

TECHNICAL NOTE

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Using Resolution Calculations to Assess Changes in Capillary Electrophoresis Run Parameters*

ABSTRACT: Capillary electrophoresis is widely used in the forensic community for the analysis of Short Tandem Repeat DNA. The CE system used in most forensic laboratories allows the user to modify standard operational protocols to accommodate some samples that fall outside of interpretational guidelines. We have made operational changes and monitored system resolution and the ability of the software to identify alleles as a result of these modifications. Increased amount of amplified product in the injection preparation, lengthened injection times or repeated injections of the same sample were all investigated along with variations in run voltages. Certain protocol modifications offer some advantages to the analysis of STRs and may allow the analyst to avoid extraction of additional portions of crime scene samples to achieve interpretable results.

KEYWORDS: forensic science, capillary electrophoresis, DNA, resolution, run modification, performance analysis

Capillary electrophoresis allows for the semi-automated analysis of forensic DNA samples. Although introduced as a single capillary instrument sequentially analyzing one sample at a time, multiple array capillary systems are becoming common in forensic laboratories. Once a sample has been analyzed, the analyst may determine that the sample needs to be re-run, accomplished through either the re-injection of the sample or by injection of a newly prepared sample. Those samples that mandate reanalysis due to peak height or peak area values outside normal analytical working ranges may be amenable to varying injection parameters or to alterations in the amount of the amplified product in the injection solution. These procedures can often yield results within the laboratory's established operating range for the instrument but obliges the analyst to provide adequate validation for such analyses. One facet of the validation required is to demonstrate that the system accommodates these experimental variations. An excellent method to assess the system's response to these changes is through measuring the resolution of the system when perturbed by the experimental variable. Resolution measures the ability of the system to separate components. Quantitative resolution measurements permit the monitoring of experimental variables upon the system and allow assessment of their impact.

In a previous, paper we discussed the use of resolution measurements to evaluate physical changes in the electrophoresis system by examining the effects polymer concentration and capillary length had on the resolution of the system. The present study examines experimental variations that may be used to produce electrophero-

grams suitable for analysis and also extends the previous work by evaluating the relationship between electric field and resolution.

Materials and Methods

The capillary electrophoresis unit employed for this study was an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). A 47 cm uncoated 50 μm ID column (Applied Biosystems) with a read length, the distance from injection end to laser, of 36 cm was used. The separation medium was the proprietary polymer product POP 4 for 310 use (Applied Biosystems) and the run buffer was a 1:10 dilution of 310 Genetic Analyzer Buffer with EDTA (Applied Biosystems). Prior to each injection the column was back flushed with new polymer by the instrument. The software employed was GeneScan® version 3.1, with the analysis parameters set to light smoothing.

Whole blood samples were collected in EDTA blood collection tubes and spotted on paper, then allowed to dry. The samples were maintained prior to extraction at -20°C or -80°C. The DNA was extracted by an organic extraction as modified by Buel et al. (1). Sample amplifications were conducted using the amplification kits AmpFℓSTR™ Profiler Plus, or AmpFℓSTR™ COfiler (Applied Biosystems) which support the amplification of nine and six short tandem repeats respectively in addition to the amelogenin sex typing locus (2,3). Amplifications were performed according to manufacturer recommendations (2,3) with one minor exception; total amplification volume was halved to 25 μL. Unless noted otherwise, the target amount of DNA amplified was 1 to 2 ng. The thermal cycler used for the amplifications was an Applied Biosystems model 9600 set to the parameters indicated by the kit instructions.

The AmpFℓSTR™ kits contain allelic ladders that represent typical alleles for each locus amplified by the respective kit. The allelic ladders or the amplified extracts were prepared for CE analysis by mixing 1 μL of the ladder or the amplified product (unless other-

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wise noted) with 0.5 μL of GS ROX 500 internal lane standard (Applied Biosystems) and 12 μL of deionized formamide (Amresco, Solon, OH). Prepared samples were denatured at 95°C for 3 min and snap-cooled in an ice-bath for 3 min. In those experiments in which the amount of amplified product added to the formamide was varied, the samples were denatured as described above with the variations in product added shown in the results section.

Samples were routinely electrokinetically injected at 15 kV for 5 s, followed by electrophoresis at a constant voltage of 15 kV and a constant temperature of 60°C. For those experiments that the injection time was modified, samples were electrokinetically injected at 15 kV while the injection times ranged from 1 to 15 s followed by the standard electrophoretic conditions described above.

For those experiments in which different electrophoresis voltages were used for the analysis, samples were electrokinetically injected as described above, 15 kV for 5 s, and the temperature remained constant at 60°C. The initial experiment employed 2.5 kV for electrophoresis voltage, while subsequent runs were incrementally increased by 2.5 kV until the final run of 15 kV was reached. As the run times were lengthened for some of these experiments due to the reduced electrophoresis voltages, the data capture times were increased accordingly. The loci visualized as “green” fluorescence in AmpF ℓ STR™ COfiler allelic ladder were used for these experiments. The above column, buffer and polymer conditions were employed for all experiments.

The evaluation of resolution may be conducted using a variety of methods. In a previous paper we described the use of resolution length, RSL, to evaluate the resolution of a system (4). The calculation of RSL allows one to easily compare changes in resolution as parameters are altered. In this work we use RSL as described previously (4) and detailed by Heller et al. (5) and by Lerman and Sinha (6) for all resolution comparisons.

Resolution measurements were conducted using a digital caliper to obtain peak widths, heights and distance measurements between peaks from printed, similarly scaled GeneScan® electropherograms. When appropriate, the base difference between peaks was expressed as bases and not base pairs since the system under consideration examines single strand DNA.

Results and Discussion

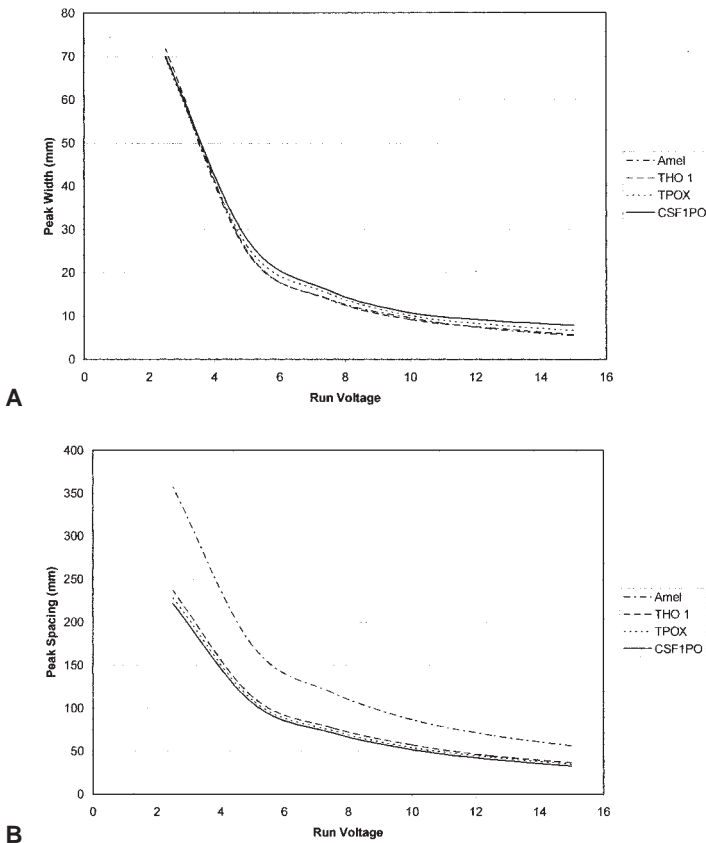
The analysis of forensic samples by capillary electrophoresis often results in electropherograms with variations in peak heights. Those samples with peaks below a certain relative fluorescent unit (RFU) level may be deemed uninterruptible. For these samples, an increase in injection time or an increase in the amount of amplified product added to the injection preparation may yield a sufficient increase in RFU of the peak to allow interpretation of the run. These minor modifications to improve the signal are changes to normal operational protocols requiring validation experiments prior to casework analysis. Investigations are important to understand the impact increased injection times or additional amplified product has on the resolution of the system. We have evaluated the effect an increase in injection time has on the resolution of an amplified sample that contains the CSF1PO alleles 10 and 11. Little variation in RSL is seen up to a 9 s injection time; beyond that point RSL increases sharply indicating a reduction in system resolution. Peaks become increasingly broad past 9 s and slightly closer together (data not shown). All the samples were correctly genotyped regardless of the injection time employed using Genotyper® software (Version 2.5, Applied Biosystems). Peak height increases in response to longer injection times. For the CSF1PO alleles, as the injection times increase from 1 to 9 s, a dramatic improvement is seen

in the resulting peak height. Although one could expect a three-fold increase in RFU when the injection time is increased from 1 to 3 s, an increase of only 2.8 is observed and only about a seven fold increase in RFU is seen when the results of the 1-s injection are compared with that obtained from a 9 s injection (data not shown). Injection times longer than nine seconds have limited affect upon the RFU. In contrast, the RFUs of the alleles found in the THO 1 locus showed an almost linear response to increases in injection time. A 15-fold increase in the injection time (1 s to 15 s) produced a 13.7 fold increase in total RFU (data not shown). The THO 1 locus represents smaller base size alleles compared with the CSF1PO locus and electrokinetic injection favors smaller ionic species in the injection process (7). This may account for the RFU difference observed between the two loci.

Another experiment examined the possibility of increasing the RFU of a sample by simply increasing the amount of amplified product in the injection preparation. Increasing the amount of amplified product gave a significant RFU response, almost linear when the amount of product added was increased from 0.5 μL to 2 μL . Beyond 2 μL a gradual increase in RFU was seen up to an addition of 8 μL of amplified product. No peak broadening was observed for any of the conditions employed and no change in RSL was observed for this set of experiments. All alleles were properly identified under all conditions evaluated (data not shown).

Those samples that have adequate peak height may still need to be re-run due to artifacts or other factors that may make interpretation of the electropherogram problematic. This can be accomplished by preparing a new injection solution with another aliquot of the sample or by re-injecting the prepared injection solution to determine if the artifacts are reproducible. We examined the effect re-injection had on the RSL and peak height of a sample subjected to five subsequent injections. The injection voltage and run conditions were those used for routine analysis. The sample had been amplified using the COfiler kit and prepared as a standard sample. No change in RSL or peak height was noticed between the first injection and the 5th injection (data not shown).

In a previous work (4), we examined the affect polymer concentration and column lengths have on system resolution. Those studies showed that system resolution could be altered through changes made in polymer concentration, POP 4 vs. POP 6, and capillary column length. Another parameter under operator control that can alter resolution is electric field strength (5). The electric field strength, defined as the voltage per unit length (8), can be modified through changes in column length or in the applied run voltage. Previously, we monitored resolution through changes in column length, here we maintained column length and examined the affect changes in run voltage have on RSL. The manufacturer's recommended running voltage is 15 kV, which is the maximum output voltage for the 310 Genetic Analyzer. Electropherograms were recorded of the COfiler allelic ladder with electrophoresis conducted at 2.5 kV and incrementally increased by 2.5 kV with subsequent runs until 15 kV was reached for the final electrophoretic run. Resolution measurements of these electropherograms were made from both amelogenin alleles, alleles 5 and 6 in the THO 1 system, alleles 6 and 7 in the TPOX system and alleles 6 and 7 and 14 and 15 to evaluate the early and late eluting alleles respectively in the CSF1PO system. The resolution of a system as calculated using RSL is based upon the peak width and the distance between peaks. Broad peaks spaced closely together will yield relatively large values indicating poor “resolution” of the system. Likewise narrow peaks widely spaced yield small values of RSL indicating a system with good resolution. As the run voltage is increased, peak width decreases, Fig. 1a, as does



FIGS. 1a and 1b—Peak widths and spacing between peaks measured from a representative peak in each of the loci contained in the COfiler ladder. The amelogenin locus is abbreviated as Amel in the legend.

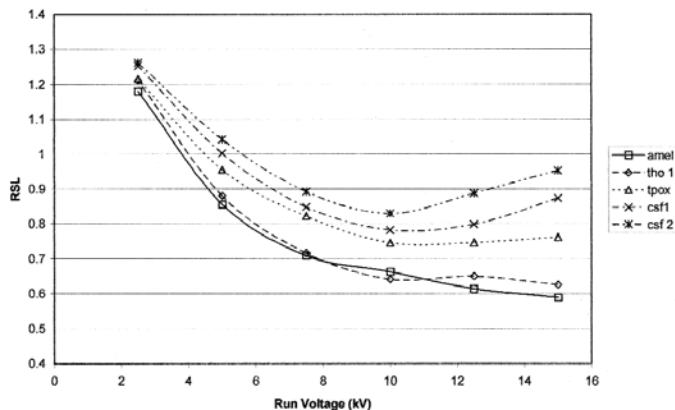


FIG. 2—Relationship between RSL and run voltage. Representative peaks were chosen in each locus of the COfiler ladder and evaluated for RSL. Two sets of peaks, early and late eluting, were chosen for CSF1PO and were termed CSF 1 and 2 respectively.

peak spacing, Fig. 1b. Peaks are wide at a low run voltage and narrow considerably as the voltage is increased from 2.5 kV to approximately 5 kV. Peak spacing responds similarly to changing run voltage, widely spaced peaks become significantly closer as the run voltage increases from 2.5 to 5.0 kV. Figure 2 shows how RSL varies with the electric field for the alleles evaluated.

The calculated RSL values show how RSL decreases (improved resolution of the system) as the run voltage is increased for the alleles evaluated for amelogenin. RSL decreases and plateaus at ap-

proximately 10 kV for the alleles evaluated for THO 1 and TPOX. At approximately 10 kV, RSL reaches a minimum and starts to increase for both sets of the evaluated alleles for CSF1PO. In evaluating an optimum value for the electric field, 10 kV appears to yield the best resolution when considering all alleles examined. To achieve this increase in resolution, the length of the run has to be increased from approximately 24 min to 40 min as the fragments migrate slower at the lower voltage. The change in resolution between the results obtained at 10 kV versus 15 kV is relatively small and may not warrant a change in protocol for routine casework analysis. Under certain circumstances it may be important to resolve a closely spaced allele falling in the base size range for CSF1PO. Reducing the run voltage from 15kV to 10kV would be an option to improve RSL requiring only minor protocol changes to include an extension of the collection run time.

There are a number of changes that can be made to the normal operational protocol to achieve improved results that do not adversely affect RSL or hamper the ability of the software to correctly identify alleles. These operational modifications allow the analyst the opportunity to improve individual sample results without the need to resample and extract precious crime scene material. However, as with any adjustment in protocol, laboratories must assess how best to apply interpretational guidelines in view of these modifications. By setting threshold RFU for analysis, many laboratories try to minimize the analysis of stochastic effects, the extreme variability of peak heights resulting from amplifications of minimal template amounts. Peak height imbalance and variability are primarily consequences of PCR, not variation in CE analysis (9). The modification of parameters that allows enhancement of peak height and for peaks to be identified above a certain RFU will still reflect the stochastic problems encountered in PCR. This must be realized and considered when interpreting electropherograms so modified.

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