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Final Report : The Development of New Analytical Buffer Systems for the Separation and Analysis of PCR Amplified DNA by Capillary Electrophoresis.

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Outline

Precis The goals of this proposal were to develop a better understanding of the mechanisms for capillary failure and to suggest new analytical procedures to mitigate these effects. Work performed during the proposal particularly focused on two major issues, sample injection effects and band shifts. A series of experiments were performed to examine the role temperature, ionic strength, buffer pH and sample concentration play in DNA typing using capillary electrophoresis. The results indicate that sample concentration and the choice of denaturing solvent can have a major effect on the quality of the output electropherogram. Band shifts clearly are the result of temperature effects and the fact that a band shift can occur at one locus and not another is due to a differential response from locus to locus as temperature changes. The reason for these temperature effects is likely the secondary structure of the DNA as changing to highly denaturing buffers at pH 11 greatly mitigates this effect.

I. Introduction

The development of methods to amplify and detect of specific regions of the DNA molecule using the polymerase chain reaction (PCR) has resulted in rapid and dramatic advances in biochemical analysis (1). With the advent of the PCR it is now possible to easily produce analytically significant amounts of a specified DNA product. In the forensic arena, PCR methods permit rapid and specific tests of evidence produced in a crime(2). The impact of the PCR has also resulted in a need for efficient and automated procedures for analysis of the reaction products. For many years it has been recognized that capillary electrophoresis has great potential to fulfill this requirement. Capillary-based systems can produce rapid and efficient separations of DNA, due to the efficient heat dissipation of the capillary when compared to standard slab gel methods.

Additionally, the capillary can be easily manipulated for efficient and automated injections. As a result of these advantages many forensic laboratories have adopted CE as the technique for the analysis of PCR amplified DNA used in genetic typing.

The systems presently being developed for DNA typing by CE are known as short tandem repeats. These are polymorphic loci with repetitive nucleotide sequences 2 to 7 base pairs in length.(3). Presently, most forensic work is performed using tetrameric repeats. The number of repeated units at a particular STR loci varies from one individual to the next, allowing the individual alleles to be separated by size. To conserve precious forensic samples amplifications of multiple STR loci are performed in a single run. To discriminate between the different STR loci, PCR primers are carefully designed to produce alleles such that different loci do not overlap in size. Additionally, individual primers can be labeled with up to 3 different fluorescent dyes. With this technique, individual alleles can overlap as long as they are labeled with different dyes. The FBI has recently designated a set of thirteen of these STRs to be used in forensic testing. Nationwide, states are beginning to collect and analyze samples from convicted sex offenders. The results of DNA analyses using these loci will be stored in a national data base known as CODIS which will be a resource to help solve cases in which no suspect has been developed. To perform such wide scale analyses, automated systems such as CE are required.

These STR analyses are generally carried out using a CE system produced by Perkin-Elmer, the PE/ABI 310 genetic analyzer. In this instrument a syringe pump is used to fill a 41cm capillary with a sieving matrix composed of a high concentration of the polymer polydimethyl acrylamide in a buffer containing TAPS, 8M urea and 5%

pyrolidinone at a pH of 8 (4). Typical analysis times are 30 minutes at voltages of 15kV. The system is equipped with an argon/ion laser with a multichannel fluorescence detector that can detect four or more colored dyes simultaneously. The instrument has a 96-sample capacity resulting in a 2-day turnaround time for a full set of samples.

A forensic DNA analyst typically sets up a series of samples on this instrument and leaves it unattended, allowing the samples to be run overnight. If a capillary failure occurs during the unattended operation of the system, all of the subsequent analyses will be ruined. This scenario can result in a major rework of the analysis and the potential loss of valuable samples and time. For CE systems composed of arrays of capillaries, random failures can result in complex problems when racks of samples must be run in a particular order.

Often the causes of these failures are completely unknown. Figure 1 shows the effect of one type of capillary failure on the performance of the instrument. Here migration times begin a dramatic shift. Determining at which point the failure occurred is critical, as separations may be effected several runs prior to the perceived failure.

II Capillary Failure Studies

There are three basic mechanisms by which failures can occur in capillary electrophoresis: separation effects, injection artifacts, and buffer depletion. Of these, separation effects may be the most common cause of failure. Separation effects can be caused by the adsorption of sample and buffer components on the capillary surface or by variations mobility resulting from temperature or osmotic flow. The theory of gel based separations in CE generally ignores the capillary wall as a contributor to the separation, as it is assumed that the thick polymer buffer coats active sites such as silanol groups on

the capillary wall, however under certain conditions wall adsorption can play a major role in the quality of the separation.

Capillary failure often occurs after a relatively long period of acceptable runs, followed by total deterioration of the results within about 3 runs. One effect which could lead to this type of behavior is electroosmotic flow. In electroosmotic flow, a bulk migration of the buffer is induced by the action of the electric field on the double layer of charged ions next to the capillary wall. Under normal conditions this phenomena does not occur because the viscous polymer solution masks charged sites on the wall and resists the bulk flow. However, with continued operation of the system, the buildup of contaminants can produce active sites along the wall. These sites produce a charged double layer along the capillary wall. This double layer can induce bulk flow, destroying the reproducibility of the migration times, and making the resultant data unreadable. The process of osmotic flow is illustrated in Figure 2. The figure shows that as positive ions begin to build up on the capillary wall, osmotic flow increases, opposing the sample's electrophoretic flow.

The net mobility of the DNA is the vector sum of these two mobilities:

$$\mu_{net} = \mu_o + \mu_e \qquad (1)$$

Note that each subsequent analysis will take longer to complete as the opposing osmotic flow becomes stronger, slowing the migration of sample DNA fragments.

Another potential problem is the adsorption of the DNA samples onto active sites on the capillary walls. One effect of adsorption is the loss of resolution as sample bands

become broad and diffuse due to this effect. Eventually this process can make it impossible to resolve mixtures or to determine peak maxima.

Band broadening effects are not always the result of wall adsorption. Similar effects can be caused by improper sample preparation. DNA samples are typically denatured in formamide, a solvent that can decompose over time into ionic species. These ions interfere with sample injections as they increase the ionic strength of the sample matrix. This can result in renaturation of the DNA samples and disrupt an injection process known as stacking by reducing the difference in ionic strength between sample and buffer.

Stacking is a process resulting from a sample introduction procedure in CE that is known as electrokinetic injection. In electrokinetic injections, an applied voltage induces the sample to migrate into the capillary orifice. The quantity of material injected Q_{DNA} onto the capillary by this technique can be described by the following equation:

$$Q_{DNA} = E\pi r^2 [DNA](\mu_{ep} + \mu_{eof}) \qquad (2)$$

where E is the field strength, r is the capillary radius, μ_{ep} is the electrophoretic flow and μ_{eof} is the electroosmotic flow. However this equation must be modified as other ions present in solution will compete with DNA for the role as electrophoretic charge carriers. Thus, the quantity of DNA injected is also a function of the ionic strength of the solution, ΣQ_i , and the corrected total quantity injected, T_{DNA} becomes:

$$T_{DNA} = Q_{DNA}/\Sigma Q_i \, . \qquad (3), (5), (6)$$

In addition, smaller ions tend to be selectively injected into the capillary and the ionic strength of the sample will alter the applied field. From the above equations, it can be

seen that differences in the ionic strength of the PCR sample or in the formamide that it is dissolved in, can have a dramatic effect upon the total amount of DNA injected.

Buffer depletion is a third possibility to explain failures in CE analyses. In the process of electrophoresis, the composition of the anode and cathode buffers can change due to electrolysis, migration of buffer ions, and the elution of sample ions. The net effect of this process is to produce composition gradients across the capillary. Such gradients can produce variations in migration rate of ions and result in miscalibration of the system.

III. The effect of capillary failure on DNA typing

The deleterious effects of capillary failure in DNA typing can range from an inconvenient loss of data to more problematic effects such as band shifts, mis-calibrations, and inconsistent results. It should be noted that under normal circumstances, failures are an infrequent occurrence. But, the precautions necessary to prevent these failures; periodic replacement of expensive capillaries and buffers, represent a significant portion of the cost of DNA typing. It should also be noted that certain of these effects occur gradually as the capillary ages, slowly reducing separation efficiency and precision. The two main observable results of capillary failure are band broadening and band shifts.

Band broadening is a common problem that results from the loss of system efficiency. The sensitivity of the assay decreases because the peak heights are reduced. As the sample peaks broaden, it becomes increasingly difficult to determine the apex of a sample peak. This results in a loss of precision in typing the data, making the system incapable of distinguishing two adjacent alleles.

The capability of the system to resolve mixtures is also adversely affected by band broadening. As resolution degrades, two adjacent peaks may not be adequately separated. The problem is exacerbated when two peak areas are dissimilar as would be the case with a mixture containing a low concentration of a second DNA source.

The most problematic type of capillary failure is the band shift. This phenomena occurs when alleles in a sample migrate differently from the estimated rate predicted by a previous calibration run. These calibration runs establish the difference in migration between an internal sizing standard and each individual allele, and are performed every 5-10 runs during a series of analyses. Subsequent analyses compare migration times of unknown alleles with those calculated from the calibration runs. If conditions such as temperature, migration velocity, or electric change during a run, the migration time differences between each allele and internal sizing standard will change. The result is a band shift in which an allele sized at 218 bp shifts to a position higher or lower in size, Figure 3. In this figure the computer miscalibrated the size of two alleles and they appear shifted in size by one base. Interpretation rules have been suggested to compensate for these effects. The sample is usually rerun, since the shift usually produces a suggested size that is extremely rare (7). However, these rules merely correct for what is in reality a misleading result.

In an attempt to answer some of these questions about capillary failure and also to help define useful procedures to ensure long term stability and reproducibility of the analysis, a series of experiments were carried out. In these experiments a study of injection effects, effects from contaminating proteins, and effects of temperature and pH were undertaken. Overall we have found the ABI 310 and capillary electrophoresis in

general to be a reliable, stable and useful platform for genetic analysis when recommended protocols are followed. Our results underscore the importance of proper training in sample and reagent preparation as well as the use of internal controls to track and maintain instrument stability.

IV. Injection Studies

The AmpF*l*STR Profiler^{+} system was used in this study to evaluate the effect of sample preparation and its injection on the electrophoretic separation by capillary electrophoresis (CE), using the PE/ABI 310. Dilution of amplified samples in water and in formamide with conductivity values ranging from 47 to 1000 μS was evaluated, as well as the effect of high DNA concentration and varying amounts of buffer salts. The resolution remained constant in the different formamides tested, while the sensitivity improved for samples diluted in deionized water and high purity formamide. An on-column sample stacking method for DNA preconcentration was evaluated to increase the sensitivity of low quality samples. The stacking method was based on the injection of NaOH immediately before sample injection(8). Following injection of base, a neutralization reaction occurs between OH^{-} and $Tris^{+}$ ions, producing a low conductivity zone at the head of the capillary. DNA fragments are concentrated at the front of this zone. Using DB-17 coated capillaries with HEC 2% (M_W 250,000) as a separation matrix, an improvement in sensitivity can be detected in all the solvents studied. The gain in sensitivity is higher for lower quality solvents and is not correlated with the size of the DNA fragments.

The goal of these studies was to evaluate the effects of sample preparation and injection on the AmpF*l*STR Profiler system. Samples were deliberately contaminated with poor quality formamide and excess concentrations of DNA and/or buffer salts.

Formamide is commonly used in the preparation of ssDNA samples for capillary electrophoresis analysis, as it is a strong denaturant. Rapid heating to 95° C and snap cooling generally used to effectively denature DNA. However it has been demonstrated that formamide is an health hazard and its toxic effects have been widely studied in animal models (9) and in humans (10). In addition, formamide produces ionic decomposition products such as formic acid which will be preferentially injected into a capillary, thus causing problems in sample injection and resolution.(11) Water has been proposed as a suitable solvent to replace formamide in the preparation of samples for capillary electrophoresis analysis (12). This could eliminate the health hazard, the cost of the chemical as well as its disposal.

In a study of sample preparation techniques, a set of samples (PE ROX 500 standard, the Profiler^{+} allelic ladder, and DNA samples extracted from FTA papers and amplified using Profiler^{+}) were deliberately mixed with poor quality formamide and excess concentrations of DNA and/or buffer salts. A second set of experiments involved a comparison between good quality formamide and water to determine if water can replace formamide in the preparation of samples for capillary electrophoresis analysis, as reported by Biega (12). For this reason, three different kinds of formamide were tested for their conductivity values, which represent an index of the quality of the solvent: low values are correlated to good quality, while high values correspond to formamides which

are not completely deionized or became ionized during storage. The conductivity of the measured formamides were 47μS, 360μS and 1000μS. While the resolution appears not to be affected by differences in sample preparation, the signal strength is higher in pure solvents and tends to decrease in lower quality formamide (Table 1).

The same results have been observed in the analysis of extracted DNA samples. The use of ionized formamides can consequently have detrimental effects on an electrophoretic separation in terms of the intensity of the signal strength. This could be due to the fact that the presence of ions interferes with the injection of the sample, thus reducing the total amount of DNA fragments entering into the capillary. There are numerous small ionic species present in the PCR reaction mixture, such as di- and deoxynucleotides and buffer ionic components (e.g., chloride) (5) as well as formic acid and ammonia resulting from formamide decomposition.(13) These small ionic species reduce the amount of DNA fragments loaded during an electrokinetic injection due to their lower charge to mass ratio.

Samples prepared in water showed approximately the same peak intensity as samples prepared in good quality formamide. This is in agreement with previous results.(12) Clearly, formamide could be replaced by water in the sample preparation, avoiding the risks connected with its use. However at high sample concentration, water presents problems in terms of renaturation of DNA fragments. Additionally, the shelf life of the sample may be reduced when it is stored in water.

To determine if sample concentration can affect the electrophoretic separation of DNA fragments, samples were deliberately prepared in different concentrations in water and formamide. In these experiments, four different dilutions of the samples were tested:

1:25 (ratio between DNA and solvent) which is the one routinely used, 1:10, 1:5 and 1:1. In these results resolution values (expressed in bases) did not significantly change for high concentrations of samples. Interestingly, samples diluted in pure quality formamide showed a slight increase of signal strength as the concentration of the sample increased, while there was no improvement in sensitivity for samples diluted in water. Samples prepared in poor quality formamide showed a larger increase in sensitivity with DNA concentration (Figure 4).

The reason samples prepared in pure formamide don't show an improvement in sensitivity as their concentrations increase, could be due to the fact that at the concentration normally used (1:25) the amount of sample injected into the capillary is maximum. For samples prepared in low quality formamide the injection of the sample into the capillary is inhibited by the ionic strength of the solvent. At higher concentrations of PCR product the ratio between DNA and the solvent ions is in favor of the DNA, so that a higher amount of DNA can enter the capillary.

Samples diluted in water showed different results: lower values of sensitivity were observed as the concentration of the PCR product increased. This effect may be due to the onset of renaturation on the DNA. This renaturation was observed at the highest concentration of samples prepared in formamide. Thus, samples prepared in formamide are more stable than those prepared in water, especially at high DNA concentrations (1:1).

The same behavior can be observed by adding salts to DNA samples. In Figure 5 an example of the renaturation process is shown that progressively increases with the concentration of $MgCl_2$ added to the sample. Thus both high sample concentrations and

high salt concentrations affect the stability of the sample. In fact it is likely that the DNA concentration effect is the direct result of the increase in concentration of buffer salts that occurs as additional PCR product is added to the formamide.

To improve the sensitivity of low quality samples, an on-column sample preconcentration method for capillary-based DNA analysis was evaluated. This method consists of injecting a small plug of NaOH immediately before the injection of the sample, so that a neutralization reaction between OH^- ions and $Tris^+$ ions of the buffer can occur creating a zone of lower conductivity in which DNA fragments concentrate, (8) Figure 6. To perform the stacking 50 mM Tris/HCl buffer containing 6M urea has been used instead of 100 mM Tris/borate with 7.1M urea. Both the concentration of the buffer and the urea concentration have been reduced to minimize the band broadening as reported by Xiong (8). Since the ABI 310 has fixed software modules that control sample injection on the 310 Genetic Analyzer, a module file was modified to permit the injection of NaOH immediately before the injection of the sample. With this stacking procedure it was possible to obtain a gain in sensitivity in all the samples analyzed and in all the solvents studied, Figure 7. The gain in sensitivity is higher for lower quality solvents and it seems not to be correlated with the size of the DNA fragments.

From the measurement of the resolution calculated for two different sets of peaks in the ROX standard it was possible to observe a decrease in resolution with stacking. This loss is more evident as the molecular weight of the DNA fragments increases. This is in contrast to the results of Xiong et al., in which the resolution remained stable while sensitivity increased(8).

The increase of the sensitivity with stacking process varied with the NaOH injection time. In our experiments NaOH injections were limited to 5 seconds as it was felt that longer injection could damage the coating of the capillary, decreasing the life time of the capillary and the reproducibility of the results.

The stacking technique was also evaluated for different sample concentrations in the different solvents. An increase in signal strength was detected with stacking in all the dilutions tested in the different solvents, however the separations were degraded at high concentrations due to renaturation. Thus this stacking technique can be applied even in the injection of high concentrated samples.

Conclusions

The electrophoretic separation of the AmpF*l*STR Profiler^{+} system is influenced by different sample preparation as well as contamination with high DNA concentrations or salts. While the resolution was not affected by differences in sample preparation, the signal strength was higher in pure solvents and tended to decrease in lower quality formamide. In highly concentrated samples resolution decreases with the concentration of the sample, while sensitivity improves in samples prepared in poor quality formamide.

The addition of salts to samples produces a change in the electrophoretic migration of the different DNA fragments. This is believed to be the result of renaturation of the DNA that progressively occurs as the concentration of $MgCl_2$ added to the sample increases.

The pH mediated base stacking presented here produces gains in sensitivity in all the solvents studied, and this gain is not correlated with the size of the DNA fragment.

Further improvement in sensitivity could be obtained by injecting NaOH for a longer time or in higher concentration, but this may affect capillary lifetime since it is commonly known that NaOH can destroy the coating of the capillary.

Future work will concern the study of the mechanism of this injection technique in the analysis of STRs using coated capillaries, in particular efforts will be focused on methods to improve sensitivity as well as resolution.

V. Contamination Studies

A series of fluorescent proteins were utilized to examine the effect of wall absorption on the separation of DNA samples using the ABI 310 capillary electropherograph instrument. It is well known that proteins can interact with the wall of the capillary, thus affecting the electroosmotic flow and influencing capillary resolution and lifetime. For this purpose, four fluorescent proteins were used, with different pI values (above and under the pI value of Ampli Taq Gold™ DNA Polymerase). While the first set of experiments have focused on the electrophoretic separation of all the proteins by CE, subsequent analyses have concentrated on the evaluation of the effects of these contaminants on the separation of DNA samples. In this case, capillaries have been flushed with a low concentration of the fluorescent protein (1pg/ml) for 5 min.

Results and discussion

The Beckman P/ACE 2050 instrument was used in the first experiments to determine the isoelectric point (pI) of Ampli Taq Gold™ DNA Polymerase. For this purpose, formamide was added to the protein sample as internal marker; since formamide is a neutral compound, its migration in this CE system results solely from electroosmotic

flow. Different pH values of the separation buffer (25 mM sodium tetraborate) were evaluated in the range between 9 and 7. The pH value of the borate buffer in which the protein has the same migration velocity of formamide represents the pI of the protein and it corresponds to 7. The knowledge of the approximate pI value of Ampli Taq Gold™ DNA Polymerase was used to choose different fluorescent proteins to be used in the contamination study: Streptavidin Alexa Fluor and Streptavidin Fluor conjugate both with pI=7, Avidin with pI=10.5 and R-phycoerythrin with pI=4.

To perform the separation of all these proteins using the ABI 310 Genetic Analyzer, it is necessary to take into account of the reverse polarity of the instrument, since the cathode is at the injection end and the anode is at the detection end of the capillary. Using 50 mM phosphate buffer at pH 2.35, all the proteins become positively charged and moved toward the cathode; so a cationic surfactant (tetrabutylammonium hydroxide 50 mM) was added to the buffer to reverse of the direction of the electroosmotic flow, forcing the proteins to move toward the anode where they are detected. Figure 8 shows an example of the separation of these proteins in the conditions described above.

Through the separation of the fluorescent proteins it was demonstrated that all proteins can be detected in a CE system, even if their separation is sometimes not correlated with what was expected based on pI value (Streptavidin Alexa Fluor and Streptavidin Fluor conj. should have the same pI value and therefore migrate at the same velocity immediately after avidin). The contamination of the capillary with solutions of these proteins gave different results: while the rinsing of the capillary with Streptavidin Alexa Fluor resulted in contamination of the sample and of the strip caps, Avidin and

Streptavidin fluor conj. resulted in contamination just of the strip caps and no contamination was observed using R-phycoerythrin. However, none of the proteins used for the capillary contamination produced alterations in the electrophoretic separation of the Rox standard.

Thus several conclusions were obtained from this study. First, moderate amounts of proteins with pIs above and below that of Taq enzyme do not appear to cause major problems with capillary resolution. Secondly, using these highly fluorescent proteins, it is possible to detect low level contamination on the caps of the septa. These caps should be thoroughly cleaned prior to reuse.

VI. Temperature Studies

The temperature studies undertaken in this proposal resulted from the anecdotal comments from various 310 users indicating that large room temperature fluctuations could result in loss of calibration and concomitant band shifts. Our hypothesis was that the primary cause of these band shifts was temperature influenced alterations in DNA secondary structure. We were particularly interested in the fact that certain loci are more prone to band shifts than others. To test this possibility we examined the effects of temperature on short tandem repeat (STR) analysis using the PE/ABI 310 Genetic Analyzer. Initial separations were performed using the Profiler + multiplex system, Additional data was also obtained later using the Promega CTTv multiplex and a Y chromosome multiplex obtained from Reliagene. (see below) The analytical separations were obtained using a POP4 (ABI) at pH 8 and an experimental buffer consisting of 3% Hydroxyethyl cellulose at pH settings ranging from 8-12. Temperatures were examined from 30-70 C. The results demonstrate the fact that highly efficient separations can be

carried out at alkaline pH. In addition improvements in temperature stability were seen when compared to results at lower pH. However, high concentrations of urea were found to be necessary to achieve optimal resolution.

To understand the effects of temperature and DNA structure on allele sizing, it is important to review the analytical methods used in genetic typing. Analytical systems used in genotyping have been developed with multiple controls to maintain precision (14). For each set of analyses, both internal and external standards are utilized. To each amplified sample an internal standard is added which consists of a mixture of DNA fragments labeled with a red (ROX) fluorescent dye. The internal standard is then used to estimate the size of each allele in the amplified samples. An external standard is also part of the analytical protocol. It consists of a mixture of the most common alleles present in each STR locus in the multiplex combined with the ROX labeled internal standard. To perform a determination, the size of an unknown allele is determined with reference to the internal standard and the identity of the allele is determined by comparing the calculated size of the unknown with the known allele sizes determined in the external standard.

The multiple comparisons between internal and external standards are necessary in order to maintain analytical precision, however, additional measures are also required. Secondary structure differences can occur between the amplified sample and the internal standard and will result in a loss of precision. These effects are caused by conformational and sequence differences and will result in poor sizing accuracy. To minimize DNA secondary structure effects, analyses are performed using elevated temperatures (60°C) and high concentrations of urea (7M).

The use of high temperature and urea in these systems necessitates careful control of instrumental parameters. High urea concentrations limit the shelf life of the buffers and can result in sublimation and crystal formation. The elevated temperatures required can be difficult to maintain in conditions where rapid room temperature shifts occur. Although these issues can be easily mitigated by proper validation of the analytical systems, a less cumbersome approach would be to perform separations at room temperature and with denaturants other than urea.

In order to investigate the plausibility of reducing the temperature and concentration of urea in these assays, we have characterized the effect of temperature, pH and urea concentration on the ABI profiler + multiplex and 2 other multiplex systems from Reliagene and Promega. To perform this work we have utilized the POP4 commercial polymer as well as an experimental sieving buffer consisting of 3% hydroxyethyl cellulose at varying temperatures and/or concentrations of urea. We have also examined the application of elevated pH as an alternative to high temperatures and urea in an effort to minimize DNA secondary structure. The elevated pH work was performed using fluorocarbon coated capillaries. Our results illustrate the effect of urea, pH and temperature on the precision and accuracy of DNA genotyping.

Results

In a paper by Rosenblum and coworkers in 1997, a technique was described for the analysis and mitigation of anomalously migrating DNA fragments (4). In their paper they identified specific fragments in a TAMRA dye labeled Genescan 500 standard

whose migration times were temperature sensitive. The effect of this temperature sensitivity was to produce size estimates for these fragments that varied with temperature. The size estimates were obtained through comparison with DNA fragemnts which were insensitive to temperature. Separations performed under stronger denaturation conditions produced size estimates closer to the true (sequenced) size.

In previous work in our laboratory we have detected a concomitant improvement in precision with increasing temperature which we believe is also associated with DNA secondary structure. It is possible that elevated temperatures and strong denaturing conditions limit the variability in migration times by melting out different DNA conformers. Certainly, analyses are more stable in regions where minor variations in temperature do not affect size estimates.

As mentioned above, current instrumentation in forensic DNA analysis utilizes a two step calibration process to produce precise estimates of DNA size based upon migration time. Allele calls are produced through a binning process that fits the data into narrow migration time windows, typically +/- 0.5 bases.

We have been interested in characterizing the environmental and experimental factors that affect this precision. Previous reports demonstrate that control of temperature and the addition of denaturants is an important parameter in the production of precise and accurate DNA size estimates. (4), (15), Thus, we undertook a study to examine the effect of temperature on size estimates of the Applied Biosystems Profiler + STR multiplex, the Promega CTTv multiplex and the Reliagne Y plex 6, three sets of forensic STR markers. In this work, we examined the effect of capillary oven temperature on the size estimates

of STR fragments. A series of temperatures from 45-70 °C were utilized to examine their effect on the size estimates for alleles in the various multiplex kits.

For these experiments we utilized the manufacturers standard protocols with the exception of the temperature, which is normally set at 60°C. To make the measurements, allelic ladders were denatured in formamide, snap cooled in an ice bath, and analyzed on a PE/ABI 310 using the POP4 polymer. POP4 polymer consists of 4% polydimethyl acrylamide in 100mM TAPS at a pH of 8.0 (4). 8M urea and 5% 2-pyrrolidinone are added as denaturants. The capillary used was a 43 cm 50μm id uncoated capillary from Polymicro technologies at a field strength of 350V/cm and all samples were injected electrokinetically at 5kV. The size in bases of each STR allele was estimated using the PE ROX 500 internal size standard fit to a global southern sizing algorithm. DNA fragments from 75-400 bases in length are included in the calibration estimate.

The selected alleles incorporate a size range from 100-350 bases in length and allowed us to probe the entire range of the calibration curve produced by the ROX standard. The results of this work are reproduced in Tables 3-5, which illustrate the differences in temperature response from 45 to 70 °C for selected alleles from each multiplex. Figure 9 and 10 demonstrate these slopes for allele 14 in the STR loci vWA and allele 8 in the STR loci DYS 385. With few exceptions most slopes were negative and quite linear in the temperature range examined.

While the sequenced size of these alleles has not been published, the response of the size estimate with respect to temperature can be used to examine the overall stability of the system. In Table 3 the results of 4 different analyses are compared. With the exception of the last two data sets, which were run in the same 2-day period, the runs

were taken at different times over a period of several months and on different capillaries. In spite of this fact the estimates of the slope of the temperature response are fairly precise with standard deviations of 0.01 to 0.02 bases/°C. Tables 4 and 5 show similar variations in slope between loci for the Promega CTTv multiplex and the Reliagene Yplex . This data also demonstrates that there is a marked consistency in temperature response for different alleles within a single locus. This is reasonable given the similarity in sequence for these alleles.

Of particular interest is the differential response in slope from one set of markers to the next. For example, a temperature change of 2°C in the capillary changes the size estimate of allele 26 in D18S51 0.36 bases while allele 36 in D21S11 changes only 0.06 bases. Another interesting pair of alleles is vWA and D13S317. These alleles occur very close to the 200 bp fragment in the ROX size standard, however the slope for these alleles is markedly different, indicating that differences in temperature response for this pair of alleles is more likely due to the amplified fragments and not the size standard. The overall results clearly illustrate the importance of temperature control on the size estimates of alleles in the multiplex.

In examining this data we were also interested in the effect of the ROX sizing ladder as temperature would be expected to affect both the unknown allele and the sizing standard. From the results it is clear that the variation with temperature is independent of the average size of a given locus, and thus the measured temperature response is the result of the allele sequence. However, we used global southern sizing, a method which uses regression to determine the best fit line for the relation between size and migration

time. This technique would tend to average out any individual contribution from a given fragment of the ROX ladder.

Thus we also examined the effect of sizing method, noting that many laboratories utilize the Local Southern sizing algorithm. Comparison of the effect of temperature on the allele size were made using Local and Global Southern sizing with the Promega CTTv multiplex and the Reliagene Yplex samples. The results, shown in tables 6 and 7 indicate a clear superiority for the Global Southern sizing method in terms of consistency of results. In general the slopes of the temperature response were consistent for a given locus with Global Southern sizing and not consistent with Local Southern. The slopes of the temperature curves varied both with locus and with allele using the Local Southern method. In one situation, shown in Figure 11, the change in sizing method resulted in loss of linearity in response of the size estimate to temperature. We believe that this variation of slope within a locus can contribute to a lack of precision in the measurement especially if the room temperature or gel block temperature varies, and that Global Southern sizing may be a superior method in such circumstances.

In controlled studies from these and other laboratories, the precision (standard deviation) of allele size estimates at 60 °C ranges from 0.24 -0.07 bases, with 0.17-0.12 bases being fairly typical. (16), (11) (17) (15), (18). An issue in forensic DNA typing of STRs is the existence of variant alleles. These alleles contain deletions of one to two bases from the standard 4 base motif. The level of precision required for reproducibly determining the difference between 2 alleles differing in size by one base is 0.15 bp(19). Thus, present CE technology is just adequate to perform this task. The appearance of these variant alleles can sometimes lead to an inconclusive result at one locus in a

multiplex. While the absence of one allele from a total of 13 sets of STR loci has minimal effect on the statistics on the analysis, (in fact the rarity of these types often results in a unique profile) improved precision would be useful in the identification of these variant alleles. Our results on the temperature dependence of sizing indicate that improved control of temperature should result in increased precision.

VII. High pH Buffers

An alternate method for affecting the conformational effects of ssDNA is to adjust buffer pH. Recent reports have demonstrated the applicability of CE for DNA analysis at elevated pH (20) (21). The denaturing effect of alkaline pH is believed to be due to the development of negative charges on guanine and thymine bases. In both these reports CE with UV detection was used to analyze DNA at pH's 11 and above. The authors found the separation mechanism in alkaline buffers to be very similar to that at lower pH using urea as a denaturant. However, DNA resolution of only 10-11 bases was illustrated and little information was given on the reproducibility of the results. In one study, separation efficiency was maximized at pH 11 although complete denaturation of DNA was expected at pH 11.5.(20).

A goal of the present work was to determine if it was possible to produce high-resolution separations under alkaline conditions using fluorescently labeled DNA. Previous studies in the literature utilized UV detection techniques and peak resolution was limited. Our expectation was that resolution would improve using higher concentrations of polymer. Our hope was that the fluorescent dye linkers would remain stable at high pH. We were also interested in how buffers at elevated pH would affect the temperature response, precision and resolution of the separation. It was our

expectation that the use of high pH buffers could obviate the need for elevated temperatures and high concentrations of urea. Initially we examined buffers at pH 11 and 12 using a 50 µm fluorocarbon coated capillary (µsil FC) filled with 3% hydroxyethyl cellulose(HEC). This specially coated capillary was necessary due to the solubility of silica at pH's above 8.5. However, the bulk of this work was performed in a 20mM CAPS buffer at pH 11, as the fluorocarbon coating was found to have limited stablity at pH 12. The HEC polymer was chosen as a result of our previous work demonstrating that high precision DNA typing can be performed with this system(15) and due to its demonstrated stability at high pH. It should be noted that these results should be fairly independent of the type of polymer system as long as the polymer remains chemically stable.

In order to characterize the effect of alkaline pH, the temperature dependence of the DNA size estimates using the HEC/CAPS buffer was compared to the results previously obtained with the POP4 system.

Experiments were performed by analyzing the profiler+ allelic ladder at pH 11 using three different concentrations of Urea: 0, 3, and 7M. The results at 0 and 7M urea are illustrated in Table 8 and 9. The slopes of the regression line between allele size and temperature in the table show a nearly flat response at all concentrations of urea. Average slopes range from 0.015 to 0.06 and show both positive and negative slopes. These results are different from the data in Table 3 in which most of the measured alleles had slopes of -0.09°C/base or greater. The results indicate that a stabilizing effect on DNA temperature response is occurring at elevated pH that is not present under standard conditions using POP4. This is demonstrated in figure 12 for allele 30 of the FGA locus.

We believe this effect to be the result of improved denaturing of the DNA fragments under alkaline conditions.

While the results at pH 11 exhibited superior characteristics to the POP4 polymer with respect to the temperature dependence of allele size, peak resolution suffered. During additional experiments to test the effect of urea concentration on the estimation of size, an interesting effect was shown. Experiments were performed at 30°C to examine the separation system at near ambient temperatures. The results showed that peak resolution improved with increasing quantities of urea. The improvement of resolution is likely to be the result of hydrogen bonding between the DNA and urea, increasing the mass of the DNA fragments. Increased urea concentrations can affect the solution viscosity and may alter the sample focusing effects during injection. Note that the presence of urea also alters the estimated allele size but has minimal affect on the slope of the temperature response, Figure 12. These results are interesting in that they illustrate that urea has a dual role in ssDNA separations. In addition to its denaturation effects, it also provides improved resolution.

The effect of the increased urea concentration is also illustrated in the migration times of the DNA fragments. For example, the migration time of allele 15 from D7S820 increased from 14.54 +/- 0.07 min. to 22.08+/- 0.07 min. as the concentration of urea was increased from 0 to 7M. In situations requiring increased sample throughput, the shorter migration times in the non-urea buffer could be considered an advantage.. Figure 13 illustrates the effect of increasing urea concentration on the separation of the blue allelic ladder from the profiler + multiplex. Urea concentration also effected the size estimates, but the temperature response as measured by the slope of the relationship between

estimated size and temperature did not markedly change, Figure 12. Thus the observed differences in size estimates at different pHs and urea content are the result of changes in DNA mobility resulting from hydrogen bonding. The effect of elevated pH on the guanine and thymine bases and the fluorescent dyes used in labeling the DNA may also affect size estimates.

Overall results from 3 different concentrations of Urea at pH 11 and 30°C are shown in Table 11. The table gives resolution values for allele sizes from 100-316 bp. Resolution is slightly degraded for late eluting peaks. Otherwise, the results are roughly equivalent to those reported for the 3%HEC and POP4 polymers at 60°C with urea but at far lower temperatures.(15) The reproducibility of the size estimates are also shown in Table 3 and range of standard deviations from 0.06 to 0.012 for a set of ten analyses using 7M urea.

In a final set of experiments, a set of 10 samples were extracted from FTA blood stain cards and the results were amplified and analyzed using POP4 at 60°C, 3% HEC in 100mM TBE buffer with 7M urea at 60°C (15)and in 20mM CAPS. The results were typed using Genotyper software and shown to be in complete concordance with each other. Figure 14 illustrates the analysis of an amplified sample at 30°C in a pH 11 CAPS buffer at 7M urea.

VIII. Suggestions for Future Work

The results from this study highlight the importance of sample preparation in obtaining precise and consistent results. If the forensic community moves towards increasing the sensitivity of current methods it is clear that further study should be made

of injection techniques as sample quality can have a major effect on this parameter. Our work also indicates the importance of controlling gel temperature in CE systems. Future molecular modeling may help to understand the underlying cause of these temperature effects. Finally further study should be made of elevated pH buffers as electrophoretic media. To accomplish this improved passive or dynamic capillary coatings may need to be developed.

XI. Conclusions

This work demonstrates that water can be considered as a useful alternative to formamide and that close attention should be paid to the conductivity of the diluent as it can have a major affect on sensitivity of the system. This work also indicates that there is a varying response to temperature in the different alleles among the forensic 10 loci studied at pH 8 and underscores the importance of temperature control when performing these analyses. The results obtained in this study also demonstrate that highly efficient separations with good resolution can be performed using elevated pH. The effect of temperature on the size estimates obtained using the internal/external standard calibrations is shown to be reduced when compared to standard procedures at lower pH. Separations near room temperature still required urea to achieve maximal resolution. This effect was believed to be the result of hydrogen bonding between the urea and DNA molecules and not the result of improvements in the denaturing ability of the buffer.

This paper demonstrates that there is a varying response to temperature in the different alleles among the forensic 10 loci studied at pH 8 and underscores the importance of temperature control when performing these analyses. The results obtained

in this study also demonstrate that highly efficient separations with good resolution can be performed using elevated pH. The effect of temperature on the size estimates obtained using the internal/external standard calibrations is shown to be reduced when compared to standard procedures at lower pH. Separations near room temperature still required urea to achieve maximal resolution. This effect was believed to be the result of hydrogen bonding between the urea and DNA molecules and not the result of improvements in the denaturing ability of the buffer.

X. References

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Figure Captions

Figure 1: An illustration of the onset of capillary failure in a CE system using repetitive injections of a ROX labeled DNA size standard. The figure shows a series of sequential analyses. As failure begins to occur the DNA standard peaks begin to elute later and later. Eventually the system cannot compensate and complete loss of calibration occurs. Conditions: POP 4 polymer in buffer run using PE/ABI 3310 Genetic Analyzer. ROX 350 DNA ladder, 15kV, 43 cm uncoated capillary.

Figure 2: The buildup of osmotic flow. (a) A coated capillary has no osmotic flow. (b) As the coating breaks down, charged ions become attracted to the capillary wall. (c) Further breakdown of the capillary coating results in the development of osmotic flow. The movement of the double layer of positive ions creates a bulk flow towards the negative electrode, which opposes the migration of the DNA, slowing it down.

Figure 3: An illustration of a band shift. In the figure shows the sample 8B is overlaid upon two allelic ladders. Alleles A1, A2 are shifted one base down from their actual size. The condition that caused this shift was unknown. Note that allelic ladders 1B and 24B are not shifted, and thus the situation that caused the shift was transient.

Figure 4: Gain in sensitivity in the ROX standard expressed as percentage of the reference value (sample diluted 1:25) in different sample concentrations in the four diluents. The results demonstrate that in low conductivity solvents it is not possible to increase the sensitivity of the system by increasing sample concentration.

Figure 5: The effect of increasing concentration of $MgCl_2$ on the electropherogram of a ABI Rox 500 Internal standard. As the salt concentration increases, the DNA renatures from single to double stranded and extra peaks appear. Top panel contains no $MgCl_2$ while the bottom panel contains 8mM $MgCl_2$

Figure 6: Illustration of stacking process. Injection of NaOH immediately before DNA injection creates a zone of low conductivity in which the field strength is high, so that DNA molecules move quickly toward the anode and concentrate between the low and high ionic strength areas.

Figure 7: Gain in sensitivity obtained with stacking process in a DNA sample extracted from a bloodstain stabilized on FTA® paper, amplified by PCR and diluted in pure formamide. Conditions: HEC 2% in 50 mM Tris/HCl pH 8, 6 M urea and 2 mM EDTA; 5 s at 15 kV NaOH 0.1 M injection, 5 s at 15 kV sample injection, 60° C, 15 kV separation voltage.

Figure 8: Separation of the four fluorescent proteins in the ABI 310 using 50 mM phosphate pH 2.35 containing 50 mM TBAH as a separation buffer.

Figure 9: The variation in estimated allele size with temperature for allele 14 in the VWA locus. Conditions: POP4 with Genescan buffer, 47cm 50μm ID capillary, 5s 15kV injection, 15kV run Global Southern sizing algorythm.

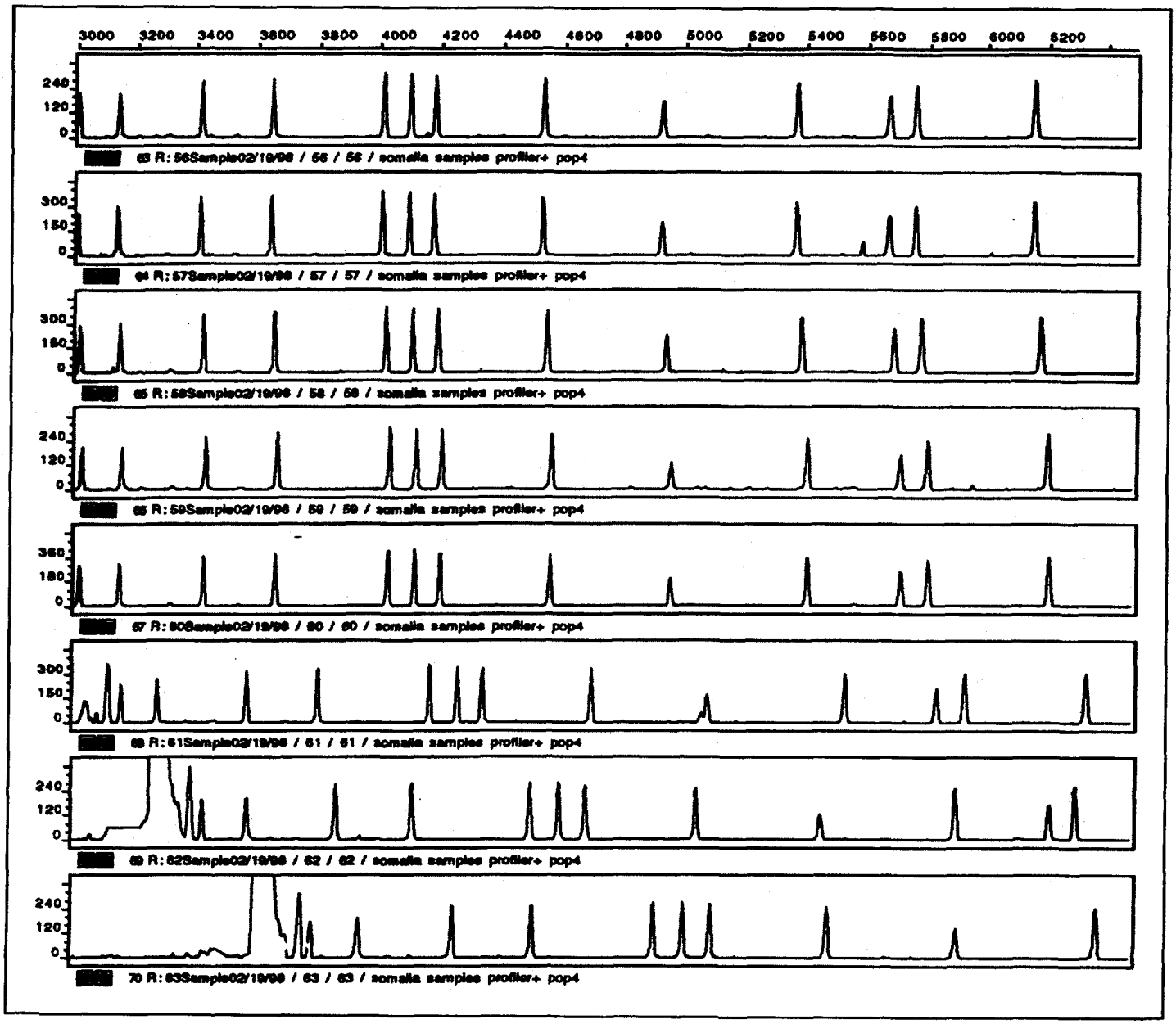
Figure 10: The variation in estimated allele size with temperature for allele 8 in the DYS 385 locus. Conditions as in Figure 9.

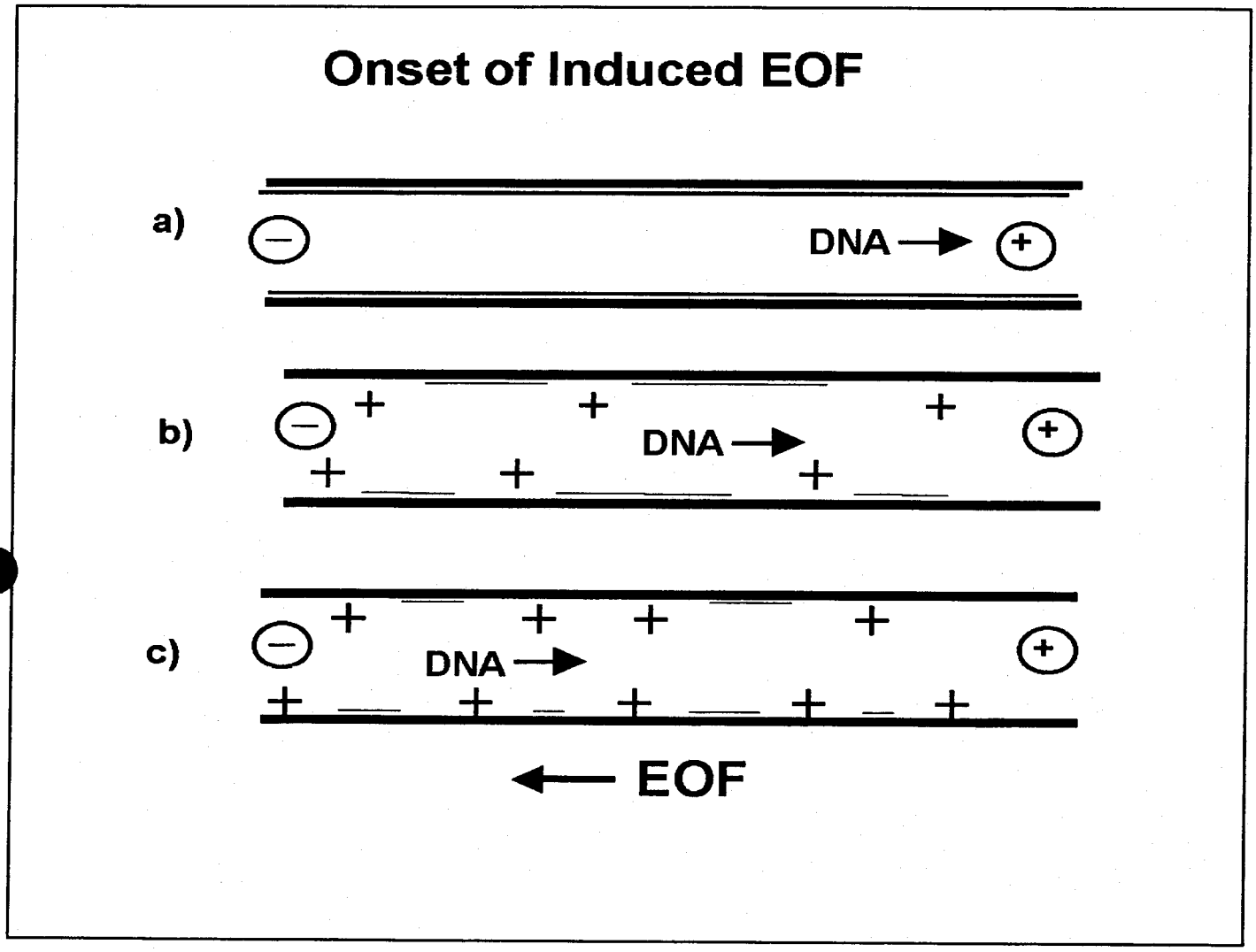
Figure 11: The effect of Local Southern Sizing on the DYS 385 locus. The variation in estimated allele size with temperature for allele 8 in the DYS 385 locus. Not the non-linearity of the plot when compared to figure 11. Conditions: POP4 with Genescan buffer, 47cm 50μm ID capillary, 5s 15kV injection, 15kV run Global Southern sizing algorythm

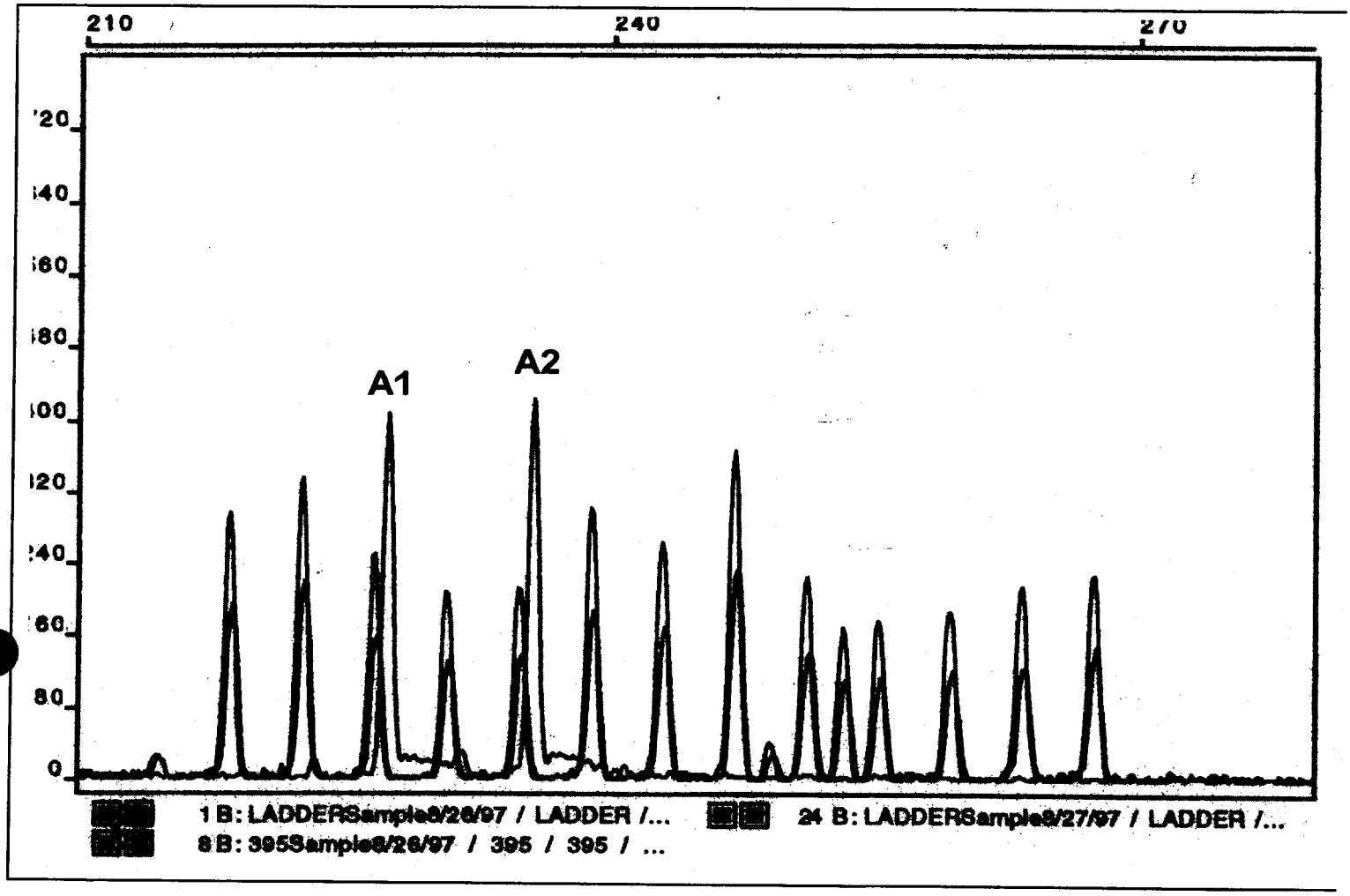
Figure 12: The variation in estimated allele size with temperature for allele 30 in the FGA locus as a function of pH and Urea concentration. All injections 5s with a run voltage of 15kV. A: Pop4 polymer, 100mM TAPS buffer, pH 8, 8M urea, uncoated capillary, B: 3% HEC polymer, 20mM CAPS buffer pH 11, no urea, J&W μSil FC 50μm, 47cm capillary C: 3% HEC, 20mM CAPS buffer 7M urea J&W μSil FC 50μm, 47cm capillary

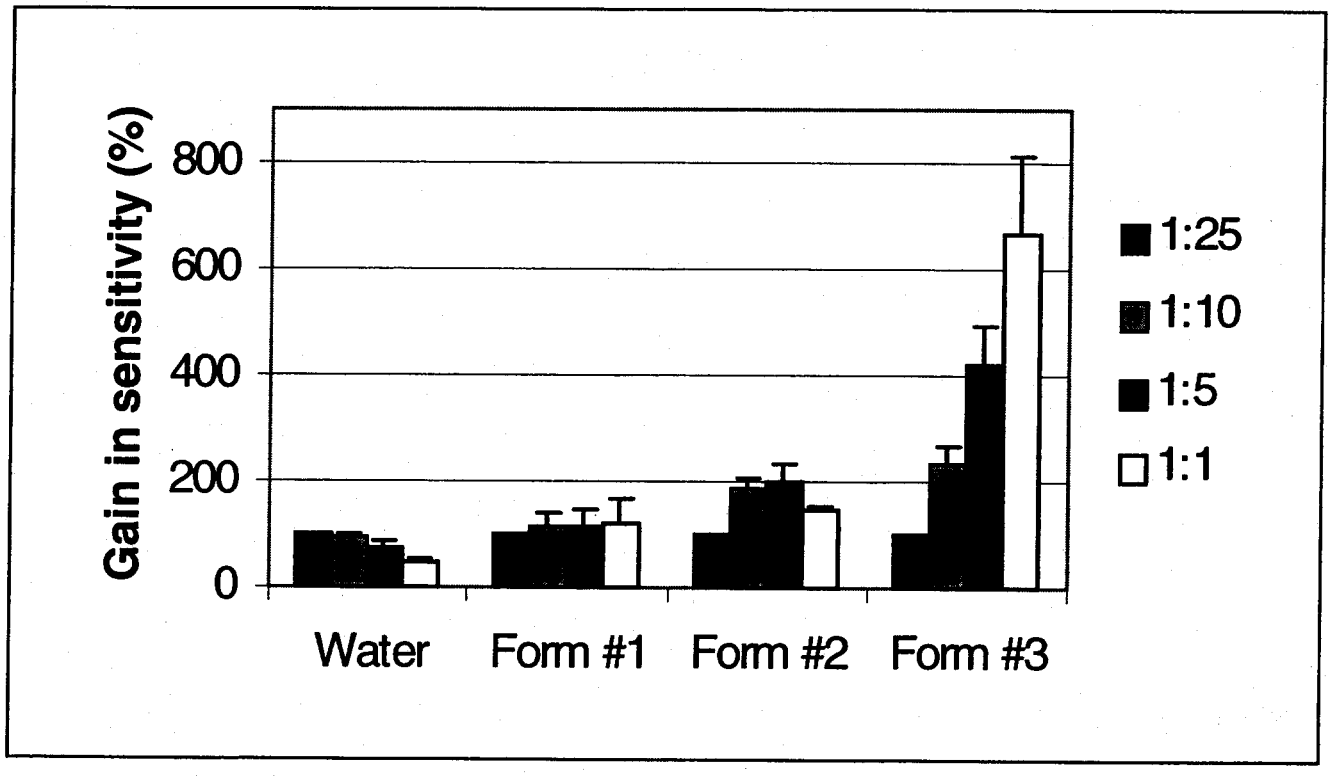
Figure 13: The Profiler + allelic ladder (blue labeled loci) showing the effect of urea on the resolution of the system. The figure demonstrates that urea not only has a denaturing effect, but also effects peak resolution. Conditions as in Figure 12.

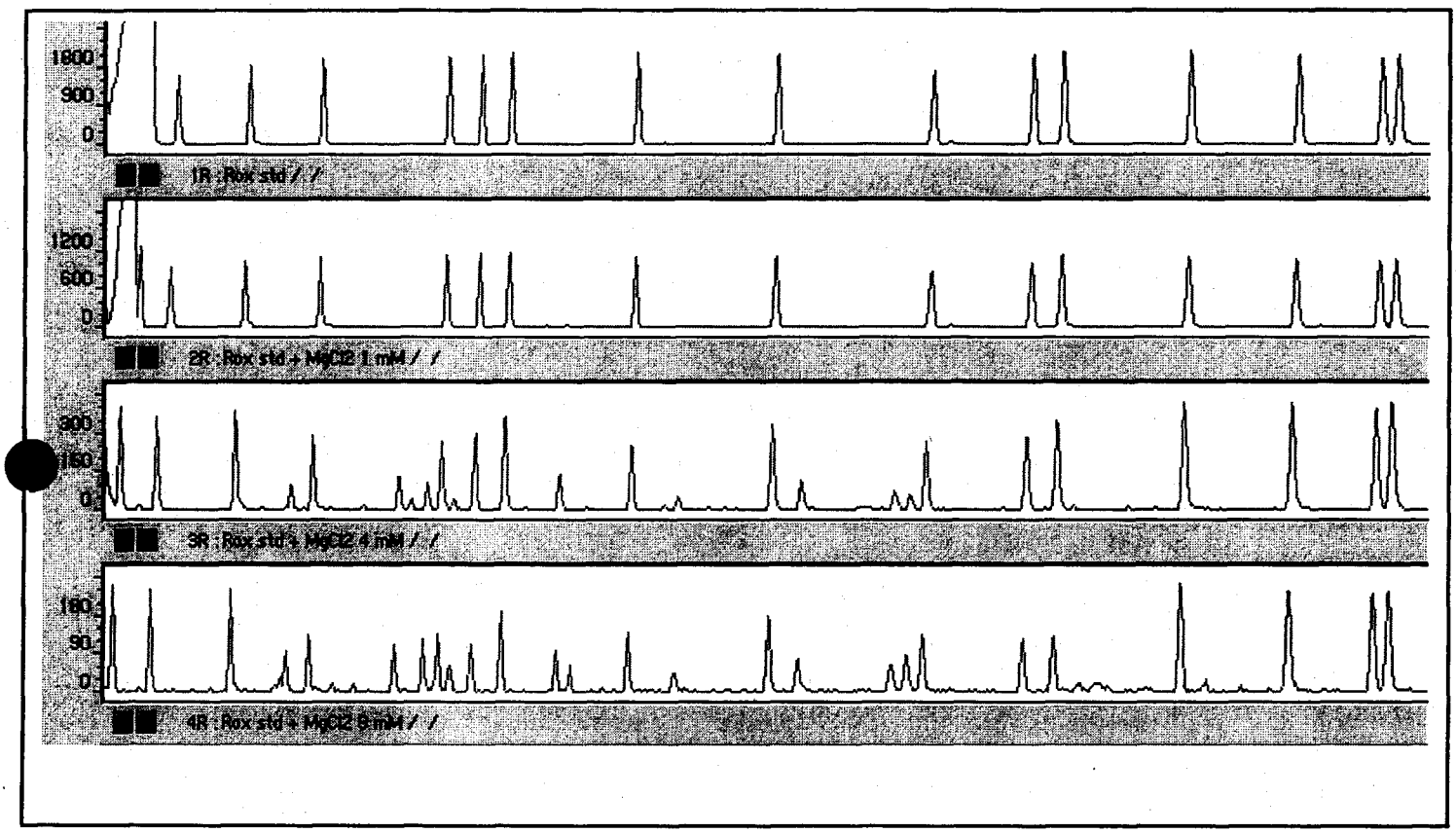
Figure 14: The electropherogram of a blood stain extracted from FTA paper and analyzed using 3% HEC, 20mM CAPS buffer 7M urea, J&W μSil FC 50μm, 47cm capillary 15kV 5s injection 15kV run voltage.

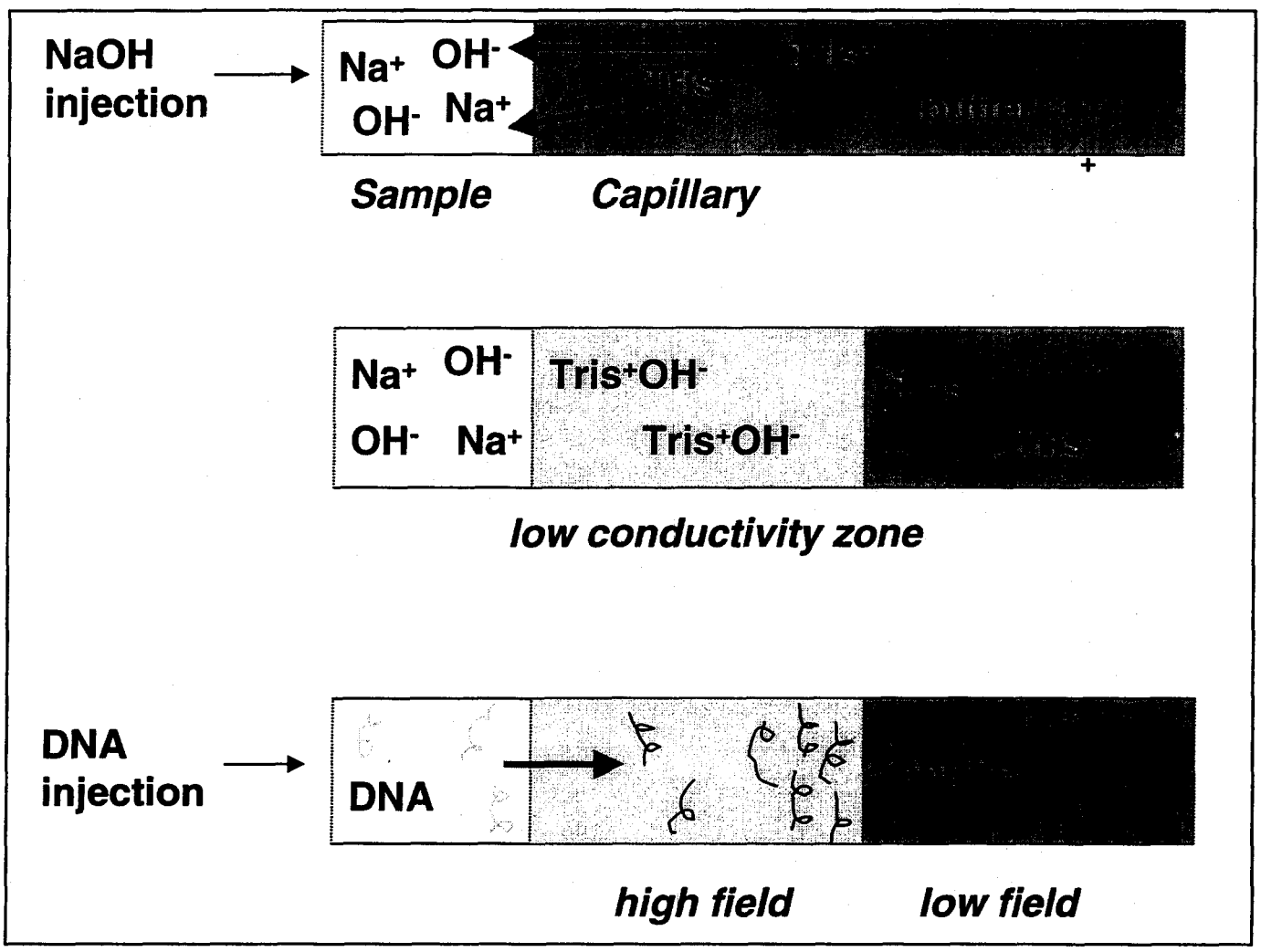


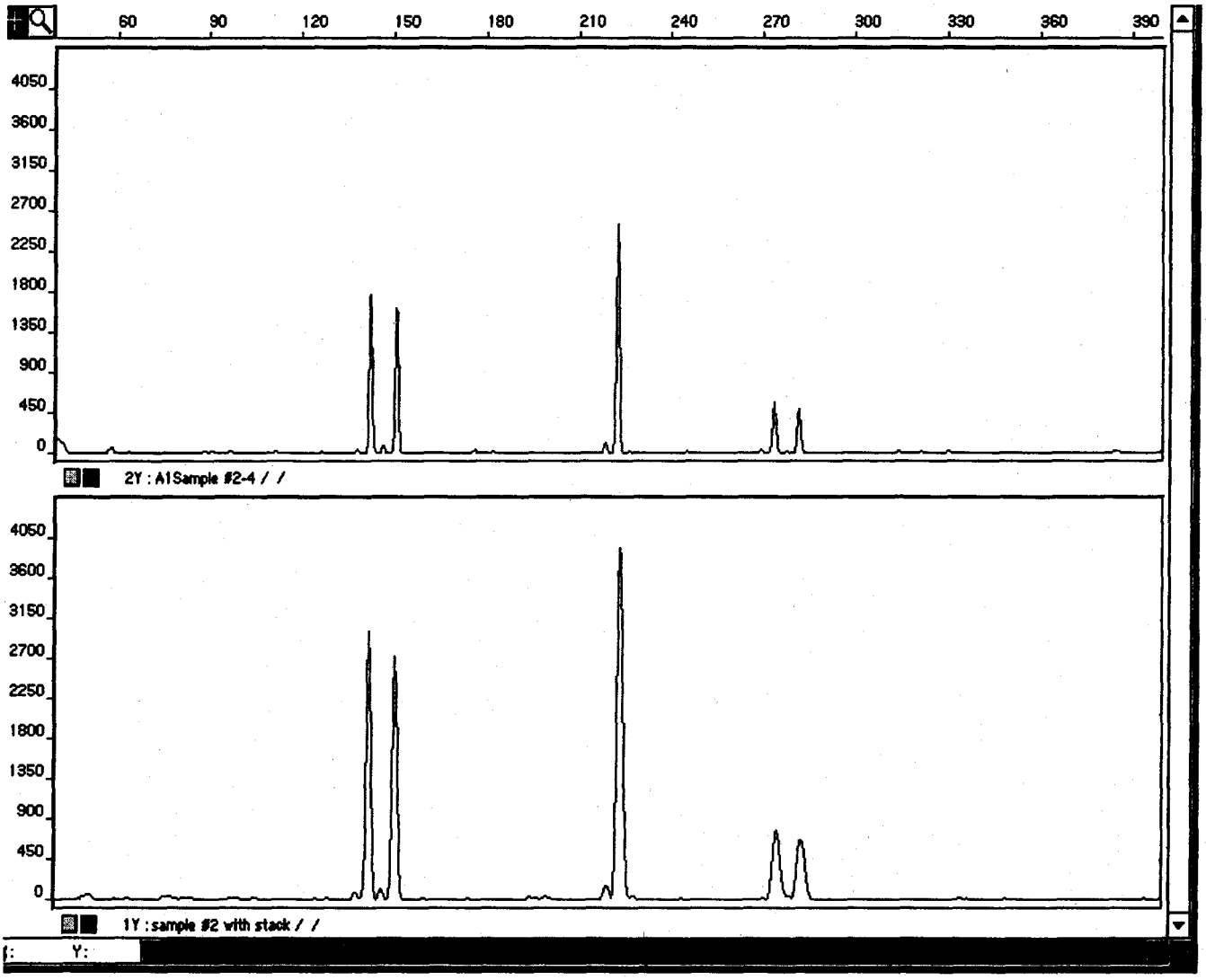


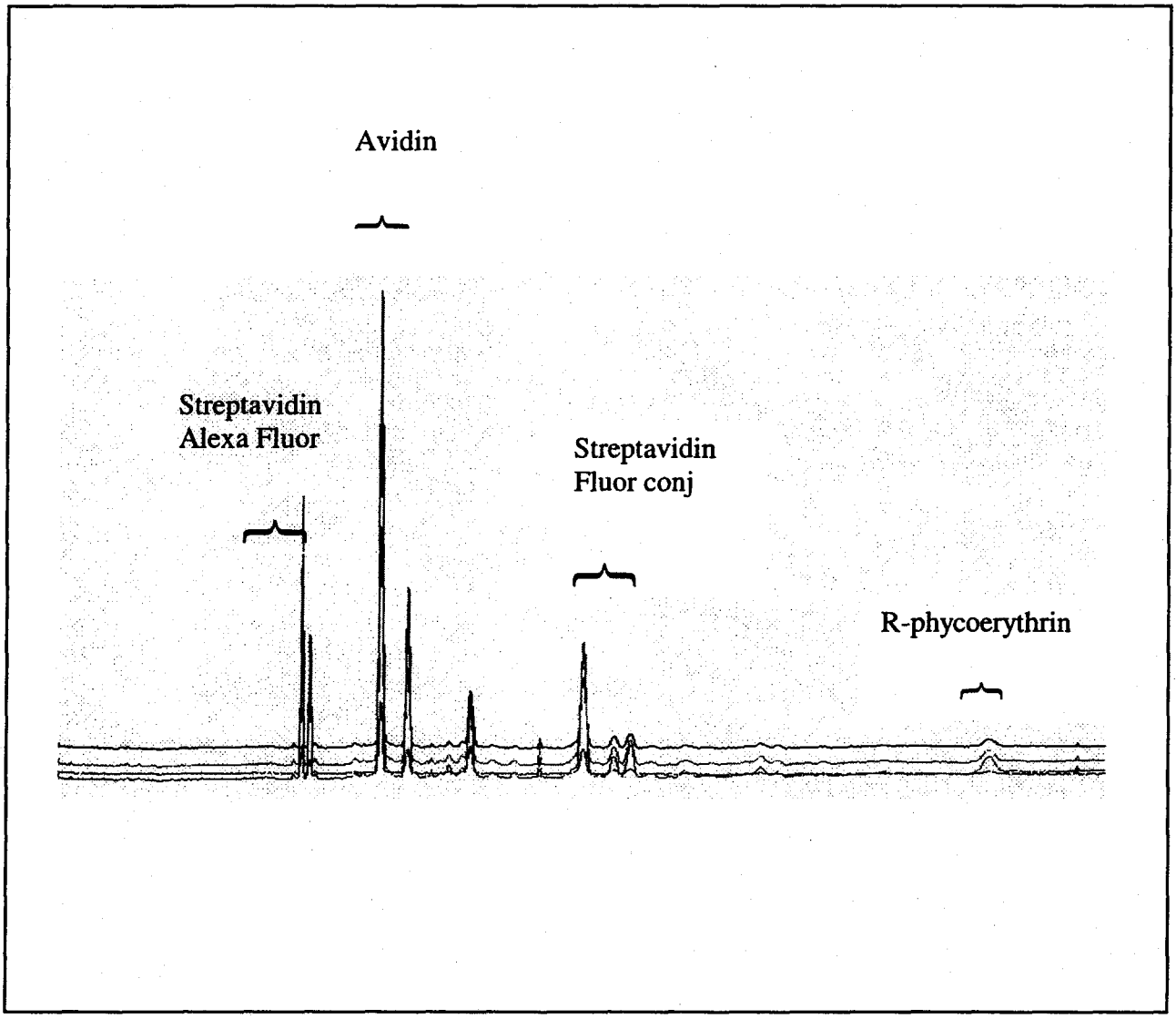


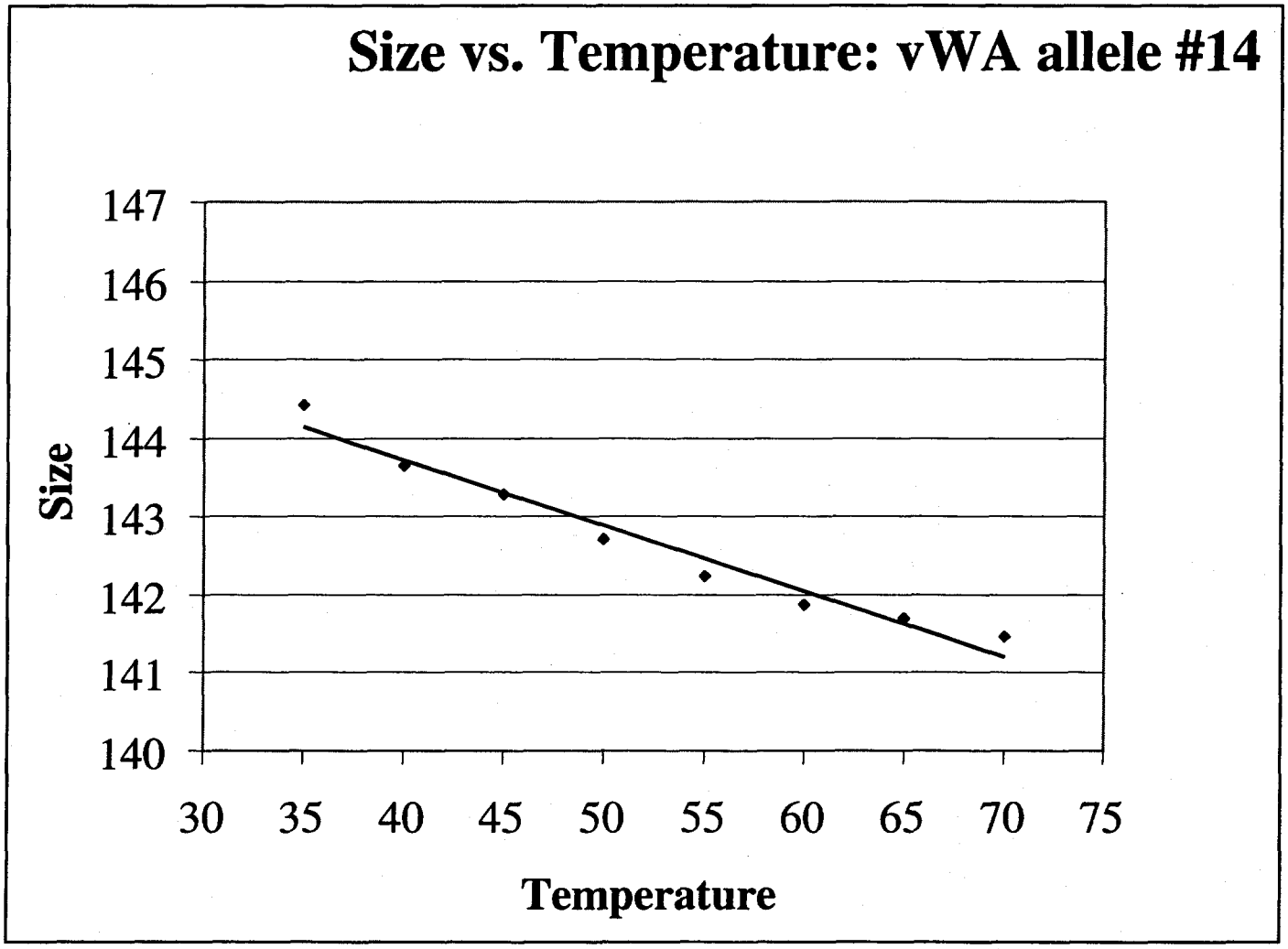


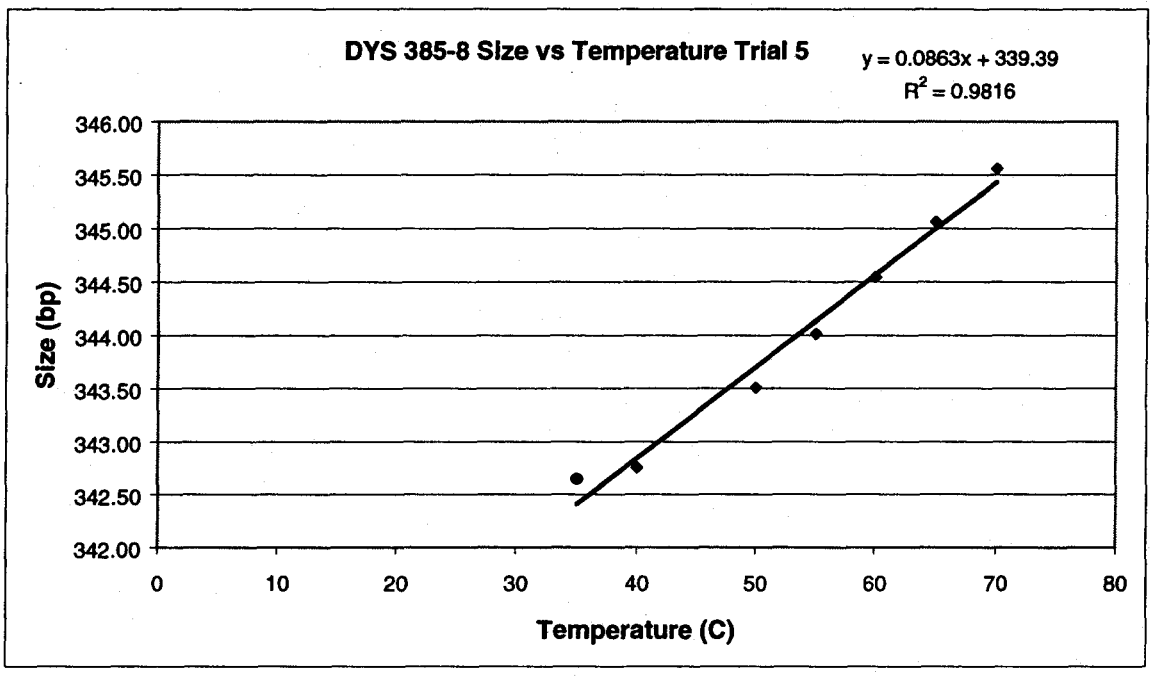


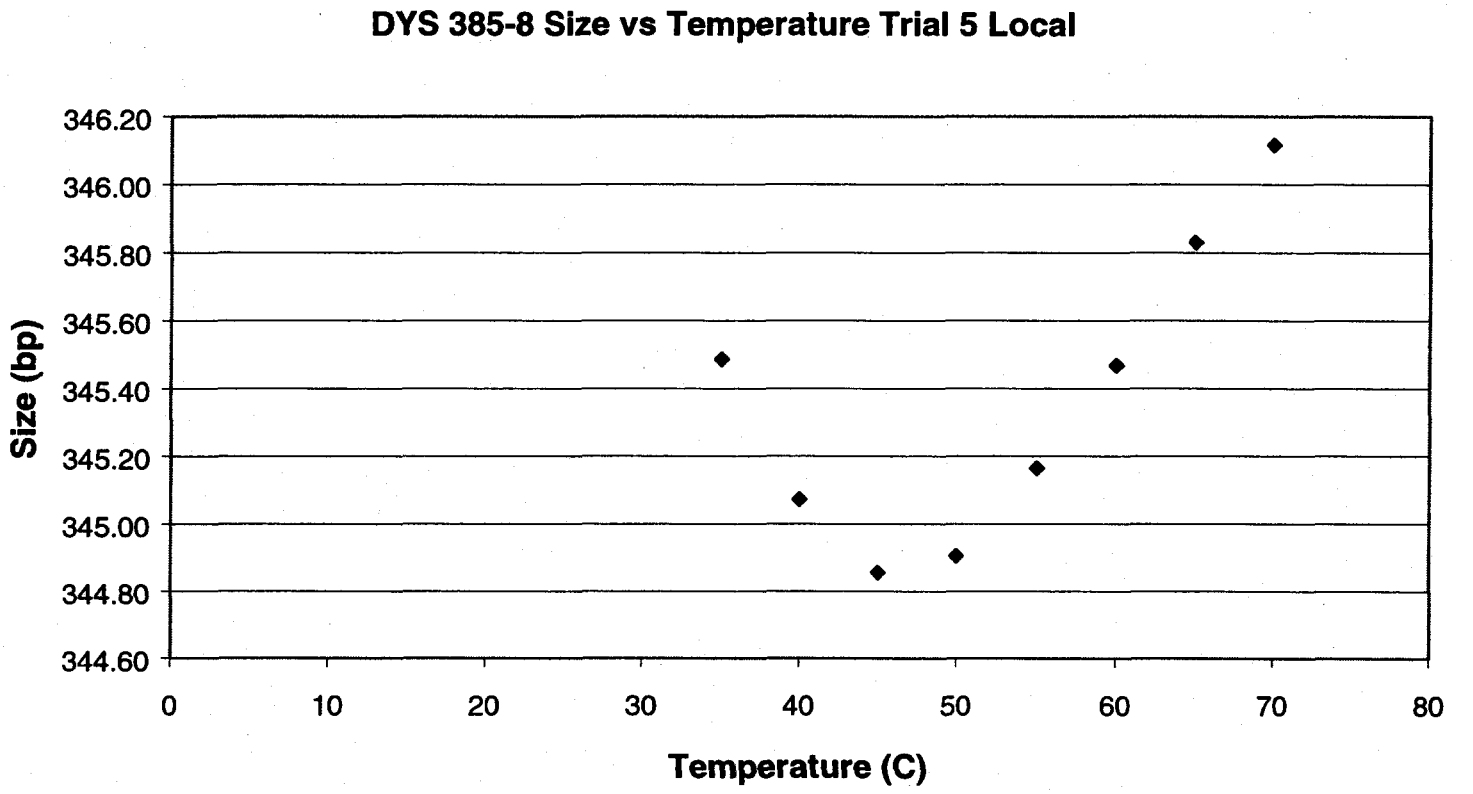


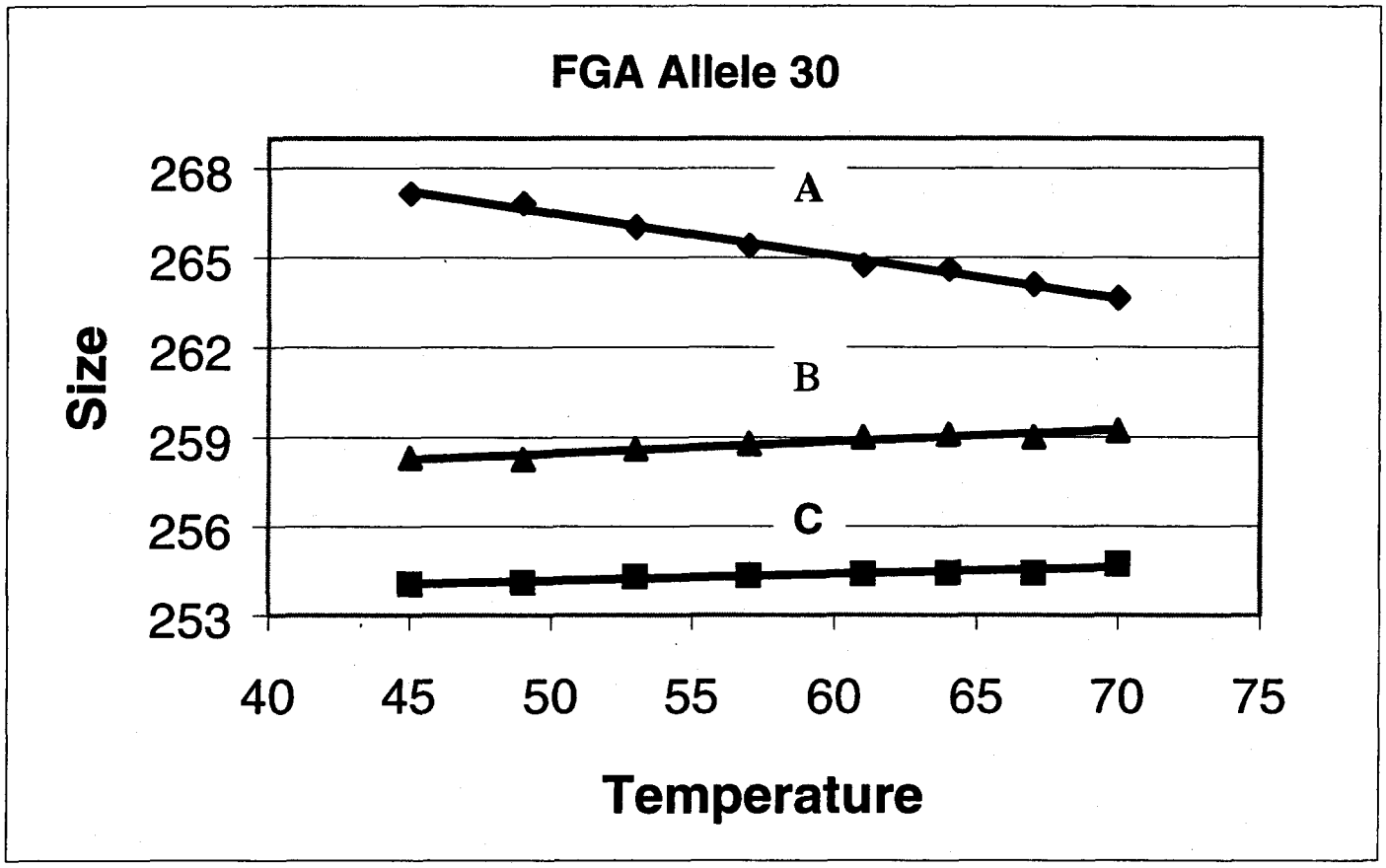


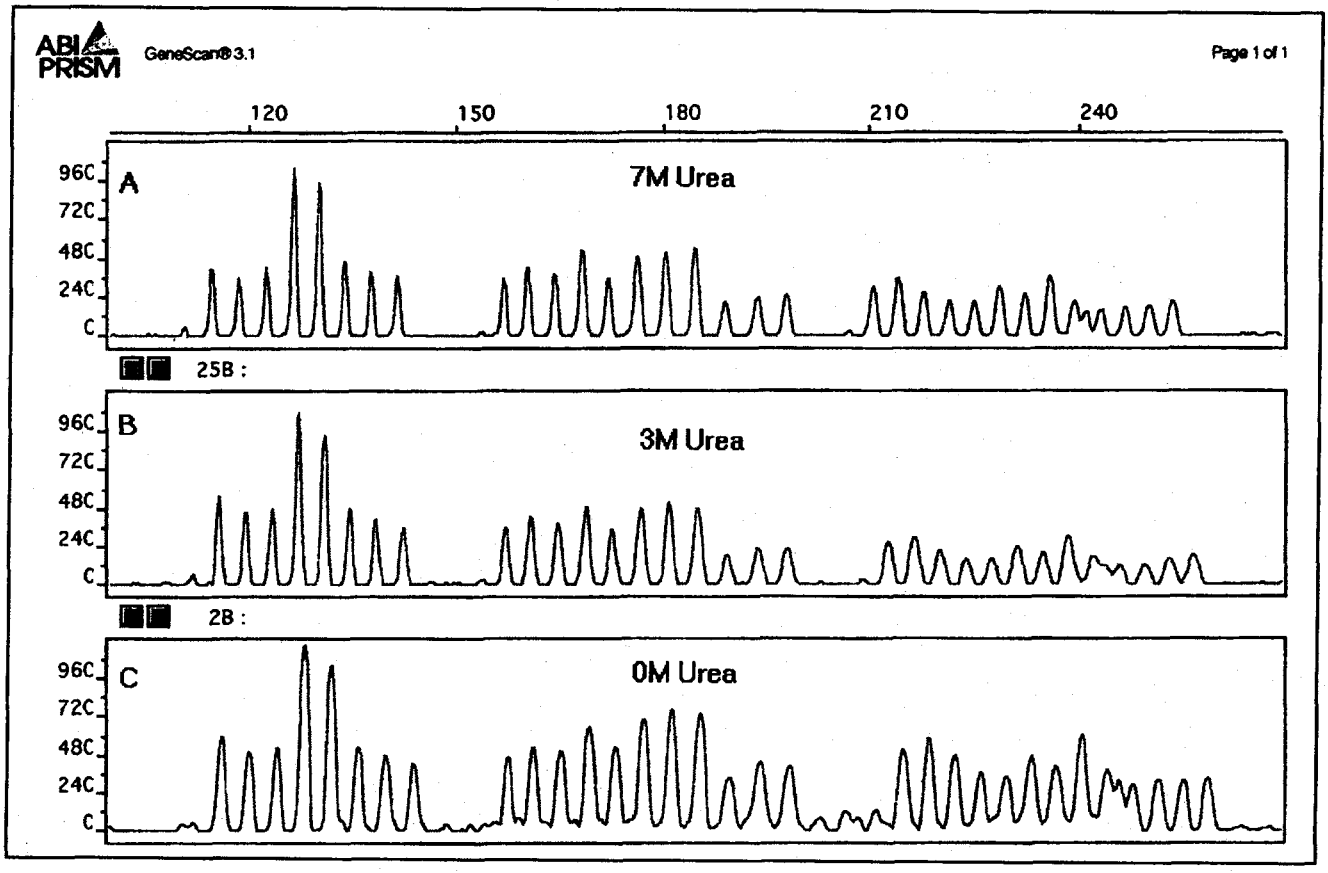












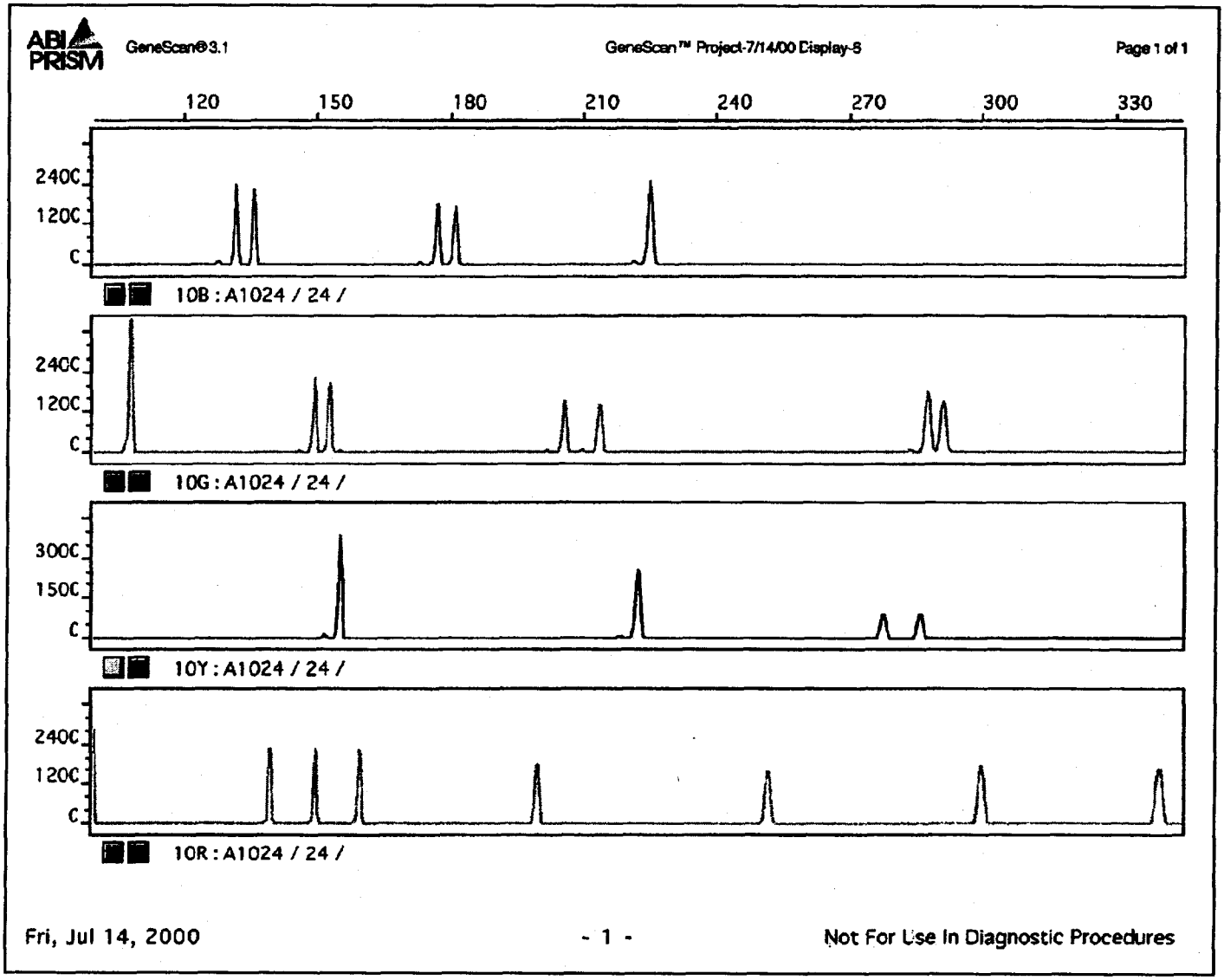


Table 1. Resolution and sensitivity values (respectively expressed in bp and peak height) for the ROX standard sample and the allelic ladder prepared in different diluents. Results are expressed as mean +/- stdev; n=5. Resolution values are expressed: * between peak 139 and 150 in ROX Std, ** peak 150 in ROX std, ° between peak 28 and 28.2 in D21S11 allele °° peak 28 in D21S11 allele.

	ROX Standard		**Allelic ladder**	
	Resolution*	***Peak height***	***Resolution°***	***Peak height°°***
Water	1.19±0.01	2713±91.91	1.18±0.07	1180±186.73
Form #1	1.15±0.05	2962±32.51	1.19±0.04	1058±101.10
Form #2	1.20±0.08	879±4.03	1.17±0.03	363±98.10
Form #3	1.20±0.06	292±13.85	1.17±0.06	250±41.19

Table 2 Comparison of the resolution values obtained without and with stacking in the analysis of ROX standard. Results are expressed as means±stdev and have been evaluated for two different sets of peaks: 139 –150 and 490-500 (bp in length). Five or more replicate analyses were performed on each measurement.

	Peak 139-150		Peak 490-500	
	Without stack	***With stack***	***Without stack***	***With stack***
Water	1.19±0.01	2.03±0.19	3.74±0.28	9.65±0.83
Form #1	1.15±0.05	1.98±0.13	3.28±0.12	7.97±1.87
Form #2	1.20±0.08	1.94±0.17	3.61±0.04	8.33±0.76
Form #3	1.20±0.06	1.87±0.20	3.55±0.10	6.59±0.65

Table 3: Temperature dependence of the Gene Scan size estimates for selected alleles in the ABI Profiler + multiplex kit. Conditions: Pop4 polymer, 47 cm 50μm ID capillary 5 sec., 15kV injection 15kVrunning voltage , 45-70 °C

Locus	Allele	Size*	Slope+	St. dev.
D3S1358	12	111.2	-0.10	0.01
vWA	21	194.9	-0.07	0.02
FGA	30	264.7	-0.14	0.02
Amel.	X	103.5	-0.13	0.01
D8S1179	19	170.4	-0.16	0.02
D21S11	36	232.4	-0.03	0.01
D18S51	26	341.9	-0.18	0.01
D5S818	7	131.2	-0.09	0.01
D13S317	8	205.0	-0.12	0.01
D7S820	15	292.8	-0.09	0.01

*Estimated size at 61°C Slope is in units of bases/°C The global southern sizing algorithm was used. Results are the average of 4 measurements.

Table 4: Temperature dependence of the Gene Scan Size estimates for selected alleles in the Promega CTTv multiplex kit. Note the consistency of slopes within a particular locus. Conditions as in Table 3.

Locus	Allele	Size*	Slope[1]	STD of slope
CSF1PO				
	7	295.6	-0.052	0.01
	10	307.7	-0.050	0.01
	12	315.8	-0.047	0.01
TPOX				
	10	238.8	-0.100	0.009
	12	246.8	-0.104	0.01
TH01				
	6	180.9	-0.053	0.005
	8	188.9	-0.054	0.007
	11	201.1	-0.055	0.007
vWA				
	14	141.1	-0.085	0.002
	17	153.3	-0.087	0.006
	20	165.5	-0.089	0.009

*Estimated size at 60°C, Slope is in units of bases/°C The global southern sizing algorithm was used.

Table 6: Global vs Local Southern Sizing for selected alleles in the Promega CTTv multiplex.Note the lack of consistency in slopes within a locus when using Local Southern sizing. Conditions as in Table 3.

Global Southern Sizing		
allele #		
CSF1PO	average slope	SD of ave.
7	-0.052	0.01
10	-0.050	0.01
12	-0.047	0.01
14	-0.048	0.01
VWA		
14	-0.085	0.002
15	-0.087	0.004
17	-0.089	0.006

Local Southern Sizing		
allele #		
CSF1PO	average slope	SD of ave.
7	-0.027	0.01
10	-0.135	0.02
12	-0.103	0.02
14	-0.156	0.01
VWA		
14	-0.060	0.009
15	-0.059	0.009
17	-0.097	0.008

Slope is in units of bases/°C

Table 7: Temperature dependence of the Gene Scan Size estimates for selected alleles in the Reliagene Yplex 6 multiplex kit using the **Local Southern** sizing algorithm. Note the **lack** of consistency of slopes within a locus. Conditions as in Table 3.

Locus	Allele	Avg. Slope	Std. Dev.
DYS 393	12	-.11	0.002
	13	-.10	0.003
	14	-.09	0.007
DYS 390	22	-.17	0.006
	23	-.15	0.004
	24	-.15	0.007
	25	-.15	0.007
DYS 385	8	non-linear	-
	10	0.10	0.01
	12	0.15	0.03
	13	0.18	0.0008

Slope is in units of bases/°C The local southern sizing algorithm was used.

Table 8: Profiler+ size estimates at pH 11. Temperature dependence of the Gene Scan Size estimates for selected alleles in the Profiler+ multiplex kit using the global southern sizing algorithm. Note the relatively low slopes produced at this pH when compared to those at pH 8. Conditions: 3% HEC, 20mM Taps Buffer pH 11, 7M Urea, 350V/cm, 45-70°C, J&W microsil FC coated capillary 47cm, 50μm ID.

STR	Allele	Size*	Slope1	Slope2
D3S1358	12	115.1	-0.02	-0.02
vWA	21	197.8	-0.04	-0.04
FGA	30	259.0	0.04	0.04
Amel.	X	108.6	-0.06	-0.07
D8S1179	19	171.1	-0.07	-0.06
D21S11	36	238.9	-0.03	-0.02
D18S51	26	333.4	0.04	0.02
D5S818	7	137.3	-0.02	-0.02
D13S317	8	207.8	-0.05	-0.04
D7S820	15	294.6	-0.01	0.02

*Estimated size at 61°C Slope is in units of °C/base,

Table 9: Profiler+ size estimates at pH 11 with no urea added. Temperature dependence of the Gene Scan Size estimates for selected alleles in the Profiler+ multiplex kit using the global southern sizing algorithm. Note the relatively low slopes produced at this pH when compared to those at pH 8. Conditions: 3% HEC, 20mM Taps Buffer pH 11, 0M Urea, 350V/cm, 45-70°C, J&W microsil FC coated capillary 47cm, 50μm ID.

STR	Allele	Size*	Slope1	Slope2
D3S1358	12	114.1	-0.02	-0.01
vWA	21	197.0	-0.03	-0.03
FGA	30	254.3	0.02	0.03
Amel.	X	106.1	-0.03	-0.03
D8S1179	19	167.2	-0.04	-0.03
D21S11	36	238.9	-0.04	-0.03
D18S51	26	328.6	0.05	0.05
D5S818	7	136.8	-0.03	-0.03
D13S317	8	203.1	-0.03	-0.02
D7S820	15	293.7	-0.01	0.02

*Estimated size at 61°C Slope is in units of °C/base.

Table 10: Promega CTTv multiplex size estimates at pH 11 with 7M urea added. Temperature dependence of the Gene Scan Size estimates for selected alleles in the CTTv multiplex kit using the global southern sizing algorithm. Note the relatively low slopes produced at this pH when compared to those at pH 8. Conditions: 3% HEC, 20mM Taps Buffer pH 11, 0M Urea, 350V/cm, 45-70°C, J&W microsil FC coated capillary 47cm, 50μm ID.

Global Southern Sizing

allele #	slope	SD of ave.
CSF1PO		
7	-0.0170	0.003
10	-0.0165	0.004
12	-0.0143	0.005
14	-0.0050	0.003
TPOX		
6	-0.0488	0.007
10	-0.0537	0.007
12	-0.0559	0.008
THO1		
6	-0.0059	0.003
8	-0.0068	0.001
11	-0.0070	0.001
VWA		
14	-0.0073	0.004
15	-0.0056	0.004
17	-0.0050	0.004
20	-0.0011	0.002

Table 11: Profiler+ size resolution at pH 11 with varying amounts of urea added. Note the improvement in resolution as the concentration of urea increases. Conditions: 30°C 3% HEC, 20mM Taps Buffer pH 11, 0-7M Urea, 350V/cm, 35°C, J&W microsil FC coated capillary 47cm, 50µm ID.

	allele	size	Resolution (bases)		
			0.0M	3.5M	7.0M
	X	111.1	2.04	1.09	0.63
vWA	20	194.9	2.91	1.82	1.26
D21S11	26	199.1	2.71	1.94	1.26
D13S317	14	237.5	2.64	2.07	1.38
D18S51	20	316.6	3.26	2.88	2.26

Appendices

Experimental Procedures

1. Injection Studies

Experimental

Chemicals

Trizma base, boric acid and HCl were obtained from Fisher Scientific (Fair Lawn, NJ); ethylenediaminetetraacetic acid (EDTA), urea and HPLC-grade methanol from EM Science (Gibbstown, NJ); hydroxyethylcellulose (HEC, average M_w ca. 250,000)) from Aldrich Chemical Co. (Catalog No. 30,863-3; Milwaukee, WI); Amberlite MB-150 from Sigma (St. Louis, MO); NaOH from Spectrum (Gardena, CA); $MgCl_2$ from J.T. Baker (Phillipsburg, NJ). Different kinds of formamide were obtained from Avocado (Heysham, Lancs.), Acros (New Jersey, USA) and Amresco (Solon, OH).

Fluorescently labeled allelic ladders (AmpF*l*STR Profiler Plus) and GS500 ROX standards were obtained from Perkin Elmer (Foster City, CA).

Conductivity measurements

Conductivity of different

An internal standard solution was prepared by mixing 55 □l of GS500 ROX standard with 1320 ul of formamide; samples were prepared by adding 12 □l of internal standard solution to 0.5 ul of allelic ladder or amplified blood sample.

Blood stains on FTA papers were extracted according to the manufacturer's protocol (GIBCO BRL) and amplified using the protocols described in the AMPF*l*STR Profiler Plus PCR amplification kit (PE User's manual, part #5).

All the samples were denatured at 95° C for 2 min. in a thermal cycler (GeneAmp PCR System 9700) and immediately cooled to 4° C to prevent reanniling.

Some samples were contaminated with $MgCl_2$ salt at concentrations ranging from 0 to 16 mM.

Capillary Electrophoresis

A capillary electropherograph, ABI Prism™ 310 Genetic Analyzer (Perkin Elmer, Foster City, CA), was used. This instrument contains a detection system capable of detecting four wavelengths simultaneously; calibrations of the detection system were performed using matrix dye standards labeled with ROX, JOE, NED and 5-FAM. J&W Scientific (Folsom, CA) DB-17 phenyl/methyl coated capillaries, 50 μm i.d. and 43 cm in total length (30 cm to detector), were used. The sieving medium was 0.2-4% (w/v) hydroxyethylcellulose containing 100 mM Tris-borate, 2 mM EDTA and 7.1 M urea or, for stacking experiments, Tris/HCl 50 mM pH 8.0, 2 mM EDTA and 6 M urea. Entangled polymers solutions were prepared as described previously {Isenberg, ALLEN, et al. 1998 } and pumped into capillaries using a power-driven syringe. New capillaries were prepared by rinsing with 100% HPLC-grade methanol for 20 min. and with water for 10 min. prior to installation. The same rinsing procedure was used after a set of 10 runs. Injections were made by electrokinetic mode at 15 kV for 5 s and, in the case of stacking, NaOH 0.1 M was injected at 15 kV for 5s immediately before the injection of the sample. Separation and run times were under 15 minutes. Data were analyzed using GeneScan software (version 3.1, Perkin Elmer) and Microsoft Excel 97. The resolution (R) between two peaks was calculated using the following formula:

$$R = (2\ln 2)^{1/2}[d/(HW_1+HW_2)]$$

where d is the distance between two adjacent peaks and HW_1 and HW_2 are the peak widths at half height for peaks 1 and 2, respectively. Dividing the resolution into the DNA fragment size difference between any two peaks produces an estimate of the resolution in units of bases.

Sensitivity determinations were carried out expressing the height of a peak as a percentage of the same peak height in the pure formamide solvent.

Viscosity measurements

For all viscosity measurements, HEC solutions were prepared by weight, rather than by volume, so that all concentrations are expressed as percentages of HEC weight contained in a given weight of water. A Gilmont Instruments Falling Ball Viscosimeter (size # 2, catalog no. GV-2200, Barrington, IL) with a tantalum ball (0.25'') has been used. Measurements were made at ambient temperature (25° C) and the time required for the ball to fall through the polymer solution was measured in triplicate with a stopwatch. Viscosity values were determined using the formula:

$$A = K(d_t-d)t$$

where A is the viscosity in centipoise (cp), K is the viscometer constant (K=3.3 for size # 2 viscosimeter), d_t is the density of the ball (d_t=16.6 for tantalum), d is the density of the

solution, and t is the time of descent for the ball. Viscosities and HEC concentrations were plotted using Microsoft Excel 97.

2. Contamination studies

Experimental

Chemicals

Phosphate buffer was obtained from Spectrum (Gardena, CA); sodium tetraborate from Acros (New Jersey, USA); tetrabutylammonium hydroxide from Aldrich Chemical Co. (Milwaukee, WI); formamide from Amresco (Solon, OH); Avidin fluorescein conjugate (Lot: 6501-1), Streptavidin, Alexa Fluor™ 488 conjugate (Lot: 7303-1) and Streptavidin fluorescein conjugate (Lot: 6501-1) from Molecular Probes (Eugene, OR); R-Phycoerythrin (Lot: 291 048) from Prozyme (San Leandro, CA); POP4, 310 separation buffer and Ampli Taq Gold™ DNA Polymerase from Perkin Elmer Applied Biosystems (Foster City, CA); GeneScan®-500 Rox Size Standard from Applied Biosystems (Warrington, England).

Capillary electrophoresis

Initial experiments were performed using the Beckman P/ACE 2050 capillary electropherograph instrument to determine the isoelectric point of the Ampli Taq Gold™ DNA Polymerase. Uncoated capillaries of 75 μm of internal diameter and 37 cm in total length were used with 25 mM borate buffer at different pH values. The protein (at a concentration of 0.25 U/μL) was injected into the capillary electrokinetically at 7 kV for 20 sec and separated applying 270 V/cm of potential. Formamide was used as neutral marker and was added to the sample at a final dilution of 1:10000.

For the contamination study, the 310 Genetic Analyzer instrument was used with uncoated capillaries (50 μm i.d.; l=41 cm). 50 mM phosphate buffer containing 50 mM tetrabutylammonium hydroxide was used for the separation of the mixture of the different fluorescent proteins. The sample was injected electrokintetically at 15 kV for 15 sec, while the separation was performed applying 366 V/cm of potential. POP4 was used for the analysis of the separation of the GeneScan®-500 Rox Size Standard before and after flushing the capillary with different protein solutions at a final concentration of 1 pg/mL. The sample was injected into the capillary by the electrokinetic mode at 15 kV for 5 sec and separated at 366 V/cm.

Quality of formamide was measured using a portable conductivity meter (model CON 100, Milwaukee Scientific Instrument). High values of conductivity (expressed in μS/cm) are correlated to poor quality of the formamide, while the pure solvents normally have low values of this parameter.

3. Temperature and pH studies

2.1 Materials

Fluorescently labeled allelic ladders and GS500 ROX standards were acquired from Perkin Elmer. (Foster City, CA) Trizma base, boric acid, CAPS, EDTA, hydroxyethyl cellulose (Aldrich Chemical Co, #30,863-3), and Amberlite MB-5 Resin obtained by Sigma Chemical Co. Urea was acquired from Spectrum (Gardena, CA). High purity formamide was obtained from Amresco, (Solon Ohio).

2.2 Sample Preparation

Allelic ladder samples (AmpFlSTR Profiler+, Perkin Elmer) were prepared by adding 11 µL of formamide to 0.5 µL of allelic ladder and 0.5 µL of the ROX standard. To denature the samples, the sample tubes were placed in a PE/ABI 9700 Thermal cycler, heated to 95°C for 2 min, and snap cooled to 4°C in an ice bath. Blood stains on FTA paper were extracted according to the manufacturer's protocols (GIBCO BRL part# 10786-010), and amplified using the protocols described in the AmpFlSTR Profiler + Users Manual. (Perkin-Elmer part# 4303812)

2.3 Capillary Electrophoresis

An ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) was used for all experiments and was calibrated using matrix dye standards labeled with ROX, JOE, NED, and FAM fluorescent dyes. J&W Scientific (Folsom, CA) µSil-FC capillary columns with 50 µm inner diameter were used. The sieving medium was 3% w/v HEC and contained 20mM CAPS, pH 11, and varying concentrations of Urea; 7, 5, 3.5, 2, and 0M. The HEC sieving polymer solution was prepared by adding various amount of urea to 25 mL of CAPS buffer and shaking until dissolved, to obtain the proper concentration of urea. 1.5 g of HEC was stirred into the solution, the solution was allowed to mix overnight and approximately 0.5 g of Amberlite mixed-bed ion exchange resin were added

and stirred for about 1 hr. using procedures similar to those described by Baskin et al.{ Bashkin, Marsh, et al. 1996}. This solution was centrifuged at 3000 rpm for 10 min and the polymer solution removed from the ion exchange resin. The solution was then filtered through a 5 μm syringe filter (Millipore). Solutions were stored in refrigerator. Buffers containing HEC in tris-borate/EDTA were prepared as described previously{ Isenberg, ALLEN, et al. 1998}, and buffers containing POP4 (Perkin Elmer, Foster City, CA) were prepared using the manufacturers suggested protocols.

Samples were electrophoretically separated using 50 μm ID capillaries 41 cm in length (30 cm to detector) and field strength of 350 V/cm. The capillaries were prepared by rinsing with the buffer for 5 min prior to running the samples. Injections were made at 15 kV for 5s. Electrophoresis was conducted at 30-70°C and data were analyzed using GeneScan software (version 2.0.1, Perkin Elmer, Foster City, CA.) Regression analysis on the slope and standard deviation of the slope was performed using the LINEST function present in Microsoft Excel (version 5.0.)

The resolution between two peaks was calculated using the standard chromatographic definition, modified to permit measurement using peak width at half height:

$$\text{Resolution} = 2(T1 - T2)/(W1 + W2) =$$

$$(2\ln 2)1/2(T1 - T2)/[(W(1/2)1 + W(1/2)2]$$

where T is the peak retention time, W is the baseline peak width and W(1/2)

is the peak width at half height{ Luckey, Norris, et al. 1993}. Dividing the resolution into the DNA fragment size difference between any two peaks produces an estimate of the resolution in units of bases.