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Improved single-strand DNA sizing accuracy in capillary electrophoresis

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ABSTRACT

Interpolation algorithms can be developed to size unknown single-stranded (ss) DNA fragments based on their electrophoretic mobilities, when they are compared with the mobilities of standard fragments of known sizes; however, sequence-specific anomalous electrophoretic migration can affect the accuracy and precision of the called sizes of the fragments. We used the anomalous migration of ssDNA fragments to optimize denaturation conditions for capillary electrophoresis. The capillary electrophoretic system uses a refillable polymer that both coats the capillary wall to suppress electro-osmotic flow and acts as the sieving matrix. The addition of 8 M urea to the polymer solution, as in slab gel electrophoresis, is insufficient to fully denature some anomalously migrating ssDNA fragments in this capillary electrophoresis system. The sizing accuracy of these fragments is significantly improved by the addition of 2-pyrrolidinone, or increased capillary temperature (60°C). the effect of these two denaturing strategies is additive, and the best accuracy and precision in sizing results are obtained with a combination of chemical and thermal denaturation.

INTRODUCTION

A variety of electrophoretic methods have been developed for determining linkage maps using inherited DNA polymorphisms where the polymorphic region varies in DNA contour length. These include restriction fragment length polymorphisms (RFLP; 1–3), variable number of tandem repeats (VNTR; 4,5), and short tandem repeats (STR; 6–11). These methods rely on transcription of electrophoretic mobility information into relative DNA size, by comparing the mobility of any given unknown fragment with the mobility of a set of size standards or allelic ladder electrophoresed concurrently with the unknown sample. The development of multiple color fluorescence detection has allowed the use of in-lane size standards for increased accuracy and reproducibility of the sizing of unknowns (12). The in-lane size standard is a convenience for slab gel analysis, allowing for more accurate relative size determination, compared with conventional out-of-lane size standard, where gel irregularities can cause uncertainty when extrapolating positional information from the standard lane to the unknown lane. For capillary electrophoresis, in-lane size standards are absolute requirements, since an out-of-lane standard evaluation would have to be done in a separate electrophoresis experiment, and would therefore be subject to much uncertainty.

A key element for accurate size determination is the development of interpolation algorithms that can convert mobility data from an unknown peak into size information, based on size versus mobility data from the standard fragment set. These algorithms generally have to identify the center of an unknown electrophoretic peak, assign a mobility to the peak's center, and convert that mobility into a size based on the mobilities of the set of standards of known sizes. Size calls of unknown peaks, therefore, are dependent on the ability to identify a peak within an envelope of peaks, and on linear relationships between DNA size and electrophoretic mobility. The algorithms tend to give incorrect size calls when poor resolution of two or more peaks results in a single broad peak, whose center will be substantially different than the actual centers of the individual peaks. Incorrect size calls will also result if unknown peaks have aberrant mobilities, giving rise to a non-linear relationship between the unknown peak's mobility and its actual size.

As two-base repeat STR markers have been discovered, they have gained in importance for developing high resolution linkage maps (13–15). Sizing errors for this type of analysis can occur from the two effects described above: poor resolution or sequence dependent anomalous mobility. The sizing of a two-base repeat requires single base resolution in order to identify a band within a size range that occupies two or fewer bases in length of uncertainty; errors from poor resolution are solved by performing the analysis with an electrophoretic separation media that is capable of single-base resolution for the desired size range. Size estimation errors due to anomalous migration of a fragment or fragments are usually attributed to sequence context secondary structure, and are solved by increasing the severity of the DNA denaturing conditions (16,17). Thus, in order to guarantee accurate and reproducible sizing for repeats where the repeat length is very short, the separating matrix must have a combination of high resolving power and good denaturing strength.

Anomalous migration of DNA due to secondary structure has been demonstrated both in slab gel electrophoresis (17,18) and in capillary electrophoresis (CE; 16,19), and is the basis of methods for identifying DNA polymorphisms such as single-strand conformational polymorphism (SSCP; 20,21) and heteroduplex mobility assay (HMA; 22,23). While DNA sequence dependent migration is most prevalent in non-denaturing electrophoretic systems, sequence dependent anomalous migration has also been observed in media classified as being denaturing (18,19). The focus of this study has been to suppress anomalous migration and

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maintain the highest possible resolution in a CE system. Our strategy is to increase denaturation of the electrophoresis system with changes in separation matrix formulation and running conditions.

The analysis of DNA by CE has become practical since the introduction of replaceable separation media (16,24–34). As a further refinement, we have developed a flowable polymer [poly(*N*,*N*-dimethylacrylamide): pDMA] capable of adsorbing to uncoated capillary walls to suppress electro-osmotic flow, as well as acting as the sieving medium (35,36). A fully automated capillary electrophoretic system, the ABI PRISM™ 310 Genetic Analyzer, was developed around the use of formulations of these flowable, glass surface coating polymers (DNA Fragment Analysis Reagent, GeneScan™ polymer, POP-4™ and POP-6™) for the analysis of double-stranded (ds) DNA and single-stranded (ss) DNA (16,37,38).

We have recently shown that the use of 6.6 M urea in the pDMA formulations provides insufficient denaturing for STR analysis: the DNA sizing data lacked both reproducibility (precision) and accuracy (38). As a result of these studies, a program to optimize the conditions needed to achieve precision and accuracy in the sizing of ssDNA fragments was initiated. We observed that improving the accuracy of sizing of anomalously migrating ssDNA also improved the run-to-run sizing precision in the capillary electrophoresis system (37,38). The following results indicate that the route to improved accuracy for sizing of ssDNA fragments in the capillary-pDMA systems includes high levels of denaturants and elevated temperature.

MATERIALS AND METHODS

Electrophoresis

Capillary electrophoretic analysis of fluorescently labeled ssDNA fragments was performed using the PE-Applied Biosystems ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA), equipped with a 47 cm, 50 µm i.d., 360 µm OD uncoated capillary (PE Applied Biosystems, Foster City, CA) and a glass syringe pump. Electrophoresis experiments were run at 320 V/cm. The separation media consisted of a 4% solution of DMA homopolymer, urea at either 0, 6.6 or 8 M either alone or in combination with 0, 2.5, 5 or 15% 2-pyrrolidinone, as shown for each experiment. POP-4 (PE Applied Biosystems, Foster City, CA), a commercially available preparation of the DMA homopolymer (4%), contains 8 M urea and 5% 2-pyrrolidinone. The separation matrix also contains 100 mM *N*-Tris-(hydroxymethyl)methyl-3-aminopropane-sulfonic acid (TAPS), adjusted to pH 8.0 with NaOH. The running buffer contains 100 mM TAPS, 1 mM EDTA, pH 8.0. The GeneScan 500 TAMRA standard (PE Applied Biosystems, Foster City, CA) was used to evaluate sizing accuracy for these studies. The DNA standard was resuspended in 20 µl deionized formamide or Template Suppression Reagent (TSR; PE Applied Biosystems, Foster City, CA) by heating to 95°C for 2 min and cooling on ice. The sample was electrokinetically injected at either 320 V/cm for 5 s or at 210 V/cm for 15 s. The standards were evaluated at 30–70°C.

Data analysis

The GeneScan 500 Tamra standard contains DNA fragments which correspond in size to 35, 50, 75, 100 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bases. Following separation in a denaturing gel, all fragments except the 250 and 340 base fragments, migrate according to their relative size by plotting size versus migration time. The 75, 100, 139, 160, 200, 300 and 400 base peaks were identified as size standards in the GeneScan analysis software (PE Applied Biosystems, Foster City, CA), and were then used to size the 150, 250, 340 and 350 base 'unknown' peaks based on their mobility relative to the identified peaks.

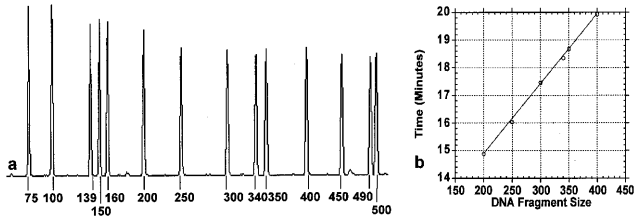

Figure 1. (**a**) Electropherogram from a capillary electrophoresis separation of GeneScan 500 Tamra standard at 320 V/cm in 4% polymer with 8 M urea. Fragment sizes (bases) are indicated. (**b**) Plot of known DNA fragment size versus migration time for fragments from 200 to 400 bases in size shown in (a).

To determine the resolution limits to the nearest nucleotide under each denaturation condition, crossover plots of peak interval and peak width were plotted against fragment size. All electrophoresis data were analyzed by converting the temporal data into spatial data with the assumption that the velocity of any peak that passes the detection window is equal to its average velocity during its migration from the sample end to the detection window. Peak interval refers to the average spatial distance between successive DNA fragments differing by 1 nucleotide in size; peak width refers to the full width at half peak height maximum (FWHM). The single-base resolution limit is defined to be the point where the peak interval curve crosses the peak FWHM curve (39). The plotted portions of the data were fitted to a second-order polynomial using KaleidaGraph™ Version 3.0.

RESULTS

Sample model

A sample of the GeneScan 500 TAMRA standard was electrophoresed using 4% pDMA with 8 M urea at 60°C. Figure 1a shows the electropherogram of the sample; below each peak is the expected fragment size. Figure 1b shows migration times of the separated fragments from Figure 1a plotted against the fragment lengths, and demonstrates that all fragments >200 bases except the 250 and 340 base fragments show a linear relationship between mobility and length, while the 250 and 340 base fragments migrate anomalously.

In this study, our goal was to evaluate suppression of the mobility deviations of the 250 and 340 base fragments compared with the other fragments of the GeneScan 500 TAMRA standard. The parameter matrix was restricted to those properties thought to affect denaturation conditions, and was further narrowed down to temperature and chemical denaturants. Relative success of a particular denaturing condition was evaluated by comparing the changes in the migration of the two anomalously migrating fragments at 250 and 340 bases with changes in the migration of 150 and 350 base fragments, two fragments of similar size that do not migrate anomalously under standard denaturing conditions. All data are expressed as size calls of the 150, 250, 340 and 350

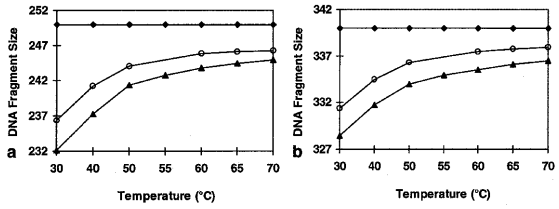

Figure 2. Plot of DNA fragment size over capillary temperature for different denaturation conditions evaluating the effect of increasing capillary temperature. (**a**) Calculated sizing for 250 base DNA fragment. (**b**) Calculated sizing for 340 base DNA fragment. ◆, Expected size of ssDNA fragment, either (a) 250 bases or (b) 340 bases. ▲, 4% pDMA with 8 M urea. ○, 4% pDMA with 8 M urea and 5% 2-pyrrolidinone.

base length fragments, using the GeneScan software, which uses the remaining peaks from the GeneScan 500 sample as size standards for the analyses.

Sample preparation

Samples of GeneScan 500 TAMRA Standard were denatured with formamide or TSR, and electrophoresed using POP-4 at 60°C to determine the effects of sample denaturation on relative migration time. No difference in electrophoretic migration of the peaks or resolution (data not shown) was observed whether the sample was denatured in formamide or TSR; however, peak heights were reduced when the sample was denatured in TSR.

Effect of temperature

Figure 2 shows the temperature effect for 4% polymer with chemical denaturants of 8 M urea and 8 M urea with 5% 2-pyrrolidinone (POP-4): increasing temperature improved the sizing accuracy for the 250 and 340 base fragments. Urea alone appeared to show some additional improvement in sizing accuracy for the 250 and 340 base fragments above 60°C (Fig. 2).

While increased temperature results in more accurate sizing, it tends to result in decreased resolution. Using POP-4, the crossover plots in Figure 3 show a resolution limit of >400 bases at 30°C, compared with 200 bases at 60°C. Examination of the plots in Figure 3 indicates the sources of the resolution loss to be a loss in peak spacing (~25% interval decrease) and an increase in peak widths (~50% increase in FWHM) with the increase from 30 to 60°C. Similar decreases in resolution limit with increasing temperature were observed for all concentrations of urea and 2-pyrrolidinone. Under all denaturing conditions, increasing temperature results in increases in peak widths and decreases in peak intervals. The observation of enhanced sizing accuracy at elevated temperatures indicates that the band broadening does not have a detrimental effect on the relative mobilities assigned to the peak centers; however, this band broadening effect tends to restrict the use of high temperature (60°C) for applications that do not require high resolution for peak identification, such as two base repeats to 200 bases and four base repeats above 200 bases.

Effect of chemical denaturation

We evaluated different combinations of the chemical denaturants, urea and 2-pyrrolidinone, in 4% polymer for sizing accuracy of the ssDNA fragments. For either the 150 or 350 base fragment, 6.6 M urea or 8 M urea provides sufficient denaturation for good

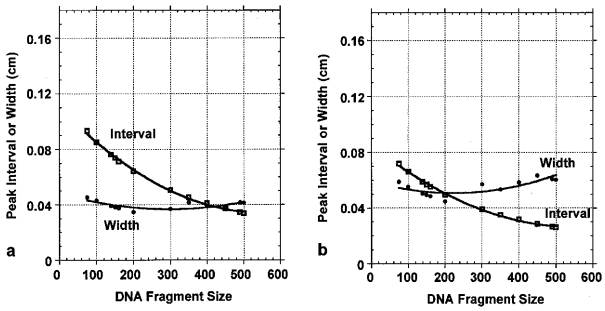

Figure 3. Resolution limit plots (crossover plot) for Genescan separation experiments using replaceable polymer and different denaturing conditions. All temporal data were converted to spatial data by assuming that the velocity of any peak as it passes the detection window is equal to its average velocity from the injection end to the detection window. Peak interval refers to the average spatial distance between successive DNA fragments spaced 1 base apart; peak width refers to the full width at half maximum (FWHM). The single-base resolution limit is defined to be the point where the peak interval crosses the peak FWMH. The data were fitted to a second-order polynomial using KaleidaGraph™ Version 3.0. All separations were performed with 4% polymer. (**a**) Separations were performed in polymer with 8 M urea and 5% 2-pyrrolidinone at 30°C. (**b**) Separations were performed in polymer with 8 M urea and 5% 2-pyrrolidinone at 60°C.

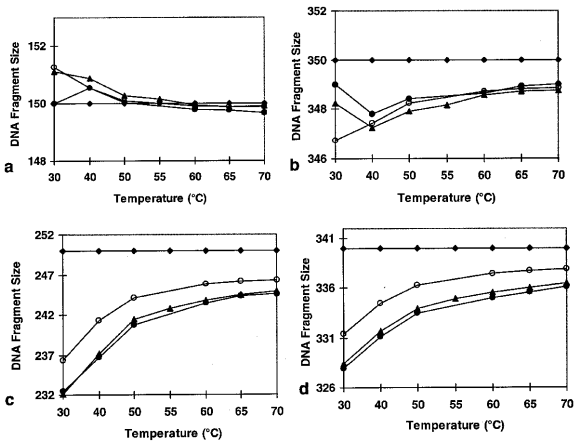

Figure 4. Plot of DNA fragment size over capillary temperature for different denaturation conditions evaluating the effect of adding 2-pyrrolidinone as a denaturant. (**a**) Calculated sizing for 150 base DNA fragment. (**b**) Calculated sizing for 350 base DNA fragment. (**c**) Calculated sizing for 250 base DNA fragment. (**d**) Calculated sizing for 340 base DNA fragment. ◆, Expected size of ssDNA fragment, either (a) 150 bases, (b) 350 bases, (c) 250 bases or (d) 340 bases. ●, 4% pDMA with 6.6 M urea. ▲, 4% pDMA with 8 M urea. ○, 4% pDMA with 8 M urea and 5% 2-pyrrolidinone.

sizing as compared with 8 M urea with 5% 2-pyrrolidinone (POP-4) (Fig. 4a and b). However, for both the 250 and 340 base fragments, 8 M urea with 5% 2-pyrrolidinone (POP-4) shows better accuracy in sizing than either 6.6 or 8 M urea alone (Fig. 4c and d).

In addition to accurate sizing, chemical denaturation has a positive effect on resolution. The crossover plots at 60°C for 6.6 M urea (Fig. 5a), 8 M urea (Fig. 5b), and 8 M urea/5% 2-pyrrolidinone (POP-4) (Fig. 3b) show increases in resolution limit from 80 bases, to 150 bases, to 200 bases, respectively. The differences in resolution between 6.6 and 8 M urea are primarily due to changes in peak interval, the peak width generally being

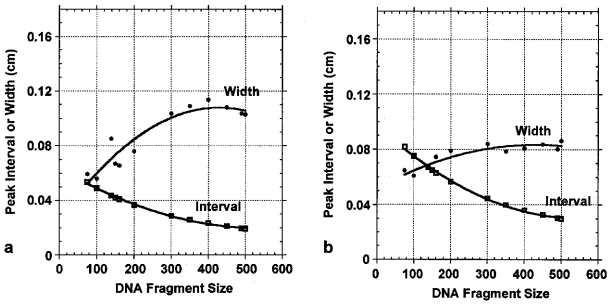

Figure 5. Resolution limit plots for Genescan separation experiments using replaceable polymer and different denaturing conditions. See Figure 3 for additional information. All separations were performed with 4% polymer. (**a**) Separations were performed in polymer with 6.6 M urea at 60°C. (**b**) Separations were performed in polymer with 8 M urea at 60°C.

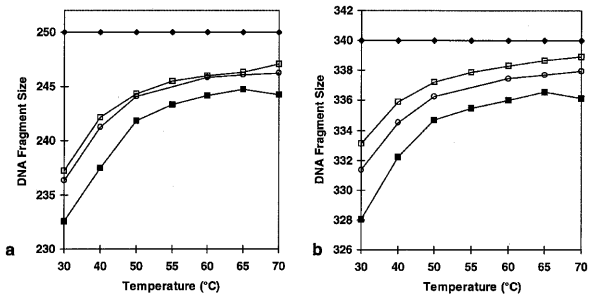

Figure 6. Plot of DNA fragment size over capillary temperature for different denaturation conditions evaluating the effect of different concentrations of 2-pyrrolidinone as denaturant. (**a**) Calculated sizing for 250 base DNA fragment. (**b**) Calculated sizing for 340 base DNA fragment. ◆, Expected size of ssDNA fragment, either (a) 250 bases or (b) 340 bases. ■, 4% pDMA with 8 M urea and 2.5% 2-pyrrolidinone. □, 4% pDMA with 8 M urea and 15% 2-pyrrolidinone. ○, 4% pDMA with 8 M urea and 5% 2-pyrrolidinone.

fairly broad. In contrast, the peak interval curves for 8 M urea (Fig. 5b) and 8 M urea with 5% 2-pyrrolidinone (POP-4) (Fig. 3b) are essentially the same, but the narrower peak widths due to the addition of 5% 2-pyrrolidinone in the formulation extend the resolution limit. At any particular temperature, 8 M urea with 5% 2-pyrrolidinone (POP-4) showed better resolution than either 6.6 or 8 M urea.

Large deviations (from 5%) of the 2-pyrrolidinone concentration showed large effects on the performance of the 4% polymer solution. At 2.5%, with 8 M urea, 2-pyrrolidinone showed a decrease in the sizing accuracy of the 250 and 340 base fragments (Fig. 6a). The decrease in 2-pyrrolidinone concentration had very little effect on the resolution of the system as compared with 8 M urea with 5% 2-pyrrolidinone system (POP-4) (data not shown). Increasing the 2-pyrrolidinone concentration to 15% improved the sizing accuracy of the 340 base fragment at all temperatures (Fig. 6b), but had little effect on the sizing of the 250 base fragment. Resolution improvements at all temperatures were observed by increasing the 2-pyrrolidinone concentration to 15% with 8 M urea. The resolution limit extended to >400 bases at 60°C (data not shown), a consequence of a substantial increase in peak interval. The requirement of urea in these systems was demonstrated with a 15% 2-pyrrolidinone, urea free, 4% polymer formulation: sizing accuracy (Table 1) and resolution were very poor. The poor resolution (data not shown) of the urea-free system was a consequence of increased peak widths.

Table 1. Sizing of DNA fragments with 4% pDMA and 15% 2-pyrrolidinone

	Temperature (°C)					
	30	40	50	60	65	70
150 bases	147.0	148.8	149.9	149.4	148.8	148.9
250 bases	217.1	230.7	233.5	239.0	241.0	241.7
340 bases	335.3	332.6	333.2	335.6	337.1	337.3
350 bases	361.6	356.5	354.3	353.6	353.7	353.7

Effect of denaturation on migration time

The effect of denaturation conditions on run time was evaluated from the migration time of the 350 base fragment (Fig. 7). As expected, increasing temperature caused fragments to migrate faster in any of the chemical denaturing systems. Also expected was the increase in migration time with increasing urea concentration. The 2-pyrrolidinone concentration had a very large effect on fragment mobility: the migration time roughly doubles with an increase in 2-pyrrolidinone concentration from 5 to 15%, at all temperatures.

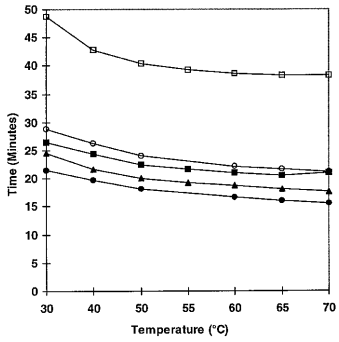

Figure 7. Plot of the migration time for the 350 base DNA fragment over capillary temperature for different denaturation conditions. ●, 4% pDMA with 6.6 M urea. ▲, 4% pDMA with 8 M urea. ■, 4% pDMA with 8 M urea and 2.5% 2-pyrrolidinone. □, 4% pDMA with 8 M urea and 15% 2-pyrrolidinone. ○, 4% pDMA with 8 M urea and 5% 2-pyrrolidinone.

DISCUSSION

The development of capillary formats for DNA analysis has historically been motivated by its promise of fast electrophoresis times (resulting in high throughput), and its sample injection process (electrokinetic injection) which is amenable to automation. As the separation matrices were developed to take advantage of this format (flowable, replenishable systems), the extrapolation of slab gel analyses onto capillaries has become suspect. The main differences between the slab and capillary formats are the basic structure of the sieving component (crosslinked versus uncrosslinked networks), and the dissipation of Joule heat, which is much more efficient in a capillary system. The fundamental differences between flowable and crosslinked sieving media seem to be transparent to the overall DNA separation mechanism (36), and the main issues in choosing a sieving matrix are those of practical importance (capillary coating for enhanced capillary life, viscosity, etc.), which need to be

optimized for a specific application or instrument format. On the other hand, very little has been published (40,41) on the subtle effects that denaturation has on the relationships between DNA fragment size, sequence and electrophoretic mobility. Our experience with both electrophoretic systems has led us to the conclusion that a slab based system is intrinsically more denaturing than an equivalent capillary system, based on the need for increased denaturation to approach the performance of slab based systems for linkage analysis of two-base repeat STRs.

Accurate sizing of two-base repeats requires a combination of high resolution and good denaturation. POP-4 (4% pDMA, 8 M urea, 5% 2-pyrrolidinone) has adequate resolution for the analysis of two-base STRs to 350 bases (37,38). However, we show that denaturation of some of the size standard DNA fragments during electrophoresis is inadequate under conditions that give accurate sizing in slab formats, resulting in a non-linear relationship between fragment size and electrophoretic mobility; this gives rise to poor sizing accuracy and precision. We have found that pDMA in capillaries requires both chemical denaturation and increased temperature for good denaturation of anomalously migrating ssDNA. In contrast to DNA sequencing, identification of these conditions is especially important in determining the size of STR fragments for a linkage analysis population, where sequence heterogeneities surrounding the repeat regions may give rise to anomalous migration and inaccurate sizing.

Urea is commonly used as the denaturant of choice in gel electrophoresis of DNA fragments. In polyacrylamide slab gels this provides sufficient denaturation of the DNA. Surprisingly, the use of only urea with the GeneScan standard in capillary electrophoresis did not provide sufficient denaturation to prevent anomalous migration of the 250 and 340 base ssDNA fragments, even with increasing temperature. We cannot conclude whether the source of this denaturing difference arises from the fundamental difference of the sieving component, the difference in buffer composition, or the much more effective heat dissipation of the capillary system.

The addition of 2-pyrrolidinone to the urea formulation yielded two advantages: the anomalous migration of the ssDNA fragments was minimized; and the resolution compared with urea alone improved at all temperatures. As a result of being able to run at higher temperatures the sizing accuracy of the anomalously migrating ssDNA fragments came close to the slab gel results. The fundamental interactions that result in good DNA sizing with this formulation are quite complex, as either urea or 2-pyrrolidinone by themselves do not constitute an adequate denaturing system.

It is clear from these studies that for the best sizing accuracy and precision, it is preferable to use the highest levels of chemical denaturation and temperature one can achieve. The penalty for the use of high chemical denaturant is an increase in migration time; the penalty for the use of high temperatures is a loss of resolution. Thus it is necessary to pick conditions that result in the best combination of sizing accuracy and precision, while maintaining acceptable resolution and migration time for the studies to be performed.

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