TECHNICAL NOTE

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Validation of STR Typing by Capillary Electrophoresis*

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ABSTRACT: With the use of capillary electrophoresis (CE), high-resolution electrophoretic separation of short tandem repeat (STR) loci can be achieved in a semiautomated fashion. Laser-induced detection of fluorescently labeled PCR products and multicolor analysis enable the rapid generation of multilocus DNA profiles. In this study, conditions for typing PCR-amplified STR loci by capillary electrophoresis were investigated using the ABI Prism® 310 Genetic Analyzer (Applied Biosystems). An internal size standard was used with each run to effectively normalize mobility differences among injections. Alleles were designated by comparison to allelic ladders that were run with each sample set. Multiple runs of allelic ladders and of amplified samples demonstrate that allele sizes were reproducible, with standard deviations typically less than 0.12 bases for fragments up to 317 bases in length (largest allele analyzed) separated in a 47 cm capillary. Therefore, 99.7% of all alleles that are the same length should fall within the measurement error window of ± 0.36 bases. Microvariants of the tetranucleotide repeats were also accurately typed by the analytical software. Alleles differing in size by one base could be resolved in two-donor DNA mixtures in which the minor component comprised ≥5% of the total DNA. Furthermore, the quantitative data format (i.e., peak amplitude) can in some instances assist in determining individual STR profiles in mixed samples. DNA samples from previously typed cases (typed for RFLP, AmpliType™ PM+DQA1, and/or D1S80) were amplified using AmpFℓSTR® Profiler Plus™ and COfiler™ and were evaluated using the ABI Prism 310. Most samples yielded typable results. Compared with previously determined results for other loci, there were no discrepancies as to the inclusion or exclusion of suspects or victims. CE thus provides efficient separation, resolution, sensitivity and precision, and the analytical software provides reliable genotyping of STR loci. The analytical conditions described are suitable for typing samples such as reference and evidentiary samples from forensic casework.

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The usage of slab gel electrophoresis to separate DNA fragments containing short tandem repeat (STR) loci is widespread. Although vertical gel electrophoresis is used for many high throughput applications, it can be time-consuming, tedious, laborious, and refractory to automation. As an alternative technique for electrophoresis of DNA, capillary electrophoresis (CE) has been used successfully with high reproducibility and efficiency (1–13).

In recent years, the commercial availability of CE instruments has expanded. One such instrument, the ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), provides a semiautomated means of typing DNA samples by CE. Following amplification of the DNA and preparation of samples for CE, a robotic mechanism enables the consecutive injection of up to 96 samples in a hands-free, computer-assisted, and user-defined fashion. Amplified fragments are separated within a narrow bore capillary containing an aqueous sieving medium. At the conclusion of each sample separation, the polymer is automatically pumped out of the capillary and replenished prior to the injection of the next sample. On the ABI Prism 310, fluorescently-tagged PCR-amplified fragments are detected by laser-induced fluorescence (LIF) as the molecules pass through a detection window near the anodal end of the capillary. Fluorescent signals from 525 to 680 nm are captured by a charged-coupled device (CCD) detection system. The sensitivity (1000-fold greater than that of UV detection) and specificity of LIF make LIF a preferred strategy for DNA detection (14,15). Greater sensitivity permits usage of narrower capillaries so that higher analysis temperatures (such as 60°C) and higher field strengths can be used, with enhanced resolution and without damage to the matrix (16). Computer-assisted data analysis is performed to estimate the size of the DNA fragments and to designate the alleles of the STR loci.

Several factors influence the efficacy of separation, resolution, precision, and sensitivity of amplified STR alleles subjected to CE. They include the following: polymer type and concentration, temperature of the capillary environment, magnitude of the electric field applied during injection and separation, duration of injection, salt concentration, volume of the sample to be injected, and capillary length. These factors impact on the facility with which samples

can be reliably typed. Furthermore, the quality, conductivity, and percentage of formamide may influence the sensitivity of detection and integrity of the DNA fragments. As a means of defining operational parameters and limits for the analysis of STRs using CE, several of these factors were evaluated using the ABI Prism 310 so that consistent and reliable STR typing results can be generated. Verification of typing results was obtained as follows: (a) samples were run multiple times on a given ABI Prism 310 instrument; (b) samples were run on multiple ABI Prism 310 instruments; (c) samples were run on both the ABI Prism 377 DNA Sequencer and the ABI Prism 310; (d) DNA from cell line 9947A was run multiple times, and (e) two or more STR kits [AmpFℓSTR® Profiler™, Profiler Plus™, and COfiler™ (Applied Biosystems); and GenePrint™ PowerPlex™ 1.1 (Promega, Inc., Madison, WI)] were used to type samples, such that, for individual samples, certain loci were typed more than once and with use of different kits. Also, analysis of reference and evidentiary samples from previously typed cases (typed for RFLP, AmpliType™ PM+DQA1, and/or D1S80) was conducted on the ABI Prism 310 with use of the AmpFℓSTR Profiler Plus and COfiler STR systems. This work demonstrates that the ABI Prism 310 Genetic Analyzer can be used for obtaining reliable STR typing results in forensic casework and other human identification applications.

Materials and Methods

DNA Samples

DNA was extracted from population samples (AC, Caucasian; AA, African American; TR, Trinidadian) (bloodstains) and case samples by an organic phenol/chloroform method followed by Microcon®-100 filtration (17). Evidentiary samples that contained semen were subjected to differential extraction to separate sperm and nonsperm DNA (18). Commercially-available DNA from human cell line 9947A (Applied Biosystems) was also used. The quantity of DNA in each extract was estimated using a chemiluminescence-based, slot blot procedure that entails hybridization of a human alphoid probe (19,20).

Amplification

AmpFℓSTR Profiler, Profiler Plus and COfiler PCR Amplification kits and the GenePrint PowerPlex 1.1 kit were used according to the manufacturer's recommendations. DNA samples were amplified in 50 μL (for AmpFℓSTR kits) or 25 μL (for GenePrint) reaction volumes using the GeneAmp® 9600 or 9700 PCR System (Applied Biosystems).

Electrophoresis and Analysis Using the ABI Prism 310 Genetic Analyzer

Ultra Pure Formamide (Life Technologies, Inc., Gaithersburg, MD) treated with AG® 501-X8 Resin (Bio-Rad Laboratories, Hercules, CA) was used to prepare samples for the following studies: DNA:formamide/GeneScan-500 ratio; sample preparation volume; sample reinjection and injection time; capillary length; resolution and precision; and GeneScan-500 250-peak. Subsequently, the following four different formamide preparations were assayed and compared: (a) Ultra Pure Formamide (Life Technologies, Inc.) treated with AG® 501-X8 Resin; (b,c) Ultra Pure Formamide (Amresco, Solon, OH) treated with AG 501-X8 Resin or with Amberlite® MB-150 (Sigma, St. Louis, MO); and (d) Ultra Pure Formamide (Amresco) as provided by the manufacturer. Analysis of case samples was then conducted using Ultra Pure Formamide (Amresco) as provided by the manufacturer. Deionization of formamide was conducted by adding approximately 5 g of ion exchange resin to 50 mL formamide and stirring for approximately 30 min (21). Conductivity measurements of some formamide preparations were made using a CON 100 conductivity meter (Milwaukee Instrument Co., Milwaukee, WI).

Some amplified samples (specified below for the following studies: formamide: DNA ratio, sample injection time, and GeneScan-500 250-base fragment) were subjected to Microcon-100 filtration, as previously described (22). Amplified products were recovered in a volume of distilled water approximately equal to the pre-filtration volume. Typically, amplified samples were prepared for electrophoresis by mixing 1 μL PCR product with 1 μL GeneScan®-500[ROX] (Applied Biosystems) and 23 μL formamide. Preparations were heated at 95°C for 3 min and cooled at 4°C for a minimum of 3 min. Using the ABI Prism 310 Genetic Analyzer, electrophoresis entailed the following, unless otherwise specified: a 5 s. electrokinetic injection at 15 kV; separation of amplified products at 15 kV and 60°C; and usage of an uncoated capillary (47 cm long, 50 μm interior diameter; Applied Biosystems) and the separation medium Performance Optimized Polymer 4 (POP™ 4) (Applied Biosystems). Table 1 specifies the instruments (CE1-5) used for the various studies. Data were collected using GeneScan Collection software (version 1.0.2; Applied Biosystems) with virtual filter set A or F. Results were analyzed using light smoothing with GeneScan Analysis software (version 2.1; Applied Biosystems) and Genotyper® software (version 2.0; Applied Biosystems).

Formamide: DNA Ratio, Sample Preparation Volume and Reinjection

One ng of DNA extracted from bloodstains from two donors (AC157, AA786) was amplified using AmpFℓSTR Profiler Plus.

TABLE 1—*Experiments performed on five ABI Prism 310 Genetic Analyzers (CE1-CE5).*

Study	# Different CEs Used	CE1	CE2	CE3	CE4	CE5
GeneScan 250 peak	3	√	√	√		
DNA: formamide ratio	3	√	√	√		
Injection time	3	√	√	√		
Reinjection/renaturation	2		√	√		
Resolution & precision	3	√	√	√		
Mixture resolution	4		√	√	√	√
Capillary length	4		√	√	√	√
Case study: Profiler Plus	3		√	√	√	
Case study: COfiler	2				√	√

TABLE 2—*Sample preparation strategy to assess the effects of varying the DNA: formamide ratio, the preparation volume, and the formamide percentage.*

	Ratio of DNA: Formamide/GeneScan-500	DNA + Formamide (μL)†	% Formamide
A*	1:24	3.5 + 84	96
	2:23	7 + 80.5	92
	4:21	14 + 73.5	84
B*	1:24	3.5 + 84	96
	1:12	3.5 + 42	92.3
	1:6	3.5 + 21	85.7

* A = Total preparation volume was constant (25 μL) and DNA volume was varied (1, 2, 4 μL). B = DNA volume was constant (1 μL) and preparation volume was varied (25, 13, 7 μL).

† Each sample was prepared (i.e., as 1:24, 2:23, 4:21, 1:24, 1:12 or 1:6) in a 3X volume and divided into three separate tubes for electrophoresis on three ABI Prism 310 instruments.

PCR products were filtered using Microcon-100 devices and mixed in various ratios with formamide (Life Technologies, Inc.; treated with AG 501-X8 Resin) as shown in Table 2. One μL of GeneScan-500[ROX] was included in each preparation. All samples were run one time on each of three different ABI Prism 310 Genetic Analyzers (CE1-3).

To examine the necessity of subsequent heat denaturation prior to reinjection of a sample, 1 μL of each of 3 DNA samples (AC648, AC694, AC764) amplified with the Profiler kit was mixed with 1 μL GeneScan-500[ROX] and 23 μL formamide (Life Technologies, Inc.; treated with AG 501-X8 Resin). The three sample preparations were heat-denatured at 95°C for 3 min and cooled at 4°C for at least 3 min. Ten injections per sample preparation and electrophoresis were conducted each of two ABI Prism 310s (CE2 and CE3). Samples were then reinjected (ten times per sample preparation) and analyzed on CE2 and CE3, without repeating the preinjection heat step, on days 0 (same day), 1 and 4.

Sample Injection Time

DNA extracted from bloodstains from six donors (AA157, AA648, AA694, AA764, AA777, AA786) was serially diluted (from 2 ng to 1, 0.5, 0.25, 0.12, and 0.06 ng) and amplified using AmpFℓSTR Profiler Plus. PCR products were filtered using Microcon-100 devices and mixed with 1 μL GeneScan-500[ROX] and 23 μL formamide (Life Technologies, Inc.; treated with AG 501-X8 Resin). Preparations were heated at 95°C for 3 min, cooled at 4°C for 3 min, and electrokinetically injected for 5, 10, 15, or 20 s. Electrophoresis and analysis of all samples were conducted on three instruments (CE1: AA648, AA694; CE2: AA764, AA777; CE3: AA157, AA786).

Capillary Length

The AmpFℓSTR Blue (D3S1358, vWA and FGA loci) and Green (TH01, TPOX, CSF1PO, and amelogenin loci) allelic ladders were used to evaluate the effects of capillary length on the resolution and typing of DNA fragments. Ten injections were conducted on each of three different instruments (CE1-3) with new capillaries. With use of a 47 cm capillary (36 cm effective length), 150 s syringe pump time and a 24 min collection time per sample injection were used with a 15-kV electrophoresis. Following the initial run, the 47 cm capillary in each instrument was replaced with a 61 cm capillary (50 cm effective length), and the same samples were run again. With use of the 61 cm capillary, a 225 s syringe pump time and a 41 or 43 min collection time per sample injection were used with a 15-kV electrophoresis.

Resolution and Precision

Resolution and precision were evaluated using both the 47 and 61 cm capillaries. Because fragment length is a primary determinant of resolution, alleles ranging in size from 177 to 301 bases were evaluated, irrespective of kit/dye format, using both allelic ladders and amplified samples. It matters little for resolution and precision analyses whether triplex or nineplex amplification systems are used. Ten injections of AmpFℓSTR Blue and Green 1 Allelic Ladders were conducted on each of three ABI Prism 310s (CE1-3). Also, the resolution and precision of PCR fragments generated with AmpFℓSTR Profiler were assessed using three DNA samples (AA648, AA694, AA764) that exhibited the following characteristics: (a) alleles that differ in size by two bases (FGA 22, 22.2); (b) alleles that differ in size by one base (TH01 9.3, 10); and (c) "n" and "$n+1$" alleles, which differ in size by one base, that were generated during a PCR in which the final extension step was omitted. Amplified samples were injected 10 times each on two different instruments (CE2 and CE3). Additionally, because microsatellite analysis is typically conducted using the 47 cm capillary and the current study was focused on the 47 cm capillary, the amplified samples were reinjected 10 times each in the 47 cm capillary on days 1 and 2 (30 injections total per sample).

Precision was calculated as the standard deviation of size estimates. Resolution is defined as the ratio of peak separation to the main peak width, as expressed by the following equation (simplified from Ref 23 for ease of use):

$$R = 1.18\,(t_2 - t_1)/(W_2 + W_1)$$

where $(t_2 - t_1)$ is the distance between the two peaks being evaluated (as determined using GeneScan data points), and W_1 and W_2 are the peak widths at half-height for peaks 1 and 2, respectively. Base resolution was calculated by dividing R by the size difference between peaks 1 and 2.

The resolution of alleles was also assessed in DNA samples containing mixtures of DNA from two donors (AC157, AA733). To assure that the alleles exhibited sufficient peak heights for resolution analysis, the quantity of DNA template was doubled for the mixture study since the individual components are diluted when mixed. The two DNA samples used to prepare the mixtures were extracted from bloodstains, and 2 ng of each DNA sample were amplified using AmpFℓSTR Profiler. One individual (AC157) exhibited TH01 8.3 and 9.3 alleles, and the other individual (AA733) exhibited TH01 7 and 10 alleles. Mixtures thus contained two alleles that differ in size by one base (TH01 9.3 and 10). The two DNA samples were amplified and analyzed twice to assess the average peak height of the TH01 9.3 and 10 alleles. Samples were diluted such that the TH01 9.3 and 10 peak heights were approximately equal and were reanalyzed prior to preparation of the mixtures to assure near equivalency of the peak heights. Mixtures were then prepared in the following ratios: 1:20, 1:10, 1:5, 1:3, 1:1, 3:1, 5:1, 10:1, and 20:1. The amplified product mixtures were run in duplicate on four different ABI Prism 310s (CE2-5) using 47 cm capillaries.

GeneScan-500[ROX] 250-Base Fragment

Migration shifts of the GeneScan-500[ROX] 250-base fragment had been previously observed (24). Factors postulated as poten-

tially affecting the mobility of the 250-base fragment were examined on each of three ABI Prism 310s (CE1-3). The factors evaluated included the following: initial injection in a new capillary, extent of capillary usage, restart of the instrument (perhaps related to run temperature), and factors related to the performance of specific instrument(s). In this study, samples from 5 DNAs (TR3062, TR3106, TR3113, TR3115, TR3117) that had been amplified using AmpFℓSTR Profiler Plus were prepared in triplicate by adding formamide and GeneScan-500[ROX]; one preparation of each DNA sample was filtered using a Microcon-100 device. Following installation of a new capillary and replacement of the polymer on each instrument (CE1-3), each of the three sets of 5 samples was heat denatured, cooled, and run on a different instrument. The instruments were then turned off and restarted after 1 h. Each of the three sample sets was then rerun on the same instrument as the initial run. Migration of the GeneScan-500[ROX] 250-base fragment was compared to that of other samples within the set of runs.

Case Studies

Samples from ten previously typed cases were analyzed with AmpFℓSTR Profiler Plus COfiler on the ABI Prism 310. Samples (Table 3) were derived from cases of sexual assault, assault, homicide, and bank robbery and comprised 15 reference samples (bloodstains) and 15 questioned items. Case samples had been previously typed using RFLP analysis (n = 9 cases) or AmpliType PM+DQA1 and D1S80 analysis (n = 1 case). One to two ng of genomic DNA were used for amplification. Amplified products (1 μL; not Microcon-filtered) were mixed with 23 μL formamide [Ultra Pure Formamide (Amresco), treated with either AG 501-X8 Resin or with Amberlite®] and 1 μL GeneScan-500[ROX]. Preparations were heated at 95°C for 3 min and cooled at 4°C for a minimum of 3 min. Samples were subjected to electrophoresis (Profiler Plus: CE2–4; COfiler: CE4 and CE5) and analysis using the ABI Prism 310 Genetic Analyzer and software under standard conditions (5 s injection at 15 kV; electrophoresis at 15 kV and 60°C; POP 4; and 47 cm capillary). Samples that exhibited off-ladder alleles or below/above-ladder alleles were rerun; samples that exhibited off-scale peaks [i.e., >8191 relative fluorescence units (rfu) in the raw data] were diluted (1:2, 1:4 and/or 1:8, depending on initial peak heights) and rerun.

TABLE 3—*Case study.*

Case	Sample Description*	Conclusion
1	Q4-1: shorts K1: victim, blood K2: suspect, blood	K1 cannot be excluded as potential contributor of Q4-1.
2	Q2-2: shirt K1: victim, blood	K1 cannot be excluded as potential contributor of Q2-2.
3	Q1-1B: manila paper K1: victim, blood	K1 is excluded as potential contributor of Q1-1B.
4	Q1-1B: cotton t-shirt Q2-1B: panties Q3-1B: denim jeans K2: suspect, blood	Q1-1B matches Q2-1B. Q3-1B inconclusive. K2 is excluded as a potential contributor of Q1 and Q2.
5	Q25-1B: cotton sheet Q27-1B: sleeping bag K1: victim, blood K4: suspect, blood	Q25-1B matches Q27-1B. K4 is excluded and K1 cannot be excluded as potential contributor of Q25-1B and Q27-1B.
6	Q1: key chain, nylon fabric Q3: glove (swab) K1: suspect 1, blood K2: suspect 2, blood	Q1 matches Q3. K1 is excluded and K2 cannot be excluded as potential contributor of Q1 and Q3.
7	Q99-1B: carpet K2: victim, blood	K2 cannot be excluded as potential contributor of Q99-1B.
8	Q1: carpet Q3: scrapings K1: suspect, blood	Q1 matches Q3. K1 cannot be excluded as potential contributor of Q1 and Q3.
9	Q1 (M,F): bathing suit crotch† K1: victim, blood K2: suspect, blood	K1 cannot be excluded as potential contributor of Q1 (F). K2 cannot be excluded as potential contributor of Q1 (M)
10	Q13-1 (M,F): panties† K1: victim, blood K6: suspect, blood	K1 cannot be excluded as potential contributor of Q13-1(F). K6 is excluded as potential contributor of Q13-1(M).

* K = known sample (reference); Q = questioned sample; M = male fraction from differential extraction; F = female fraction from differential extraction.

† Semen detected by P30 assay.

Results and Discussion

The purpose of this study was to define the utility and working parameters of the ABI Prism 310 Genetic Analyzer for CE of STRs for forensic casework and other human identification studies. The data demonstrate that STR typing of biological materials using the ABI Prism 310 yields reliable results under various conditions. The results of performance testing and manipulation of analytical conditions are presented to assist the forensic laboratory in developing and using protocols suitable for forensic casework.

Sensitivity of Detection

Different ABI Prism 310 instruments can have different sensitivities due, for example, to differences in CCD (charged coupled device) detectors, laser effectiveness and alignment, and cleanliness and alignment of optical components. Given the possible inter-instrument variation, determination of the stochastic threshold line, or "S-line," for the interpretation of STR data (22) should be performed following in-house studies to define the zone of potential stochastic effects. The sensitivity differences among CE instruments, however, did not impact on accuracy or precision of STR typing with use of the ABI Prism 310.

Formamide

The accuracy and precision of sizing of DNA fragments and of allelic designations require that the amplified DNA remain denatured during electrophoresis. To denature the DNA and maintain single-stranded conformation, urea (in the polymer), formamide, and heat are used during sample preparation and CE. In this study, four different formamide preparations were assayed. Highest peak amplitudes were obtained with the Amresco formamide (Fig. 1), as compared with the Life Technologies formamide. This observation is consistent with the lower conductivity measurement of the former [ca. 100 μmhos (without in-house deionization), as compared to ca. 200 μmhos (with or without in-house deionization) for the latter]. Our studies showed that, provided the formamide exhibits a conductivity value of <100 μmhos (as determined with a conductivity meter), further in-house deionization with ion exchange resin is not necessary. Furthermore, the preparation and/or lot of formamide may affect the integrity of the DNA fragments, the efficiency of injection relative to fragment size, and/or the sensitivity of detection, as shown in Fig. 1. Breakdown products of formamide, which are known to degrade DNA, may increase conductivity in the sample and/or degradation of DNA. As an example

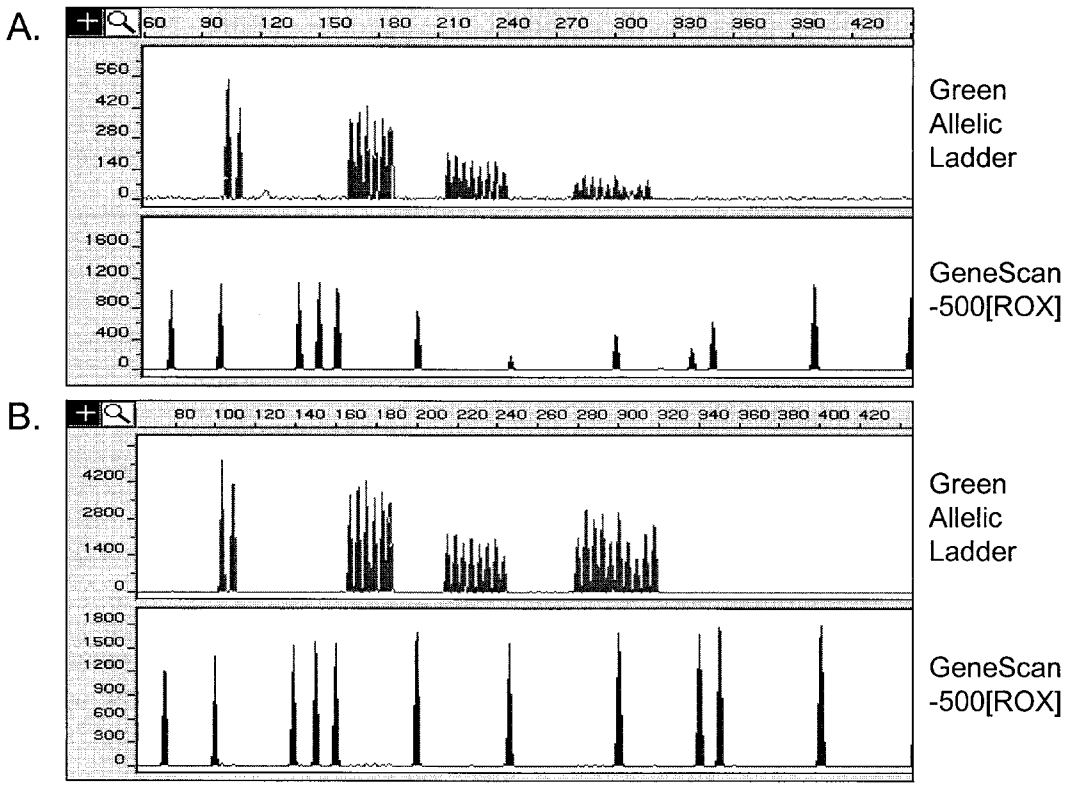

FIG. 1—*Comparison of two formamide preparations used in capillary electrophoresis: (*A*) Life Technologies formamide treated with AG 501-X8 (ca. 200 μmhos) and (*B*) Amresco formamide (ca. 100 μmhos), as provided by the manufacturer. The AmpFℓSTR COfiler allelic ladder (green: Amelogenin, TH01, TPOX, CSF1PO loci) is shown in the upper panel of each set (*A *and* B*), and GeneScan-500[ROX] is shown in the lower panels. A reduction in peak height and reduction of overall peak balance is observed between 200–350 bases with use of the lower-conductivity formamide (*A*).*

(Fig. 1), samples mixed with one formamide preparation (Life Technologies formamide, treated with AG 501-X8) demonstrated reduced peak height in the range 200 to 350 bases, as compared with peaks from samples mixed with Amresco formamide. The observed imbalance of the alleles in the green allelic ladder (Fig. 1*A*) is consistent with degradation of the DNA sample; however, in the electropherogram of the coelectrophoresed GeneScan-500[ROX], degradation or reduced peak height of fragments >350 bases was not apparent. This phenomenon (termed the "Golden Gate Effect" due to the pattern in the GeneScan-500[ROX] electropherogram) is perhaps due to reduced injection efficiency with use of the lower-conductivity formanide.

Prior to analysis on the ABI Prism 310, a DNA sample is placed in a denaturing solution (96% formamide) and heat-denatured. The preparation is then placed in an autosampler tray on the instrument. Reinjection of a sample(s) can be performed simply by creating a new injection list (or by modifying an existing injection list during a run) on the computer. To assess whether heat denaturation is required prior to sample reinjection, three samples were reinjected ten times each without reheating at the following time intervals: on days 0, 1 and 4. There was no evidence of DNA renaturation in any of the reinjections, and resolution was maintained [base resolution: 1.34 ± 0.03 (day 0); 1.36 ± 0.04 (day 1); and 1.30 ± 0.02 (day 4), comparing TPOX 6 and 8 alleles in samples subjected to electrophoresis using a 47 cm capillary and POP4]. These data suggest that samples left at room temperature (such as in the instrument's autosampler) for up to at least four days can be reinjected without repeating the preinjection heat step. Thus, additional treatment and/or manipulation of the sample is not necessary.

Various DNA:formamide ratios and sample preparation volumes were assayed (Table 2). Because the efficiency of sample injection relative to the method of sample preparation is consistent among kits (i.e., AmpFℓSTR Profiler, Profiler Plus, COfiler) (personal observation), samples amplified using a single kit (AmpFℓFlSTR Profiler Plus) were used in the assessment of sample preparation. First, the preparation volume (25 μL) was held constant while the DNA volume varied (1, 2, and 4 μL). An increase in peak magnitude was observed, as expected, with increasing amounts of input DNA. However, the response was not linear (i.e., 4-fold increase in DNA resulted in ca. 2-fold increase in peak height). Second, the DNA input amount was held constant (1 μL) while the formamide/GeneScan-500[ROX] volume was varied (6, 12, and 24 μL) (Fig. 2). A linear response in peak height was observed, whereby approximately 2-fold and 4-fold increases in peak height were observed with usage of the 1:12 and 1:6 ratios, respectively, relative to the 1:24 ratio. These data suggest that increasing the DNA concentration in the sample preparation by reducing the formamide volume can substantially enhance peak magnitude. The percentage of formamide (84 to 96%) in the various preparations assayed did not impact on the extent of denaturation.

Sample Injection

The amount of DNA injected into a capillary is a function of the injection method, injection time, salt content of the sample, and

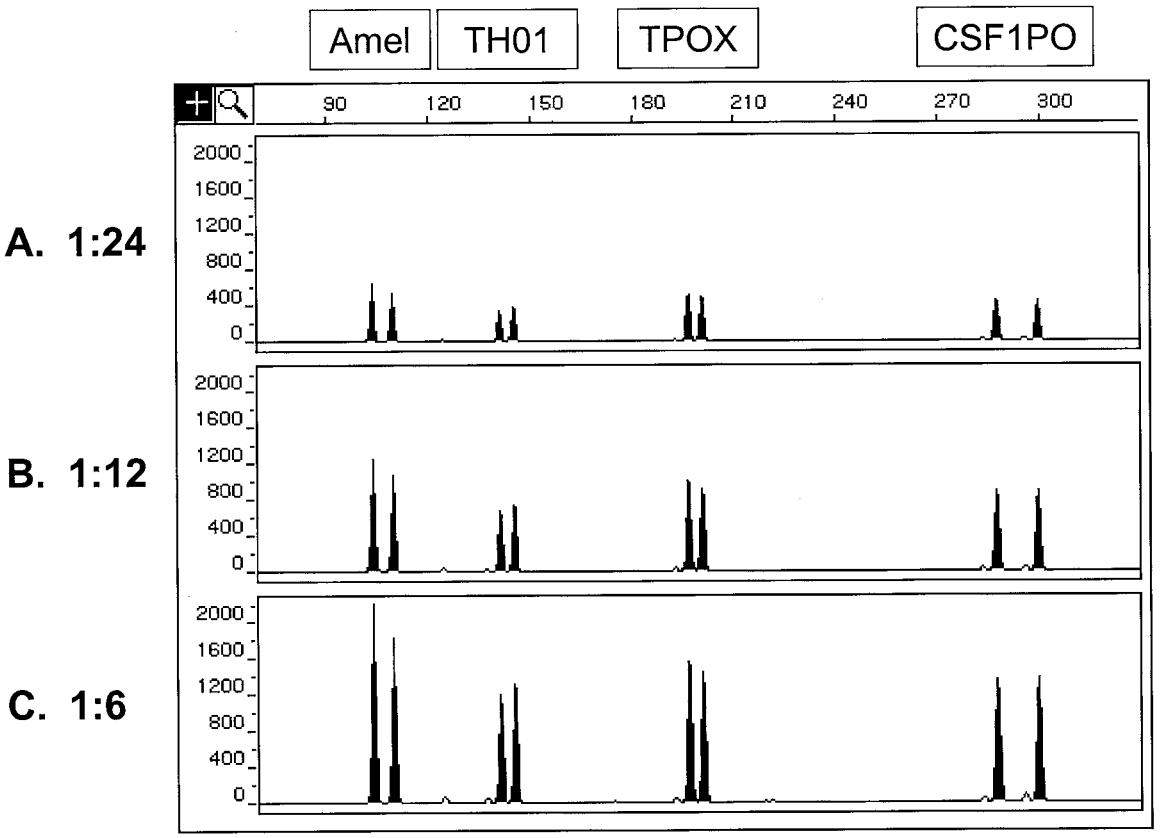

FIG. 2—*Evaluation of sample preparation volume. One μL of amplified product was added to 24 μL* (A)*, 12 μL* (B)*, and 6 μL* (C) *of formamide/GeneScan-500[ROX]. Peak height increased with reduced preparation volume. Amel = amelogenin.*

voltage (25). Compared to hydrodynamic injection (in which physical force is used), electrokinetic injection generally results in sharp, well-resolved peaks and greater sensitivity (25). The difference between the ionic strength of the sample and that of the polymer in the capillary results in a "stacking effect" that produces a narrow injection zone of the sample (26). Efficient sample stacking can generate narrow peaks.

Some ions may be injected preferentially over DNA due to their higher charge-to-mass ratio (27,28). Although PCR-amplified samples typically contain 1.5 mM $MgCl_2$, 10 mM Tris-HCl, and 50 mM KCl, a 1:24 dilution of the sample in formamide/GeneScan-500[ROX] effectively lowers the ionic strength of the sample such that the DNA can be efficiently injected. Filtration of PCR products with Microcon-100 devices removes ions from the sample. In the current study, the injection efficiency of PCR products that had been Microcon-filtered (Fig. 3*B*) was compared with that of unfiltered samples (Fig. 3*A*). An approximate 1.5-fold increase in peak height was observed in the filtered samples (Fig. 3). However, if low-conductance formamide is used (i.e., Amresco formamide, $<$100 μmhos), the efficiency of injection and the sensitivity of detection is generally such that filtration of the sample is unnecessary.

As expected, increasing the injection time (from 5 to 10, 15 and 20 s) increased peak magnitude (Fig. 4). However, the sample injection zone is broadened with increased injection time. With 15 and 20 s injections, peak broadening and lowering of resolution were exhibited, particularly at the larger-sized fragments in GeneScan-500[ROX] ($\geq$300 bases) (Fig. 4*A*) and larger-sized STR loci, such as FGA (Fig. 4*B*). The peak widths and resolution exhibited in 5 and 10 s injections were not appreciably different.

Capillary Length and Resolution

Capillaries of two different lengths (47 and 61 cm) are available from the manufacturer of the ABI Prism 310 for usage in CE. The 47 cm capillary is typically used for GeneScan applications, such as microsatellite analysis. The 61 cm capillary is typically used for DNA sequencing. As with gel length in slab gel electrophoresis, it was expected that increasing the length of the capillary might enhance the separation of DNA fragments. Enhanced resolution may occur with usage of the 61 cm capillary because of the lower field strength in the longer capillary, which results in a concomitant reduction in heat generation and dispersion of DNA. However, longer runs may result in increased diffusion. Results showed that sufficient resolution (Table 4) was achieved with usage of the shorter capillary, making the 47 cm capillary suitable for forensic applications. However, the resolution was improved in the 61 cm capillary, as compared to the 47 cm capillary (Table 4).

Although most alleles generated with the AmpF*ℓ*STR Profiler Plus and COfiler systems differ in size by a factor of four bases, alleles that differ by 1, 2, and 3 bases are encountered. For example, the TH01 9.3 allele is relatively common in most populations. Both alleles in a TH01 9.3,10 heterozygote were readily resolved with use of POP4 and either the 47 or 61 cm capillary (Fig. 5). When the same applied voltage is used (i.e., 15 kV), migration time is in-

creased with usage of the longer capillary, since samples must travel farther to reach the detection window and because of a lower field strength. The data collection time used with the 61 cm capillary is 43 min, as compared to 24 min for the 47 cm capillary. Given the single-capillary design of the ABI Prism 310 and the throughput needs of the laboratory, the difference in sample collection time may be significant to the laboratory.

In addition to resolving and accurately designating alleles in single-source samples, the analysis of forensic samples may require the identification of components of mixtures of DNA from two or more donors. In particular, the resolution of a fragment that: (a) is present as a minor component in a mixed DNA sample, and (b) differs in size by one base relative to the major peak can be challenging. In our study, TH01 9.3 and 10 alleles, which differ in size by one base, were therefore evaluated in DNA mixtures that were prepared in ratios ranging from 1:20 to 20:1. Although resolution in the 61 cm capillary was slightly better than that in the 47 cm capillary, the minor component in these mixtures containing TH01 9.3 and 10 alleles could be resolved at 1:20 (67 and 893 rfu, respectively) (and 20:1) in the shorter capillary (Fig. 6) as well as in the longer capillary (data not shown). However, at some larger loci, it could be expected that alleles that differ in size by one base occasionally may not be resolved. Nonetheless, the analytical parameters used on the ABI Prism 310 are effective operationally, and comparisons in forensic casework can be reliably made. The data support that the use of the POP4 polymer and the 47 cm capillary can resolve components in most mixed samples that may be encountered in forensic casework.

Precision of Sizing

Precision of the sizing of DNA fragments separated by CE is defined as the ability to reproducibly estimate fragment sizes from run to run. Size estimates for a given allele can vary across different runs and/or different instruments (of the same and different model) (6). An internal size standard (GeneScan-500[ROX]) is therefore coinjected with each sample to normalize migration differences among runs (6,25,27–30). The internal size standard is used to generate a calibration curve based on the migration of known fragments. Unknown fragments are sized by comparison to the calibration curve. However, even with usage of an internal size standard, sizing precision of the same allele generated from different DNA samples is expected to be lower than that obtained from repetitive analysis of the same sample. For example, variation in the ionic strength of different sample preparations may decrease the sizing precision among samples. Based on the precision of the assay, alleles are therefore binned by the Genotyper software and can be characterized by comparison to an allelic ladder (which also contains the same internal size standard). Since size estimates of alleles originating from the same donor can vary slightly across multiple runs, allelic designations provide a better means for reliable comparisons both within and among laboratories.

The alleles evaluated in the current study ranged in size from 121 bases (D3S1358 allele 13.3) to 317 bases (CSF1PO allele 15) (32); those at D3S1358, TH01, TPOX and FGA were 1 or 2 bases different in size and had an ancillary usefulness for resolution analyses. Sizing precision for samples analyzed at 60°C using a 47 cm

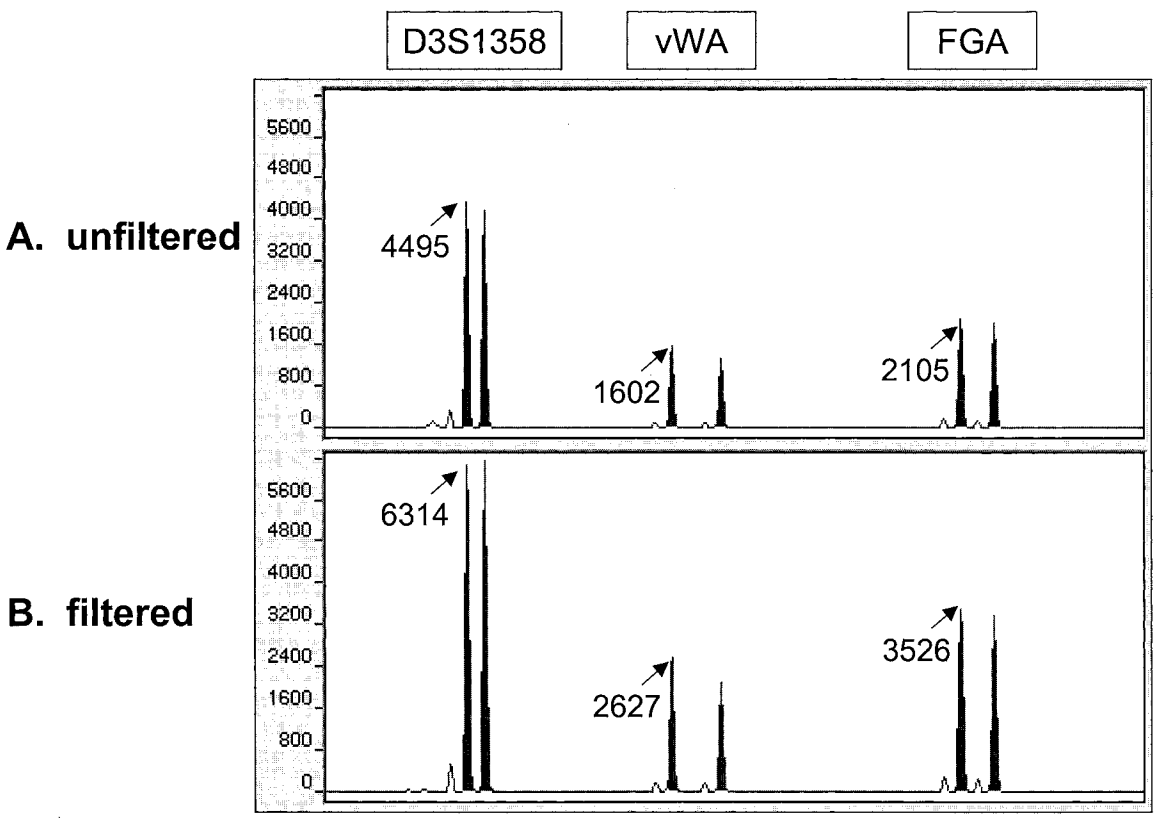

FIG. 3—*Comparison of* (A) *unfiltered and* (B) *Microcon-100-filtered PCR products generated using AmpFℓSTR Profiler Plus. Because injection of sample into the capillary is achieved electrokinetically, the removal of ions from the preparation by Microcon-100 filtration results in greater injection efficiency and increased peak heights* (B). *Numbers indicate peak heights.*

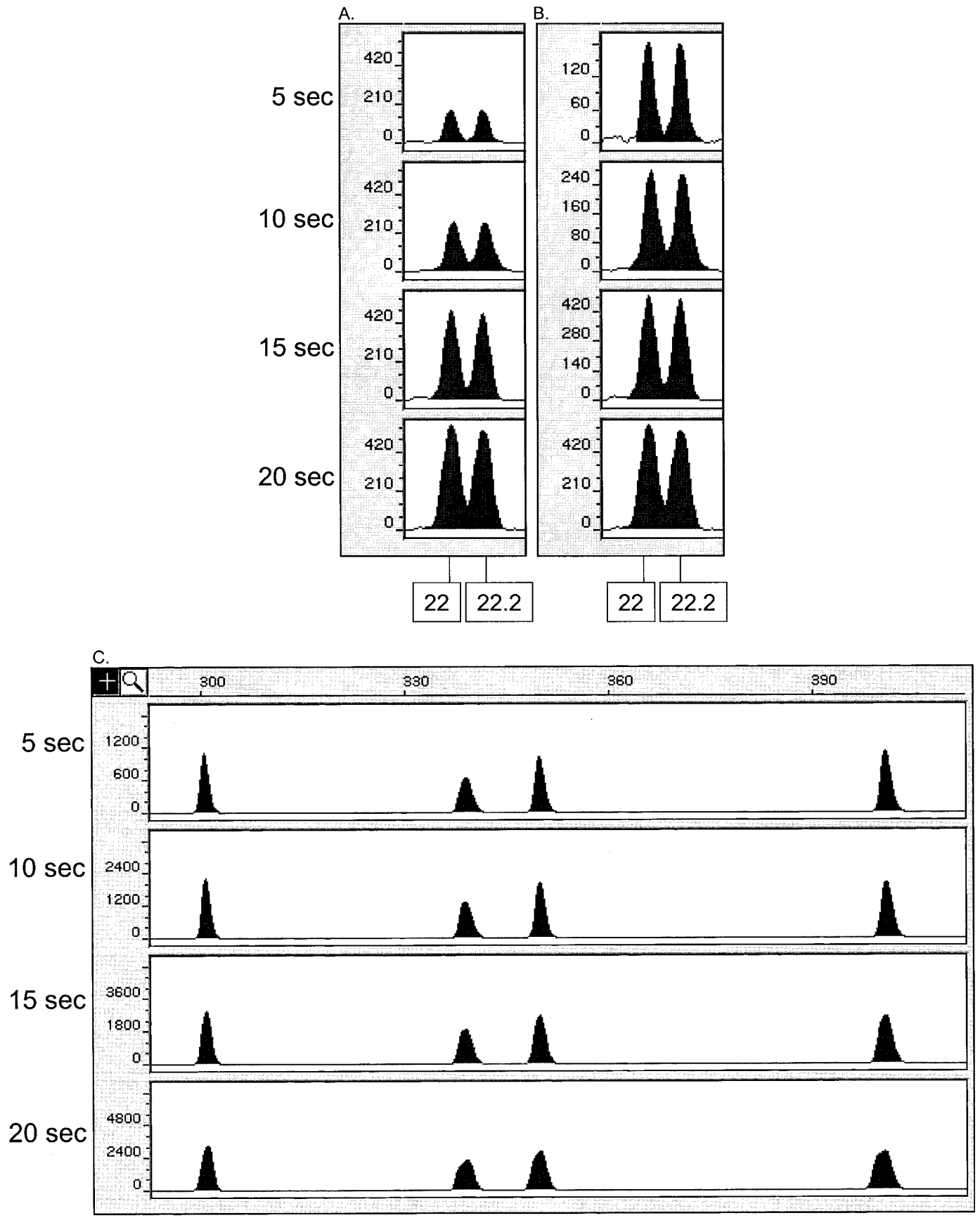

FIG. 4—*Variation of sample injection time prior to capillary electrophoresis. Samples were injected at 15 kV for 5–20 s. FGA alleles 22 and 22.2 (*A, B*) that were co-electrophoresed with GeneScan-500[ROX] (*C*) are shown. Increasing the injection time resulted in increased peak height (*A*, all panels are exhibited on the same vertical scale). However, peak broadening was observed with the longer injection times (*B*, all panels are exhibited on different vertical scales;* C*). Peak broadening was more pronounced for the larger DNA fragments (*C*).*

TABLE 4—*Comparison of base resolution using 47 and 61 cm capillaries on the ABI Prism 310. DNA fragments were generated by amplification with AmpFℓSTR Profiler.*

Alleles	Fragment Length (Bases)*	47 cm Capillary	61 cm Capillary
TH01 7, 8	177, 181	1.13 ± 0.05	0.99 ± 0.04
TPOX 9, 10	230, 234	1.34 ± 0.06	1.14 ± 0.07
CSF 10, 11	297, 301	1.49 ± 0.06	1.24 ± 0.04

* Length of the designated alleles, respectively, according to (32).

capillary on the ABI Prism 310 (Table 5) ranged from a standard deviation of 0.02 to 0.12. Plus/minus three standard deviations is ±0.36 bases. These data therefore support at most a ±0.36-base range for binning alleles and thus the ability to generally distinguish alleles that differ in size by one base.

GeneScan-500[ROX] 250-Base Fragment

Anomalous migration of the 250-base fragment in GeneScan-500[ROX] has been intermittently observed on the ABI Prism 310. Usually, the mobility shift occurs during the initial run in a series. It has been postulated that the 250-base fragment is sensitive to temperature fluctuations due to its sequence characteristics (24). A mobility shift may therefore be indicative of a substantial temperature difference during a run as compared with other runs. The following factors were evaluated for potential influence on migration of the 250-base fragment: (*a*) first usage of a new capillary, (*b*) temperature of initial run in a series, and (*c*) instrument-specific issues (assessed on multiple instruments). Shifting of the 250-base peak (Fig. 7) occurred sporadically yet infrequently throughout the run series (that is, in both initial and subsequent runs). Shifting could not be attributed solely to: (*a*) the extent of capillary usage, (*b*) whether the instruments had reached the 60°C set point before the first run, or (*c*) any specific instrument. Because this fragment is not used in sizing, anomalous migration of the 250-base peak does not affect the sizing precision of alleles. However, it may be a useful indicator of temperature fluctuations that may occur during a run. For example, if some alleles in a particular sample fail to fall within the bins designated by Genotyper [i.e., are called "OL (off-ladder) Allele"], the positioning of the 250-base peak may suggest that the temperature varied substantially during the analyses. Such samples should be reinjected for reliable typing.

Pullup and Spikes

"Pullup" refers to the observation of a single peak in more than one color. It typically may occur when a true DNA peak is off-scale [i.e., on the ABI Prism 310, >8191 relative fluorescence units (rfu) in the raw data]. A similar phenomenon, called bleed-through, has occasionally been observed on some ABI Prism 310 instruments for peaks that are not off-scale. The spectral emissions of the fluorescent dyes (FAM, JOE, NED, and ROX) in the AmpFℓSTR systems overlap; if the spectra are not adequately separated by the software during multicomponent analysis, pullup may be observed. Typically, pullup peaks occur more so in spectrally adjacent colors (i.e., blue more so into green, than blue into red) and have identical base sizes and scan numbers in the different colors. The shape of a pullup peak is similar to that of the true allele, but the true allele typically has a greater peak magnitude than the pullup peak. Pullup peaks are often reproducible if rerun under identical conditions; however, if the sample is diluted (such that the peaks are no longer off-scale), the pullup is eliminated (data not shown). Because pullup peaks can be identified due to off-scale counterparts, often the presence of pullup peaks does not impact on the ability to reliably type DNA samples.

In contrast, "spikes" which are sporadically observed in capillary electrophoresis (and not in slab gel electrophoresis) are not influenced by the intensity of the peaks. As of this writing, the cause of spikes is unknown. It has been hypothesized that they may be

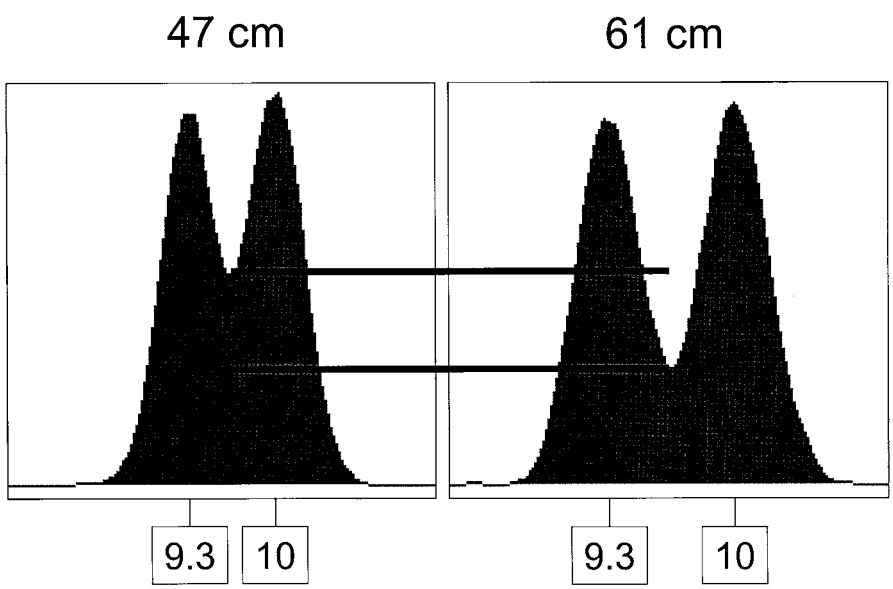

FIG. 5—*Resolution of DNA fragments relative to capillary length. TH01 alleles 9.3 and 10 (which differ in size by one base) are shown as separated in a 47 cm capillary (36 cm effective length) and a 61 cm capillary (50 cm effective length).*

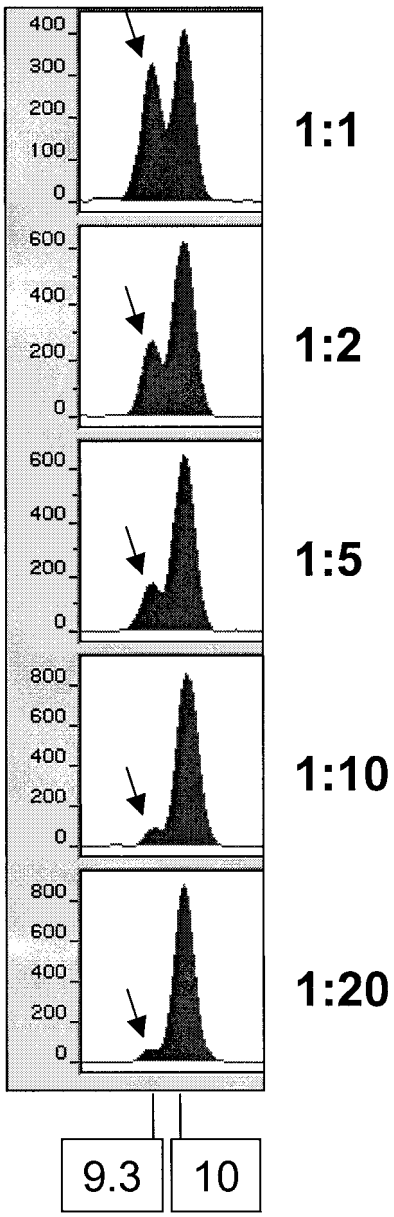

FIG. 6—*Resolution of alleles in mixtures of DNA from two donors, using a 47 cm capillary. The DNA samples are shown in mixture ratios ranging from 1:1 to 1:20. The alleles shown [i.e., 1:20:TH01 9.3 (arrow) (67 rfu), 10 (893 rfu)] differ in size by one base. The minor component in the mixtures used in this study were resolved at ≥5% of the total DNA.*

due to electrical disturbances; particulate matter in the polymer (such as urea crystals), buffer or sample; or the passing of dust particles in front of the detector. Generally, spikes occur in all four colors (blue, green, yellow, and red), but spikes in only two or three colors have been observed (Fig. 8). Like pullup peaks, the multicolor components of a spike typically have identical base sizes and scan numbers. However, they generally occur intermittently and are not reproducible. That is, if a spike occurs in a given position in one run, it typically does not occur in the rerun. If a spike is observed in the rerun, it is unlikely to occur in the same position as that of the initial spike. When spikes are observed in different positions in an initial run and a rerun, it is not generally necessary to run the sample a third time. In the position of the first spike, the absence of an allele may be confirmed in the rerun; and the presence of a true allele in the position of the second spike may be discounted by the first run. Depending on the position of a spike relative to a potential allele(s), sometimes it may not be necessary to rerun a sample in which a spike occurred.

Case Studies

Ten previously reported criminal cases (which had been previously typed using RFLP, or PM+DQA1 and D1S80) were analyzed with the AmpFℓSTR Profiler Plus and COfiler kits on the ABI Prism 310 Genetic Analyzer. The results are summarized below:

- Twenty-eight out of 30 samples yielded typable results at all loci (i.e., Fig. 9*A*). In, Case #3, K1 (blood, victim) (Fig. 9B), some of the larger loci were not typable due to DNA degradation; and in Case #4, Q3-1B (denim jeans), two amplification attempts (each of which was run twice) failed.
- Three samples (that represent three different individuals) exhibited off-ladder alleles within the expected range of alleles (as defined by the allelic ladders) as follows: in one experiment, Case #6, Q1 sample exhibited heterozygous OL alleles at D7S820, and allele 18.2 and an OL allele at FGA; Case #6, K1 sample exhibited allele 8 and an OL allele at D7S820; and Case #8, Q3 sample exhibited homozygous OL alleles at D13S317, and heterozygous OL alleles at FGA. Review of the profiles indicated that it was unlikely that the OL alleles were true microvariants. Reruns demonstrated that these alleles were not microvariants (i.e., were on-ladder). In addition, two samples (reference sample and questioned sample that represent one individual: Case #7, Q99-1B and K2) exhibited a below-ladder FGA allele (Fig. 9*C*). The samples were rerun and then reamplified/rerun, confirming the result of a FGA allele 16.2, which is two bases smaller than FGA allele 17 (the smallest allele in the ladder).
- Occasional spikes were observed. Spikes that fell outside of the expected range of alleles did not impede the ability to accurately type the sample. Generally, spikes that fell within the range of potential alleles were called "OL Allele" by Genotyper, and all samples exhibiting spikes within the range of potential alleles were reinjected. If a spike(s) reoccurred, it did not reoccur in the same position as the original spike, and the samples could be accurately typed.
- Nine out of ten cases yielded sufficient information from STR typing results to determine whether a suspect (s) could be excluded as a potential contributer of a given questioned sample. In one case (#4, Table 3), sufficient information was not obtained from STR typing due to amplification failure (in two attempts) from a questioned item (denim).
- Finally, compared with previous typing of nonSTR loci using the same case samples, there were no discrepancies as to the inclusion or exclusion of suspects or victims with the use of the ABI Prism 310 for STR analysis.

Conclusions

In all studies, allelic designations for samples that were run multiple times by capillary electrophoresis were concordant, and 9947A (control DNA) results were in agreement with the known genotype. Furthermore, correct typing of the loci D3S1358 and D7S820, which can be analyzed with both the Profiler Plus and

TABLE 5—*Sizing precision on the ABI Prism 310 Genetic Analyzer. DNA samples extracted from bloodstains of three donors (A, B and C) were amplified using AmpFℓSTR Profiler. Ten injections of amplified samples were conducted (in each of three experiments using a 47 cm capillary, and in one experiment using the 61 cm capillary). The alleles evaluated ranged in size from 121 bases (D3S1358 allele 13.3) to 317 bases (CSF1PO allele 15) (32); those at D3S1358, TH01, TPOX and FGA were 1–2 bases different in size and had an ancillary usefulness for resolution analyses.*

			47 cm					61 m			
			CE2		CE3			CE2		CE 3	
Locus	Alleles	DNA	*N*	S.D. Allele 1	S.D. Allele 2	S.D. Allele 1	S.D. Allele 2	*N*	S.D. Allele 1	S.D. Allele 2	S.D. Allele 1	S.D. Allele 2
D3S1358	13.3, 14	A	10	0.03	0.04	0.04	0.06					
			10	0.06	0.03	0.04	0.06	10	0.03	0.03	0.04	0.03
			9*	0.03	0.03	0.07	0.05					
TH01	9.3, 10	B	10	0.05	0.03	0.03	0.04					
			10	0.05	0.06	0.04	0.03	10	0.07	0.04	0.04	0.04
			10	0.09	0.08	0.02	0.03					
TPOX	11.3, 12	A	10	0.08	0.04	0.09	0.07					
			10	0.08	0.08	0.07	0.05	ND	ND	ND	ND	ND
			10	0.10	0.05	0.06	0.04					
FGA	22, 22.2	C	10	0.11	0.12	0.10	0.11					
			10	0.05	0.06	0.05	0.05	10	0.05	0.05	0.04	0.03
			9*	0.06	0.05	0.05	0.05					
TH01	7, 8	Ladder	8	0.04	0.04	0.05	0.03	8	0.04	0.04	0.03	0.03
TPOX	9, 10	Ladder	8	0.03	0.04	0.07	0.04	8	0.15	0.17	0.03	0.02
CSF1PO	10, 11	Ladder	8	0.04	0.04	0.06	0.05	8	0.18	0.03	0.03	0.03

* One run failed to provide an interpretable result. Calculation was therefore based on nine out of ten runs.
ND = not determined (peak height was below threshold for analysis); S.D. = Standard deviation; CE2, CE3 = CE instruments 2 and 3.

COfiler kits, was confirmed for each sample by comparing the Profiler Plus-generated types with the COfiler-generated types. Also, when samples were typed using multiple kits that share loci (i.e., vWA, D5S818, D7S820, and D13S317 can be typed using Profiler Plus or PowerPlex 1.1), the typing results were consistent among kits [although rare occurrences of allelic dropout have been described (31)]. There was negligible variation in resolution among the five ABI Prism 310s used in this study. Sensitivity differences among CE instruments were noted but did not impact on accuracy of STR typing. These results support the reliability of the ABI Prism 310 Genetic Analyzer for the electrophoresis and detection of DNA samples amplified using the AmpFℓSTR Profiler Plus and COfiler PCR Amplification Kits and of the GeneScan and Genotyper software for sizing and designating alleles.

In conclusion, operational parameters have been developed for the analysis of PCR-amplified STR loci using capillary electrophoresis. Consistent and reliable typing results were generated with samples from previously typed cases. The data support that

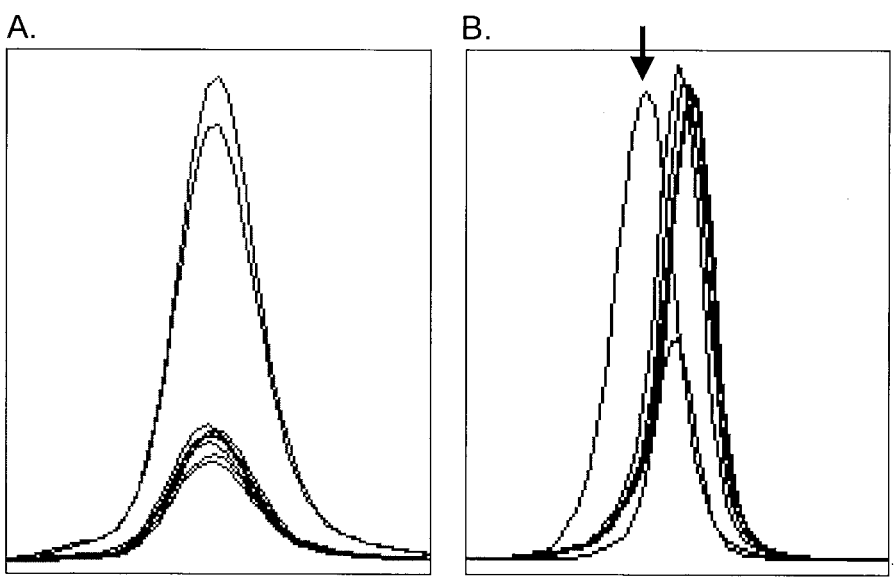

FIG. 7—*GeneScan-500[ROX] 250-base fragment. The 250-base peaks from multiple runs are overlaid (*A, B*). Anomalous migration of the peak is demonstrated in one run by a peak shift (arrow) in panel* B.

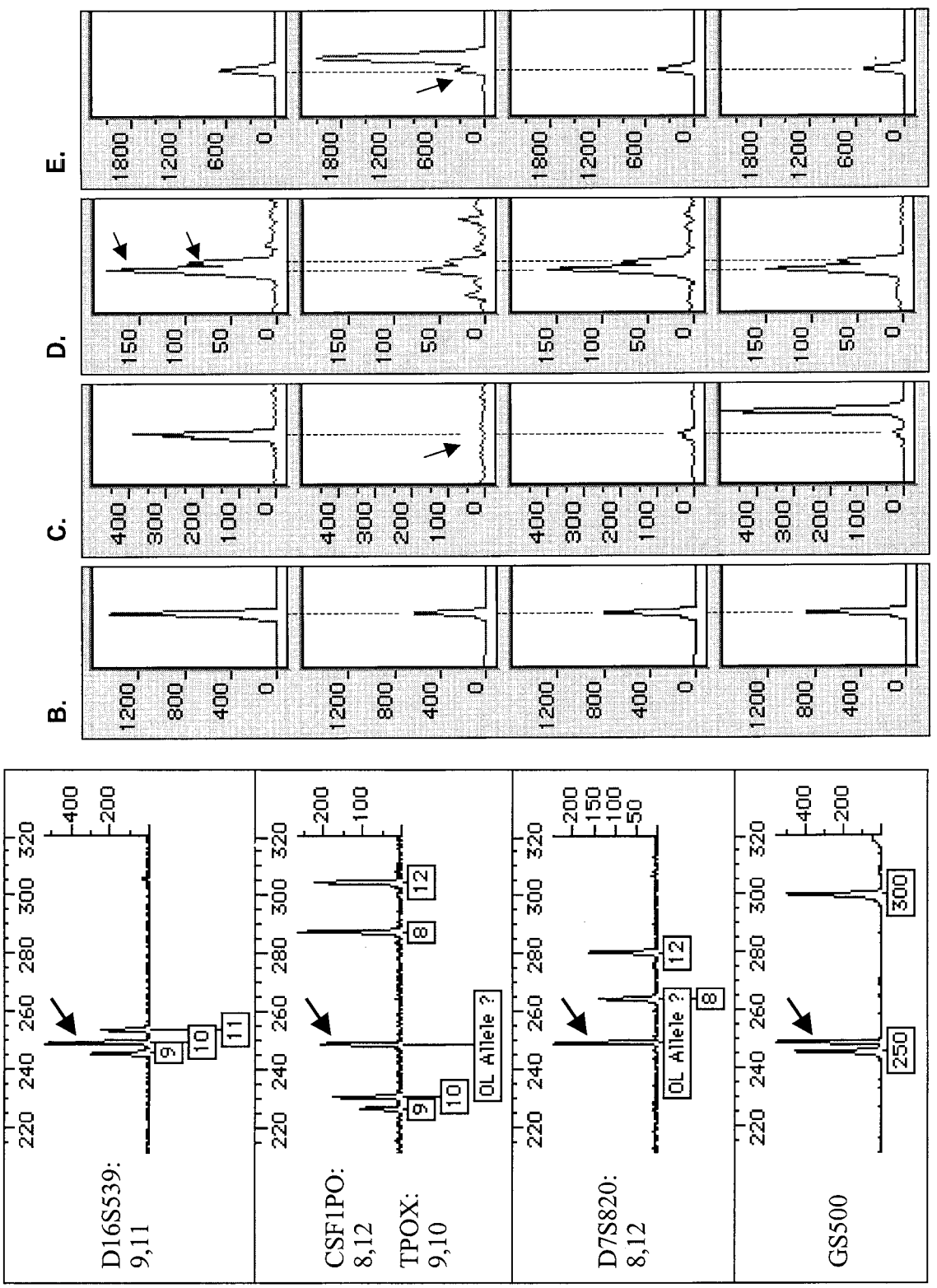

FIG. 8—*"Spikes" occurring during capillary electrophoresis. (A) A spike is shown (arrow). Spikes are typically designated as off-ladder alleles by the Genotyper software. However, if a spike falls within an allele bin (as shown at D16S539, "allele 10"), it is given an allelic designation. (B) A typical spike. (C) Spikes with adequate fluorescence intensity may be observed in all four colors. However, weaker spikes may not occur in some colors (arrow). (D) "Double spike" (arrows) appearing as two distinct yet unresolved peaks. (E) A spike residing in the position of a "minus A" (failure of nontemplated nucleotide addition) peak.*

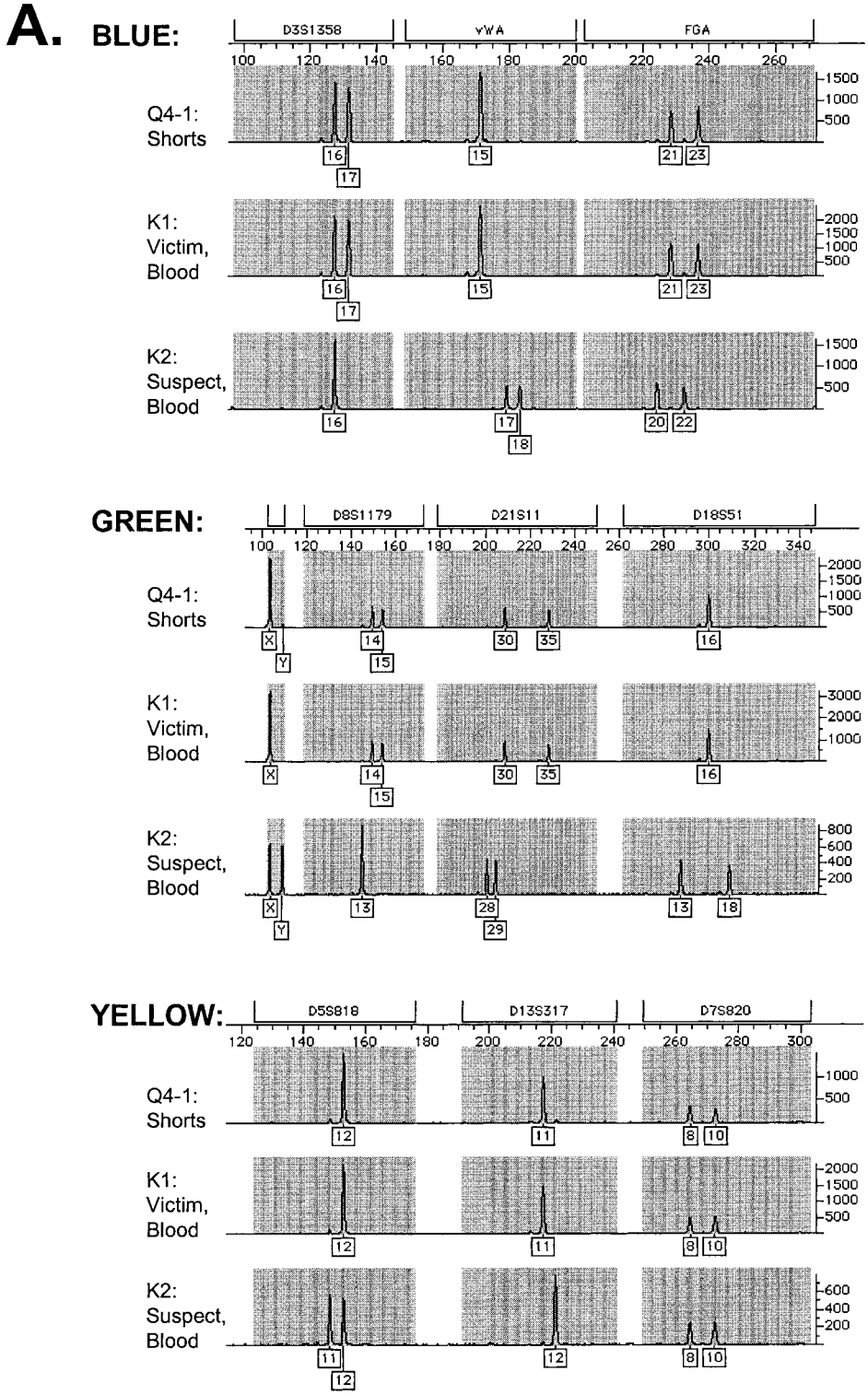

FIG. 9—*Case study examples. STR typing results were generated using AmpFℓSTR Profiler Plus.* (A) *The profile obtained from the evidentiary item (Q4-1, shorts) matches that of the victim (K1) at all nine STR loci. K2 is excluded as a major contributor of the profile, and K1 cannot be excluded as a major contributor.* (B) *Despite apparent degradation (as observed by amplification failure/reduced peak height of larger-sized loci) of the victim's DNA (K1), K1 is excluded as a potential contributor of the biological material found on Q1-1B.* (C) *The profile obtained from the evidentiary item (Q99-1B, carpet) matches that of the victim (K2) at all nine STR loci and the amelogenin locus (X,Y). The off-ladder (OL) allele at the FGA locus is present in both the reference and questioned item and, although not necessary, was confirmed by a subsequent amplification and CE analysis.*

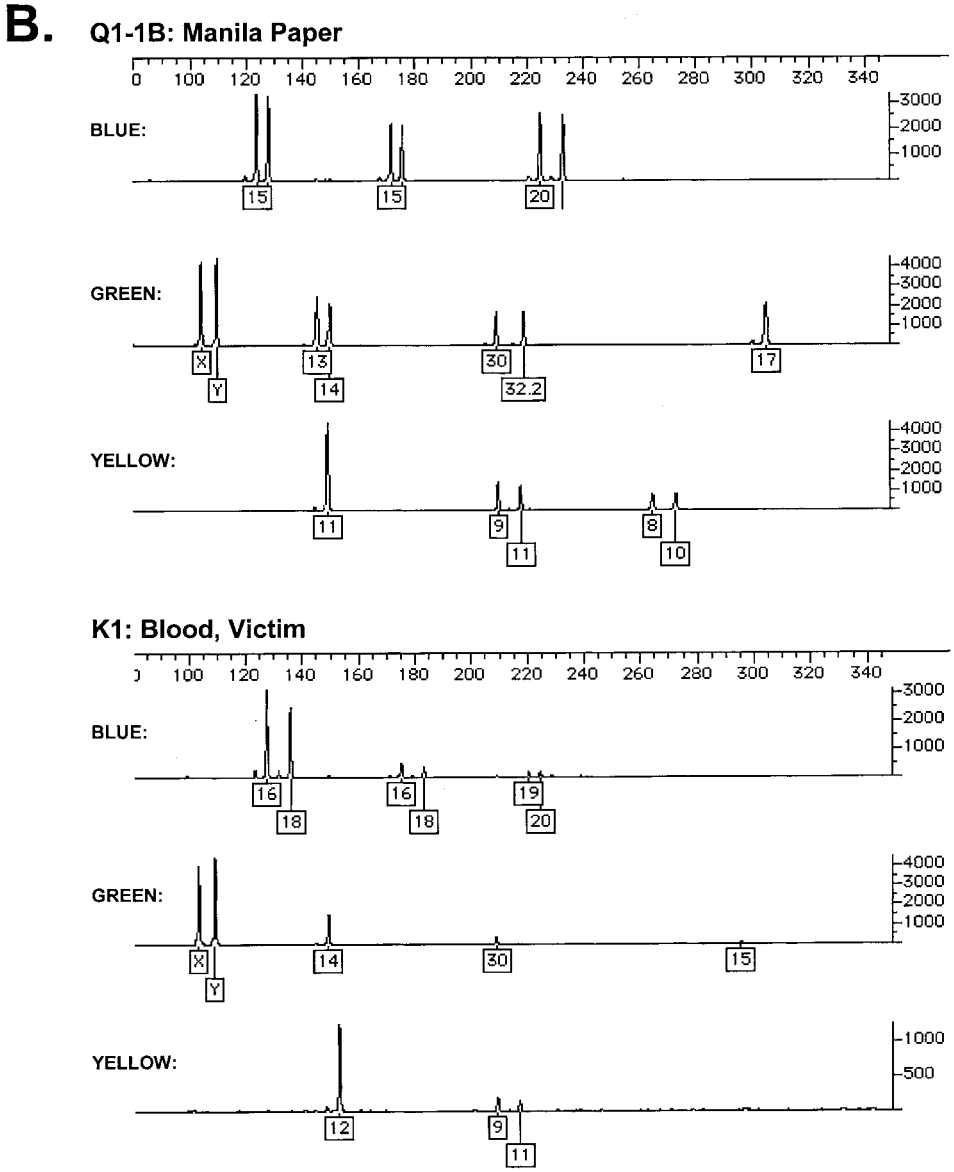

FIG. 9 *(continued)*

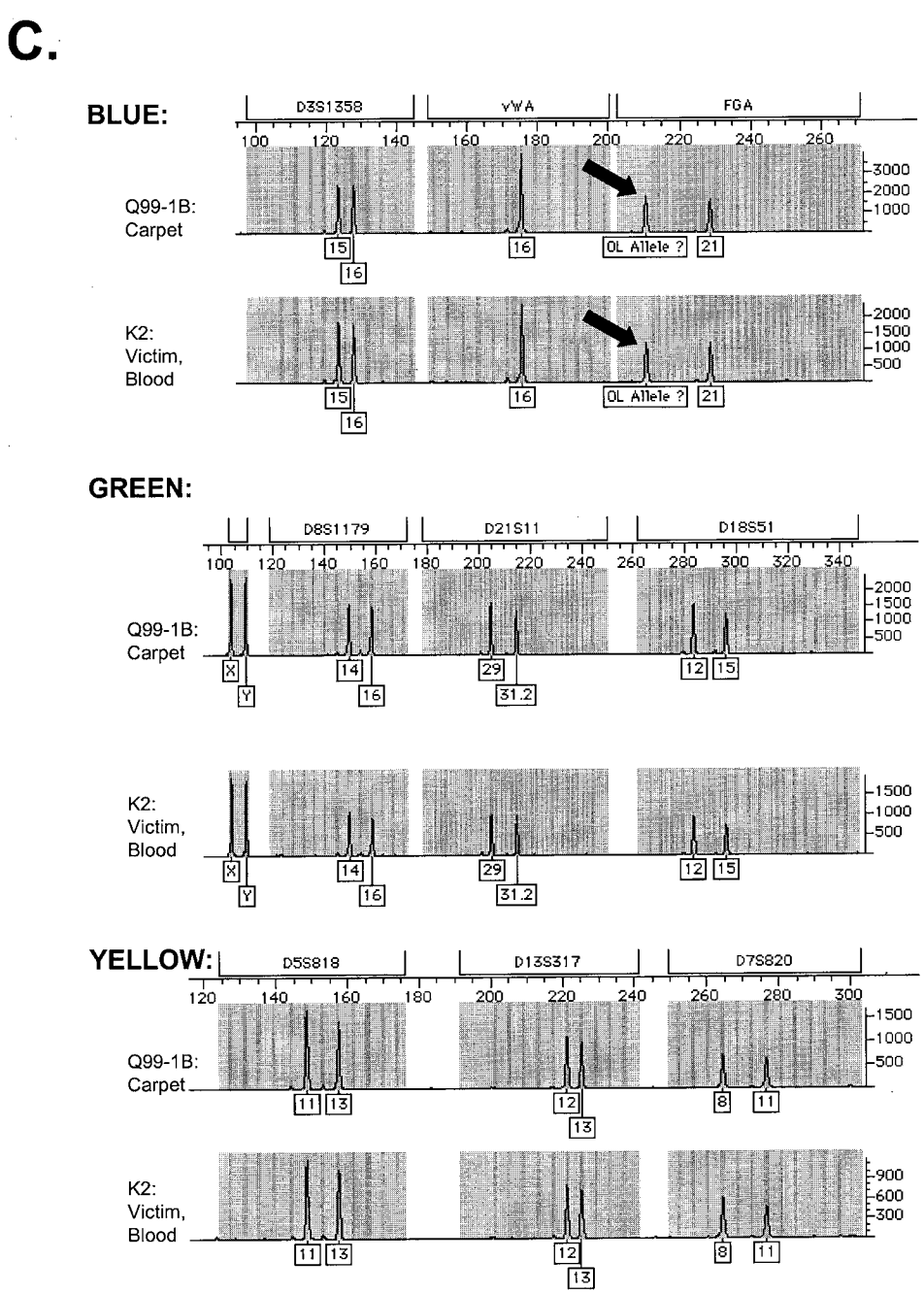

FIG. 9 *(continued)*

DNA derived from known samples and forensic biological materials can be reliably characterized by capillary electrophoresis following PCR amplification. As an alternative to slab gel electrophoresis, capillary electrophoresis provides a relatively simple, semiautomated means of DNA typing that is suitable for implementation in most application-oriented laboratories.

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