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Research Article

An optimized microchip electrophoresis system for mutation detection by tandem SSCP and heteroduplex analysis for p53 gene exons 5–9

With the complete sequencing of the human genome, there is a growing need for rapid, highly sensitive genetic mutation detection methods suitable for clinical implementation. DNA-based diagnostics such as single-strand conformational polymorphism (SSCP) and heteroduplex analysis (HA) are commonly used in research laboratories to screen for mutations, but the slab gel electrophoresis (SGE) format is ill-suited for routine clinical use. The translation of these assays from SGE to microfluidic chips offers significant speed, cost, and sensitivity advantages; however, numerous parameters must be optimized to provide highly sensitive mutation detection. Here we present a methodical study of system parameters including polymer matrix, wall coating, analysis temperature, and electric field strengths on the effectiveness of mutation detection by tandem SSCP/HA for DNA samples from exons 5–9 of the p53 gene. The effects of polymer matrix concentration and average molar mass were studied for linear polyacrylamide (LPA) solutions. We determined that a matrix of 8% w/v 600 kDa LPA provides the most reliable SSCP/HA mutation detection on chips. The inclusion of a small amount of the dynamic wall-coating polymer poly-*N*-hydroxyethylacrylamide in the matrix substantially improves the resolution of SSCP conformers and extends the coating lifetime. We investigated electrophoresis temperatures between 17 and 35°C and found that the lowest temperature accessible on our chip electrophoresis system gives the best condition for high sensitivity of the tandem SSCP/HA method, especially for the SSCP conformers. Finally, the use of electrical fields between 350 and 450 V/cm provided rapid separations (<10 min) with well-resolved DNA peaks for both SSCP and HA.

Keywords: Heteroduplex analysis / Linear polyacrylamide / Microchip electrophoresis / Mutation detection / p53 DOI 10.1002/elps.200600358

1 Introduction

Within the past decade, there has been a great increase in the understanding of how mutations in genetic material correspond to certain diseases such as cancer. While direct DNA sequencing or hybridization arrays can be used to determine the exact location and nature of a mutation in a patient sample, these methods are expensive and have limitations for the accurate characterization of large gene regions for which a large number of different single-base substitutions and/or insertion/deletion mutations are relevant to the disease, such as in the p53 gene. We have chosen to work with the p53 gene because of its prevalence in human cancers (it is the most commonly altered gene [1]) and because the genetic alterations are better characterized than in many genes. Our study also focused on exons 5–9 of the p53 gene because this is where >90% of p53 mutations have been reported to exist. Several notable reviews have been published [1–4] as well as recent article [5] containing further information on the p53 gene.

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Abbreviations: CAE, capillary array electrophoresis; **HA**, heteroduplex analysis; **LPA**, linear polyacrylamide; M_w, weight-average molar mass; **PHEA**, poly-*N*-hydroxyethylacrylamide; **μCE**, microchip electrophoresis

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Heterozygous patient samples can also create difficulties in data analysis and interpretation for automated DNA sequencing and hybridization methods. Alternative mutation screening methods based on mobility shift assays such as single-strand conformational polymorphism (SSCP) analysis can be used to inexpensively determine whether a sequence alteration is present or not in a specific gene or gene region. Only DNA samples found to differ in sequence from the common wild-type gene need to be subjected to sequence analysis. The decreased cost and rapid analysis times that screening methods can provide could allow a wider screening of multiple genes or gene regions for the presence of mutations, which could then be further analyzed by sequencing or microarrays, if desired. Two mobility shift assays, SSCP and HA, have been widely implemented and are generally held to be extremely useful in a slab gel format; however, slab gels are not practical for clinical assays. Both SSCP and HA offer a great potential for rapid, cost-effective, and sensitive mutation detection in a microfluidic chip electrophoresis format, if the influence of important analysis parameters can be better understood.

The SSCP method is a genotyping technique first developed by Orita *et al.* in 1989 [6]. There are several different commonly used methods for preparing SSCP samples. In the clinical setting, homozygous or heterozygous DNA is extracted from a tissue source, such as tumor tissue, then typically PCR-amplified with fluorescently labeled oligonucleotide primers, and denatured at 95°C followed by snap-cooling on ice. If the DNA derives from a "more pure" or easy-to-work-with sample, such as from cultured tumor cells, the "mutant" (or unknown) DNA amplicons can then be mixed with wild-type (or normal) DNA amplified with the same primers, and then denatured at 95°C and snap-cooled on ice. This heating, followed by quick cooling in dilute solution, causes DNA single-strands to fold upon themselves into unique, internally base-paired conformations that depend sensitively on the sequence. The conformers can then be separated by electrophoresis in a dense polymer matrix or gel, if the conformations of the wild-type and mutant conformers in a given DNA strand (sense or antisense) are sufficiently different, so that mutant DNA conformers have a detectable mobility shift. Often a low concentration of denaturant is also added to the sample to prevent duplex formation. While the SSCP method is highly used and reported in the literature, with thousands of publications since its inception, it is so far not used clinically. The main problem in translating this technology to clinical use has been that it has not yet been proven to have a consistently high sensitivity of mutation detection (>97%) and that many of the research papers only report the testing of a small number of samples (*i.e.*, 5–10 samples rather than the hundreds of samples needed to prove clinical validity).

The sensitivity of mutation detection can also be improved by combining SSCP with a complementary mobility shift assay such as heteroduplex analysis (HA). In the preparation of HA samples in a clinical setting, homozygous or heterozygous DNA is extracted from a tissue source, such as tumor tissue, amplified with fluorescent primers, and then denatured at 95°C followed by slow cooling. If the DNA comes from a more pure mutant sample, such as cell lines, the mutant DNA can be mixed with wild-type DNA directly at that point and then heat-denatured (95°C) and allowed to cool slowly. By slowly cooling, the denatured DNA is allowed to reanneal into double-strands, not only forming wild-type and mutant homoduplexes, but also forming some heteroduplexes between a mutant single strand of DNA and a wild-type single strand of DNA. The sequence mismatches or "bulges" in the heteroduplexes then allow them, sometimes, to be separated from the homoduplexes by electrophoresis in a polymer matrix or gel [7]. The mobility shifts seen in HA are typically smaller than what can be seen for SSCP.

Kozlowski and Krzyzosiak [8] first demonstrated the usefulness of combining CE-based SSCP and HA on 21 different mutant samples derived from the BRCA1 and BRCA2 genes. They found that by combining SSCP and HA they could achieve an apparent sensitivity of mutation detection of 100%, which was higher than SSCP or HA alone (90 and 81%, respectively). Kourkine *et al.* also demonstrated the ability of a tandem SSCP/HA-capillary array electrophoresis (CAE) method to achieve 100% sensitivity of mutation detection for 32 different mutant samples derived from exons 7 and 8 of the p53 gene. Interestingly, the sensitivity of the tandem SSCP/HA approach was also substantially higher in this study than that of SSCP or HA alone (93 and 75%, respectively) [9], and so the two studies are in excellent agreement.

Although SSCP and HA samples can be prepared separately and combined before analysis, we also demonstrated a method for preparing the samples in tandem before analysis by CAE [10]. Our method involved the use of an appropriate buffer salt concentration (10 mM Tris-HCl) and heating and snap-cooling the sample without the use of a denaturant, the latter of which was found to reduce the efficiency of the electrokinetic injection and increase the variability of the results. Recently, however, Vahedi *et al.* [11] also published a tandem SSCP/HA method on a microchip, in which they denatured the samples using formamide. They claim that by adding a small amount of formamide to their samples, they were able to produce both SSCP and HA conformers reproducibly.

A wide range of different polymer matrices have been examined for use in microchannel SSCP including methylcellulose (MC) [12], polyacrylamide [9, 13],

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hydroxyethylcellulose (HEC) [14], and GeneScan™ (Applied Biosystems) [15, 16]. It has generally been shown that more concentrated polymer matrices provide better conformer resolution; however, these more concentrated matrices are more difficult to load into microchannels and also increase analysis times [17, 18]. Less attention has been paid to the importance of the physical characteristics of the polymer, *i.e.*, the average polymer molar mass.

A polymer wall coating is also necessary to suppress EOF and analyte adsorption. A coating can either be covalent or be "dynamic", where the coating polymer forms an adsorptive coating on the glass surface of the microchannel. The most widely used covalent coating for CE was a polyacrylamide coating developed by Hjertén in 1985 [19]. Although this coating works well, like any covalent coating, it has significant drawbacks. The coating must be polymerized *in situ*, which can make it difficult to get a homogeneous coating, and the coating has a limited lifetime after which the capillary must be replaced. An alternative is to use dynamic or adsorptive coating polymers. These coatings can be externally synthesized and their deposition is theoretically reversible, which allows the coating to be replenished or reapplied as it degrades overtime. A variety of dynamic coatings have been used, including poly(ethylene oxide) (PEO) [20], polydimethylacrylamide (PDMA) [21], and PVP [22].

In previous studies, we determined the optimum polymer matrix and wall coating for CAE tandem SSCP/HA. In this study, we found that 6% w/v 600 000 g/mol linear polyacrylamide (LPA) was the optimum concentration and molar mass to allow highly resolved SSCP conformers and highly sensitive mutation detection by tandem SSCP/HA-CAE [9]. We also found that the novel, dynamic coating, poly-*N*-hydroxyethylacrylamide (PHEA) was necessary for sharp resolution of the SSCP conformers, and worked better than a covalently applied LPA coating [9].

In order to develop a very rapid, scalable, clinically useable mutation screening method, there is a need to translate SSCP and HA from the slab gel and capillary formats onto a microfluidic chip. Microchip electrophoresis (μCE) offers the potential to be much more rapid, cost-efficient, and integrated than the other formats. The first published report of SSCP on a chip was by Tian *et al.* [14] who examined mutations in the BRCA1 and BRCA2 genes. They were able to detect three different mutations in less than 2.5 min on the microchip, which was four times faster than their mutation detection separations by CE [14]. The first published report of HA on a microchip was also by Tian *et al.* [23] who looked at six mutations in the BRCA1 and BRCA2 genes. They found that they could detect the same mutations by μCE as by CE, but four to six times faster. However, they also noted that the single-base substitution mutations were less well resolved when analyzed by μCE-HA. The first report of combined SSCP and HA on a microchip was by Vahedi *et al.* [11] who looked at samples from the BRCA1 and HFE genes. They used formamide (~66%) to denature and form the SSCP and HA conformers. In addition, they used an intercalating dye (POPO-3) for on-chip labeling, although they found that in order to achieve high-resolution separations they needed to prepassivate their channel with adsorbed Sytox Orange, a different intercalating dye. It is important to note that these studies were oriented more toward proof-of-concept than toward optimization of the chip electrophoresis methods for clinical use.

After these initial studies, there have been other reports of SSCP separations and HA on microchips. Tian and Landers [24] examined the use of HEC as an effective polymer matrix for HA on uncoated glass microchips. They were able to detect four mutations in the BRCA1 gene and found that the uncoated chip had a longer useful lifetime than an uncoated silica capillary (>70 runs *vs.* ~40 runs) with the same polymer loading. They hypothesized that this could be due to differences in the physical and chemical properties of the microchip and capillary surfaces. It is important to note that although they compared the performance of HEC to other polymers (PEO, PDMA, and LPA) on capillaries, they did not perform any analyses on the influence of the polymer matrix's physical properties (*i.e.*, concentration and molar mass). Tian and Landers compared the performance of μCE-HA to mutation detection by denaturing HPLC (DHPLC) [25]. They found that both methods had similar mutation detection abilities for their panel of BRCA1 and BRCA2 mutants; however, the DHPLC method had been highly optimized, while the HA method was designed only to produce amplifiable targets. In another study, Footz *et al.* [26] combined HA with RFLP analysis, by μCE, in order to improve the overall mutation detection sensitivity. Although SSCP is often run in the slab gel format at more than one temperature, a recent study by Tian and Landers [25] found that it was possible for their chip electrophoresis system to detect 21 mutations in the HFE, MYL2, MYL3, and MYL7 genes with 95% sensitivity using just one electrophoresis temperature (25°C) and with 100% sensitivity using two temperatures (25 and 40°C). This not only indicates the potential for SSCP to be used in a clinical setting, but also emphasizes the need to understand the important variables for mutation detection by SSCP and HA so that one highly sensitive and specific analysis condition can be used.

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Although the usefulness of SSCP and HA mutation detection by μCE have been demonstrated, there is still a significant need to study these systems and to understand how parameters such as polymer matrix and coating influence the sensitivity of mutation detection before these methods can be considered optimized for routine clinical analyses. Our group was particularly interested in understanding what polymer matrix and wall coating would be best suited to the tandem SSCP/HA method on glass microchips. We envision the eventual creation of a highly sensitive, rapid, integrated microfluidic electrophoresis device that can perform automated SSCP/HA analyses of many parallel samples (*i.e.*, in many parallel electrophoresis channels), and believe that such a system would offer great promise for use in a high-throughput setting such as a clinical laboratory. However, such a vision can only be realized if the best polymer matrix and dynamic wall-coating system for SSCP/HA on borosilicate glass chips is identified, and that is the purpose of the present study.

2 Materials and methods

2.1 Cell line samples

Mutant DNA specimens of the p53 gene were supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, MD) in the form of plasmids or PCR amplicons. The cell line-derived plasmids were created at NIST as described previously [27, 28]. The presence of a mutation in these samples was confirmed by sequencing at NIST. Amplicons of the p53 exons 6–9 were produced using a PCR protocol previously described by O'Connell *et al.* [29] for generating SSCP fragments. Exon 5 was amplified using the same PCR protocol, but modified to use different primers (forward primer TGCCCTGACTTTCAACTCTGT and reverse primer GCAACCAGCCCTGTCGTCTCT) [30]. The primers were fluorescently labeled with FAM on the forward strand primers and with JOE on the reverse strand primers. This labeling scheme allowed for two-color LIF detection, which we have previously found to allow the unambiguous assignment of DNA peaks in the electropherograms [9, 10].

2.2 DNA sample purification, quantitation, and storage

Crude PCR products were purified by the Qiaquick-spin PCR purification kit following the manufacturer's instructions. Quantitation of purified cell line DNA was determined either spectrophotometrically or with the use of the Agilent 2100 Bioanalyzer and Agilent DNA 500 or 1000 kit (Agilent Technologies, Palo Alto, CA). Typically, the DNA samples were prepared at a concentration of 1 or 10 ng/μL in 10 μL of 10 mM Tris-HCl. These stock solutions were stored at −20°C.

2.3 SSCP and HA sample preparation

In order to mimic clinical samples, well-characterized cell-line-derived, pure mutant and wild-type DNA samples were mixed together. For preparation of tandem SSCP/HA samples, 1–3 μL of the fluorescently labeled mutant and wild-type PCR products were combined with buffer (10 mM Tris-HCl) to a total sample volume of 10 μL, denatured at 95°C for 3 min, and snap-cooled on ice for 3 min. For preparation of wild-type alone samples, 1–3 μL of fluorescently labeled wild-type PCR products were combined with buffer (10 mM Tris-HCl) to a total volume of 10 μL, denatured at 95°C for 3 min, and snap-cooled on ice for 3 min. The samples were stored at 0–4°C.

2.4 Polymer matrix synthesis and characterization

Two samples of short-chained (weight-average molar mass distribution (M_w) 300 000 and 600 000 g/mol) and one sample of long-chained LPA (M_w 1 000 000 g/mol) were synthesized using free-radical polymerization as previously described by Kourkine *et al.* [9]. Briefly, an aqueous solution of acrylamide was degassed by bubbling with N_2 gas for 30 min. The solution was then placed into a water bath kept at 50°C and isopropanol, ammonium persulfate (APS), and TEMED were added in amounts to produce a polymer with the desired molar mass. The solution was kept at 50°C under N_2 flow for 1.5 h to ensure anaerobic conditions until the polymerization was complete. Once the polymerization was complete, the polymer was dialyzed, lyophilized, and characterized to confirm its M_w by tandem gel permeation chromatography (Waters, Milford, MA)/multiangle laser light scattering (Wyatt Technology, Santa Barbara, CA). DNA separation matrices were prepared by dissolving dried polymers in electrophoresis buffer, 1× TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) with 10% glycerol at the desired concentrations.

2.5 Coating polymer synthesis and characterization

PHEA (Cambrex Bio Sciences, Walkersville, MD) polymer (M_w 4–5 MDa), which was used for dynamic capillary coating, was prepared, purified, and characterized in our laboratory as described by Albarghouthi *et al.* [31].

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2.6 Microfluidic chips and dynamic microchip coating

Borosilicate glass microfluidic chips (Micronit, The Netherlands) with the following properties were used in the experiments: double-T injector with an offset of 100 μm, channel width of 50 μm, channel depth of 20 μm, and separation length of 80 mm. Prior to being used for μCE - SSCP/HA runs, the uncoated glass microchips were conditioned and dynamically coated by rinsing them with the following: HCl (aq. 1 M) for 15 min, PHEA (coating reagent, aq. 0.1% w/v) for 15 min, followed by a water rinse [31].

2.7 Polymer matrix loading into microfluidic chip

Owing to the relatively high viscosity of the polymer matrices and the small channel dimensions of the microfluidic chips, a high-pressure loading device was designed for loading the microchips. The device consisted of a pneumatic press (Carver, Wabash, IN), two metal plates, and stacks of neoprene rubber gaskets (McMaster-Carr, Atlanta, GA) cut in such a manner as to hold the chip in place. One well of the microchip was filled with the desired polymer and the chip was placed into a "hole" cut into the stacks of neoprene rubber gaskets, with the well containing the polymer being toward the center of the neoprene gaskets, and the wells not filled with the polymer being left outside the neoprene gaskets. The neoprene gaskets with the chip were then placed between the metal plates. The bottom metal plate had a hole drilled into it that was attached with copper tubing to a nitrogen tank, which allowed the area containing the microchip between the neoprene gaskets to be pressurized with nitrogen. The pneumatic press was then used to clamp the metal plates tightly around the neoprene gaskets, and once firmly held together, the system was pressurized until the channel was filled with polymer. A release valve was used to release the nitrogen pressure once the microchip channel was filled with polymer. The microfluidic chips were loaded with the LPA separation matrices under positive N_2 pressure (~50–100 psi).

2.8 μCE

PCR samples prepared for tandem SSCP/HA were injected into the chips at 800–1000 V/cm for 20 s (with isotachophoretic stacking afforded by the lower ionic strength of the sample relative to the polymer matrix) and typically electrophoresed at 350–450 V/cm and at ambient temperature (17–20°C). The μCE system has been described previously [32]. Briefly, the experiments were conducted in a μCE system custom-built by ACLARA BioSciences (Mountain View, CA). The system consisted of a high-voltage power supply with the ability to independently control four electrodes, a 488-nm argon ion laser (JDS Uniphase, San Jose, CA) that was directed to the focal site with mirror to produce a laser spot ~10 μm, a high-quantum efficiency, 532 × 64 pixel CCD cooled to −15°C (Hamamatsu, Bridgewater, NJ), and analysis software from ACLARA BioSciences written in LabView (National Instruments, Austin, TX).

3 Results and discussion

3.1 Polymer selection and optimization

LPA was determined in a previous CAE-SSCP/HA study to be a good polymer matrix for achieving high-resolution separations for samples from p53 exons 7 and 8 [9]. LPA is a hydrophilic polymer known to have good DNA-sieving abilities from its use in DNA sequencing and is relatively simple and inexpensive to synthesize. Therefore, we selected LPA as our polymer matrix to optimize for μCE-SSCP/HA. The CAE-SSCP/HA study used the Amersham MegaBACE 1000 instrument to examine LPA matrices with four different average molar masses (300 000, 600 000, 1 000 000, and 2 000 000 g/mol) and three different concentrations (2, 4, and 6% w/v). For the μCE-SSCP/HA polymer optimization study, we chose to examine three molar masses (300 000, 600 000, and 1 000 000 g/mol) and four concentrations (4, 6, 8, and 10% w/v). We decided not to work with the 2 000 000 g/mol LPA because the high viscosity of its solutions made loading the matrix into the small microchip channels very difficult, and our previous CAE-SSCP/HA study indicated that it did not improve resolution beyond that of the 1 000 000 g/mol polymer [9]. In addition, we found that it was necessary to include higher concentrations (8 and 10% w/v) of the polymer matrix for chip-based studies to gain back some of the resolution that is lost, possibly, owing to the decreased separation length in the microchips (8 cm), compared with capillaries (40 cm) [9].

Initially we tried to load the polymer matrices into the narrow microchannels using vacuum (or negative) pressure (<12.5 psi). However, we found that in general, for concentrations >4% the polymer matrices were much too time-consuming to load in this manner (>1 h), and in many cases could not be loaded even after several hours. Therefore, we built a high-pressure loading device that allowed all of the polymer matrices tested to be loaded in <30 min, and more typically in under 5–10 min for the optimal matrices.

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3.2 Dynamic microchip wall coating

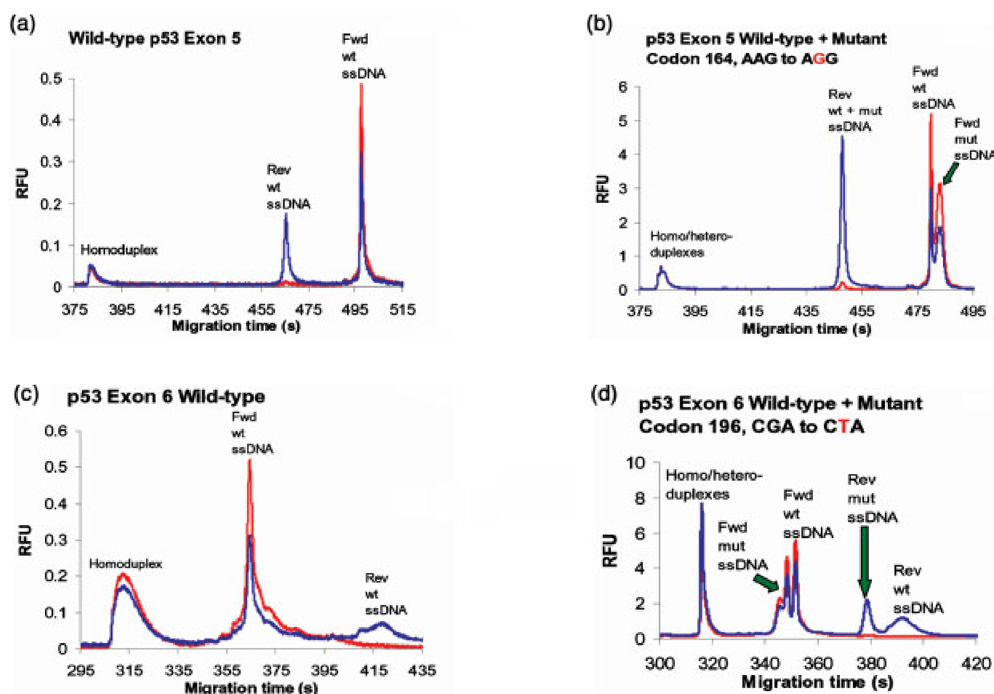
In our previous CAE-SSCP/HA work, we emphasized the importance of the wall coating for the resolution of the peaks, especially for separation of the SSCP conformers, and found that the novel, dynamic, hydrophilic coating PHEA seemed to provide the best performance of the coatings tested [9]. Therefore, we chose to use PHEA for the translation to the μCE platform. For CAE-SSCP/HA, Kourkine *et al.* also found that high quality separations could be obtained for 10–14 runs in series. For this μCE-SSCP/HA study, we found that the coating for the microchips lasted much longer (>95 polymer loads) without significant loss of resolution (data not shown). We believe this may be due to the inclusion of a small amount of the coating polymer (0.25% w/v) in the polymer matrix, a modification that was not included in the CAE studies.

3.3 Characteristics of analyzed mutations

For this methodical study, we examined five single-base substitution mutations, one from each of the p53 exons 5–9. These amplicons ranged in size from 102 to 263 bp (exon 5, 263 bp; exon 6, 182 bp; exon 7, 139 bp; exon 8, 200 bp; exon 9: 102 bp). The correct selection of the optimum polymer was later confirmed by the ability to detect 52/53 mutations from p53 exons 5–9 in a blinded study of 106 total samples, the details of which will be published in a future article.

3.4 Analysis of SSCP/HA electropherograms

Typical tandem SSCP/HA electropherograms obtained in this study are presented in Fig. 1. In the electropherogram, the dsDNA peaks elute first and can be easily identified by the overlapping of emission peaks from the two dyes (FAM on the forward/sense strand (red peaks), JOE on the reverse/antisense strand (blue peaks)), as expected when the two DNA strands are migrating together. The ssDNA peaks elute later, and can easily be identified by the predominance of one of the two fluorescent dyes, where the presence of some red/blue peak overlap for the forward SSCP strand is due to a small overlap in the spectral emission of the two dyes being detected. The *x*-axis has been adjusted to highlight the region of the electropherogram that shows the DNA peaks of interest and to allow the degree of resolution of the mutant peaks to be clearly seen, as indicated by the green arrows. It is interesting to note that several of the DNA peaks are broad, especially the exon 6 peaks and the exons 7 and 8 forward ssDNA peaks. These peak shapes were reproducible, and it is remarkable that in the same electropherogram one or two peaks may be broad while others will be quite sharp. Our previous work with p53 exons 7 and 8 using CE did not show this peak-broadening effect [9]. Although the exact reason for the peak broadening (for some, but not other DNA peaks in a given electropherogram) is currently unknown, we theorize that it could be due to the difference between microchip and capillary glass properties, channel geometry, or



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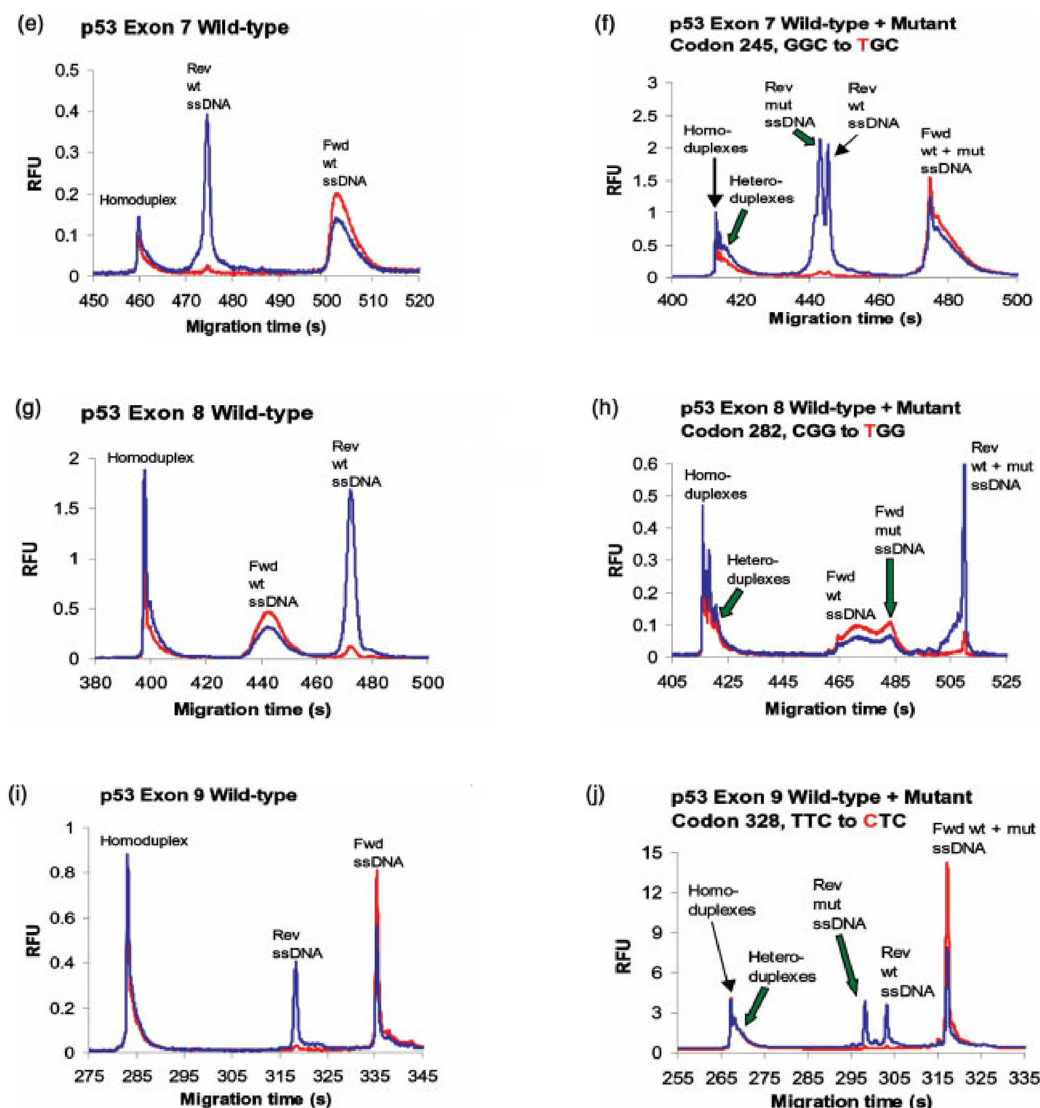

Figure 1. Representative electropherograms showing the analysis of p53 exons 5–9 wild-type and wild-type + mutant samples. The wild-type alone electropherograms are on the left and the wild-type + mutant electropherograms are on the right, with the detection of the mutations highlighted by green arrows. Mutations were detected by tandem SSCP/HA on a glass microfluidic chip using the following conditions: ambient temperature (~20°C), two-color LIF detection (FAM on the forward/sense strand (red peaks) and JOE for the reverse/antisence strand (blue peaks)), 0.1% w/v aqueous PHEA dynamically coated channel, 8% w/v 600 kDa LPA separation polymer matrix, 350–450 V/cm applied electric field strengths. dsDNA peaks are easily identified by the overlap of the two fluorescent dyes. ssDNA peaks are easily identified by the predominance of one of the fluorescent dyes where the presence of some red/blue peak overlap for the forward SSCP strand of a small overlap in the spectral emission of the two dyes being detected. Baselines were adjusted to zero value to remove the background noise. The *x*-axis has been adjusted to highlight the elution of the DNA peak and to emphasize the mutation detection.

separation length. It also seems to be strongly related to the sequence of the DNA fragment, and may be related as well to the particular fold of the SSCP conformers, *i.e.*, how many hydrophobic DNA bases are exposed for a given conformer.

3.5 LPA optimization for high-resolution μCE-SSCP/HA mutation detection

The resolution between the mutant and wild-type peaks was determined by the commonly used equation

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$$R = 2\frac{|t_{wt} - t_{mut}|}{(w_{wt} + w_{mut})} \quad (1)$$

where t_{wt} is the elution time of the wild-type peak, t_{mut} is the elution time of the mutant peak, w_{wt} is the wild-type sample's peak width at half maximum, and w_{mut} is the mutant sample's peak width at half maximum. For each sample, elution time and peak width at half maximum were determined by plotting the electropherogram in Origin (Microcal Software, Northampton, MA) and using a Gaussian fit to the peaks. The final resolution was calculated as the average of at least three repeats and the SDs were used to make the error bars.

3.6 LPA optimization for sensitive μCE-SSCP/HA mutation detection

For the matrix study, we initially experimented with LPAs of three different M_w's – 300 000, 600 000, and 1 000 000 g/mol. We found that regardless of polymer M_w, polymer concentrations below 6% w/v did not provide sufficient DNA separation for good mutation detection, and that concentrations above 10% w/v were extremely difficult to load into chip microchannels. The higher the molar mass of the polymer, the more difficult it was to load a high-concentration solution of the polymer. On the basis of these preliminary results, we performed extensive optimization experiments with the following six polymer matrices, which could be reasonably loaded and showed good mutation detection: 6, 8, and 10% w/v 300 000 g/mol LPA, 6 and 8% w/v 600 000 g/mol LPA, and 6% w/v 1 000 000 g/mol LPA.

The optimum polymer matrix was determined based on both resolution and repeatability (as indicated by small SDs in our ability to resolve neighboring DNA peaks). Plots of resolution (R) *versus* LPA concentration and LPA molar mass for the sample from each p53 exons 5–9 were constructed for the most common mutation detection method (SSCP or HA) for that exon, as shown in Fig. 2. The height of each bar on the bar graph represents the average of at least three repeats and the SDs are given as error bars for the resolution. As might be expected, resolution typically increases linearly with increasing polymer matrix concentration. Interestingly, resolution seems to have a more complicated relationship with molar mass. Although resolution often increases with molar mass, there are several cases where the 600 000 g/mol LPA gives a higher resolution than the 1 000 000 g/mol LPA at the 6% concentration. A somewhat surprising result of this methodical study we have undertaken is the sensitivity of the method to the average molar mass and concentration of the polymer: it is clear to us that these parameters must be controlled carefully if good results are to be obtained in a repeatable fashion.

Overall, we determined 8% w/v 600 000 g/mol LPA to be the best matrix overall for the separation of DNA from p53 exons 5–9, because it often gave the highest resolution and because the resolution from run to run was highly repeatable. After the initial optimum matrix was determined, we found that adding a small amount (0.25% w/v) of the wall-coating polymer PHEA often improved the resolution. We believe that the primary reason that the resolution was improved with the addition of PHEA was because it improved the durability of the wall coating, which reduced the interactions of the sample molecules with the microchannel wall. We plan to report the use of this optimum polymer matrix (8% w/v 600 000 g/mol with 0.25% PHEA) to analyze the sensitivity and specificity of mutation detection in a large-scale study of more than 100 samples from exons 5–9 of the p53 gene.

3.7 Temperature

The effect of temperature on mutation detection can be complex, but generally the optimum results for SSCP are obtained at lower temperatures, probably because of the increased stability of the conformers at lower temperatures [33, 34]. Some reports have also indicated that using multiple temperatures is important for improving mutation detection sensitivity [35]. For this study, tandem μCE-SSCP/HA was performed at ambient temperature (17–20°C), which falls near the range of temperatures that have most often been reported as favorable for CE-SSCP (20–30°C) [34, 36]. HA, on the other hand, has been reported to detect mutations at temperatures of up to 65°C and has been indicated to provide acceptable mutation detection in the BRCA1 and BRCA2 genes at 30°C [8].

We did not have a cooling device for our microchip system available to conduct studies at temperatures below ambient; however, we did have a home-built heating device available. Therefore, we decided to test the effects of increasing temperature. In addition to ambient temperature, we also tested 25 and 35°C. For samples of p53 exon 8 wild-type, increasing the temperature decreased the separation between the ssDNA peaks as seen in Figs. 3a–c. This decrease in separation between the ssDNA peaks could complicate mutation detection by SSCP. For the exon 8 wild-type + mutant, we noted that increasing the temperature sharpens the forward ssDNA peak, as shown in Figs. 3 d–f. In addition, at 25°C the mutation detection by SSCP in the forward strand is lost. Mutation detection is possible up to temperatures of 35°C by HA. Therefore, it seems that mutation detection by SSCP is much more sensitive to temperature than HA. We found the optimum analysis temperature for mutation

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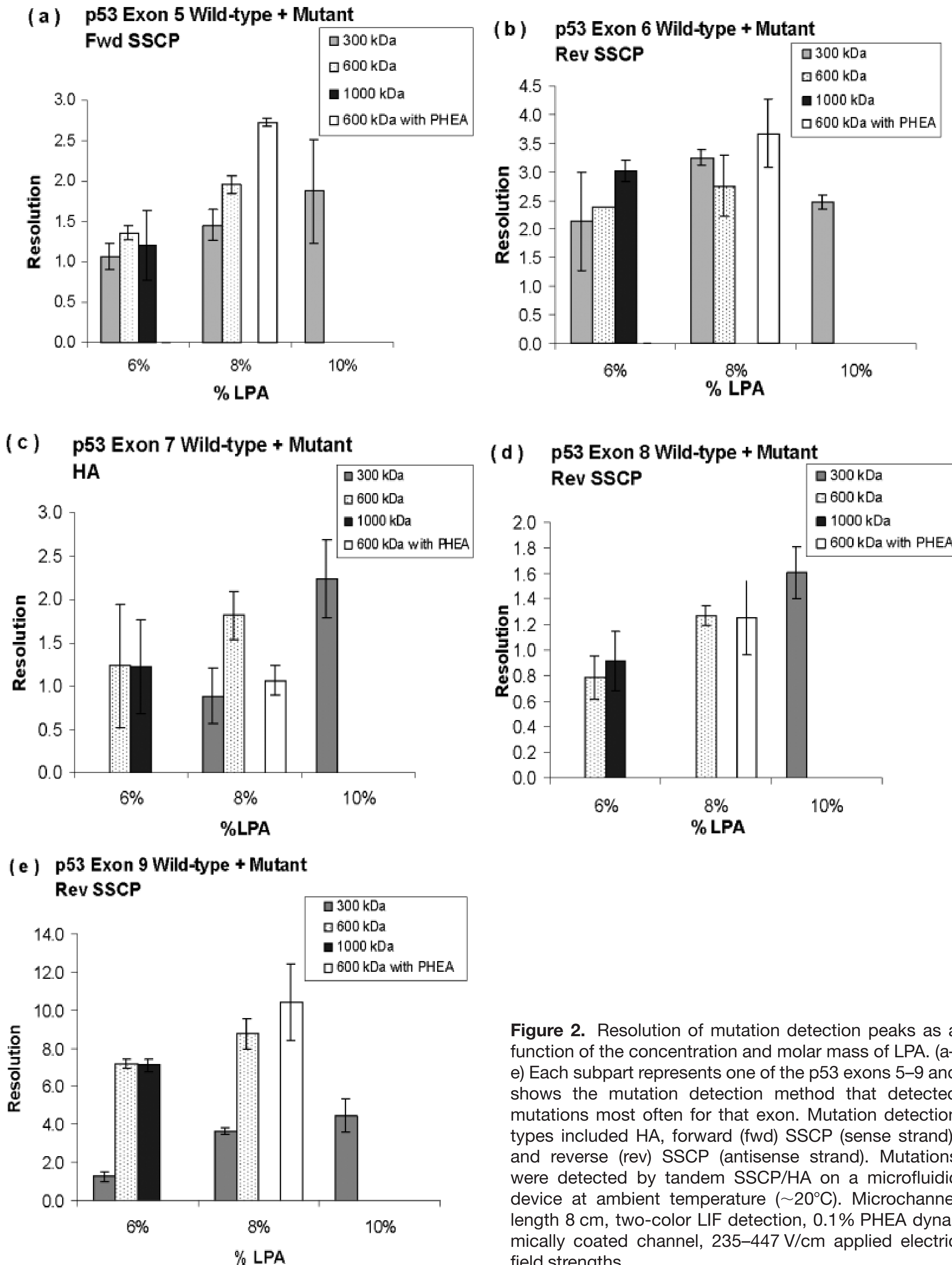

Figure 2. Resolution of mutation detection peaks as a function of the concentration and molar mass of LPA. (a–e) Each subpart represents one of the p53 exons 5–9 and shows the mutation detection method that detected mutations most often for that exon. Mutation detection types included HA, forward (fwd) SSCP (sense strand), and reverse (rev) SSCP (antisense strand). Mutations were detected by tandem SSCP/HA on a microfluidic device at ambient temperature (~20°C). Microchannel length 8 cm, two-color LIF detection, 0.1% PHEA dynamically coated channel, 235–447 V/cm applied electric field strengths.

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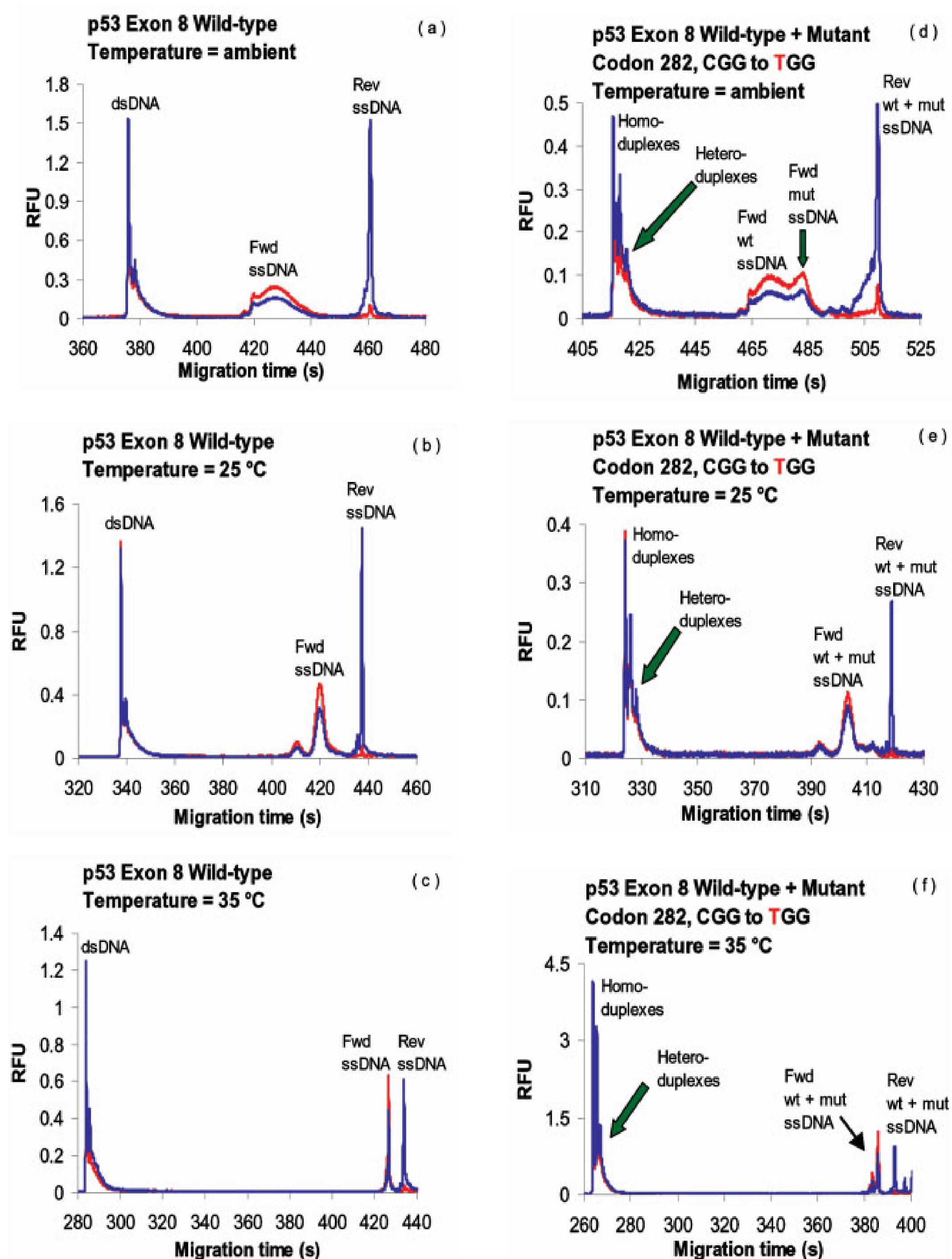

Figure 3. Electropherograms showing the analysis of p53 exon 8 amplicons, demonstrating the effects of temperature on mutation detection by μCE-SSCP/HA. Detection of the mutations by tandem SSCP/HA is highlighted by green arrows. Mutations were detected on a microfluidic device using the following conditions: ambient temperature (~20°C), two-color LIF detection (red dye FAM for the forward strand and blue dye JOE for the reverse strand), 0.1% PHEA dynamically coated channel, 350–450 V/cm applied electric field strengths. dsDNA peaks are easily identified by the overlap of the two fluorescent dyes. ssDNA peaks are easily identified by the predominance of one of the fluorescent dyes, and the presence of the other dye is because of a small overlap in the spectral emission of the two dyes being detected. Baselines were adjusted to zero value to remove background noise.

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detection by both SSCP and HA to be the lowest temperature available to us with the current system setup (ambient, 17–20°C).

3.8 Electric field strengths

The effects of electric field strengths on the resolution of this mixture of single-stranded and dsDNA species are also complex. At electric field strengths that are too low, sample diffusion will occur, leading to band-broadening and difficulty in detecting the subtle mobility shifts necessary for sensitive mutation detection. On the other hand, if the applied electrical field is too high, current flow-induced Joule heating can occur, also leading to band dispersion or potentially a change in the conformers. There have been multiple studies on the effects of electric field strengths on resolving DNA of different sizes. For gel electrophoresis, the electric field strengths are generally ~10–30 V/cm. A study by Luckey and Smith [37] determined that for DNA of ~200 bp (an optimal size for SSCP and HA fragment), an electric field strengths of ~250 V/cm was optimal for a 50-μm id capillary with a separation length of 40 cm. However, to our knowledge there has been no study looking at the effect of electric field strengths on resolving DNA molecules of different conformations in rectangular chip microchannels.

We were limited in the range of electric field strengths we could investigate by the capabilities of the high-voltage power supply that is part of our inhouse-built μCE system. For the T8050 glass Micronit microchip used in our experiments (with an 8-cm separation length), electric field strengths of up to ~450 V/cm could be tested. We therefore worked with electric field strengths ranging from around 140 to 450 V/cm. We found that in general, there was no significant difference in DNA resolution for these electric field strengths, as can be seen in Fig. 4. As the use of low electric field strengths greatly increases the analysis time as shown in Fig. 5, and allows a greater amount of time for evaporation of the sample within the small chip wells to occur, higher fields were considered to be optimal. Therefore, for the T8050 chip we selected 350 V/cm as the optimized running condition for p53 exons 5–8 and 450 V/cm for exon 9 (the smallest exon).

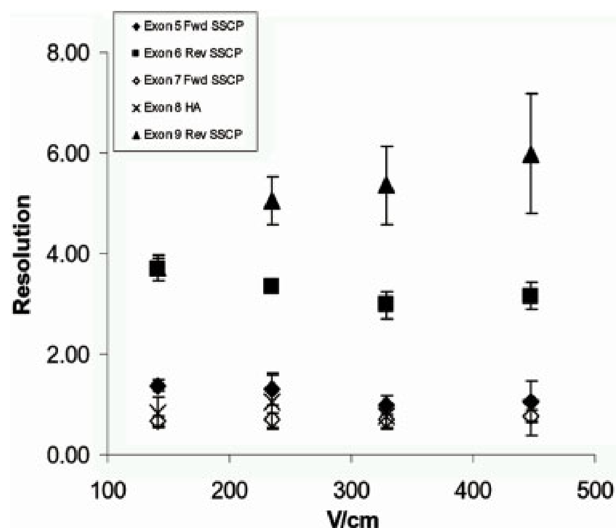

Figure 4. Resolution of mutation detection peaks as a function of the electric field strengths. Each set of data on the graph represents the effect of electric field strengths for one of the p53 exons 5–9 and typically shows the mutation detection method that detected mutations most often for that exon. Mutations were detected by tandem SSCP/HA on a microfluidic device at ambient temperature. Microchannel length 8 cm, two-color LIF detection, 0.1% PHEA dynamically coated channel, 141–447 V/cm.

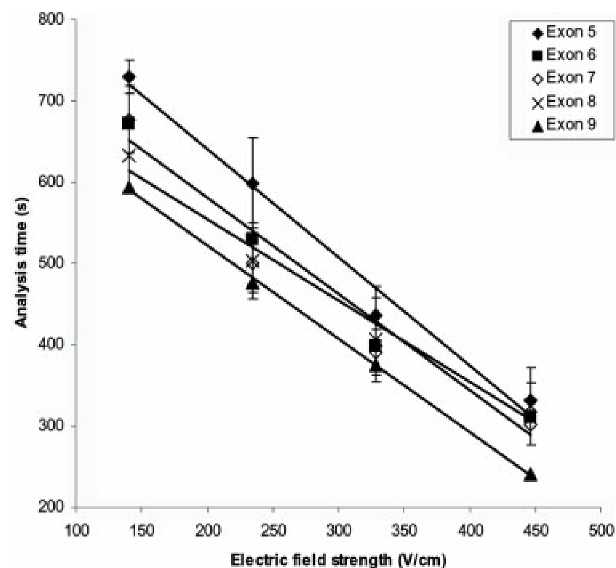

Figure 5. Effects of electric field strengths on total analysis time for mutation detection by μCE – SSCP/HA. Each set of lines on the graph represents the effect of electric field strengths on analysis time for one of the p53 exons 5–9. The lines have been generated using a linear fit with an R^2 value greater than 0.97. Mutations were detected by tandem SSCP/HA on a microfluidic device at ambient temperature. Microchannel length 8 cm, two-color LIF detection, 0.1% PHEA dynamically coated channel, 141–447 V/cm.

4 Concluding remarks

In order to develop a routine clinical mutation detection method using μCE-SSCP/HA, there is a critical need to understand better the role of various parameters such as the polymer matrix, wall coating, and electric field strengths. In this study, we explored the effect of concentration and molar mass on the resolution and repeat-

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ability of mutation detection by μCE-SSCP/HA. Similar to a previous CAE-SSCP/HA study [9], we found that 600 000 g/mol was the optimum molar mass for the LPA polymer matrix, but that a higher concentration (8% w/v) was necessary to maintain a reasonable resolution of the DNA conformers. We also found that a novel, hydrophilic wall coating, PHEA, was a durable and effective wall coating that allowed for highly sensitive mutation detection [31]. In addition, adding a small amount of the wall-coating polymer (0.25%) to the polymer matrix improved the peak resolution, possibly because of an increase in coating stability.

Although electric field strengths has a complicated relationship with resolution, we found that for the range of electric field strengths permitted by our instrument (140–450 V/cm), there was no significant impact on resolution, although lower electric field strengths increased the analysis times and increased the effects of evaporation in the small microchip wells. Therefore, we used electric field strengths of 350–450 V/cm for rapid but still high-resolution analyses.

Generally, lower temperatures have been shown to be favorable for SSCP analyses. As we did not have a cooling device, we tested the effects of increasing the temperature above 20°C and found that although there was little effect on the ability of HA to detect mutations with increasing temperature, than the ability of SSCP to detect mutations was greatly reduced.

This study outlines the approaches our group has taken to optimize μCE-SSCP/HA to develop a highly sensitive and rapid mutation detection method that has the potential to be used for clinical screening of the p53 gene, the most commonly mutated gene in human cancers [1]. We have developed and optimized our system using a single-channel, borosilicate glass microfluidic chip, but clearly the matrix and wall-coating combination could easily be implemented in a multichannel chip, which would offer much higher throughput. Moreover, a more automated (nonmanual) method of pressurized matrix loading into chip microchannels is clearly necessary. Although these and other improvements and developments will be necessary before this chip electrophoresis-based mutation screening method would be used in a clinical setting, such as further integration and automation of the microchip system, we feel that careful examination of these parameters in the μCE system creates an important foundation for a clinically useable, highly sensitive, and specific mutation detection method that could be used to screen patient samples for DNA sequence alterations.

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