TECH TIPS

Troubleshooting Capillary Electrophoresis Systems

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INTRODUCTION

The development of capillary electrophoresis (CE) has played a key role in bringing about the modern application of DNA typing. Forensic laboratories are the beneficiaries of this new technology, but many practitioners are not fully aware of the underlying principles of the CE system. This article attempts to address the important issues in CE separations to aid analysts in troubleshooting problematic separations. The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele. These points are addressed below.

SEPARATION

DNA analysis by CE is performed using entangled polymer buffers (Figure 1). These buffers can be easily pumped into a capillary prior to a separation and pumped out at its conclusion, providing a fresh separation matrix for each run. A typical buffer for forensic DNA separation contains 4% polydimethyl acrylamide (pDMA), buffered to pH 8 (1). This polymer provides good separation of DNA and tends to coat the capillary wall. This coating is important because the capillary wall can become charged at high voltages, producing irreproducible migration shifts. Wall effects can also affect peak resolution, and it is a good idea to monitor a pair of peaks in your electropherograms to make certain that resolution is consistent from run to run (2).

In addition to the polymer and buffer, strong denaturants, such as urea and pyrolidinone, are added to the solution to keep the DNA single-stranded. Single-stranded DNA (ssDNA) is preferred because it interacts more efficiently with the sieving polymer, and its size is proportional to its length, a relationship that is the key to accurate genotyping by CE. Denaturants also help prevent the formation of DNA secondary structure, a phenomena in which complementary sequences within ssDNA molecules bind to each other. This can create loops and hairpins, which cause the DNA molecules to migrate in an anomalous fashion. Elevated temperatures can minimize the formation of secondary structure, but temperature must be carefully controlled, as this parameter strongly influences DNA mobility and the calculated allele size (3). Temperature also affects separations by altering the viscosity of the polymer solution.

To help mitigate problems with temperature and secondary structure, all DNA separations are performed with an internal sizing standard. The use of this standard ensures that, if temperature or current fluctuates, the resultant shift in mobility will affect both the sizing ladder and sample. Two different methods can then be used to estimate size: a point-to-point method known as local Southern and a regression fit known as global Southern. Global Southern sizing is generally less affected by temperature change, but both methods produce highly precise results (4). Regardless of which method is used, the allele size estimate produced by the computer is a relative size and not necessarily the true sequenced size. To find the true size, the result is compared to the allelic ladder.

Because the analysis for a set of CE samples can take a day or more, size estimates may slowly change over time. To correct for this effect, run an allelic ladder every 10 runs or so. If loss of calibration of later samples occurs due to room temperature or other factors, simply switch from calibrating with an earlier allelic ladder to an allelic ladder run at a later time.

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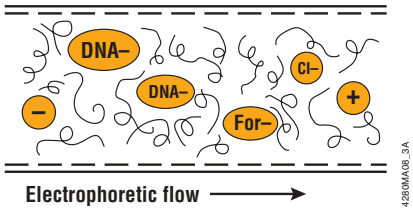

Figure 1. Denatured DNA migrates through entangled strands of polydimethyl acrylamide. DNA mobility is related to its charge and the average size of the fragment as it twists and tumbles through the gel. Small ions such as chloride (Cl^-) and formate (For^-) have a greater charge-to-size ratio and higher electrophoretic mobility. They can compete with DNA during sample injection, reducing the peak intensity.

INJECTION

DNA samples are typically injected into a CE system using an applied voltage of 5–15kV for a few seconds. This process attracts the negatively charged DNA fragments in the sample tube and moves them into the capillary. Injection problems usually occur due to excess PCR buffers masking the charge of the DNA. In addition, buffer ions, such as Cl^-, move faster in an electric field than bulky DNA. Thus to inject PCR mixtures directly into a CE capillary, the samples must be diluted to reduce the ionic strength. Samples can be diluted with formamide or water (5). Formamide, while toxic, is generally used, as it denatures the DNA on contact. Heat denaturation is usually not necessary. The DNA sample is also more stable in formamide. A disadvantage of formamide is its tendency to decompose into formic acid over time or through exposure to moisture in the air. The negatively charged acid directly competes with DNA for injection and dramatically reduces sensitivity. To avoid this problem, measure the conductivity of the formamide with a meter prior to use. Best results are obtained using formamide solvents with conductivity values under 50 microsiemens. Once measured, the formamide should be dispensed into small aliquots and frozen. An additional indicator of poor-quality formamide is an inconsistent appearance of the internal standard due to sample renaturation.

Injection time is also an important parameter that affects the sensitivity of CE injections. Forensic laboratories typically use 5-second injections at 15kV, although longer injection times are possible and will enhance sensitivity. Longer injection times must be validated before use, as stochastic effects will become more evident when low-copy-number DNA is detected (6).

DETECTION

Capillary electrophoresis-based DNA sequencers use laser-induced fluorescence to detect DNA. In many CE systems, a 488nm argon ion laser is focused onto a window burned in the polyimide coating of the capillary, providing efficient access to the sample. The laser universally excites dyes added to the 5′ end of each DNA fragment. Once excited, the dyes emit fluorescent light at different wavelengths. The emitted light is separated into its constituent wavelengths and captured by a photoarray detector. Each dye has a characteristic emission spectrum. However, the dye spectra overlap, making direct analysis of mixtures impossible. A set of 4 equations, known as a matrix, is used to determine the contribution of a given dye at a particular wavelength range (or virtual filter set). A computer is used to separate the individual contributions from the dyes at each data point along the electropherogram (7).

Because the quality of the results depends on properly defining the matrix, the analyst must carefully evaluate each electropherogram to make certain that the spectral corrections are working properly. While poor matrices can cause baseline disturbances, the most common phenomena associated with this problem is known as pull-up. Pull-up can be easily identified by bleedthrough of peaks from one dye lane into another (Figure 2). Pull-up is particularly noticeable when larger peaks are present, as these tend to accentuate the effect. Matrix effects are a function of the optical system, and any process that affects the optics of the system will affect the matrix. This can include

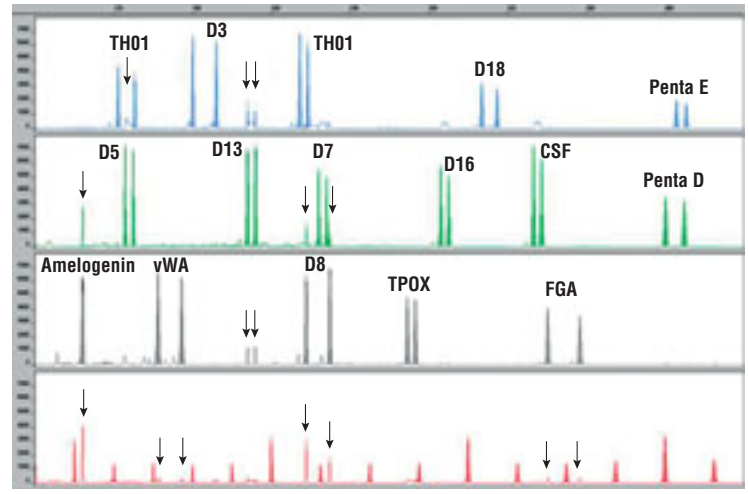

Figure 2. An overloaded PowerPlex® 16 System[a,c,d] sample, demonstrating the problem of pull-up. The matrix cannot compensate for the elevated signal level. Peaks from the green, blue, and yellow lanes "bleed" into the other dye lanes, creating pull-up peaks. (Arrows indicate this effect.) Figure courtesy of Jiri Drabek, Ohio University.

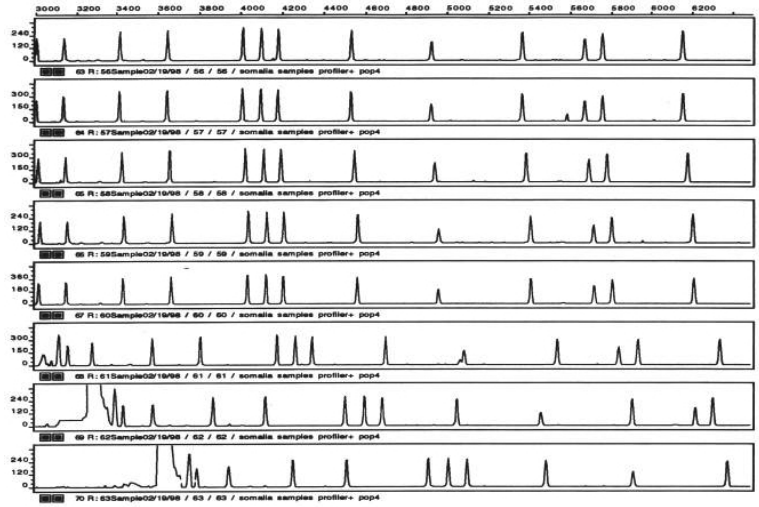

Figure 3. Temperature changes, syringe leaks, clogs or deterioration of capillary wall can create mobility shifts in the internal standard, resulting in loss of system calibration. Monitoring the run current can help the analyst troubleshoot these problems. Note the peak shifts on the late-eluting runs.

switching capillaries, changing buffers or simple aging of the instrument.

The sensitivity of CE can vary from one lab or instrument to the next. Most laboratories use peak height as the primary measurement of sensitivity; however, the analyst should understand that the signal-to-noise ratio, not the absolute intensity of a peak, determines sensitivity. An instrument showing low signal with minimal noise is just as sensitive as an instrument showing larger peak heights with higher amounts of noise. Different instruments will not always have similar sensitivities, and therefore laboratory results must be validated on each system. Unfortunately, as instruments age, peak intensity can drop due to dying lasers, dusty optics or other problems. To keep track of this effect, monitor the signal-to-noise ratio of the internal standard over time.

TROUBLESHOOTING

Capillary electrophoresis systems work at extremely high voltages and low currents. When an inconsistent result is obtained, the analyst should immediately check the instrument log to examine the current during the run. Clogs or inadequate buffer replacement (the result of system leaks or syringe problems) will produce dramatic shifts in mobility and run current (Figure 3). The entire sampling process should be observed to make certain the capillary tip is fully immersed during sample injection. If the capillary moves in its holder or buffer volumes become low, air bubbles may be introduced into the system, producing anomalous currents.

The high voltages used in CE analysis can also cause problems. Because such voltages will always seek alternative pathways to ground, the system must be clean and free of buffer crystals at all times. Listen carefully during a run for sounds of sparking between the sample block and the base of the autosampler. This will create spikes during a run and will eventually destroy the instrument electronics. To avoid spikes, all vials placed into a CE instrument must be clean and dry; clean all exposed surfaces of the instrument with distilled water and alcohol as necessary.

CONCLUSIONS

The key to proper maintenace of CE systems is to understand the effects of various parameters on separation. Through careful study of the detection, separation and injection systems, the analyst can more fully appreciate how to avoid problems with instrument operation. Observe and keep track of the currents produced during the run and keep your equipment clean. You will be rewarded with trouble-free operation and exceptional results.

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