically, the commercial laboratory will perform their analysis and requisite quality control analysis and forward the profiles to the sending laboratory for their review. The process involved in this second review is very time-consuming and delays the uploading of convicted offender profiles into the database. Much work has been conducted to assist in this second review through the use of what have been termed "expert systems". These systems evaluate the electropherogram using specific criteria detailed by the examining laboratory to make allelic determinations from the electropherogram. Once fully validated, the system could be used to read the electropherogram and make the allelic calls and "flag" those samples that require human intervention. Some states have begun validation efforts with these systems and may soon be in a position to implement them for database use.

7 Increasing sample throughput

7.1 Capillary array electrophoresis systems

The ABI 310 uses a single capillary and as such cannot match the parallel processing potential throughput of a multilane slab-gel system. At its maximum capacity, the ABI 310 can run about 48 samples in a 24 h time period since each run takes close to 30 min. However, a number of capillary array electrophoresis (CAE) instruments are now commercially available [18]. These CAE systems offer from 8 to 384 capillaries run in parallel (Table 3). Thus, sample throughputs can be greatly increased by running many samples in parallel. However, it should be kept in mind that each capillary is an independent environment and thus not directly analogous to a multilane slab gel.

Table 3. Size of arrays in commercial CAE systems

	No. of capillaries
Applied Biosystems (Foster City, CA, USA)	
ABI 3100 Avant	4
ABI 3100	16
ABI 3700	96
ABI 3730	96
Amersham Biosciences (Piscataway, NJ, USA)	
MegaBACE 500	48
MegaBACE 1000	96
MegaBACE 4000	384
SpectruMedix Corporation (State College, PA, USA)	
SCE 2410	24
SCE 9610	96
SCE 19210	192
Beckman Coulter (Fullerton, CA, USA)	
CEQ 8800	8

STR typing by CAE has been reported in a number of publications. Early demonstrations of CAE for STR typing were performed in the laboratory of Rich Mathies at UC-Berkeley [63, 64] and at Molecular Dynamics [65, 66]. CAE systems have used different detection formats including a sheath flow cuvette, moving capillaries over a fixed laser beam, moving laser beam and detector over the capillaries, and a split beam approach to illuminate all of the capillaries simultaneously. Since the ABI 310 has been so widely used by the forensic DNA community, many labs will likely look to the ABI 3100 (16-capillary) and ABI 3700 or ABI 3730 (96-capillary) instruments in order to increase their sample throughput capabilities [67].

Precision studies conducted on the ABI 3100 [68] and the ABI 3700 [67] demonstrates that reliable results can be obtained with a multicapillary CE system. Table 4 illustrates the high degree of precision observed with more than 4600 allele measurements across all 16 capillaries over a six-month period on the same ABI 3100 instrument [69]. Note that the maximum spread in observed allele sizes was 0.83 bases for DYS389II allele 30 with 215 measurements. Most of the standard deviations for these Y-STR allele measurements are below 0.10 bases.

A high degree of resolution is needed with STR typing in spite of the fact that most of the markers are tetranucleotide repeats with expected nearest-neighbor alleles being

Table 4. Summary of 4651 Y-chromosome STR allele measurements observed on an ABI 3100 Genetic Analyzer across all 16 capillaries over a six-month period

Loci	Allele	Observed range (bp size relative to GS500 LIZ)	Spread in bp size	Sample No. (*N*)	Mean (bp)	SD (bp)
DYS19	13	243.36–243.81	0.45	47	243.62	0.096
	14	247.30–247.89	0.59	316	247.64	0.097
	15	251.38–251.88	0.50	198	251.68	0.079
	16	255.50–255.90	0.40	69	255.73	0.073
	17	259.65–260.02	0.37	50	259.81	0.068
DYS385	10	248.20–248.55	0.35	10	248.33	0.092
	11	251.78–252.42	0.64	275	252.21	0.083
	12	255.90–256.33	0.43	46	256.13	0.082
	13	259.89–260.28	0.39	104	260.05	0.078
	14	263.71–264.19	0.48	302	263.95	0.083
	15	267.42–268.13	0.71	156	267.89	0.098
	16	271.60–272.03	0.43	138	271.82	0.079
	17	275.49–276.03	0.54	118	275.76	0.088
	18	279.51–279.96	0.45	69	279.72	0.084
	19	283.44–283.85	0.41	30	283.65	0.102
	20	287.35–287.59	0.24	9	287.50	0.117
DYS388	10	148.96–149.13	0.17	10	149.04	0.055
	12	155.10–155.58	0.48	537	155.43	0.089
	13	158.29–158.71	0.42	55	158.58	0.088
	14	161.42–161.81	0.39	46	161.67	0.096
	15	164.63–164.86	0.23	19	164.76	0.059
	16	167.55–167.88	0.33	11	167.74	0.123
DYS389I	12	152.35–152.95	0.60	126	152.74	0.115
	13	156.53–157.22	0.69	421	157.00	0.103
	14	160.79–161.38	0.59	128	161.16	0.103
	15	165.22–165.36	0.14	8	165.28	0.049
DYS389II	26	262.23–262.54	0.31	3	262.44	0.179
	28	270.24–270.91	0.67	91	270.55	0.152
	29	274.21–275.03	0.82	230	274.63	0.147
	30	278.35–279.18	0.83	215	278.78	0.154
	31	282.52–283.20	0.68	108	282.90	0.155
	32	286.77–287.32	0.55	22	286.99	0.156
	33	291.11–291.22	0.11	4	291.17	0.046
DYS390	20	200.76–200.93	0.17	5	200.83	0.071
	21	204.56–205.09	0.53	157	204.86	0.085
	22	208.63–209.12	0.49	70	208.84	0.104
	23	212.57–213.09	0.52	138	212.82	0.112
	24	216.54–217.13	0.59	243	216.83	0.127
	25	220.52–221.10	0.58	67	220.84	0.109

Adapted from [69], Table 4.8

4 bp apart. In a recent population study involving approximately 12 000 allele measurements at 15 autosomal STRs [70], we observed 160 instances where heterozygous alleles were present that required a 1, 2, or 3 bp resolution up to about 300 bp due to microvariant alleles. Figure 8 shows several examples of these closely spaced alleles.

7.2 Microchip CE systems

Running single or multiple samples faster may also increase sample throughputs. By micromachining channels in glass, researchers have miniaturized CE systems with demonstrated DNA separations of less than a minute [71]. A major reason that microchip CE systems can achieve

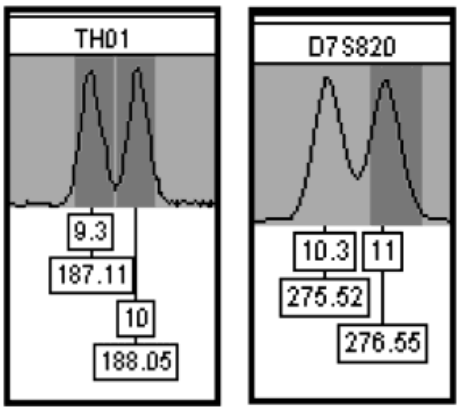


Figure 8. Resolution of STR variant alleles containing single-nucleotide spacing on ABI 3100 with POP-6 polymer. These TH01 alleles 9.3/10 and D7S820 alleles 10.3/11 were observed when typing samples in a previous study [70].

faster separation times is that the injection plug can be kept extremely small. Unfortunately, as of December 2003, no group has succeeded in producing routine and reliable STR typing data with 4 or 5-dye detection on a microchip CE device that is ready for "prime time" in a forensic laboratory setting. Caliper Technologies and Agilent Technologies both sell microchip CE devices such as the Agilent Bioanalyzer 2100, but these systems do not have the resolution or the multiwavelength detection capability necessary to perform modern STR typing. Rich Mathies' group at UC-Berkeley [72–74] and Dan Ehrich's group at the Whitehead Institute have made progress in this area [71, 75, 76].

7.3 Future methods for DNA typing with STR markers

Future analysis systems that wish to enable more rapid or easier STR typing will need to match or exceed the capabilities of currently available analytical systems such as the ABI 310 single-capillary CE system or the multicapillary ABI 3100. These capabilities include analysis of PCR reactions that contain at least four or five spectrally resolvable fluorescent dyes without significant pull-up between the various colors. Many current microchip CE platforms fall short in this regard. Future STR typing systems must maintain single-base resolution over a size range that extends from 50 bp to 250 bp or even 500 bp. Time-of-flight mass spectrometry approaches, while making substantial strides in recent years [77], currently fail in this regard.

Future electrophoretic systems need to maintain good temperature control to enable a high degree of precision from run to run. Throughput must be increased without compromising data quality. Due to the time invested in validating current STR kits and typing methodologies, many forensic laboratories will likely be reluctant or slow to change to a new technology even if substantial improvements can be demonstrated [7]. Rather an evolution to a multicapillary environment on a familiar platform is more likely than a radical change in technologies.

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