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Research Article

# Simultaneous detection of 19 K-*ras* mutations by free-solution conjugate electrophoresis of ligase detection reaction products on glass microchips

We demonstrate here the power and flexibility of free-solution conjugate electrophoresis (FSCE) as a method of separating DNA fragments by electrophoresis with no sieving polymer network. Previous work introduced the coupling of FSCE with ligase detection reaction (LDR) to detect point mutations, even at low abundance compared to the wild-type DNA. Here, four large drag-tags are used to achieve free-solution electrophoretic separation of 19 LDR products ranging in size from 42 to 66 nt that correspond to mutations in the K-*ras* oncogene. LDR-FSCE enabled electrophoretic resolution of these 19 LDR-FSCE products by CE in 13.5 min ($E = 310$ V/cm) and by microchip electrophoresis in 140 s ($E = 350$ V/cm). The power of FSCE is demonstrated in the unique characteristic of free-solution separations where the separation resolution is constant no matter the electric field strength. By microchip electrophoresis, the electric field was increased to the maximum of the power supply ($E = 700$ V/cm), and the 19 LDR-FSCE products were separated in less than 70 s with almost identical resolution to the separation at $E = 350$ V/cm. These results will aid the goal of screening K-*ras* mutations on integrated "sample-in/answer-out" devices with amplification, LDR, and detection all on one platform.

**Keywords:**
Drag-tag / Free-solution microchip electrophoresis / FSCE / LDR
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## 1 Introduction

With the completion of several Cancer Genome Atlas project studies [1–3], the genomic basis for cancer continues to be a focus for potential diagnosis, prognosis, and treatment decisions [4, 5]. Direct genome sequencing is the "gold-standard" for mutation detection, but single-base mutation assays are valuable tools to probe for point mutations. Single-base mutation assays frequently utilize an enzyme to discriminate the presence of a mutation and a bioanalytical method to detect and identify it. Possible detection methods include DNA microarrays, mass spectrometry, and fluorescence, to name a few. Cancer samples frequently contain a large amount of wild-type DNA along with mutated genes, thus requiring a highly sensitive and specific detection method.

One technique with a proven ability to detect and identify low-abundance single nucleotide mutations is the ligase detection reaction (LDR) [6]. In the LDR protocol, a PCR-amplified region of interest is mixed with two complementary primers that align side-by-side with the mutation site in the middle. When mixed with a highly specific, thermally stable DNA ligase, the "discriminating" primer (whose 3′ end lies at the mutation site) is only ligated to the neighboring "common" primer if the mutation is present. Ligation does not happen without the mutation because a mismatch at the 3′ end of the discriminating primer is not subjected to ligation by the highly specific ligase enzyme. The concentration of

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**Abbreviations: CRC**, colorectal cancer; **FSCE**, free-solution conjugate electrophoresis; **LDR**, ligase detection reaction; **NMEG**, poly(*N*-methoxylethyl glycine; **pHEA**, poly(*N*-hydroxyethylacrylamide); **sulfo-SMCC**, sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate

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**Colour Online:** See the article online to view Fig. 3 in colour.

 

LDR products is then linearly amplified through temperature cycling. LDR products have been detected by microarrays, fluorescence resonance energy transfer and slab gel, capillary, and microchip electrophoresis [7–13].

LDR has been used to probe single-base mutations in the K-*ras* oncogene that are indicative of colorectal cancer (CRC). These K-*ras* single-base mutations are ideal genotyping targets because they occur early in cancer development, are preserved throughout the course of the disease, and can be detected noninvasively in stool and plasma samples [10, 14]. One or more of 19 known mutations in the K-*ras* gene are present in up to 50% of tested CRC samples [15, 16]. These K-*ras* mutations are localized to codons 12, 13, and 61 [17–21]. In CRC samples, cells containing the mutant alleles are usually in low abundance even at the primary tumor site; LDR has been shown to accurately detect mutations in samples with up to a 500-fold excess of wild-type DNA to mutant DNA [9]. Multiplexing the LDR assay to test for multiple mutations at once simply requires the ability to resolve the products. The importance of testing for all known K-*ras* mutations in CRC patients is highlighted by the advent of epidermal growth factor receptor (EGFR)-targeted therapies. Patients without K-*ras* mutations have significantly improved outcomes with EGFR-targeted therapy while patients with K-*ras* mutations typically do not benefit from the therapy [22, 23].

By incorporating a fluorescent dye on one of the two primers, the LDR product can be detected by laser-induced fluorescence. When the mutant is present, the LDR product is detected as the combined length of the discriminating and common primers; without a mutation, the primers are not joined and only the shorter fluorescently labeled primer is detected. Electrophoresis is a standard method for separating DNA fragments by size, and the LDR fragments ($<$ 100 bases) have been separated using cross-linked slab gels and gel electrophoresis in both capillaries and microchips [7, 10]. However, the size range of LDR products requires stringent conditions to achieve adequate resolution between the unligated primer and the longer, double-length ligation product. Capillary-based separations require more than 1 h for sufficient resolution [7]. Microchip-based electrophoresis reduces the analysis time and sample volume, but loading the highly viscous polymer matrix necessary for DNA sizing of fragments less than 100 bases is challenging due to the pressure limit of both glass and plastic microchips (50–200 psi). Previous work demonstrates only the separation of individual LDR products by gel electrophoresis on microchips, and with limited resolution [7]. Polymer-based separations also lose resolution with increased electric field strength, limiting the speed of the separation. To achieve a rapid, multiplexed separation where all 19 possible K-*ras* mutations are probed, a novel method of electrophoresis must be used.

Proof-of-concept experiments showed the promise of LDR fragments separated by electrophoresis without a polymer matrix, using free-solution conjugate electrophoresis (FSCE) [24]. When DNA molecules are separated in buffer, without a polymer matrix, their elution time is independent of length due to linear scaling of both the charge and friction of DNA. By creating a conjugate molecule of DNA and an essentially charge-neutral perturbing entity ("drag-tag"), the free-draining properties of DNA in an electric field are disrupted, and the mobility of DNA becomes size dependent [25, 26]. Using FSCE, DNA sequencing of approximately 265 bases has been achieved by CE [27] along with genotyping sixteen p53 mutations by a single-base extension assay [28]. Initial LDR-FSCE experiments used four small, chemically synthesized peptoid [29, 30] drag-tags to accomplish separation of four LDR products by capillary and microchip electrophoresis; the microchip separations achieved adequate resolution in less than 85 s [24]. (See Supporting Information Fig. S1 for an illustrated schematic of how LDR-FSCE works.)

In this study, the LDR-FSCE assay is expanded to detect all 19 of the K-*ras* mutants associated with CRC simultaneously by electrophoresis in free solution in both capillaries and glass microchips. To achieve resolution of 19 LDR-FSCE products, longer drag-tags were necessary than the previously used peptoids. Three practically monodisperse, genetically engineered, highly repetitive "protein polymer" drag-tags (110, 141, and 204 amino acids in length) [31, 32] were used for this purpose. A more stable linker molecule was used for these drag-tags to eliminate extra peaks from degradation of the maleimide linker that were seen in the previous LDR-FSCE separations [24]. All 19 LDR-FSCE samples were separated individually by CE to ensure adequate resolution of all neighboring peaks was achieved through appropriate pairing of primer and drag-tag lengths. Multiplexed separations of these 19 mutations were performed using both capillary and microchip electrophoresis in free solution with no polymer network. Additionally, microchip separations highlighted an advantage of FSCE over polymer matrix-based separations; no loss of resolution occurred when the electric field strength was doubled [33]. The 19 LDR peaks were separated in less than 75 s on a glass microchip at the highest field strength possible. These results are a significant advance toward the goal of screening K-*ras* mutations on integrated "sample-in/answer-out" devices where amplification, LDR, sample clean-up, and separation are all on one platform, without requiring loading of a viscous polymer gel.

## 2 Materials and methods

### 2.1 Drag-tag production

Three different methods were used to produce the peptoid and protein polymer drag-tags that were used. The peptoid drag-tag was chemically synthesized by the submonomer method of solid-phase synthesis, which has previously been described in detail and was accomplished using an ABI 433A automated peptide synthesizer [30, 34]. This peptoid was a 36 unit long poly(*N*-methoxylethyl) glycine (NMEG) molecule, and it was capped with an N-terminal maleimide group after cleavage from the solid-phase resin and purified to monodispersity by RP-HPLC. Instead of using maleimidopropionic acid as the maleimide moiety, the more stable heterobifunctional linker sulfo-SMCC (sulfosuccinimidyl

 

4-*N*-maleimidomethyl cyclohexane-1-carboxylate, Thermo Fisher Scientific, Waltham, MA) was added. One method of "protein polymer" production and purification (described thoroughly in [31, 35]) was used to produce two protein polymers. The proteins consisted of a repeating sequence of the amino acid unit "GAGTGSA," where 1 of every 9 serines was mutated to an arginine. These proteins had 18 and 27 repeats of this sequence (141 and 204-aa total length, respectively). The last method (described thoroughly in [32]) produced a "highly charged" protein polymer that was 110 amino acids in length. The 110-aa protein had the amino acid sequence $(GTAGSAGTAGSATGAGSAGSRGTAGSGATGASGTGR)_3$-GA. To activate the protein polymer drag-tags with a maleimide, the single amine at the N-terminus of each protein was activated with sulfo-SMCC by mixing each protein polymer with a 10:1 molar excess of sulfo-SMCC in pH 7.5 0.1 M sodium phosphate buffer. The mixture was gently vortexed for 1 h at room temperature and then lyophilized after removing excess sulfo-SMCC with a CentriSep gel filtration column (Princeton Separations, Adelphia, NJ).

### 2.2 ssDNA templates

To successfully genotype all 19 mutations in codons 12, 13, and 61 of the K-*ras* gene that are relevant to CRC diagnostics and treatment, templates with each of the mutations were necessary. Due to the infrequency of several mutations, cell line samples with each mutation could not be procured. Nineteen oligonucleotides (ssDNA) that were 60 bases long and contained sequences of K-*ras* codon 12, 13, or 61 were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa) as templates; their sequences are provided in Supporting Information Table S1.

### 2.3 Primer design and conjugation of drag-tags

The choice of total LDR fragment length was determined by the ability of each drag-tag to separate short DNA fragments with sufficient resolution. Additional nonpriming bases were added to the end of either the common or discriminating primers for extra length only when absolutely necessary. The sequences of the common and discriminating primers are in Supporting Information Table S2. The discriminating primers are modified at the 5' end with a thiol reactive group; the common primers are modified at the 5' end with a phosphorylation and at the 3′ end with fluorescein (FAM). All DNA was purchased from IDT. The discriminating primers were reduced and conjugated to the drag-tags specified in Table 1 following a previously described protocol [28, 35, 36]. The thiol group of the primer was reduced by incubating with a 20-fold molar excess of TCEP (Tris(2-carboxyethylphosphine)) in a 0.1 M sodium phosphate buffer (pH 7.5) for 100 minutes at 40°C. The reduced primer was then mixed with a 100-fold molar excess of maleimide-terminated drag-tag in the same sodium phosphate buffer for 4–18 h at room temperature.

**Table 1.** LDR-FSCE product lengths, drag-tags, and resolution between neighboring peaks by CE

| Elution order | Mutant | LDR product length | Drag-tag | Resolution |
|---|---|---|---|---|
| 1 | c13.4 V | 42 | NMEG-36 | |
| 2 | c61.5 K | 66 | 110-aa | 12.69 |
| 3 | c61.5 E | 63 | 110-aa | 2.69 |
| 4 | c61.7 HT | 58 | 110-aa | 6.07 |
| 5 | c13.3 C | 55 | 110-aa | 3.77 |
| 6 | c61.6 R | 52 | 110-aa | 5.42 |
| 7 | c12.1 R | 54 | 141-aa | 7.4 |
| 8 | c12.2 V | 44 | 110-aa | 5.18 |
| 9 | c12.1 S | 46 | 141-aa | 5.50 |
| 10 | c13.3 R | 44 | 141-aa | 3.28 |
| 11 | c13.4 D | 42 | 141-aa | 2.50 |
| 12 | c61.6 P | 59 | 204-aa | 4.10 |
| 13 | c61.7 HC | 56 | 204-aa | 3.72 |
| 14 | c13.3 S | 53 | 204-aa | 3.72 |
| 15 | c61.6 L | 50 | 204-aa | 4.89 |
| 16 | c12.1 C | 46 | 204-aa | 3.52 |
| 17 | c12.2 A | 45 | 204-aa | 5.59 |
| 18 | c12.2 D | 43 | 204-aa | 3.50 |
| 19 | c13.4 A | 42 | 204-aa | 2.43 |

### 2.4 LDR reaction and clean-up

The reaction mixture consisted of 50 nM each of the discriminating primer—drag-tag conjugate and the 3′ fluorescein-labeled common primer, 5 nM of the ssDNA template, 40 U of *Taq* DNA ligase (M0208 New England Biolabs, Ipswich, Massachusetts), and 1 X *Taq* ligase buffer (20 mM Tris-HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1.0 mM NAD, and 0.1% Triton X-100 at pH 7.6) in a final volume of 20 μL. The reactions were cycled 30 times for 15 s at 94°C and 2 min at 65°C. The reaction was quenched by rapid cooling to 4°C, and the samples were desalted using CentriSep spin columns prior to electrophoresis.

### 2.5 Capillary and microchip electrophoresis conditions

CE separations were performed using an Applied Biosystems Prism 3100 Genetic Analyzer with four-color LIF detection. The 16-capillary array of bare fused-silica capillaries has an inlet-to-detector length of 36 cm (total length to outlet 47 cm) and 50 μm id. Electrophoresis was performed in 1X TTE buffer (89 mM Tris, 89 mM TAPS, 2 mM EDTA) plus 7 M urea and a 1:200 dilution of POP-6™ ("Performance-Optimized Polymer™," ABI, Life Technologies, Carlsbad, California) for dynamic wall-coating purposes [24]. The drag-tagged samples were introduced into the capillary array by electrokinetic injection at 22 V/cm for 2–20 s, and separation was carried out at 55°C and $E = 250$–$310$ V/cm (12–15 kV applied voltage). Fresh buffer was flushed into the array between each run, and buffer reservoirs were refilled every 1–5 runs.

 

Microchip electrophoresis separations were performed on a home-built electrophoresis system that was previously described [33]. A 488 nm solid-state laser was used to excite the fluorescent dye-labeled DNA, and the emitted light passed through a dichroic filter before being detected on a cooled CCD. LabVIEW software was used to control the custom high voltage power supply and record the fluorescent signal. Commercially available borosilicate glass microchips (Micralyne, SC-100) with a simple-cross injection (50-μm width injection zone) were used. The chips were dynamically coated with poly(*N*-hydroxyethylacrylamide) (pHEA) to minimize EOF and reduce interactions between the drag-tags and the walls using a previously described procedure [37]. A custom copper heating plate and chip caddy was used to maintain the channel temperature at 55°C and increase the well volume to 100 μL. The chip and all wells were loaded with the same 1X TTE plus 7 M urea buffer that was used for CE; a 1:100 dilution of a 0.3% w/w solution of pHEA was added to the buffer for additional dynamic wall coating. Separations were performed by loading the sample into one of the side arms of the microchip and electrophoresing across the separation channel for 20–50 s at $E = 350$–$500$ V/cm. The applied voltage was then switched and the sample in the injection cross was electrophoresed down the separation channel at 350–700 V/cm. The laser was positioned at a distance of $L = 7.2$ cm from the injection cross.

## 3 Results and discussion

The initial LDR-FSCE experiments used four lengths of peptoid drag-tags [24]. FSCE separations of the LDR products with the peptoid drag-tags were successful yet limited. The maleimide used for conjugation was unstable during temperature cycling, and the longest peptoid used (56 units of NMEG) is one of the longest possible due to the limits of solid-phase synthesis. Achieving adequate resolution of all 19 LDR-FSCE products requires the use of longer drag-tags with significantly larger hydrodynamic drag as well as a better maleimide linker that would not hydrolyze during temperature cycling. To support this multiplexed separation, a panel of 3 monodisperse "protein polymer" drag-tags (110, 141, and 204-amino acids long) [31, 32] was used along with one peptoid (36 mer NMEG) drag-tag. The drag-tags were conjugated to the discriminating primers using the heterobifunctional linker sulfo-SMCC, which has an internal cyclohexane ring that stabilizes the maleimide and prevents ring-opening hydrolysis.

### 3.1 CE separations

To test the design of the primers and drag-tags, each of the LDR-FSCE products was produced by the LDR protocol, cleaned up with a CentriSep spin column, heat denatured, and snap-cooled on ice. The samples were injected onto the 16-capillary ABI 3100 CE instrument to test for the presence of each sample and the resolution between each peak. In order to separate all 19 samples simultaneously on the 16-capillary CE instrument, three of the capillaries contained two LDR-FSCE products. The smallest three samples were coinjected with the largest three samples (i.e. the first sample to elute was run with the 17th sample, the 2nd with the 18th, etc.) in order to have the largest possible space between the coinjected samples. Running all 19 samples simultaneously eliminates injection variation and allows individual peaks to be directly compared to determine if the drag-tags achieve adequate separation for a multiplexed separation. Electropherograms from all the individual CE separations demonstrate that this combination of drag-tags and LDR product lengths yields a well-resolved multiplexed separation with no polymer-sieving network (Supporting Information Fig. S2).

To evaluate the separation between each of the neighboring peaks, resolution was calculated using the following equation [38]:

$$R = \frac{\sqrt{2 \cdot \ln(2)} \cdot (T_1 - T_2)}{(W_1 + W_2)} \quad (1)$$

where $T$ is the elution time of the peak ($T_1 > T_2$), and $W$ is the full width at half the maximum height (FWHM). When used to determine single-base resolution for sequencing separations, this equation typically has a term in the denominator that is the size difference in bases between the two peaks. As these separations involve four different drag-tags, and this measurement is simply looking for resolution between two neighboring peaks, that term is not included. Resolution more than 0.5 is considered well resolved [38]. Resolution was determined for the 19 FSCE-LDR products separated individually by CE, and all resolution values are significantly more than 0.5 (Table 1).

In addition to separating each fragment individually by CE in buffer (with no polymer network present), one LDR product was produced with a nondrag-tagged discriminating primer. This "negative control" reaction was separated by free-solution electrophoresis to demonstrate the pronounced effect of the drag-tag. Figure 1 shows this effect; the LDR product with no drag-tag (top) coelutes with the common primer due to the free-draining properties of DNA in free-solution electrophoresis. The movement of the LDR product with the drag-tag (bottom) in the electric field is retarded relative to that of the common primer due to the added friction of the essentially uncharged drag-tag. This mirrors the data presented previously for this method, where nondrag-tagged LDR fragments were unable to be detected without a polymer matrix [24].

In addition to separating all 19 LDR products individually by CE, the 19 fragments were produced separately and mixed together such that the multiplexed sample was separated in one capillary. Figure 2A shows the separation of all 19 LDR fragments in one capillary by free-solution electrophoresis. The numbered peak labels in Fig. 2A (and all figures herein) identify the K-*ras* mutant genotype using the corresponding peak order listed in Table 1. The first peak in the electropherogram is excess dye-labeled common primer, and the second

 

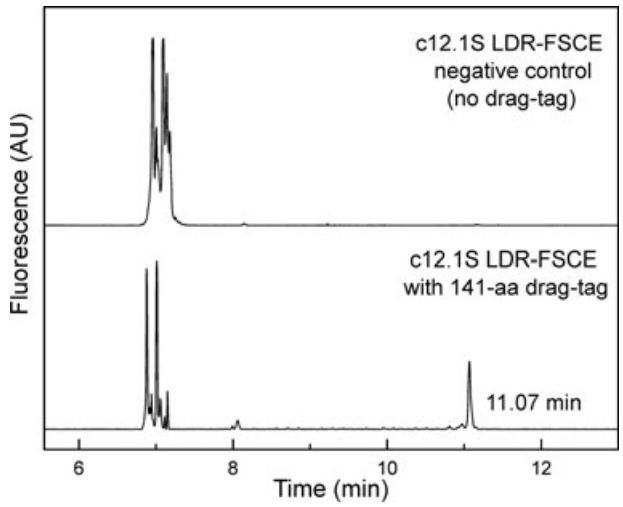


**Figure 1.** CE of LDR-FSCE products for mutant c12.1S. The negative control (no drag-tag conjugated to the discriminating primer) is on top, and the positive control (with drag-tag) is on bottom. No LDR product is detected in the negative control because it coelutes with the excess common primer. Separations were performed at 15 kV (310 V/cm), 55°C, in a 36-cm capillary filled with buffer (1X TTE, 7 M urea).

peak is a system peak that appears consistently in all of the multiplexed separations. This system peak is sufficiently separated from the product peaks and thus does not cause any harm or distraction from genotyping of the K-*ras* mutants by LDR-FSCE.

All 19 peaks are separated with essentially baseline resolution in 13.5 minutes at an electric field strength of $E = 310$ V/cm. Minor noise in the baseline is likely due to the slight polydispersity of the protein drag-tags but does not negatively impact the multiplexed separation. With the exception of a gap between the LDR product peaks labeled "1" and "2", all 19 peaks are approximately evenly spaced, which was enabled by the choice of drag-tags and LDR fragment lengths. Also, the same fluorescent dye was used on all 19 LDR products. By using a single fluorophore, this system can be adapted to any laser by simply changing the dye at the 3′ end of the common primer; a multicolor CCD and a complex set of dyes is not necessary.

The multiplex mixture of LDR fragments was also separated at decreasing electric field strengths by CE to determine if increased resolution would occur if the fragments were separated over a longer time. The results (Supporting Information Fig. S3) show that separation resolution remains fairly constant across the three electric field strengths without trending toward better resolution at lower field strengths. An advantage of FSCE separations is that increased electric field strength is not expected to bring a loss of resolution like traditional separations in a polymer matrix. This will be further tested with the microchip electrophoresis separations where the field strength can be increased above the maximum 310 V/cm of the ABI 3100 CE instrument.

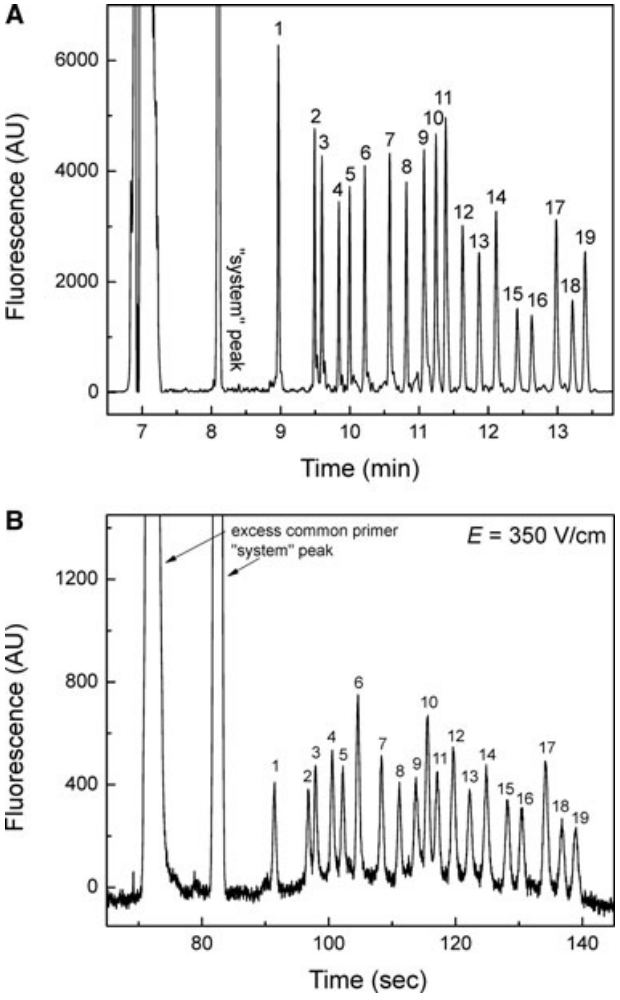


**Figure 2.** Free-solution electrophoretic separations of a mixture of all 19 LDR-FSCE products. (A). CE separation of the 19 LDR-FSCE samples; separation conditions are the same as Fig. 1. (B). Microchip separation of the same 19 LDR-FSCE samples. Separations were performed at 350 V/cm and 55°C on a glass microchip with channels were filled with buffer (1X TTE with 7 M urea).

### 3.2 Microchip electrophoresis separations

Individual separations of each LDR-FSCE product were first performed on chip. The commercially available glass microchips have a separation channel with a total length of 8.5 cm, and a length of $L = 7.2$ cm from injection cross to the detection point. A solid-state 488-nm blue laser was used to excite the fluorescein dye; the emitted light passed through a dichroic filter and onto a CCD for detection. The glass microchips have grid lines near the channel that enable the laser to be positioned at almost exactly the same place for each separation. All individual microchip electrophoresis separations of the LDR-FSCE products were successful. An example in Supporting Information Fig. S4 shows that the genotyping fragment was injected and showed no evident signs of band broadening due to interactions between the protein drag-tag and the glass microchip walls. The glass microchip walls were

 

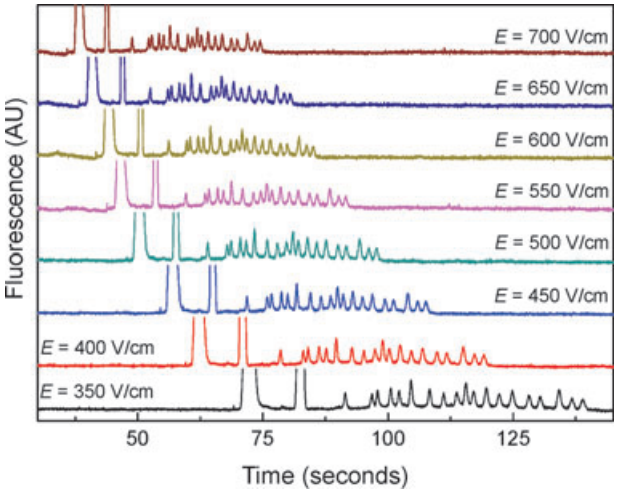


**Figure 3.** Microchip separation of all 19 LDR-FSCE products at increasing electric field strengths ($E = 350$–$700$ V/cm). All 19 peaks are visibly separated at each electric field strength tested. All other separation conditions are the same as in Fig. 2.

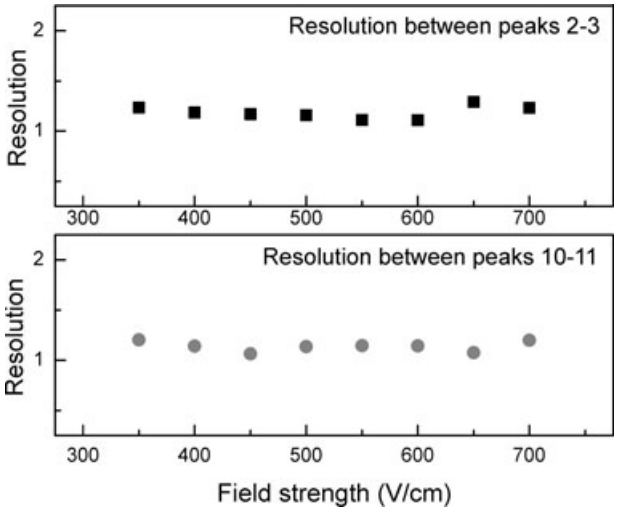


**Figure 4.** Resolution of two pairs of peaks (peaks 2 and 3, peaks 10 and 11) from Fig. 3, plotted versus electric field strength.

dynamically coated with pHEA, using a previously developed method that has been shown to essentially eliminate EOF [24]. The chips were coated with pHEA instead of the POP™ polymer that was used as a dynamic coating for the fused silica capillaries because POP™ does not coat borosilicate glass sufficiently while the pHEA coating has been very successful on borosilicate glass [37, 39].

After confirming that the LDR products could be injected by microchip electrophoresis individually, all 19 LDR-FSCE fragments were mixed together and injected simultaneously on the glass microchip. Initial separations were performed at an electric field strength similar to the capillary separations while the injection protocol was optimized. A multiplexed separation of all 19 LDR products on a glass microchip by electrophoresis in buffer alone (with no polymer matrix) at $E = 350$ V/cm is shown in Fig. 2B. All 19 fragments eluted and were separated in less than 2.5 min, almost six times faster than the same separation performed by CE.

As mentioned previously, FSCE theory predicts that no loss of resolution is expected as electric field strength is increased [33]. Theory predicts that as the field strength increases, peak width decreases faster than the peak spacing. In addition to separating the 19plex mixture of all LDR-FSCE products by microchip electrophoresis at $E = 350$ V/cm, the 19plex mixture was separated at increased electric field strength (controlled by increasing the applied voltage). This is demonstrated in Fig. 3, where the 19plex LDR-FSCE mix is separated at electric field strengths ranging from 350 V/cm up to 700 V/cm (which is the limit of the high voltage power supply used for the microchip experiments). A close-up view of the separation at $E = 700$ V/cm is shown in Supporting Information Fig. S5; when compared to Fig. 2B, the separation looks almost identical with the exception of the different time scale. The fastest separation in Fig. 3 offers very similar resolution in less than 75 s, which is almost an 11-fold decrease in separation time from the CE separation in Fig. 2A. In addition to simply comparing the electropherograms by eye, the resolution was also calculated between two pairs of peaks that elute close to each other (peaks 2–3 and 10–11) for all the separations in Fig. 3. Resolution between these two peak pairs is plotted versus field strength in Fig. 4. The resolution for each of the peak pairs is more or less constant across all the field strengths, which is a drastic improvement over matrix-based separations.

## 4 Concluding remarks

The ability of FSCE to separate DNA genotyping fragments with high efficiency on glass microchips with no polymer matrix was demonstrated using the hybrid LDR-FSCE method of genotyping. Nineteen loci where mutations in the K-*ras* gene are indicative of disease state or response to chemotherapeutics were genotyped by this method. The flexibility of the FSCE method enabled primers to be designed with the minimum number of added bases to achieve adequate resolution between each peak. To achieve simultaneous separation of all 19 LDR genotyping products, long protein polymer drag-tags with significantly larger hydrodynamic friction than the previously used chemically synthesized peptoid drag-tags were required. These large drag-tags were produced with a different maleimide linker moiety that eliminated the noise that was seen in the previous separations due to hydrolysis of the linker molecule [24].

The 19 FSCE-LDR genotyping products were separated individually by capillary and microchip electrophoresis to test the design of the drag-tag-primer pairings. Once adequate separation was ensured, the 19 LDR molecules were combined and separated simultaneously by CE and microchip electrophoresis on a glass microchip with a 7.2-cm separation channel. Separation with near baseline resolution was

 

achieved in both the capillary and microchip separations, and separations in glass microchips decreased the analysis time to be almost 11 times faster than capillary separations. Microchip separations of the mixture of 19 LDR-FSCE fragments were achieved at increased electric field strengths without loss of resolution; separation of all 19 fragments was finished in less than 75 s at $E = 700$ V/cm.

Further work will focus on developing a fully multiplexed LDR with all primers and templates in one tube and then transitioning this method to an integrated microchip where sample processing, thermal cycling, clean-up, and separation are all performed on a "sample-in/answer-out" microfluidic device. The sensitivity of FSCE-LDR in the presence of large amounts of wild-type DNA, as is typical in colorectal cancer samples, has already been shown [7, 9, 24]. This rapid method also utilized only one fluorophore, giving flexibility for the optimal fluorescent dye to be determined by the choice of microfluidic device material or laser. The elimination of the need for a viscous sieving matrix will greatly enable the transition of FSCE-LDR onto integrated devices and into clinical settings.

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