applied biosystems by life technologies

FAS Corner July 2010

Maximizing the Performance of Capillary Electrophoresis Systems

The Maximizing Data Quality Series—Part 4

Introduction

Since the introduction of the ABI PRISM® 310 Genetic Analyzer in 1996, capillary electrophoresis (CE) instruments have become the dominant platform on which analysis of forensic DNA data is performed. Being such an important component in the overall forensic workflow, these instruments play a key role in determining the quality of the data produced, and there are many simple ways to ensure they are kept in pristine running condition. Comprehensive information on some of the most simple preventative maintenance activities and common troubleshooting scenarios associated with use of a CE platform can be found in a previous article, "Troubleshooting Amplification and Electrophoresis of the AmpFℓSTR® Kits", published in the October 2007 issue of *Forensic News*. In this article, we will focus on other features of CE platforms that frequently generate questions from our users, and highlight the improvements we believe the new Applied Biosystems® 3500 Genetic Analyzer will bring to overall data quality.

Factors Influencing Baseline Instrument Noise Levels on Capillary Electrophoresis Platforms

All systems for analysis of chemical or biochemical applications are designed to minimize the amount of overall baseline or background noise produced, thereby minimizing any impact on the analysis of data. However, no instrument-based analysis, including capillary electrophoresis (CE) of STR profiles, can eliminate all sources of noise. The role of the forensic scientist is to understand the sources, evaluate their prevalence within the system, and establish interpretation guidelines that reflect their observations.

Crosstalk

When dealing with single-capillary systems such as the 310 Genetic Analyzer, localizing the fluorescent signal from each injection to the individual capillary is a simple and straightforward task. With the advent of multi-capillary CE instruments, however, achieving the same degree of separation becomes more of a challenge, one which increases as the number of capillaries on the platform increases. The goal on a multi-capillary platform is to maximize confinement of the signal from each individual capillary and thereby minimize signal encroachment into neighboring capillaries, which may have the potential to affect data interpretation. Engineering requirements inherent in the optical system on multi-capillary CE systems sometimes permit very small amounts of signal transference from one capillary into another directly adjacent to it. This phenomenon is termed 'crosstalk'. In a properly functioning and well-maintained instrument, the level of crosstalk is almost always at or below the baseline noise level of the instrument and certainly below the common thresholds used in forensic laboratories.

So how does crosstalk occur in practice? As the signal from the dye-labeled STR fragment fluoresces, the resulting signal is directed toward an exact region of the CCD camera as determined by the spatial calibration. The vast majority of the signal for a given capillary is contained within the specific region identified for that capillary. However, a small amount of signal, generally much less than 0.5%, may be imparted onto the regions allocated to the capillaries directly adjacent to it. Crosstalk can be evaluated by measuring the peak height of the primary peak (5000 RFU in blue, for example) and evaluating the blue dye channel in the adjacent capillaries for signal at the same data point (crosstalk peak). The percentage of crosstalk can be calculated by dividing the peak heights of the primary and crosstalk peaks.

Continued next page

Minimizing the Occurrence of Crosstalk

Excessive amounts of crosstalk generally represent a hardware or calibration failure within the instrument, or an overloaded DNA sample resulting in excessive signal (i.e., off-scale data). To minimize the occurrence of these events:

- Ensure the instrument is regularly maintained by a qualified engineer, according to the recommended service schedule
- Ensure a valid spatial calibration is available for the currently installed array to accurately determine the appropriate region for collection of data from each individual capillary
- Follow recommended and validated guidelines for the amount of DNA added to the amplification reaction to minimize the occurrence of off-scale data
- Use the appropriate injection mixture chemistry (size standard, formamide, PCR product/allelic ladder) to optimize the signal intensity of all analysis components

Following these simple guidelines will help ensure that the level of crosstalk does not rise above the level of the inherent background noise of the instrument and is therefore effectively inconsequential with regard to data interpretation.

Carryover

In contrast to crosstalk, which affects only adjacent capillaries within a single injection, carryover is defined as the physical transfer of DNA from one injection to the next. Therefore, carryover can affect both single and multi-capillary platforms and, unlike a crosstalk evaluation, the evaluation of carryover is performed by evaluating the subsequent injection of a single capillary for any sign of signal from a previous injection.

Some examples of possible explanations for carryover are as follows:

1. *Defective Septa*

Following sample injection, the capillaries are moved from the electrophoresis plate to the water container to rinse off any excess sample. Before they enter the water, the capillaries must pass through the septa covering the wash containers which are designed to squeeze liquid off the capillaries as they exit. It is possible, however, that the septa may remove liquid from the capillaries as they enter the wash container, depositing a minute amount of sample on the septa. During the following injection, the capillaries are rinsed once again, in this case to remove the high salt run buffer. After this wash step, the capillaries move to the new sample wells. If droplets from the previous sample were left on the water tray septa, the residue could be picked up by the tips when exiting the water tray and transferred into the next sample.

Continued next page

Whether or not this signal is sufficient to manifest itself in the subsequent injection is dependent on factors such as:

- The concentration of the DNA in the source well - excessive amounts of DNA in the source well could lead to a greater likelihood of DNA transfer
- The effectiveness of the septa to perform as intended - defective septa may not remove liquid effectively from the tips of the capillaries, and this is one of the reasons why long-term usage of the septa is not recommended
- The integrity of the tip of the capillary - chipped or cracked capillary tips may provide harbors for liquid to linger in during the wash step

2. Incomplete Filling of the Capillary/Capillary Array

As part of each injection cycle, the polymer used during each injection is replaced with fresh polymer for the next injection. During this process, exhausted polymer containing electrophoresed PCR products is expelled into the waste container and replaced during a fill step, which pumps more than the minimum fill requirement of polymer through the capillaries. This ensures that the contents of the capillary are completely replaced and that no PCR products remain in the capillary to be re-electrophoresed with fresh PCR product.

The most common factors affecting filling of the capillary/capillary array are:

- Incorrect installation of capillary/capillary array - insufficient tightening of the capillary ferule/array knob around the capillary/capillary array may lead to leaks during the fill process and insufficient polymer reaching the capillaries
- Arcing in the lower polymer block - this may affect the integrity of the seal between the buffer pin valve and the buffer channels, leading to leaks and reduced array fill pressure
- Exhausted polymer syringe - on 310 and 3100 platforms, natural wear and tear can lead to leaks around the syringe plunger, which affect the efficiency of the capillary/capillary array fill process
- Alteration of capillary/capillary array fill parameters in the instrument run module - reducing the fill volume parameter may lead to incomplete replacement of the contents of each capillary following each injection
- Instrument hardware failure - e.g., polymer pump on the 310 platform

Laboratories, may test the occurrence of carryover in their system by injecting alternating source wells containing amplified STR fragment DNA followed in a subsequent injection by blank wells containing only formamide and size standard. The percentage of carryover may then be evaluated by calculating the relative peak heights of the fragments observed in the source wells and blank wells as described for the crosstalk calculations. It is important to remember, however, that evaluation of the level of carryover should always be assessed within the normal operational protocols of the laboratory (i.e., using extraction blanks, PCR negatives, samples, allelic ladders, etc.). Totally blank samples (e.g., formamide only with no size standard present) are not generally representative of the normal types of samples run within the laboratory and therefore do not provide an adequate benchmark to perform the carryover calculations.

Continued next page

Minimizing the Occurrence of Carryover

Significant amounts of carryover may be a symptom of an instrument hardware failure or the result of poor maintenance of the instrument platform. To minimize the occurrence of these events:

- Ensure the instrument is regularly maintained by a qualified engineer, according to the recommended service schedule
- Replace plate/tube and reservoir septa at the recommended intervals to ensure optimum function
- Ensure the capillary ferule/array knob is tightened correctly to create an effective seal between the polymer block and the capillaries
- Clean up any buffer spills from around the lower polymer block and remove bubbles from polymer channels to prevent arcing in the lower polymer block
- Replace polymer syringes according to the recommended schedule to prevent leaks
- Utilize recommended run module parameters

In general, when all components are functioning correctly the amount of carryover is extremely small. In most instances, this carryover occurs at levels below the background noise level of the instrument and therefore has a negligible impact on data interpretation.

Understanding & Accommodating Baseline Instrument Noise

All analytical instruments have inherent sources of noise, but understanding them and setting the appropriate interpretation guidelines allows the user to eliminate it as a hindrance to data analysis. Laboratories should attempt to minimize any source of noise within their system to maximize their ability to interpret data effectively, especially when the source of input DNA is limited.

Comprehensive validation studies, which include evaluation of a baseline interpretation RFU threshold appropriate to the samples being analyzed, are a fundamental requirement prior to implementation of any new technology including CE instrument platforms. The evaluation of the overall noise level will incorporate all of these factors described above, and allow the laboratory to confidently set an appropriate interpretation threshold to increase the likelihood of obtaining a reliable profile for interpretation.

Continued next page

applied biosystems by life technologies™

Environmental Temperature

Laboratory temperature is a critical parameter to control to avoid aberrant peak morphology of STR alleles, including split peaks and broad peaks, and shifting of electrophoresis data points, which compromises the ability to accurately size the PCR alleles. Running the capillary electrophoresis platforms in a laboratory environment that is too cold can have a marked effect on the appearance of the data, as demonstrated in the October 2007 *Forensic News* article, "Troubleshooting Amplification and Electrophoresis of the AmpFℓSTR® Kits." It is generally accepted that split peaks such as this are the result of incomplete denaturation of the fragments, caused by lower ambient temperatures. Some instruments specify wide ranges for acceptable operation, such as 15-30 degrees Celsius; however, these ranges are not application-specific and do not reflect acceptable ranges for HID chemistries. In most cases, laboratories have been able to eliminate split peaks caused by room temperature issues by maintaining laboratory temperatures above 21-25 degrees Celsius. The optimum room temperature will vary based on many factors, but each laboratory will need to evaluate the optimum temperature for their protocols and workflows. Some factors which will affect the ideal room temperature include instrument variables, STR chemistry, and the quality or viability of the consumables. This optimum room temperature can only be determined empirically within each laboratory by systematically increasing the room temperature until the deleterious effects are eliminated.

Optimization of Sample Injection Parameters

During development of each new CE platform, Life Technologies performs an extensive developmental validation study. This study is designed to identify the optimum run conditions for the instrument in combination with the AmpFℓSTR® family of PCR Amplification Kits. Historically, a single, optimum set of injection parameters has been recommended and supported for Human Identification applications for each CE platform in an effort to promote consistency and deliver, as much as possible, consistent peak heights both within and between different instrument models. However, as forensic laboratories have expanded to contain multiple CE units, experience has shown that the recommended injection parameters may not produce the expected peak heights on all instruments within a laboratory, which can present challenges when attempting to establish standard operating procedures. Also, individual instruments with sensitivity levels towards the low end of the normal spectrum can cause difficulties with sample analysis if they do not produce peak heights within the normal analytical working ranges of the laboratory. In this situation, samples may need to be re-run, consuming valuable time and resources.

We frequently receive inquiries regarding whether injection parameters may be modified under certain circumstances, and requests for guidance on the best way to achieve this. In order to help answer these questions more effectively, we conducted a study to evaluate a range of injection parameters. The study was designed to develop a more comprehensive understanding of the capabilities and limitations of the system, and investigate whether laboratories could effectively normalize sensitivity between instruments by making small alterations to the parameters contained in the standard run modules.

Overview of the Injection Parameter Optimization Study

The study focused on the 3100 & 3130 series instrument platforms, and a range of injection parameters were tested to determine the limits of performance of the instruments (Table 1). Replicate injections of the Identifiler® Kit allelic ladder were performed under each set of conditions and the results evaluated for peak height, resolution, and sizing precision. Based on these results, a subset of conditions was chosen for further evaluation of sensitivity and ability to detect the minor contributor in a 9:1 mixture.

Continued next page

Injection Module	Injection Voltage (kV)	Injection Time (secs)	Injection Product (volts x time)
1	1	5	5
2	1	10	10
3	1	15	15
4	1	20	20
5	1	25	25
6	3	5	15
7	3	10	30
8	3	15	45
9	3	20	60
10	3	25	75
11	5	5	25
12	5	10	50
13	5	15	75
14	5	20	100
15	5	25	125
16	7	5	35
17	7	10	70
18	7	15	105
19	7	20	140
20	7	25	175
21	10	5	50
22	10	10	100
23	10	15	150
24	10	20	200
25	10	25	250

Table 1: A total of 25 different sets of injection conditions were tested on a single instrument to establish the limitations of the system. Five sets of optimal injection conditions were identified for further testing on a wider range and number of instruments (hilighlighted in orange). Injection Module 6 represents the standard injection conditions for the 3100-*Avant* and 3130 platforms; Injection Module 7 represents the standard injection conditions for the 3100 & 3130*xl* platforms.

Continued next page

Summary of Injection Optimization Results

Of the 25 different sets of injection conditions tested, 5 were identified as producing optimum results across the full range of 3100 and 3130 instrument platforms. Unsurprisingly, the set of 5 contains the standard recommended run parameters for the 4- and 16-capillary instruments, illustrating why these conditions were selected as the standard run conditions during the original validation of these platforms. We still anticipate, therefore, that for the majority of instruments and sample types, the recommended conditions will continue to provide the required level of performance.

For those laboratories looking to normalize signal on outlying instruments that generate signal which is either higher or lower than the main population of instruments within the laboratory, increasing injection time and/or voltage does cause a corresponding increase in peak heights (and vice versa), but the increase is non-linear and, therefore, no exact formula is available to allow the user to establish which parameters would deliver the required increase (or decrease) in peak height according to their current signal level. It is highly recommended that laboratories looking to adjust their injection parameters run a comprehensive validation study to establish the best injection modules for obtaining the desired peak heights, based on samples containing a range of starting input DNA amounts. The five optimum sets of conditions highlighted in Table 1 would be a sensible starting point for any such study.

For laboratories looking to investigate a wider range of injection parameters, acceptable resolution was obtained for injection modules with an injection product of less than 100. Laboratories are recommended not to exceed this value, as injection modules with a product of greater than 100 are more likely to fail to generate the 1 bp resolution required for analysis of forensic STR data.

New Developments to Improve Data Quality

At Life Technologies we continue to strive to ensure that forensic laboratories are able to obtain maximum performance across all our instrument platforms. Conscientious attention to the recommended maintenance routines detailed in our instrument user guides is a very simple but effective way to help ensure smooth running of the instrument and maximize data quality. Understanding all aspects of the DNA profile and developing sound interpretation guidelines to accommodate all factors inherent in the process will also simplify as much as possible the time-consuming and often challenging practice of data interpretation.

At the same time, we continue to investigate new technologies with the intention of producing even higher-quality data and increasing the confidence with which even the most difficult samples can be analyzed. The next generation of AmpF*ℓ*STR® chemistries (Identifiler® Direct, Identifiler® Plus, and NGM™ kit) are manufactured using the latest primer synthesis techniques, which help eliminate the presence of dye artifacts, producing cleaner baselines than ever before. The next-generation CE instrument platforms, the Applied Biosystems 3500 Series Genetic Analyzers, are the first capillary electrophoresis platforms from Applied Biosystems to be designed specifically for Human Identification applications. These platforms offer a unique combination of hardware, software, and reagent enhancements, all designed to promote smoother and more efficient production of the highest-quality data. In combination, these new instrument and chemistry developments offer the potential to increase still further the confidence with which forensic DNA profiles can be interpreted, and maximize the contribution of each profile to the criminal justice system.