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Research Article

Ligase detection reaction for the analysis of point mutations using free-solution conjugate electrophoresis in a polymer microfluidic device

We have developed a new method for the analysis of low abundant point mutations in genomic DNA using a combination of an allele-specific ligase detection reaction (LDR) with free-solution conjugate electrophoresis (FSCE) to generate and analyze the genetic products. FSCE eliminates the need for a polymer sieving matrix by conjugating chemically synthesized polyamide ''drag-tags'' onto the LDR primers. The additional drag of the charge-neutral drag-tag breaks the linear scaling of the charge-to-friction ratio of DNA and enables size-based separations of DNA in free solution using electrophoresis with no sieving matrix. We successfully demonstrate the conjugation of polyamide drag-tags onto a set of four LDR primers designed to probe the *K-ras* oncogene for mutations highly associated with colorectal cancer, the simultaneous generation of fluorescently labeled LDR/drag-tag conjugate (LDR-dt) products in a multiplexed, single-tube format with mutant:WT ratios as low as 1:100, respectively, and the single-base, high-resolution separation of all four LDR-dt products. Separations were conducted in free solution with no polymer network using both a commercial capillary array electrophoresis (CAE) system and a PMMA microchip replicated *via* hot-embossing with only a Tris-based running buffer containing additives to suppress the EOF. Typical analysis times for LDR-dt were 11 min using the CAE system and as low as 85 s for the PMMA microchips. With resolution comparable to traditional gel-based CAE, FSCE along with microchip electrophoresis decreased the separation time by more than a factor of 40.

Keywords:
Drag-tag / Free-solution conjugate electrophoresis / Ligase detection reaction / Microchip electrophoresis / Mutation detection DOI 10.1002/elps.200800197

1 Introduction

The stochastic nature of molecular alterations during tumorigenesis makes cancer diagnosis and prognosis using molecular profiling an arduous task. Cancers often possess multiple mutations embedded within several different genes with varying frequencies; in most cases, these mutations must be thoroughly probed to accurately identify the presence or risk of developing a particular phenotype. For example, 30–50% of all colorectal adenomas are marked by the presence of one or more of the 19 known mutations found in the *K-ras* oncogene alone [1–4]. Most *K-ras* mutations are localized to codon 12 and, to a lesser extent, codons 13 and 61; these mutations are well preserved throughout tumor progression. Testing for these mutations is difficult because the percentage of cells with mutated DNA fluctuates greatly with respect to the stage of tumorigenesis and the location and proximity of the sampling site with respect to the primary tumor site [5–10]. For example, colorectal cancer (CRC) sampling of plasma from CRC patients found that only 0.01–1.7% of the 47 800 adenomatous polyposis coli molecules collected *per* mL of plasma contained mutant alleles [11]. Even at the primary tumor site, the predominant cells are normal stromal cells (WT) found at levels as high as 70% [12]. Successful and accurate genotyping for CRC therefore not only depends on the utilization of a multi-

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Abbreviations: CAE, capillary array electrophoresis **CRC,** colorectal cancer; **ELFSE,** end-labeled free-solution electrophoresis; **FSCE,** free solution conjugate electrophoresis; **LDR,** ligase detection reaction; **LDR-dt,** LDR/drag-tag conjugate; **MHEC,** methyl hydroxyethyl cellulose; **NMEG,** *N*-methoxyethylglycine; **TCEP,** Tris(2-carboxyethylphosphine)

plexed analysis format, but also the ability to detect low copy numbers of mutated sequences in a vast majority of WT DNA.

One technique that can distinguish low abundant mutant DNA from WT DNA in a multiplexed format is the ligase detection reaction (LDR) coupled to a primary PCR [13–20]. Following PCR amplification of the appropriate genes containing the loci of interest, the amplicon is mixed with two complementary primers (a common primer and a discriminating primer) that flank the mutation locus of interest. Conventionally, the discriminating primer contains a base at its 3′ end that coincides with the single-base mutation site. Facilitated by a highly specific thermally stable ligase, the two primers are covalently joined to form an LDR product only if the nucleotide at the potential mutation site is complementary to the 3′ end of the discriminating primer. This process then linearly amplifies LDR products during subsequent thermal cycles; the products that correspond to the presence of a mutation are approximately twice as long as the original LDR primers. The flexible design of the primers used for the LDR assay has allowed for the detection of successful ligation events *via* a variety of analytical formats, such as low-density DNA microarrays, high-resolution electrophoresis and spectroscopic techniques employing fluorescence resonance energy transfer [12, 13, 20–22].

When electrophoresis is used to analyze LDR products, stringent conditions have been employed, including cross-linked slab gels or CGE, which typically require >1 h to achieve separations with adequate resolution for sorting the products that are commonly <100 bp [13]. Recently, microchip electrophoresis has successfully been used to score the presence of mutations using LDR [22]. The use of microchips as a miniaturized electrophoretic platform allows reduced analysis times and provides a platform for the integration of front-end molecular processing to realize an autonomous lab-on-a-chip system, which can reduce the potential for sample contamination and expand the user base of genetic molecular analyses due to the automated nature of sample processing. Using inexpensive polymeric substrates for the fabrication of the fluidic elements will dramatically reduce the overall cost of the analysis and make these platforms viable for one-time use diagnostic applications [21, 23–27].

An obstacle for the translation of capillary-based electrophoretic technologies to microchips is the highly viscous sieving gels required for size-dependent separation of DNA. Viscous media lengthen the preparation and separation times and limit the electric field strength that can be used and also demand robust devices to fill the channels. Gel loading devices and port assemblies constructed to address these issues have had moderate success, but the relatively low pressures that microchips and assembly interconnects can sustain make these solutions tenuous [28]. Furthermore, maintaining the integrity of the assembled microchip becomes more difficult as pressure drops increase due to increases in channel length for improvements in separation resolution and multi-channel designs for high-throughput applications [29]. The use of polymer-based electrophoresis exacerbates this problem due to its inability to withstand high gel loading pressures. Moreover, the chemical composition and/or concentration of the sieving matrix must often be modified to accommodate only a limited size range of DNA to be sorted [30].

In efforts to circumvent the necessity of sieving matrices for DNA separations, end-labeled free-solution electrophoresis (ELFSE), also referred to as free-solution conjugate electrophoresis (FSCE), was theorized and later demonstrated [31–33]. FSCE is an attractive separation technique for sorting charged biopolymers without the need of a sieving medium. In FSCE, a monodisperse, uncharged polypeptide or polypeptoid ''drag-tag'' is conjugated to DNA to disrupt the free-draining behavior of DNA in an electric field [33]. In this regime, the size of the DNA determines its electrophoretic driving force, which is countered by the frictional and hydrodynamic drag from the appended drag-tag. The conjugates of DNA and drag-tags can then be separated by size using electrophoresis in free solution (with no polymer matrix present); the molecular weight and other properties of the drag-tag determine the length of DNA that can be separated with single-base resolution by FSCE possible. With the size range of monodisperse drag-tags now available, DNA separations of $\sim$180 bases have been demonstrated and this electrophoretic approach has proven useful for sequencing and also single-base extension genotyping with free-solution separations performed in both capillaries and glass microchips [34–36].

Here, we present the combination of LDR and FSCE (LDR-FSCE) to create a novel, multiplexed electrophoretic method to screen low-copy-number mutations (in a high abundance of WT DNA) using PMMA microchips without a sieving matrix. Because the desired fragment lengths of LDR products in addition to their fluorescent probes are relatively small and require single-base separation performance (42–46 bp), a combination of viscous matrices and relatively long column lengths are usually needed to sort them. In our previous work involving PMMA microchip separations of LDR products, a few commercially available polymer matrices optimized for capillaries and glass microchips were investigated [22]. Although effective, none exhibited the degree of performance required to separate multiple LDR products in this particular size range, especially in a high excess of WT DNA. For the LDR-FSCE, LDR primers were conjugated to polypeptoid drag-tags to efficiently resolve fluorescently labeled LDR products generated from *K-ras* mutations with a high diagnostic value for CRC [37, 38]. Using this FSCE approach, rapid separations ($\sim$85 s) of LDR/drag-tag conjugates (LDR-dt) were achieved in PMMA microchips using only a Tris-based buffer containing an EOF suppression additive. In addition, we will demonstrate the ability to detect point mutations harbored in mutant DNA found in samples containing large excesses of WT DNA, a prevalent challenge in most diagnostic applications especially those geared toward cancer molecular diagnostics.

2 Materials and methods

2.1 DNA template preparation

Genomic DNA was extracted from cell lines of known *K-ras* oncogenic expression associated with the onset of CRC (HT-29, WT; SW1116, G12A; SW620, G12V; LS180, G12D; and DLD1, G13D) (ATCC, Manassas, VA, USA) using a Qiagen DNeasy kit (Valencia, CA, USA). Here, the nomenclature of the given mutations (*i.e.* G12D) denotes the DNA base substitution (*G*) within a particular codon (12; G*G*T) in exon 1 of the *K-ras* gene, which alters the amino acid translation from glycine to aspartic acid (*D*). PCR amplifications were carried out to generate 290 bp amplicons of each template in 50 μL volumes containing 10 mM Tris–HCl buffer (pH 8.3, 10 mM KCl, 4.0 mM $MgCl_2$), 250 μM dNTPs, 1 μM forward and reverse primers (50 pmol of each primer) and between 1 and 50 ng of genomic DNA extracted from the cell lines. The gene-specific primer sequences were: exon 1.3 forward – 5′ AAC CTT ATG TGT GAC ATG TTC TAA TAT AGT CAC 3′ and exon 1.4 reverse – 5′ AAA ATG GTC AGA GAA ACC TTT ATC TGT ATC 3′. After a 2-min initial denaturation, 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) was added under hot-start conditions and amplification was achieved by thermally cycling for 35 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 1 min with a final extension at 72°C for 3 min.

2.2 Drag-tag synthesis and primer conjugation

The synthesis of the four, linear poly *N*-methoxyethylglycine (NMEG) drag-tags (length = 20, 32, 44 and 56 monomers) utilized in this study (see Fig. 1) was achieved using a solid-phase submonomer synthetic protocol, which has previously been described in detail and was accomplished

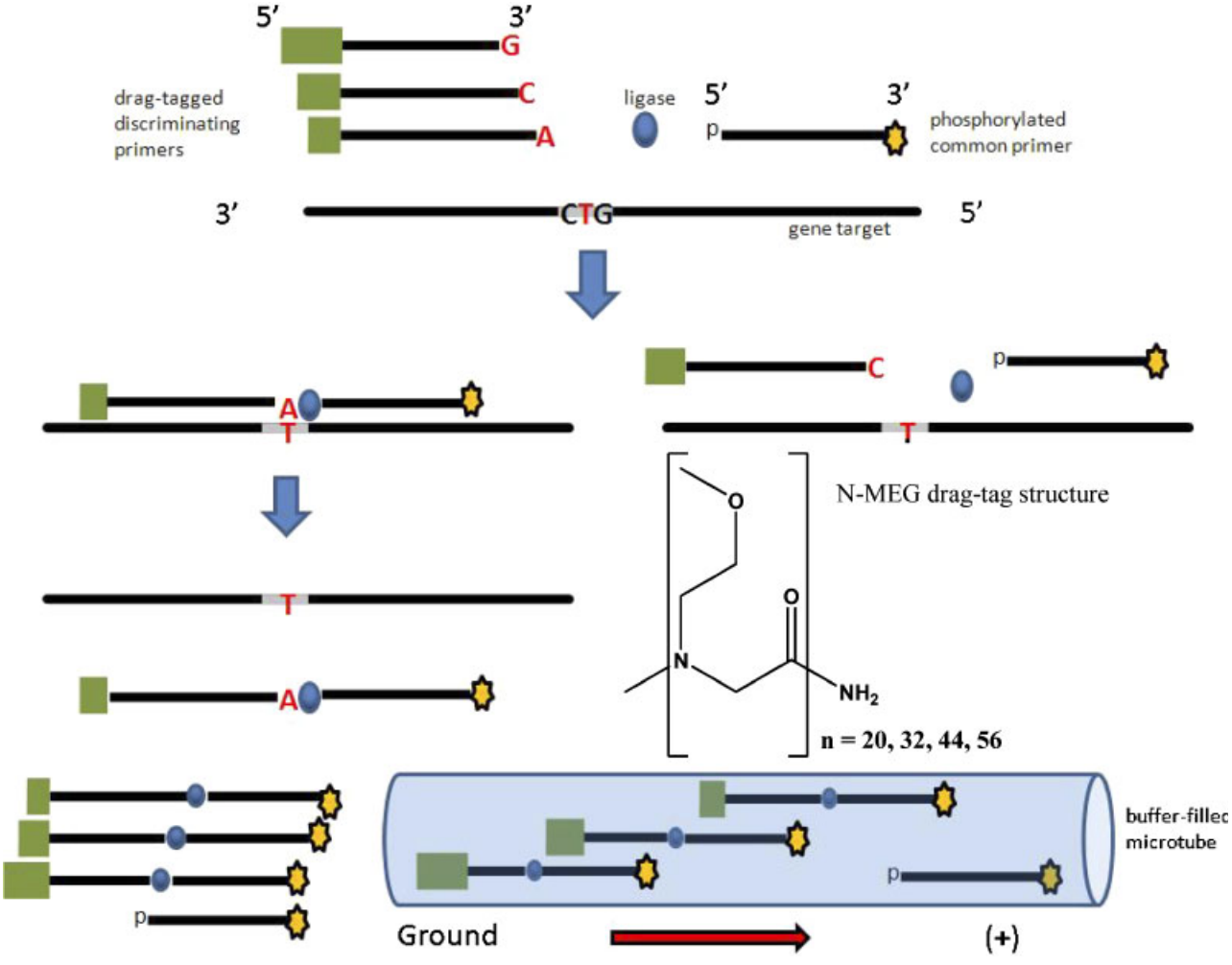

Figure 1. Diagram showing the generation of drag-tagged LDR products and their subsequent separation profile from a mixed population of these LDR-dt products. LDR primers having single-base differences upon successful ligation are inversely paired with drag-tags of different sizes (largest drag-tag to smallest ligated-primer pair). Under an electric field, each LDR-dt has a unique electrophoretic mobility in free solution, whereas the non-conjugated primers have a mobility that is independent of size. Larger LDR-dt products having greater overall charges migrate faster than LDR products composed of smaller oligonucleotides.

Table 1. Design of primers for LDR-ELFSE

LDR primer	Sequence	Length[a)]	T_M (°C)	Drag-tag
K-ras c12.2V	x[b)]-ACA AAA ACT TGT GGT AGT TGG AGC TGT	27	63.6	NMEG-20
K-ras c12.2A	x[b)]-CAA AAC TTG TGG TAG TTG GAG CTG C	25	63.9	NMEG-32
K-ras c12.2D	x[b)]-TGT GGT AGT TGG AGC TGG TGA	24	67.6	NMEG-44
K-ras c13.4D	x[b)]-ACT TGT GGT AGT TGG AGC TGT	21	67.2	NMEG-56
K-ras c12 Com-2	p[c)]-TGG CGT AGG CAA GAG TGC CT-z[d)]	20	69.8	
K-ras c13 Com-4	p[c)]-CGT AGG CAA GAG TGC CTT GAC A-z[d)]	22	69.8	

a) The length of an LDR product (bp) is the sum of a discriminating primer with its corresponding common primer.
b) x, drag-tag attachment.
c) p, phosphorylation.
d) z, fluorescent label.

using an ABI 433A automated peptide synthesizer [39, 40]. All drag-tags were capped with an N-terminal maleimide and purified to monodispersity by RP-HPLC. The discriminating primers used for the LDRs were purchased from IDT (Coralville, IA, USA) and contained a C-6 spacer and thiol linker modifications on their 5′ terminus to facilitate the attachment of the drag-tags (see Table 1). Common primers were synthesized bearing either 6-carboxyfluorescein (FAM) (excitation/emission = 492/517 nm; IDT) or infrared dye (IRD-800) (excitation/emission = 780/816 nm; LI-COR, Lincoln, NE, USA) fluorescent labels (z) on their 3′ ends with phosphorylation on their 5′ termini (see Table 1). The thiol groups on the discriminating primers were reduced by incubating the primers with a 20× molar excess of Tris(2-carboxyethylphosphine) (TCEP), (Acros Organics, Morris Plains, NJ, USA) in pH 7.2 100 mM sodium phosphate buffer at 40°C for 90 min. The drag-tags were conjugated to the 5′ termini of the discriminating primers using a 1:20:28 primer:TCEP:drag-tag concentration ratio in pH 7.2 100 mM sodium phosphate buffer that was incubated at room temperature for 3 h [35]. The largest polypeptoid drag-tags (56 monomers) were paired with the corresponding smallest discriminating primers (21 bp), and *vice versa*, to generate the greatest possible resolution between the LDR products (see Table 1) [35].

2.3 LDR of drag-tag/DNA primer conjugates

LDR assays were carried out using conditions similar to those described elsewhere with slight modifications [22]. Briefly, appropriate ratios of the aforementioned WT and mutant amplicons were added to a solution containing 1× Taq DNA ligase buffer (20 mM Tris–HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1.0 mM NAD) at pH 7.6 (New England Biolabs, Beverly, MA, USA) and 25 nM of each discriminating and common primers aken to a final volume of 20 μL with ddH_2O. After an initial 2-min denaturation at 94°C, 40 U of Taq DNA ligase (New England Biolabs) was added to the cocktail under hot-start conditions and the reactions were thermally cycled 20 times for 15 s at 94°C and 2 min at 65°C. The reaction was then quenched by rapid cooling to 4°C followed by the addition of 0.5 mM EDTA. Prior to electrophoretic analyses, samples were desalted using CentriSep spin columns (Princeton Separation, Adelphia, NJ, USA).

2.4 Microchip fabrication

Microchips were fabricated using methods previously developed and reported by our group [41]. Briefly, the procedure involved machining a molding die by milling raised microstructures onto a brass plate. These microstructures formed a separation channel with dimensions of 70 μm (depth) × 30 μm (width) and 9.5 cm in length (total) with 0.5 cm intersecting side channels offset by 500 μm, which provided a defined 105 pL volume for sample injection. These microstructures were embossed into PMMA wafers (MSC, Melville, NY USA) using an embossing system consisting of a PHI Precision Press Model Number TS-21-H-C (4A)-5 (City of Industry, CA,USA) and a vacuum chamber connected to the press to remove air (pressure <0.1 bar). The microchannel pattern was transferred into a PMMA wafer at 155°C and 1000 lb for 4 min. After hot-embossing, the press was opened and the polymer wafer was cooled to room temperature. Reservoirs were added to the microchips by drilling 1.25 mm holes centered at the end of each channel. After an ethanol rinse and sonication in ddH_2O for debris removal, the final device was assembled by annealing a PMMA cover plate to the open face of the device by clamping between glass plates (∼10 lb) and heating to 107°C in a circulating air oven for 20 min.

2.5 LIF and high-voltage control system

An LIF detection system was constructed in-house with an epi-illumination configuration. A diode 780 nm laser filtered with a band-limiting line filter (Thorlabs, Newton, NJ, USA) was directed onto a dichroic mirror, which reflected the excitation beam through a 40× microscope objective (Newport, Fountain Valley, CA, USA) into the microchannel, which

was situated on an *X–Y–Z* micro-translational stage. The fluorescence emission was filtered through a filter stack and focused onto a single photon avalanche diode (Model SPCM-AQR-12, Optoelectronics, Vaudreuil, Canada). The filter stack consisted of an 825 nm bandpass filter (Oriel, Stratford, CT, USA) and an 800 nm long-pass filter (Edmund Scientific, Barrington, NJ, USA). The LIF signals were acquired on a personal computer using a 32-bit counter/timer board (Model PCI-6601, National Instruments, Austin, TX, USA).

A custom Labview program was used to control the applied voltages for the microchip electrophoresis. The unit included three internal high-voltage power supplies (EMCO, Sutter Creek, CA, USA) capable of receiving inputs of 0 or + 5 V from a digital-to-analog converter output of a CYDDA 04P board (CyberResearch, New Haven, CT, USA). These power supplies were capable of delivering 0 to +2 kV to sample and waste reservoirs (EMCO Model C20) and +0.3 to +5 kV to the anodic reservoir (EMCO Model G50), all of which could be dynamically altered throughout the separation.

2.6 Capillary and microchip LDR-FSCE conditions

Separations of the LDR products were performed on an ABI 3100 (Applied Biosystems) using 36 cm (47 cm total separation length) capillaries filled with 1× TTE (89 mM Tris, 89 mM TAPS, 2 mM EDTA) and 7 M urea buffer containing 0.5% (v/v) POP6 (Applied Biosystems) to dynamically coat the capillary walls to suppress the EOF. Samples were electrokinetically loaded into the capillaries by applying 43 V/cm for 20 s and electrophoresed at 320 V/cm. For the microchip analysis, separations were performed at room temperature using microchannels filled with 1× TTE (50 mM Tris, 50 mM TAPS, 2 mM EDTA) and 7 M urea buffer containing 0.05% (w/v) methyl hydroxyethyl cellulose, MHEC (Sigma Aldrich, St. Louis, MO, USA), to dynamically coat the PMMA channel walls for EOF suppression [42]. To generate a volume-defined injection plug, 347 V/cm was applied for 50 s from the sample reservoir (ground) to the waste reservoir (+3.5 kV). Electrophoresing of the sample was conducted using 365 V/cm, while adduction field strengths of 345 and 276 V/cm were applied to sample and waste reservoirs, respectively, to prevent extraneous sample leakage into the separation channel.

3 Results and discussion

3.1 LDR-FSCE genotyping

In order to conduct FSCE separations of LDR products, LDR primers were reconfigured to allow for the addition of drag-tags onto the 5′ terminus of the discriminating primers. LDR primers intended for electrophoretic separation in a sieving medium are typically designed with discriminating primers bearing fluorescent labels on their 5′ termini [18]. In this case, LDR primer sets were modified in a similar manner to those previously used for microarray studies [12]. As illustrated in Fig. 1 and Table 1, the fluorescent label was placed on the 3′ terminus of the common primer, which also contained a 5′ phosphorylation modification to facilitate covalent coupling of the two primers in the event of successful ligation due to primer complementarity with the mutant allele. A thiol group was positioned on the 5′ terminus of the discriminating primers, which served as the reactive site for drag-tag attachment.

LDR assays were initially performed using thermocycling conditions and primer concentrations previously optimized for conventional primer-based procedures to establish LDR parameters suitable for drag-tagged primers. To verify successful conjugation of the drag-tag with the thiolated oligonucleotides as well as the ability to generate LDR products without interferences stemming from the presence of the drag-tag, single primer set positive control reactions (4) were conducted consisting of one drag-tagged modified discriminating primer (G12D-dt, G12A-dt, G12V-dt and G13D-dt, see Table 1) and its respective dye-labeled common primers with individual *K-ras* mutant templates. In addition, the same reactions were performed with discriminating primers with no drag-tags using the G12D mutant template for comparison.

3.2 CE analysis of the LDR-dt products

FSCE separations of the LDR-dt control samples were conducted using an ABI 3100 system for optimizing the LDR-dt reaction parameters. Figure 2 shows the successful generation of LDR-dt products for all four K-ras mutant templates. As can be seen, all LDR-dt products were effectively resolved from excess dye-labeled common primers, which tended to mask LDR products due to their much higher concentration. The FSCE using this capillary-based machine separation required ~11 min. Each product possessed a unique electrophoretic mobility yielding resolution between the dye-labeled common primer and the LDR-dt products of 4.33, 6.54, 8.50 and 12.27 for the G12V-dt, G12A-dt, G12D-dt and G13D-dt products, respectively. As expected, the relative migration times of the LDR-dt corresponded to the size of the drag-tag units appended to the given LDR-dt products in that the LDR-dt product with the largest drag-tag eluted last and that with the smallest drag-tag eluted first (see Table 1).

In a direct comparison, the separation of the non-drag-tagged G12D LDR sample is shown in Fig. 2. In this case, no LDR product was visible after the separation as the LDR product co-migrated with the unincorporated labeled common primer in free solution, thus illustrating the pronounced effects of the drag-tags on the mobility of the LDR products. However, the presence of a G12D LDR product was illustrated upon the separation of the sample using gel electrophoresis (data not shown), but

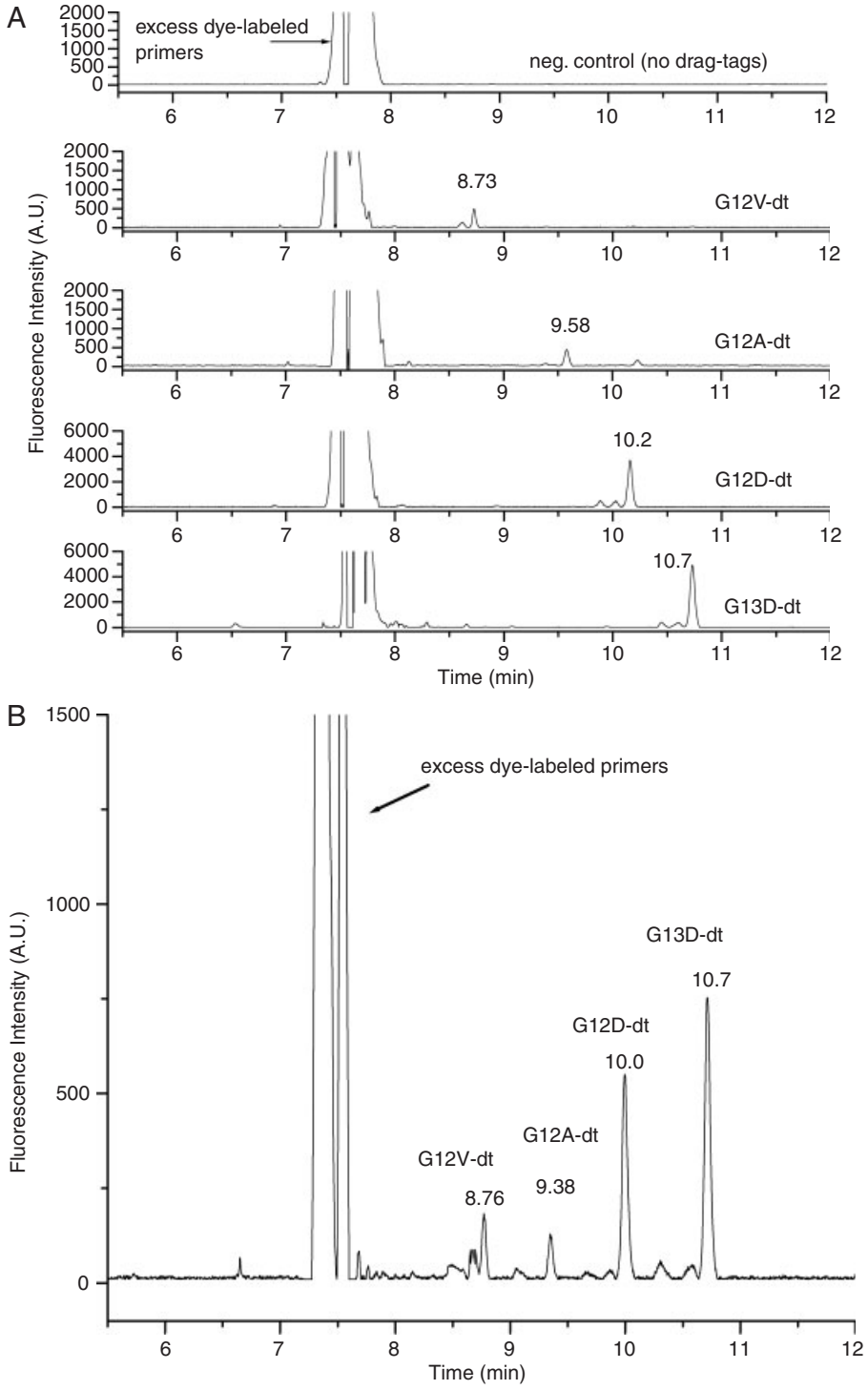

Figure 2. Free-solution CAE electropherograms of positive control samples and multiplex LDR-dt samples that are probing four *K-ras* mutations. (A) Four positive control LDR assays consisting of each mutagenic template paired with its respective discriminating (drag-tagged) and common primer. A negative control LDR assay excluding drag-tags was conducted and analyzed *via* FSCE for comparison. (B) An FSCE separation of four *K-ras* LDR-dt products generated simultaneously in a single-tube reaction using the entire panel of mutant templates and LDR primers (see Table 1 for sequences). The reactions consisted of 1 nM of each mutagenic template with 25 nM each of the common and discriminating primers *per* reaction in 1 × Taq ligase buffer and 40 U Taq ligase (20 μL total reaction volume). Thermocycling conditions included 20 cycles of 95°C for 20 s and 65°C for 2 min following a 2-min initial denaturation at 95°C. Electrokinetic injections and separations were performed in 1 × TTE buffer containing 7 M urea and 0.1% POP6 (for EOF suppression) at $E = 416$ V/cm for 10 s for injection and $E = 375$ V/cm for the separation.

required a development time of ~36 min with a resolution of 3.14 [22].

It was discovered that extending the denaturation time during the LDR cycling beyond 2 min at 95°C leads to partial hydrolysis of the maleimide linker between the primers and the drag-tags, which caused the formation of minor LDR product peaks with slight mobility shifts. To alleviate this artifact, LDRs were performed by adding the drag-tagged discriminating primers after the initial denaturation step used in the LDR phases of the assay to minimize primer-dt degradation. Also, it was determined that the incorporation of drag-tags did not appreciably affect the melting temperature of the LDR primers by comparing product yields from experiments performed at different annealing temperatures (data not shown). During initial experiments, the relative peak areas indicated that LDR products generated for G12D and G13D mutations, which are the most commonly found *K-ras* mutant alleles, were approximately 20-fold greater than LDR products generated for the less frequently occurring G12A and G12V mutations [43]. To increase their visibility in the electropherograms in subsequent results, the relative amount of G12A and G12V LDR-dt products was enhanced by increasing their genomic template concentrations from 50 to 100 pmol prior to PCR amplification.

Once optimal conditions were established for the LDR-dt assays, a single-tube multiplexed reaction was conducted using FSCE for sorting the LDR-dt products. The reactions consisted of concurrent generation of all *K-ras* amplicons (multiplexed PCR) from which a measured aliquot was then LDR cycled with a mixture containing all four drag-tagged discriminating primers (100 nM each) and two common primers (50 nM each) to demonstrate the ability of this mutation detection scheme to probe mutations within the same locus or having close proximity with high fidelity. The electropherogram depicted in Fig. 2B shows the separation of the simultaneously generated LDR-dt products whose unique migration times approximately matched those established in the initial control experiments. Likewise, the relative peak areas for G12D and G13D mutant alleles were approximately ten-fold greater than those for the G12A and G12V mutant allele signals consistent with the relative abundance of these mutations.

3.3 PMMA microchip LDR-dt separations

PMMA microchip FSCE separations of positive control samples were performed using the described in-house-constructed LIF and high-voltage control system. Two important factors that must be considered when using polymer microfluidic devices as electrophoretic platforms for genotyping are the pressure tolerance of the device and the ability to manage the EOF of the polymer substrate surface, which sometimes varies from batch to batch in different feedstocks of PMMA. Here, both issues were addressed through the use of free-solution electrophoresis, which eliminated the need for pressurized introduction of highly viscous sieving media into the microchannels of the devices, and a dynamic EOF suppression coating, which required the inclusion of 0.05% MHEC (w/v) in the running buffer, well below its entanglement threshold [42]. EOF measurements for the PMMA microchip showed that the running buffer containing MHEC attenuated the EOF to $1.49 \pm 0.07 \times 10^{-5}\,cm^2/V\,s$, which was consistent across a number of different PMMA chips and one order of magnitude lower than that obtained for untreated PMMA microchips ($1.77 \pm 0.11 \times 10^{-4}\,cm^2/V\,s$).

Figure 3 illustrates the separation of *K-ras* LDR-dt products using a PMMA microchip. Similar to the capillary separations, the results for each mutant allele were baseline resolved from the free dye-labeled primers. The separations using the PMMA microchips were completed in $\leqslant 85$ s with an effective separation length of 3.5 cm and a field strength of 365 V/cm, which provided resolution values of 2.13, 2.32, 2.77 and 3.25 between the dye-labeled common primer and the LDR-dt products G12V-dt, G12A-dt, G12D-dt and G13D-dt, respectively. As many as five consecutive separations could be performed on the same microchip without requiring the replenishment of the sample or changing the buffer used for the electrophoresis. No carryover contamination from run to run was observed when adduction voltage fields were applied to the sample reservoir during injection. Using these electrophoretic parameters, migration times were highly reproducible

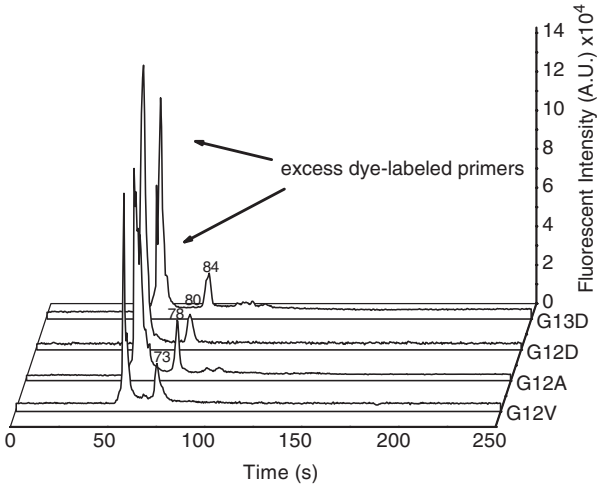

Figure 3. Microchip FSCE separations of positive control *K-ras* LDR-dt reactions. The custom-micro-milled PMMA microchip was hot-embossed from a brass master (9.5 cm length, 30 μm width and 70 μm depth) utilized to separate the LDR-dt products generated using the same conditions given in Fig. 2. The volume-defined cross injection (105 pL) and separation were performed in 1× TTE containing 7 M urea and 0.05% MHEC (for EOF suppression) at $E = 365$ V/cm for 50 s (injection) and $E = 375$ V/cm for the electrophoretic separation. Typical analyses used an effective channel separation length of 3.5 cm.

(average migration time = 81 ± 1.8 s for the G12D-dt LDR product) over five successive runs using the same chip with a similar migration time reproducibility observed inter-chip as well.

Microchip FSCE separations of multiplexed LDR-dt samples were also conducted for the simultaneous detection of all four mutant alleles. The experiment was carried out using the same conditions given in Fig. 2B, except that the genomic G12A and G12V template quantities were increased from 50 to 100 pmol prior to PCR amplification in order to provide peak intensities similar to the predominant G12D and G13D alleles. As seen in Fig. 4, all four LDR-dt products were separated within ~165 s using a field strength of 450 V/cm at an effective distance of 6.0 cm to provide near baseline resolution of all products. At this high field strength, no physical alterations of the microchannels were observed under magnification as well as no deleterious effects on separation performance in terms of plate numbers due to Joule heating.

In order to evaluate the ability to score the presence of these mutations when present in low abundance compared with the WT alleles, LDR-dt assays were conducted using G12D and G13D *K-ras* mutants at biologically relevant levels (1 mutant copy *per* 100 WT copies) by adding 5.0 nM WT template to 0.05–5.0 nM of mutant templates to construct 1:1, 1:10 and 1:100 excesses of mutant-to-WT samples. LDR-dt products were visible in the electropherograms for both *K-ras* mutant templates at levels up to 1:100 at an SNR = 7.1 (see Fig. 5). Attempts to detect LDR-dt products lower than 1:100 were not successful due to the limited detection sensitivity of the LIF system and electrophoretic masking resulting from the high peak intensities of the unligated fluorescently labeled primers. A negative control reaction excluding the *K-ras* mutant templates conducted on 5.0 nM WT DNA showed no visible peaks indicative of the formation of misligated products. Thus, there is minimal probability that this LDR-FSCE assay will give false-positive results. The PCR/LDR technique is capable of maintaining high specificity in low abundance conditions due to: (i) the thermostable ligase, which has the ability to rapidly dissociate from junction sites containing mismatches; and (ii) early misligation events are not further amplified [13]. For example, in the case of amplification techniques based on allele-specific PCR, fluorescent mutant sequences are directly produced, which can generate false positives from misincorporations of nucleotide bases by the polymerase. Here, the PCR serves only to amplify target DNA sequences and the presence of mutations within these DNA sequences is discerned by a follow-up allele-specific ligation.

4 Concluding remarks

We have demonstrated the successful genotyping of four clinically relevant *K-ras* markers important for diagnosing CRCs by a hybrid LDR-FSCE method that generated fluorescently labeled LDR-dt products. The versatility of the LDR-FSCE method allows for multiplexed, highly specific mutation profiling of samples containing up to 100-fold excess of WT sequences on a plastic microchip. The LDR-dt products were separated in 11 min on a

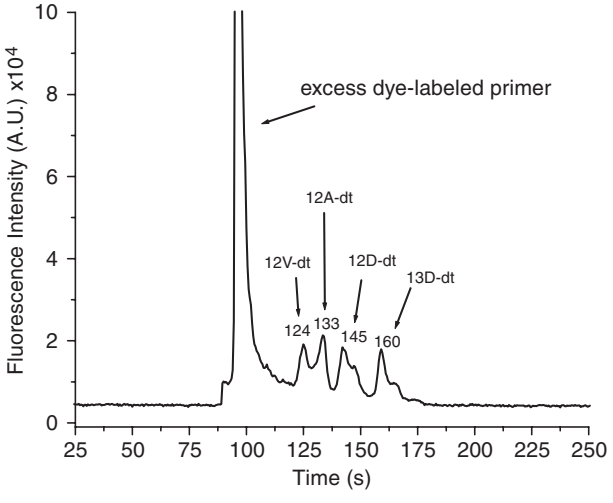

Figure 4. Microchip FSCE separation of a multiplexed LDR generating LDR-dt products. Following PCR of the mutagenic *K-ras* loci in a single-tube reaction, a multiplexed single-tube LDR was conducted using the conditions stated in Fig. 2 with an increase in genomic DNA for G12A and G12V templates to 100 pmol prior to PCR. The effective channel separation length of 6 cm and $E = 450$ V/cm were selected to optimize the resolution of all LDR-dt products. The other separation conditions are similar to those described in Fig. 3.

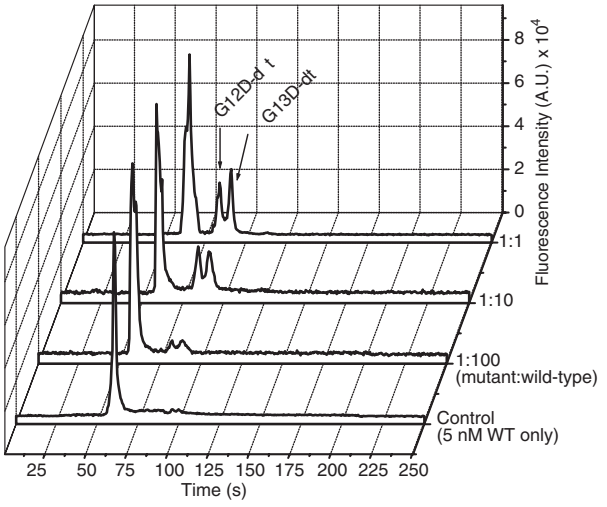

Figure 5. Microchip FSCE separations of multiplexed *K-ras* LDR-dt reactions probing the G12D and G13D mutant alleles with changes in the relative abundances of WT alleles with respect to the mutant alleles. The reaction compositions consisted of 5.0 nM WT template to 0.05, 0.5 and 5.0 nM of the mutant templates to construct 1:1, 1:10 and 1:100 excesses of mutant-to-WT samples, respectively. Separations used the same conditions as those given in Fig. 3.

commercial capillary array electrophoresis (CAE) system and ~85 s on a dynamically coated separation channel of a PMMA microchip that was replicated *via* hot-embossing. LDR-FSCE is a highly flexible method; drag-tags are ideal for genotyping applications that require high-resolution separations of short oligonucleotides. Moreover, the absence of a highly viscous sieving matrix also simplified the operation of the chip-based electrophoresis by eliminating the need for gel filling prior to the electrophoretic analysis. Thus, narrower channel dimensions to improve electrophoretic efficiency or increasing the channel number within the device to improve throughput could be realized without the constraints of high pressure that can result in device failure due to disassembly.

Future work will include the integration of genomic sample pre-processing, including DNA extraction and thermal cycling, onto a polymeric wafer to produce autonomous systems appropriate for genotyping applications that do not require sieving matrices or gel-filling apparati [44]. In addition, improvements in FSCE performance to handle highly multiplexed assays can be realized with the utilization of polyamide drag-tags of larger sizes. The degree of multiplexing will also be increased to probe the entire panel of 19 *K-ras* mutations associated with the development of CRC as well as other well-characterized disease states by the incorporation of a larger array of drag-tags.

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