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Revolutionizing genomic analyses: mutation analyses using novel enzyme-based assays with laser-induced fluorescence and polymeric microfluidic devices as electrophoretic platforms

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REVOLUTIONIZING GENOMIC ANALYSES: MUTATION ANALYSES USING NOVEL ENZYME-BASED ASSAYS WITH LASER-INDUCED FLUORESCENCE AND POLYMERIC MICROFLUIDIC DEVICES AS ELECTROPHORETIC PLATFORMS

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I dedicate this work and accomplishment to my parents, Leroy and Delphine Sinville Taylor, and grandparents, Charles and Escille Sinville, and Huey Lee and Alma Ruth Taylor. It has been a long time coming, but we have finally made it.

"If I have seen further it is because I am standing on the shoulders of giants".

- Isaac Newton

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Finally, I thank myself. Make no mistake about it; this was not easy. Thank you, Doctor Rondedrick DeShaun Sinville!

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List of Commonly Used Abbreviations and Acronyms

bp – base pair

CAE – capillary array electrophoresis

CE – capillary electrophoresis

CGE – capillary gel electrophoresis

DNA – deoxyribonucleic acid

dNTP – deoxynucleoside triphosphate

dsDNA, ssDNA – double stranded and single stranded DNA

dt – drag-tag

EOF – electroosmotic flow

ELFSE – end-labeled free solution electrophoresis

Endo V – endonuclease V

FSCE – free solution conjugate electrophoresis

HDA – heteroduplex assay

K-ras – Kirsten rat sarcoma

LDR – ligase detection reaction

LIF – laser-induced fluorescence

LPA – linear polyacrylamide

MHEC – methyl hydroxyethyl cellulose

N - efficiency

PCR – polymerase chain reaction

R – resolution

PMMA – poly (methylmethacrylate)

SNP – single nucleotide polymorphism

SSCP – single-strand conformational polymorphism

TBE – TRIS borate EDTA

TTE – TRIS TAPS EDTA

µTAS – micro total analysis system

(w/v) – (weight per volume)

Abstract

Polymer-based microelectrophoresis was investigated to analyze known (mutation detection) and unknown (mutation scanning) low-abundant mutations in genomic DNA with high diagnostic value for colorectal cancers. For our mutation detection assays, point mutations in the *K-ras* oncogene were identified using the ligase detection reaction (LDR). For the mutation scanning assay, which searches for sporadic mutations, an EndoV-LDR assay was utilized with mutations in the p53 tumor suppressor gene used as a model.

A poly(methylmethacrylate), PMMA, microchip filled with a 4% linear polyacrylamide (LPA) gel was used to electrophoretically sort products formed from LDRs, which produced oligonucleotides <65 bp in length. Using microchip electrophoresis with the LPA, a 44 bp ligation product was resolved from a 100-fold molar excess of unligated primers (25 bp) in approximately 120 s, which was ~17 times faster than conventional capillary gel electrophoresis.

In order to simplify the electrophoretic process and further reduce development time, the LDR products were sorted in the absence of the sieving gel using free solution conjugate electrophoresis (FSCE). FSCE incorporated polyamide “drag-tags” onto LDR primers, which provided DNA fragment mobilities in free solution that were dependent upon their size. LDR/drag-tagged (LDR-dt) products could be formed in a multiplexed format for mutant-to-wild-type ratios as low as 1 to 100 with single base resolution. Separations were conducted using capillary array electrophoresis (CAE) and PMMA microchips filled with only a TRIS buffer. Analysis times for the LDR-dt products were less than 11 min using CAE and ~85 s for PMMA microchips with high reproducible migration times within and between microchips.

PMMA-based microchips were also evaluated for the identification of sporadic mutations using an endonuclease V – LDR (Endo V/LDR) technique. Endo V cleaves heteroduplexed DNA one base 3’ of single-base mismatched sites as well as nicking DNA at some matched sites as LDR reseals miscleaved sites to reduce false positive signals. Results suggested that Endo V/LDR products from p53 mutations could be successfully separated and detected using a PMMA microfluidic chip filled with a sparsely cross-linked replaceable polyacrylamide gel in less than 6 min, which was approximately 10-fold shorter compared to CAE.

Chapter 1: Advances in Genotyping

1.1 Overview of DNA

All of the tremendously diverse biological attributes of life are governed by the mere sequence of a simple, four-alphabet genetic code – that of deoxyribonucleic acid (DNA). Housed in the nuclei of cells in dense bundles known as chromosomes, DNA is a biopolymer containing the genetic instructions that transcribe ribonucleic acid (RNA) molecules, which are in turn translated into functional, regulatory enzymes or highly organized structural components ascending from proteins to organelles to cells. DNA exists in long strands comprised of four unique nucleosides: adenine (A), cytosine (C), guanine (G) and thymine (T) (see Figure 1.1). Each strand is held together by a sugar-phosphate backbone, which gives the macromolecule a net negative charge. Together, a single sugar-phosphate group and nucleoside comprise DNA monomer units known as nucleotides. Through Watson-Crick pairing, complementary DNA strands conform into a double helix via hydrogen bonding between coordinated G/C and A/T bases as shown in Figure 1.2. These weak hydrogen bonds permit two complementary strands

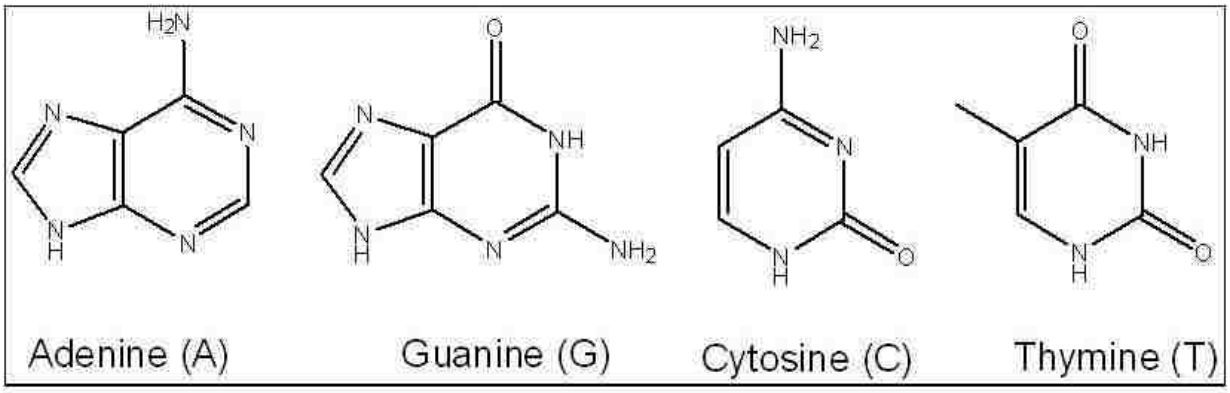


Figure 1.1. The chemical structures of the adenine, guanine, cytosine and thymine DNA nucleosides.

of DNA to easily separate and reestablish pairings under certain physiological conditions, such as the process of mitosis whereby two daughter cells are created with the exact genetic code as the parent cell.

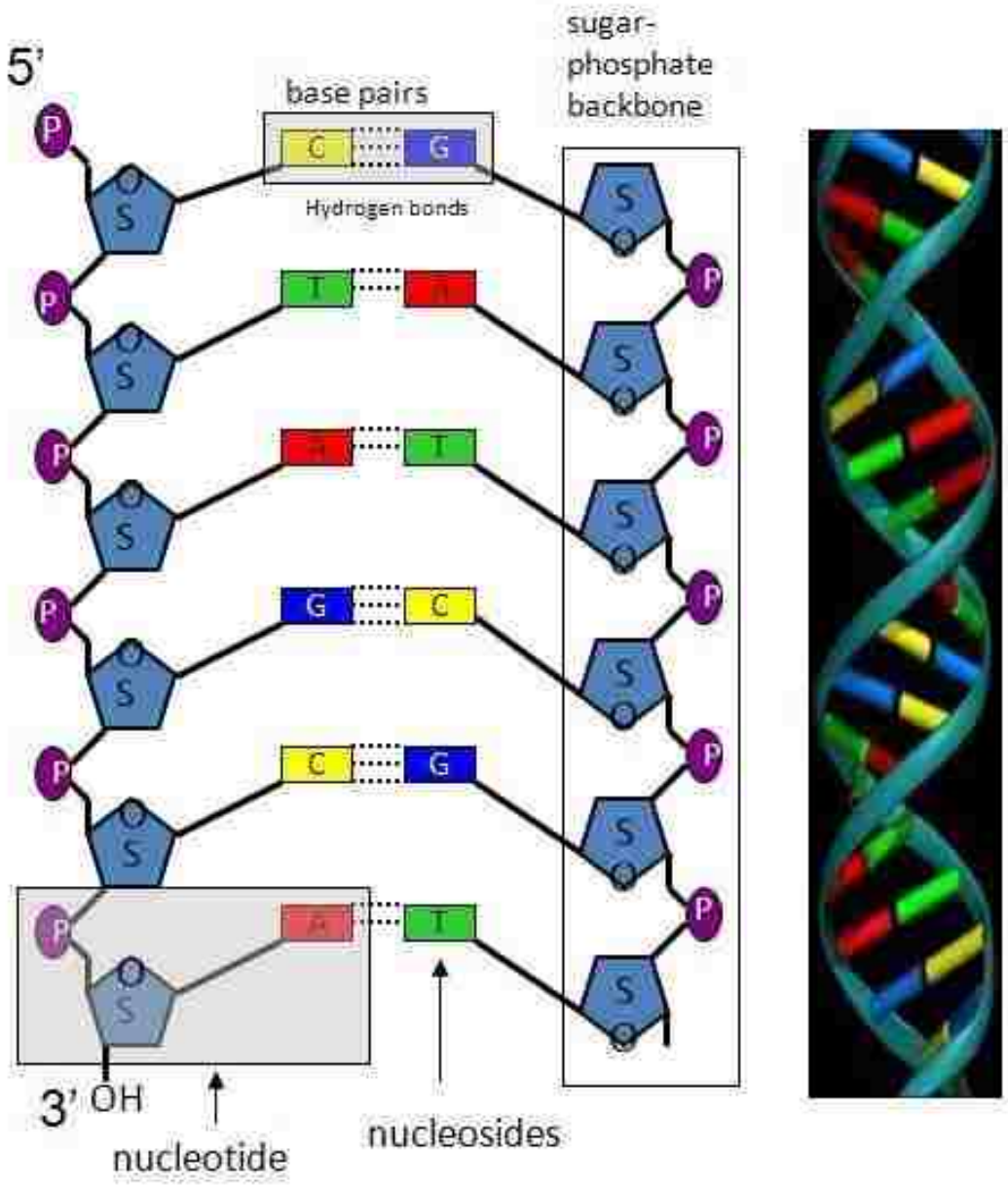


Figure 1.2. An annotated diagram depicting the DNA double helix highlighting its fundamental components. Adapted from http://cnx.org/content/m12382/1.5/ with permission.

1.2 Overview of the Human Genome

In June 2000, some 50 years after the discovery of the DNA double helix, the rough draft sequencing of the approximate three billion bases in the human genome was completed due to the monumental undertaking of the Human Genome Project (HGP). Upon the initial deciphering of the human genome, we now know that our genomes are nearly 99% identical and that they only contain approximately 30,000 – 40,000 genes instead of the hypothesized 100,000 (one gene per protein), leaving about 1% of the human genome that codes for functional components that regulate various cellular machinery and processes. However, this small percentage of the human genome accounts for over 90% of all sequence variations that make each individual unique [1, 2].

While this rough draft of the human genome has already aided biomedical research, much more sequencing detail is needed. In fact, it has been predicted that medical care will one day be tailored to each individual's genomic sequence [3]. Aside from the discovery of the remaining unidentified genes, the challenge that now remains is identifying (genotyping) the subtle differences within various regions of the genome known as polymorphisms that are produced from either environmental or inherited conditions. Upon identification, numerous polymorphisms - base substitutions, deletions, insertions, or frameshifts (see Figure 1.3) - within an active gene-coding region can be used as markers to monitor for the presence of or even susceptibility to developing a given disease. Most polymorphisms are classified as single nucleotide polymorphisms (SNP)s wherein a single base is substituted, which can potentially lead to genetic mutations and thus, the development of deleterious conditions such as sickle cell anemia, muscular dystrophy and many forms of cancer to name just a few. For

instance, muscular dystrophy, a highly debilitating and fatal X chromosome-linked muscle disorder affecting approximately 1 in 3,500 new-born males, has been traced to irregularities in a single gene, dystrophin, measuring 2.4 Mb making it the largest found in humans [4].

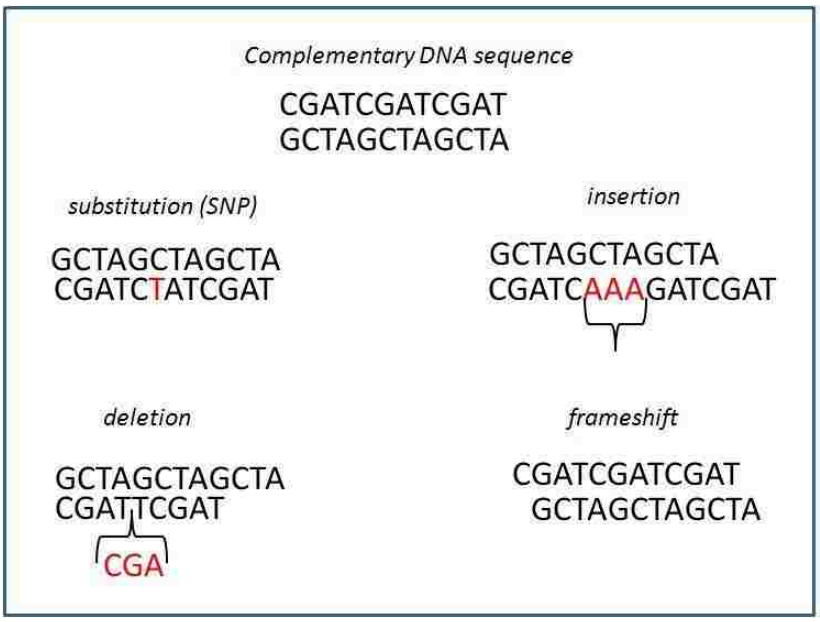


Figure 1.3. Depiction of base substitutions, insertions, deletions and frameshifts comprising the different types of DNA sequence polymorphisms.

Genetic sequencing information also plays a key role in the development of presymptomatic screening assays for the early diagnosis of disease states. Cancers, uncontrolled cell growth initiated by an accumulation of point mutations in functional gene regions promoting tumorigenesis, are of particular interest as the relationship between time of discovery and survival rates has been well documented in the scientific literature [5].

The onset of colorectal cancer, for example, is highly associated with 19 known SNPs within codons 12, 13, and 61 of the *K-ras* gene, which convert the amino acid

glycine to valine in these critical positions, making the gene oncogenic. The ras gene is a signal transduction protein producer (GTPase), which under normal functionality is activated by guanine exchange factors and inactivated by GTPase-activating proteins via GTP hydrolysis of ras back to its GDP form. However, the *K-ras* oncogene continually encodes 21 Kb hydrolysis-resistant GTPase analogs (cell growth signals), thus stimulating growth and differentiation of cells autonomously [6, 7]. On the other hand, tumor suppressor genes, such as the p53 gene, are also highly associated with the onset of colorectal cancer. The p53 tumor suppressor protein is a multi-functional transcription factor that regulates cellular processes affecting proliferation and induces growth arrest and apoptosis via several pathways [8]. These mutations occur sporadically throughout large spans of the gene, mostly in exons 5 – 9 [9]. Therefore, both oncogenic and tumor suppressor mutations must be assessed for accurate diagnosis of the disease.

1.3 Genotyping with Fragment Analysis Methods (More information provided in Chapter 2)

Equipped with vast insight from the HGP including the locations of genes and specific DNA bases within them responsible for the onset of particular disease states, small fragments (~100 – 500 bp) encompassing these regions are typically generated and probed. There is an array of fragment analysis genotyping methods including single-strand conformational polymorphism (SSCP), heteroduplex analysis (HA), minisequencing, short tandem repeats (STR)s, restriction fragment length polymorphisms (RFLP)s, and the ligase detection reaction (LDR) to name a few, all of which will be discussed in great detail in the following chapter. Although sequencing is

still considered the genotyping gold standard, these methods are often used in lieu of sequencing because it is a relatively time-consuming, a laborious process.

Each fragment analysis method has its advantages and disadvantages with respect to simplicity, sensitivity, ease of multiplexing, throughput, and cost. Therefore, the choice of SNP genotyping method varies, depending on the specific needs and resources of each laboratory. Central to most fragment analysis techniques is the utilization of the polymerase chain reaction (PCR), which has the ability to exponentially amplify a single copy of a gene fragment of interest into millions of synthetic copies as shown in Figure 4 [10]. Thereafter, normal and mutagenic DNA fragments are directly deciphered by either conformational differences due to sequence composition (i.e., SSCP and HA) or size differences elucidated via electrophoretic-based separations (i.e., STR). Other methods require subsequent treatments with enzymes that either cleave DNA fragments with endonucleases (e.g., RFLP) or conjoin DNA fragments using ligases (e.g., LDR) and are highly dependent on the ability of such enzymes to recognize and act only upon DNA bases of interest within a sequence. Consequently, thousands of novel enzymes have been discovered, characterized, and utilized in DNA treatments according to their specificity [11].

1.4 Genotyping Technology (More information provided in Chapter 2)

1.4.1 Electrophoresis of DNA

Gel electrophoresis is the method of choice for sorting DNA fragments. In general, electrophoresis is a method used to separate a heterogeneous population of charged particles in a conductive medium under an electric field. Given DNA is a free-draining molecule that has a constant size-to-charge ratio that will not permit size-sorting in free-solution, a porous sieving material must be suspended in the conductive

medium in order to induce frictional drag on DNA molecules [12]. Under such parameters, an applied electrical field results in the electrophoretic migration of DNAs with a frictional force acting in the opposite direction. The electrical force (F_e) is given by the equation:

$$F_e = qE \qquad (1.1),$$

where q is the net charge on the molecule and E is the applied electric field. The frictional force (F_f) is described by the equation:

$$F_f = f\frac{dx}{dt} \qquad (1.2),$$

where f is the translational friction coefficient and dx and dt are the distance and time

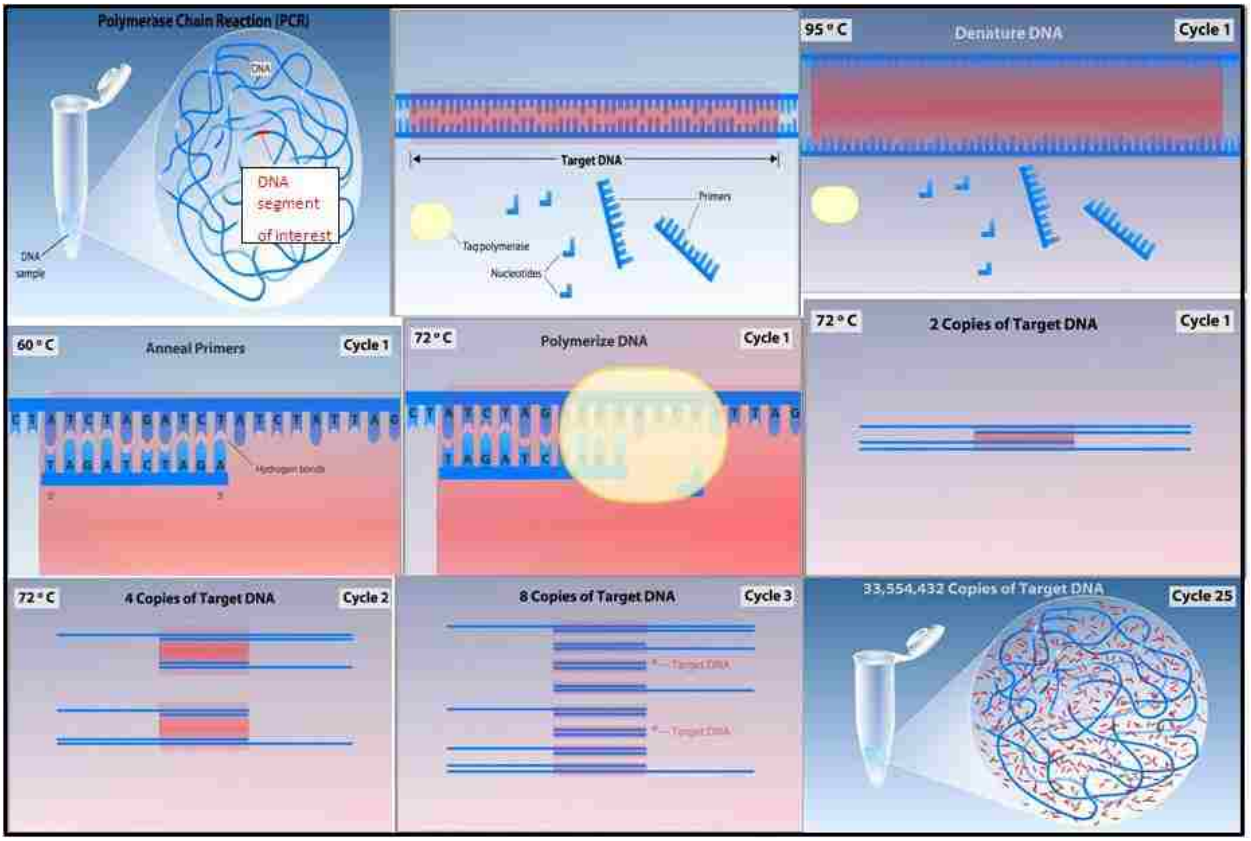


Figure 1.4. Illustration of PCR process used to amplify segments of DNA from http://www.sumanasinc.com/webcontent/animations/content/pcr.html., 2007.

increments, respectively. Under steady-state conditions where F_e and F_f are counterbalanced, DNA migrates with a steady-state velocity (v);

$$v = \frac{Eq}{f} \qquad (1.3).$$

Therefore, the movement of DNA molecules can be quantified in terms of its electrophoretic mobility (μ) from the equation:

$$\mu = \frac{v}{E} \qquad (1.4).$$

Substituting equation (1.3) into (1.4) yields:

$$\mu = \frac{f}{q} \qquad (1.5),$$

which describes the electrophoretic mobility of a DNA molecule in terms of its charge and frictional components. As mentioned previously, DNA is a net negatively charged molecule with a constant size-to-charge ratio. Since q α (N), the number of base pairs in a DNA chain, and each of these units contribute proportionally to the overall frictional drag exerted on the molecule (f α N):

$$\mu = \frac{q}{f} = \frac{N}{N} = N^0 \qquad (1.6).$$

Thus, the electrophoretic mobility of a population of DNA molecules in free solution is uniform, which explains the necessity of porous sieving materials for their electrophoretic separation into well-resolved zones or bands as shown in Figure 1.5.

1.4.2 DNA Sieving Media

It was first reported in 1967 that separation profiles utilizing gels occur because smaller DNA fragments traverse porous media at greater velocities than larger DNA chains, which are subjected to greater friction under an electric field [13]. With gel electrophoresis, the extent to which a mixture of DNAs is resolved depends heavily on the pore size of a gel medium relative to the DNA size distribution. For DNA genotyping

separations, a host of entangled polymer in solutions of varying concentrations (see Table 1.1) or chemically cross-linked networks in solution is commonly utilized. Entangled polymers in solution are ideal for automated capillary array electrophoresis(CAE) systems because they are easily replaced between runs to prevent contamination during successive analyses (see Figure 1.6).

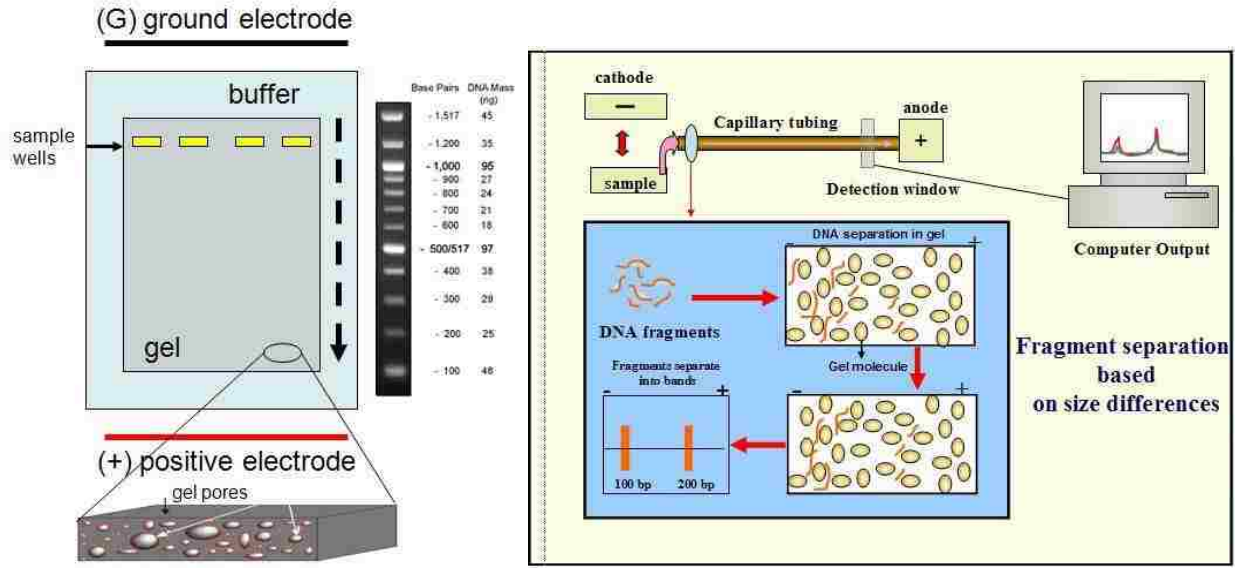


Figure 1.5. An illustration of the use of a porous gel medium to electrophoretically sort a mixed DNA population into bands in a slab gel and capillary gel format. Capillary gel format from www.chnola-research.org/.../capilary.htm, 2004.

Such matrices include poly (ethyleneoxide), PEO, hydroxyethyl cellulose and linear polyacrylamide (LPA) to name a few [14]. LPA and its derivatives having ultra-high molar mass are the most popular sieving material due to their high hydrophilicity and excellent DNA separation ability [15]. On the other hand, non-replaceable cross-linked polyacrylamides in solution are typically used in slab gel formats and have the ability to resolve DNA ranging from 6 to 1,000 bp given the appropriate concentration and degree of cross-linking [16].

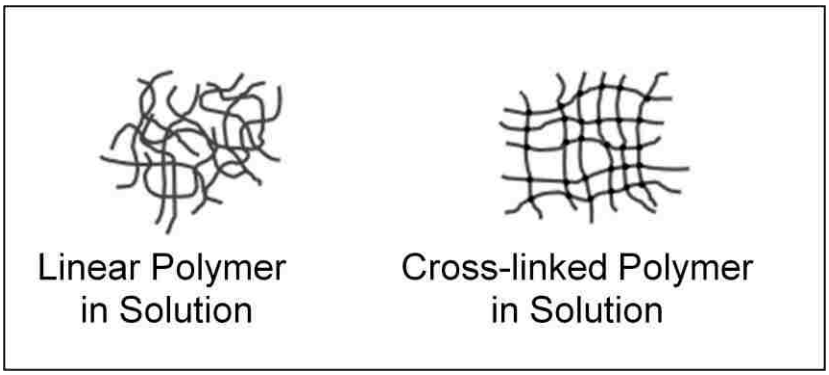


Figure 1.6. Schematic representation of linear and cross-linked polymers in solution.

1.4.3 Dispersion and Band Broadening

The ability to resolve a mixture of solutes into homogeneous zones or bands depends on the length of each band, which in turn is dependent on the dispersion processes that act on them. This dispersion leads to band broadening, which causes migrating bands to overlap and become indistinct from each other. Such dispersion processes include: longitudinal molecular diffusion, thermal or convective diffusion, nonspecific analyte-wall interactions, sample injections and noise presence in the detector. Dispersion can be described as the total variance within the system, σ_{TOT}^2, which is given by the sum of several contributing factors:

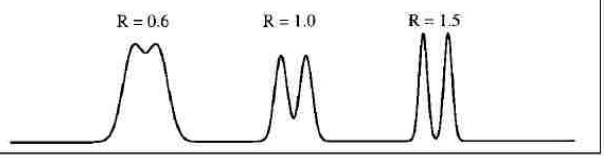
$$\sigma_{TOT}^2 = \sigma_{Dif}^2 + \sigma_{TEMP}^2 + \sigma_{INJ}^2 + \sigma_{ADS}^2 + \sigma_{DET}^2 \qquad (1.7),$$

where the subscripts refer to diffusion, temperature gradients, injection, adsorption and detection, respectively. In an ideal system, longitudinal diffusion is the major contributor to the total zone variance and all other dispersion factors are negligible. For electrophoretic separations of DNA performed with polymer matrices, longitudinal and radial molecular diffusion do not contribute as much to band broadening as in free solution separations because the rate at which DNA molecules diffuse in viscous media (*i.e.*, ~10^{-8} – 10^{-10} cm^2/s) [17] is relatively low compared to the rate of diffusion in free

Table 1.1. Compilation of experimental conditions with replaceable polymers for high performance DNA separations using capillary electrophoresis. Reprinted with permission (see Appendix for references within Table) [14].

Polymer	Ref.	M_{wt}	MHS C^*M_{wt} (w/v)*	Concentration used (w/v)†	Electric Field (V/cm)	Sieving length (cm)	Internal diameter (μm)	Buffer & Denaturants‡	Temp. (C°)	Cap. Coating§	Sample Load Conditions#	$R_{apparent} \cong 0.6$ (bases/min)**	LOR (bases)§§
HEC	12	100,000	1.17%	2.0%	190	41	75	- 1xTBE - 6M Urea - 10% formamide	26	LPA type A	~ 15 sec @ 190 V/cm	~510 bases / ~62 min	500 - 600
LPA	14	339,000	1.49%	6.2%	218	40	50	Buffer: 220ml water, 40 ml methanol, 5.6 g Tris titrated with 85% Phosphoric acid Denaturant: 8M Urea	—	LPA type B	~ 5 sec @ 109 V/cm	~370 bases / ~81 min	450
LPA	39	1,000,000	0.63%	8.0%	100	12	100	- 1xTBE - 3.5M Urea - 30% formamide	Rm.	LPA type A	~ 20 sec @ 154 V/cm	~375 bases / ~84 min	—
LPA	13	≥ 5,500,000	0.16%	2.0%	150	30	100	- 50 mM Tris - 50 mM Taps - 2 mM EDTA - 7M Urea	50	PVA	~ 15 sec @ 200 V/cm	~685 bases / ~61 min	—
LPA	9	—	—	5.0%	150	39	50	- 1xTBE - 7.0M Urea	60	LPA type A	~ 40 sec @ 100 V/cm	~550 bases / ~106 min	—
LPA	10	—	—	9.0%	100	40	75	- 0.1M TBE - 7.0M Urea	Rm.	LPA type A	~ 40 sec @ 213 V/cm	~520 bases / ~430 min††	—
DMPA	8	100,000	0.96%	6.5%	200	50	50	- 100mM TAPS - 8M Urea - pH = 8	45	Self Coat	~ 40 sec @ 50 V/cm	~600 bases / ~130 min‡‡	650
PEO	11	600,000 + 8,000,000	0.6% with 600,000 & 0.08% with 8,000,000	1.4% 600,000 + 1.5% 8,000,000	267	35	75	- 1xTBE - 3.5M Urea	Rm.	Self Coat	~ 12 sec @ 133 V/cm	—	—

Note: $R_{apparent}$ was determined by the author's observation of where adjacent DNA peaks (of the same base type) in an electropherogram consistently appeared having a resolution, R, equal to 0.6. Gaussian peaks representing resolution equal to 0.6, 1.0, and 1.5 are shown to the right. The relationship, $R = \Delta t/\bar{w}$, was recast as $R = \Delta t/(1.6986*fwhm)$ assuming the Gaussian's average base width, $\bar{w}$, is measured when the peak has diminished to $1/e^2$ of it's original value ($R \cong \Delta t/4\sigma$). Fwhm corresponds to the full width half maximum of the Gaussian, and Δt, the peak spacing.



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solution (*i.e.*, ~10^{-7} cm^2/s) [18]. As a result, higher efficiencies are achieved with gel electrophoresis.

1.4.3.1 Electroosmotic Flow

Electroosmotic flow (EOF) is the bulk flow of a solution towards a cathode or anode upon voltage application due to the formation of an electric double layer that develops at the capillary surface/solution interface. This phenomenon occurs because a plane of shear between the fixed and mobile layers creates a potential difference between the layers known as the zeta potential, ζ, which is determined by the charge of

a conduit wall. This phenomenon is dependent upon the surface charge of a conduit as well as the pH and the ionic strength of buffered solutions. EOF can be expressed in terms of mobility using the equation:

$$\mu_{eof} = \frac{\varepsilon\zeta}{\eta} E \qquad (1.8),$$

where ε is the dielectric constant. The electrophoretic mobility of a solute in the presence of the EOF is termed the apparent mobility (μ_{app}), which is expressed as:

$$\mu_{app} = \mu_{eof} - \mu_e \qquad (1.9).$$

Both electroosmotic and electrophoretic flow within a microchannel have a flat profile as shown in Figure 1.7, which is beneficial for band efficiencies. However, EOF is undesirable when performing DNA separations in capillaries because its mobility is toward the cathode, opposing the electrophoretic mobility of negatively charged DNA, which is toward the anode. Moreover, highly charged surfaces that lead to EOF also result in unwanted analyte-wall interactions. Thus, surface modifications are administered to suppress EOF and analyte-wall interactions.

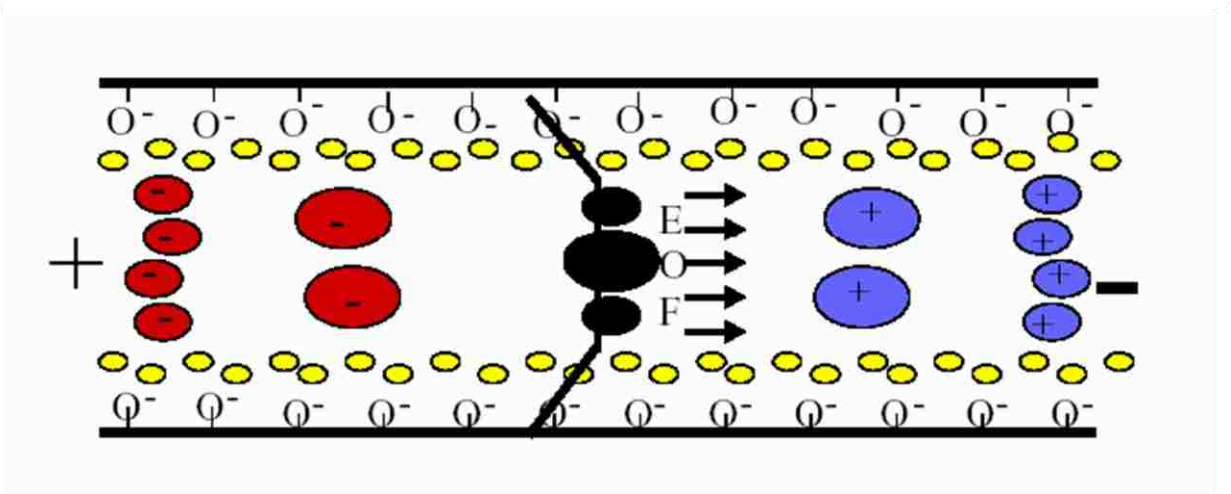

Figure 1.7. Plug flow profile and EOF in a capillary tube during electrophoresis adapted from http://micromachine.stanford.edu/~dlaser/images/eof_capillary.jpg, 2006.

1.4.3.2 Surface Modification for Suppression of EOF

To reduce surface charge influences on the separations, the EOF in capillaries must be either significantly reduced or eliminated. This is accomplished by coating channel surfaces with polymeric materials. These coatings can either be static through means of covalent attachment of the coating materials to the surface, or dynamic, whereby a coating is adsorbed onto the wall. Both methods provide a homogeneous surface to reduce dispersion effects, which is especially important as DNA separations are dramatically affected by inhomogeneities in surface charges [19].

One of the earliest reports concerning chemical modifications of capillary surfaces is the Hjerten coating method. This modification involves the coating of channel surfaces with a non-cross-linked polyacrylamide to eliminate the EOF in silica capillaries through attachment of a thin layer of polyacrylamide groups by reacting the SiO- groups with γ methacryloxypropyltrimethoxysilane (γ-MAPS) prior to polymerization [20].

Dynamic coatings are advantageous because they do not require time-consuming chemical modifications. Passivation is typically achieved by either pre-rinsing the microchannel with a solution containing the adsorptive polymer or additive, or by including the coating agents in the run buffer or separation medium. Dynamic coating with self-coating sieving matrices such as poly(dimethylacrylamide) (PDMA) and polyvinylpyrrolidone (PVP) are attractive since the coating is regenerated with each refilling of the microchannel, which increases the reproducible of separations over several analyses [21, 22].

1.4.4 Characterization of Electropherograms

Upon electrophoretic separations, electropherograms reveal bands referred to as peaks, whose dispersion can be measured by evaluating the width of the peak at its base, w_b. For a Gaussian peak,

$$w_b = 4\sigma \quad (1.10),$$

where σ is the standard deviation of the peak. From this expression, efficiency, N, can then be expressed as theoretical plates by:

$$N = \left(\frac{l}{\sigma}\right)^2 \quad (1.11),$$

where l is the effective length of the capillary (cm). Also, efficiency can be directly related to the molecular diffusion by:

$$\sigma_{TOT}^2 = 2Dt = \frac{2DlL}{\mu_e V} \quad (1.12),$$

where D is the diffusion coefficient of the molecule, L is the total length of the capillary (cm) and t is time. Substituting 1.12 into 1.11 yields the fundamental electrophoretic expression for plate number:

$$N = \frac{\mu_e V l}{2DL} = \frac{u_e E l}{2D} \quad (1.13).$$

Efficiency can also be calculated directly from peak parameters in an electropherograms as shown in Figure 1.8 with the following expression:

$$N = 16\left(\frac{t_m}{w_b}\right)^2 \quad (1.14),$$

where t_m is the migration time of the peak (see Figure 1.8). The resulting value is then normalized to an effective column length of one meter (plates/m). For gel electrophoresis, the major contributor to band broadening for a well-designed system is

longitudinal diffusion of the solute molecules. In this instance, N can be directly related to the molecular diffusion by:

$$N = \mu \frac{El}{2D} \qquad (1.15).$$

Resolution is the degree of separation between the target molecule(s) and other molecules within a sample. It is the result of two factors: selectivity (the relative difference in the retention of two peaks) and efficiency. Resolution is given by the expression:

$$R = 2\frac{\left(t_{m2} - t_{m1}\right)}{w_{b2} + w_{b1}} \qquad (1.16),$$

the difference in migration times divided by the average width of the two peaks at the base. Resolution can also be calculated in terms of efficiency and mobility with the expression:

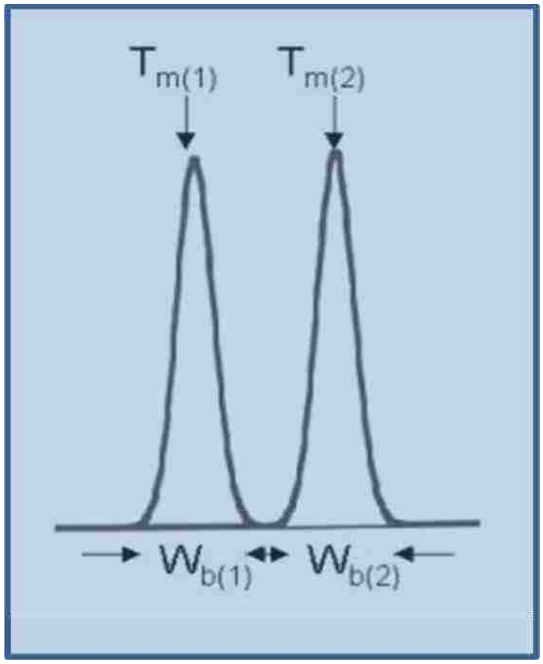

Figure 1.8. Adjacent peaks in an electropherogram marking migration times and peak widths from which efficiency and resolution can be calculated adapted from [23].

$$R = \frac{1}{4}\frac{\Delta\,\mu_{app}}{\mu_{app,avg}}N^{\frac{1}{2}} \qquad (1.17),$$

where $\mu_{app,avg}$ is the mean mobility of two neighboring components.

1.5 Evolution of Electrophoretic Genotyping Platforms

Driven by the enormous demand for electrophoretic systems with high throughput capabilities, automated CAE systems were devised to replace cumbersome slab gel systems. Due to a capillary's ability to dissipate heat, higher electrical fields can be sustained, and thus, faster separations can be performed. Though these commercial instruments remain effective, they are still relatively expensive to operate and slow considering the ever expanding post-HGP genotyping demands including disease, forensic and ongoing genome sequencing studies [24]. Meanwhile, miniaturized microfluidic platforms have gained traction as electrophoretic devices with the potential to satisfy these growing genotyping needs [24]. Just as CAE systems improved upon slab gel systems for DNA sorting in terms of reduction in sample consumption and speed of analysis, microfluidics have improved upon these same factors of CAE systems by orders of magnitude (see Table 1.2). In addition, this

Table 1.2. Comparison of typical parameters associated with the given electrophoretic formats.

	Slab Gel	Capillary	Microchip
Analysis Time	2 – 8 h	1 – 2 h	0.5 – 10 min
Field Strengths	50 – 80 V/cm	100 – 300 V/cm	100 – 300 V/cm
Sample Load Vol.	1 – 10 μL	1 – 10 nL	10 – 100 pL

burgeoning miniaturized (microchip) platform is even more attractive as front-end sample processing (*e.g.,* PCR cycling), back-in signal read-out and electrophoretic sorting abilities can be combined onto a single wafer creating a micro-total analysis system (μTAS), also referred to as a lab-on-a-chip [25].

1.5.1 Microfluidic Devices (Detailed microchip genotyping applications provided in Chapter 2)

Microfluidic devices, commonly referred to as microchips, were first used as electrophoretic platforms in the early 1990s and they were fabricated with glass and silicon substrates because their surface properties and optical clarity were essentially identical to capillary tubing [26]. Recently, polymeric (plastic) substrates having manageable surface chemistries and adequate clarity have gained favor due to their relative low cost and ease of machining, which further enhances the outlook for the aforementioned development of μTAS units [27]. Replicates of plastic microchips can be mass-produced from a metal mold master containing raised micro-features

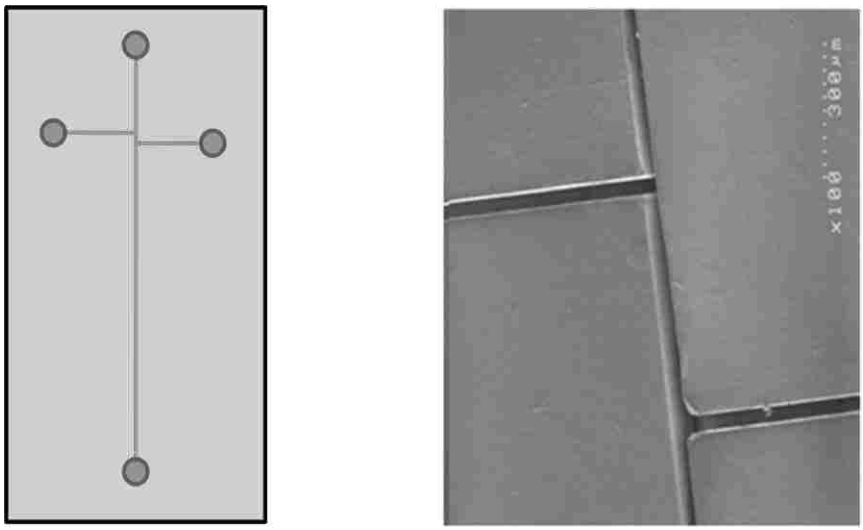

Figure 1.9. (Left) Schematic of a “twin T” microchip typically used for electrophoresis. (Right) A x100 magnified image of microstructures hot-embossed in a plastic substrate.

(channels) by injection molding or hot-embossing into an amorphous substrate (see Figure 1.9) [28].

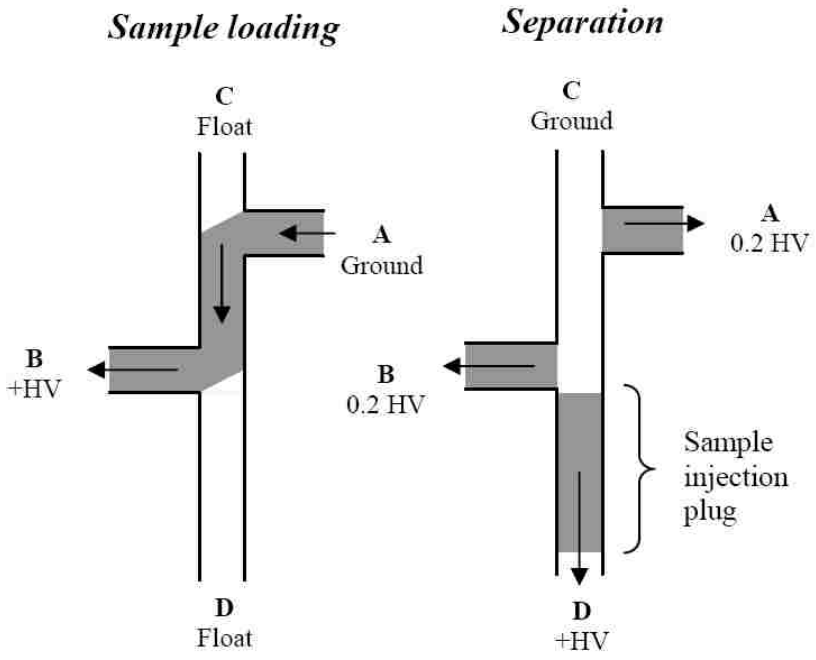

Figure 1.10. Microchip sample loading and corresponding voltage applications through a twin T injection zone and the initial separation of the volume-defined sample plug.

Electrophoretic separations carried out on microfluidic devices are similar in manner to those performed using a capillary. The major operational difference lies in the sample plug introduction prior to separations. Capillary injections involve placing a tube in a sample vial and electrokinetically pumping sample into it over some given period of time, and then returning the capillary to a system buffer vial to perform separations. Alternatively, microchip injections are performed by electrophoretically attracting a sample placed in an on-chip reservoir across a separation channel, at which point the sample volume within intersecting zone – the sample plug – is simultaneously electrophoresed as the residual sample is drawn back into the sample reservoir (see Figure 1.10). Unlike capillary injections, microchip injections do not suffer electrokinetic

injection biases whereby smaller charged species are loaded onto a conduit in larger quantities than larger, slower moving charged species, thus leading to unrepresentative sample loading, particularly for DNA samples [29].

1.6 Research Focus

In the following chapters of this dissertation, the research efforts conducted to advance the development of genetic analysis methods on polymer microchips will be described. The focus of this work was the adaptation and transition of DNA mutation product separations currently limited to CAE system processing onto inexpensive polymer microchips manufactured by our research group. These separation adaptations included two novel enzyme-based genotyping methods having high fidelity for correctly identifying known and unknown mutations present in low abundances; the ligase detection reaction (LDR) and endonuclease V – ligase detection reaction (EndoV/LDR) assays, respectively. The purpose of this work was to set the stage in part for the future completion of disposable μTAS units suitable for clinical genetic analyses.

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Chapter 2: High Resolution DNA Separations Using Microchip Electrophoresis for Genotyping Applications*

2.1 Introduction

The completion of the Human Genome Project (HGP) approximately 50 years after the discovery of the DNA double helix has unveiled a multitude of genetic information, which will and has begun to shape future directions in biomedical research and others as well. For instance, we now know that the sequences of human genomes are nearly 99% identical and that our genome contains only approximately 30,000 – 40,000 genes instead of the hypothesized 100,000 (one gene per protein), leaving about 1% of the human genome that codes for functional components that regulate various cellular machinery and processes. However, this small percentage of the human genome accounts for over 90% of all sequence variations that make each individual unique [1, 2]. Aside from the discovery of the remaining unidentified genes, the challenge that now remains is identifying (genotyping) the subtle differences within various regions of the genome known as polymorphisms that are produced from either environmental or inherited conditions and can be linked to a number of diseases. Most polymorphisms are classified as single nucleotide polymorphisms (SNPs) in which a single base is substituted, which can potentially lead to genetic mutations and thus, the development of deleterious conditions such as sickle cell anemia, muscular dystrophy and many forms of cancer to name just a few.

The undertaking of unraveling the primary structure of our genome (DNA sequencing) or looking for unique sequence structural differences within the genome (DNA genotyping) has driven the advancement of DNA-based electrophoretic technologies significantly beyond the performance metrics that could be realized using slab gels, which were the dominate electrophoresis platform used up until the late

* Reprinted with permission from the *Journal of Separation Sciences*. [22]

1990s. In order to relieve the labor intensive demands associated with slab gels, automated high throughput formats of gel electrophoresis were required to meet the demands predicated by the HGP. This was satisfied to a certain extent with the development of capillary array electrophoresis (CAE).

To further reduce the time and overall cost of DNA analysis, planar microfluidic devices have been projected as a viable alternative electrophoretic platform. This has lead to the production of a few commercial instruments using glass microchips instead of capillaries or slab gels, which have been discussed elsewhere in detail [4]. In Table 2.1 is provided a basic operational and performance comparison between the 3 electrophoretic platforms typically used in DNA analysis namely, slab gel, CAE and microchip electrophoresis (microchip CE).

Table 2.1. Performance and operational comparison between three different electrophoresis platforms used for DNA sequencing.

	Number Of Lanes	Injection Volume (nL)	Analysis Time (h)	Avg. Read-length (bp)	Field Strength* (V/cm)	Through-put (bases/ per 8 h)	Gel Pouring	Lane Tracking
Slab[a] *gel*	96	500 - 1,000	6 -8	700[a]	20-50	67,200	Yes	Yes
Capillary	96[b]	1-5	1-3	650[b]	100-300	~166,400[b]	No	No
Microchip	96[c]	0.1-0.5	0.1-0.5	430[c]	100-300	~660,480[c]	No	No

a) ABI 377A [73].
b) based on ABI 3730xl using standard sequencing conditions [74].
c) based on research from [65].

As seen from the data presented in Table 2.1, CAE and microchip CE offer some attractive capabilities, such as the elimination of gel pouring and lane tracking due to the boundary conditions imposed on the separations by the walls defining the separation channel. In addition, the small diameter of the separation columns utilized in these micro-separation platforms permit the use of higher electric field strengths to

significantly reduce electrophoretic development time. As a result of the performance capabilities associated with these micro-column separation platforms, they can improve throughput compared to slab gel electrophoresis by nearly 3-fold for the CAE format, but by nearly an order of magnitude for microchip CE. The challenge with the micro-column separation techniques is that they require load volumes that are 3 – 4 orders of magnitude smaller than slab gel electrophoresis and as such, place severe demands on detection and/or sample pre-processing prior to the separation to build sufficient concentrations of the targets for detection.

In this post-genomic era, DNA separation-based technologies are shifting from primarily a research tool into a more diffuse user pool targeting such as clinical diagnostics, forensic applications, and small-scale sequencing of individual genomes for personalized medicine initiatives. To realize this transition, DNA-based analysis systems must attain certain performance characteristics such as: (1) *Development of low cost instrumentation that can be easily operated by novice users.* Typically, the equipment required for processing DNA samples is expensive, difficult to operate and maintain and performs only one step of the many steps that are required for DNA processing. For example, many of the CAE-based electrophoretic instruments require expensive laser systems for reading fluorescence from the capillary tube(s) in a finish line format, gel sieving matrices that are expensive that must be changed after every single electrophoretic run and finally, perform only the separation step. DNA samples typically require significant amounts of pre-processing prior to the electrophoresis such as purification, amplification via PCR, and labeling of the generated DNA fragments. Unfortunately, different instruments are required for each step of the processing pipeline, which significantly increases the capitol equipment costs for performing genetic

assays as well as demanding high levels of operator expertise. (2) *Automation of the entire DNA processing pipeline preferably into a single instrument.* As noted above, the analysis of DNA typically requires many processing steps, with each step dedicated to a different instrument. This requires manual handling and transfer of samples from instrument-to-instrument, which leads to contamination, sample loss and potential errors in reporting answers from assays in which the copy number of the input DNA is low. (3) *Disposable fluid handling devices that can be mass-produced at low-cost.* The containers or reactor vials for the sample must be discarded after a single use to prevent sample carryover from one assay to the next potentially giving rise to false negatives, which is critically important in such areas as DNA diagnostics and DNA forensics. At the present time, titer plates are used for sample containment, which are then poised on instruments that carry out the required active processing such as thermal cycling reactions. In the case of the electrophoretic sorting step, the processed samples (typically 96 – 384) are either pipetted into a well of the slab gel or electrokinetically injected into an array of glass capillaries. The slab gel is used once and discarded while capillaries are flushed after a single use and filled with fresh sieving matrix.

Microfluidic chips hold the potential to allow expansion of DNA-based assays into a broader application base due to their ability to directly address the 3 issues cited above. For example, microfluidic chips can be constructed from moldable polymer (plastic) materials to fabricate monolithic devices that are not only potentially low-cost, but can be populated with many of the processing steps required for DNA analyses on the same chip used to carry out the electrophoretic separation. This can create

functional platforms that provide high levels of automation at low cost and reduce the number of peripherals (*i.e*., instruments) built around the intended application.

Early microfluidic efforts were focused on developing devices specifically for the electrophoretic step of DNA analyses and used predominately glass-based devices. This was spawned by glass' high optical clarity, which accommodated nicely to optical fluorescence readout, and surface properties that were similar to that of capillaries, which allowed simple transitioning of many of the surface chemistries from capillary-based instruments to microchips.

In the past few years, many polymeric materials have been extensively evaluated for their potential use as micro-electrophoretic devices and several have been found to possess favorable qualities [5]. Besides the lower cost of the material, the manufacturing methods now available to produce plastic microfluidics allow for much faster production and at lower cost to meet the anticipated demands imposed by the clinical and forensic markets. However, it should be noted that many of the established surface chemistries that have functioned well for glass or fused silica, such as the dynamic coatings used to suppress the electro-osmotic flow (EOF) or minimize solute-wall interactions, do not transition well to polymer-based devices due to profound differences in surface chemistry. In addition, the optical clarity of many polymers does not compare favorably to that of glass or fused silica, especially in the UV and/or visible region of the electromagnetic spectrum [4].

In this review, we will focus specifically on recent progress made in the area of microfluidic chips for electrophoretic-based genomic analyses including genotyping, DNA sequencing, and forensic applications. For reviews that cover a variety of different biological applications of microchips we would refer the reader to [6-10]. In addition, for

reviews that have focused on the integration of various DNA pre-processing steps into the micro-separation chip, several reviews have appeared in which the reader can refer [11, 12]. The areas that we will discuss include genotyping applications (mutation scanning and mutation detection) for diagnostics, DNA forensics and finally, DNA sequencing.

2.2 Genotyping: Mutation Scanning Applications

Mutational scanning strategies are used to search for sequence polymorphisms in which the locus or location of the polymorphism is not known or the sequence variation occurs sporadically within a certain section of the genome. Common scanning techniques that are used in conjunction with electrophoresis to type DNA include single-strand conformation polymorphism (SSCP), heteroduplex analysis (HDA), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE). These methods are based on conformationally-induced mobility shifts of Watson-Crick matched or mismatched single or double-stranded DNA (dsDNA) fragments and represent an alternative to DNA sequencing, which is still impractical for routine clinical uses due to its relatively high cost, labor-intensive processing and slow turnaround time. Moreover, scanning methods have the ability to score DNA regions containing large insertions and/or deletions. Genomic interrogations of this sort are typically used for the detection of unknown SNPs within PCR amplified regions; however, vital information regarding the exact sequence location and characterization of SNPs are not rendered using most mutation scanning assays. With the exception of DGGE, all of the methods described above have been adapted to a microfluidic format.

2.2.1 SSCP

SSCP is widely applied for DNA mutation scanning due to its relative simplicity as only PCR amplification and subsequent denaturation of the double-stranded amplicons to generate single-stranded DNA is required for sample preparation prior to the electrophoretic sorting. Single-stranded DNA fragments, under the appropriate experimental conditions, possess sequence-specific 3-dimensional conformations resulting from intra-strand base pairing. Heterozygous alleles have the exact same size as homozygotes, but possess one or more altered bases at loci that can be scored through conformationally-induced perturbations to the electrophoretic mobility. Samples subjected to mutation scanning surveys using SSCP, and all mutation scanning assays for that matter, typically include the mutagenic DNA as well as their known wild-type counterparts from a PCR-defined region. As such, the electrophoretic technique must be able to afford high resolution capabilities to observe the presence of the mutation in a high level of background from “normal” or wild-type DNA sequences.

Closely following the formats adopted for SSCP analysis using slab and capillary gel electrophoresis (CGE), researchers have established microfluidic separation methods for SSCP. For example, the first published research exploring SSCP on a microchip was by Tian *et al.* who displayed the usefulness of this platform to distinguish three common mutations in BRCA1 and BRCA2 genes (185delAG, 5382insC, and 6174delT) among the Ashkenazi Jewish population, which possess high risk for breast cancer. Their glass microchip profiling methodology, which paralleled a CGE method that was also presented in the report, used 2.5% hydroxyethylcellulose (HEC) containing 10% glycerol as a sieving matrix, which discriminated all mutant alleles from their wild-type counterparts with a four-fold reduction in analysis time (~130 s)

compared to the CGE separation [13]. However, they noted a substantial decrease in resolution in the microchip SSCP profiles and attributed it to possible inadequate surface passivation of the microchip using polyvinylpyrrolidone (PVP) as the dynamic coating for EOF suppression and a lack of adequate temperature control in the microchip format.

As with the case of SSCP analysis employing capillaries, the most scrutinized aspect of microchip SSCP separations remains the selection of appropriate separation parameters to impart large mobility differences for subtle sequence variations such as the type of sieving matrix, stability of the additive, wall coatings, temperature, and field strength. As such, parametric studies placing emphasis on these parameters have recently emerged. For example, Endo and co-workers presented a systematic optimization of SSCP separation conditions using a glass microchip, which they ultimately used to probe for a set of seven p53 mutations. Evaluating a wide array of sieving and glycerol concentrations, they found 1.5% methylcellulose excluding glycerol to be the most effective sieving matrix as the two commercial systems used (Agilent 2100 Bioanalyzer and Hitachi SV1100) did not possess microchip cooling capabilities to attenuate the Joule heating produced when glycerol was present [14]. Using the optimized set of sorting parameters, the authors were able to analyze the full panel of markers in under 1 min. Kang *et al*. used another sieving combination (1.75% PVP, 1.0% poly(ethyleneoxide), 5% glycerol including 0.01 - 0.025% NaOH) to enhance selectivity of SSCP profiles for ghrelin (childhood obesity-related) Leu72Met point mutations using commercial glass microchip-based instruments [15]. They were able to resolve the full panel of markers using the aforementioned microchip conditions in under 85 s. A high-throughput SSCP analysis of genes associated with hereditary

hemochromatosis (metabolic iron storage disorder) and hypertrophic cardiomyopathy (heart muscle abnormality) has also been demonstrated using a 48-lane section of a 384-lane glass-based microdevice, which yielded 100% selectivity for the 21 mutations being scanned [16]. The 200 mm diameter high density microchip contained 8.0 cm length straight channels that were radially-configured about a centered common anode. In this study, they performed separations in their preferred sieving material (5.0% polydimethylacrylamide, PDMA, 10% glycerol, 15% urea) at two running temperatures (25ºC and 40ºC) to realize full separation of the marker panel. The authors also noted that using a delayed back biased electrokinetic injection scheme improved sample loading resulting in higher plate numbers and improved resolution.

2.2.2 HDA

HDA is an electrophoretic mutation scanning method that is based on relative conformational changes between heteroduplexed and homoduplexed PCR amplified DNA fragments. This method differs from SSCP because it relies on differences in conformational structures of nearly complementary (heteroduplexed) and completely complementary (homoduplexed) double-stranded DNA (dsDNA) fragments instead of single-stranded intra-molecular folding as is used in SSCP. Formed heteroduplexes contain bulges within the rehybridized DNA fragments where bases are mis-matched and are typically accompanied by an abundance of homoduplexes that possess hybridions that are fully matched (*i.e.*, no bulges). When electrophoresing a mixture of homoduplexes and heteroduplexes using the appropriate separation parameters, slight mobility shifts between these species are often observed. This method is often used for DNA species containing insertions and deletions, which typically yield more pronounced

mobility shifts due to significant bulges resulting from mismatched base pairs in duplexed DNA.

Tian *et al.* first brought attention to the potential of microchip HDA separations for differentiating six different heterozygous breast cancer associated mutations (185delAG, E1250X (3867GT), R1443G (4446CG), 5382insC, 5677insA and 6174delT) within BRCA1 and BRCA2 genes. This was one of the first reports of HDA separations in which the microchip CE method was directly compared to CGE formats [17]. The authors found a four- to six-fold reduction in analysis time for the microchip trials using the same buffer and sieving reagents as that used for CGE (1xTBE buffer with 15% urea, 2.5% and 4.5% HEC with 10% glycerol). The reduced analysis time was associated with the use of an effective separation distance for the microchip (5.5 cm) that was shorter than that used for the CGE format. However, this separation channel length was insufficient to provide the same level of resolution incurred with the capillary columns.

Another microchip-HDA study involving the detection of five BRCA1 and BRCA2 mutations was conducted and directly compared to a denaturing (d)-HPLC method [18]. These authors found that while the glass microchip separations could not approach the remarkable selectivity provided by (d)-HPLC, the microchip method could provide more information on different mutations within the same amplicons. Moreover, the authors emphasized the microchip's operational advantages including simplicity of the separation protocol, versatility and lack of sample preparation constraints.

Due to the similarities in the electrophoretic conditions used for HDA and SSCP, separations of various DNA species can be performed using both HDA and SSCP simultaneously. The complementarity of the two is often advantageous in circumstances

when the selectivity of one is inadequate to score the presence of the full panel of potential mutations. Therefore, most adaptations of these techniques on microfluidics have been performed in a tandem format. The first microchip-based HDA-SSCP report of this type was given by Vahedi *et al.*, who described an integrated on-chip labeling of HFE and BRCA1 amplicons with fluorescent dyes prior to separation of the complementary species using a commercial polymer [19]. A similar integrated microchip-based HDA/SSCP approach by Manage *et al.* was reported with the exception of the on-chip labeling scheme as noted for the Vahedi *et al.* report [20]. The authors detailed the profiling of three specific HFE mutations having high clinical relevance (C282Y, H63D, and S65C) for hemochromatosis. The separation required a 4 min electrophoresis development time using microchip CE. An exhaustive study to elucidate the optimal parameters for glass microchip CE analyses using a combined HDA/SSCP method was conducted by Hestekin and co-authors [21]. They found that a combination of 8% w/v 600 kDa LPA and a dynamic wall-coating of poly(N-hydroxyethylacrylamide) as opposed to a wall coating using covalent methods provided the best performance for p53 conformer separations, which could be completed in less than 10 min of electrophoresis development time.

Diverging from the use of a combined HDA and SSCP approach, an integrated on-chip HDA/restriction fragment length polymorphism (RFLP) methodology was demonstrated. Footz *et al.* demonstrated the discrimination of both heterozygous and homozygous mutations present in the HFE gene using microchip electrophoresis, which the HDA method alone was incapable of detecting [22].

To date, all HDA-related assays have been conducted on glass-based microchips. For clinically-based assays that require one-time or disposable use

devices, transitioning these mutation-scanning electrophoresis assays to polymer-based chips will be critical, since polymer devices can be produced at lower cost in higher scale production modes compared to glass chips.

2.2.3 TGGE and DGGE

TGGE and DGGE are two analogous techniques used to discriminate heteroduplexes containing mismatched base pairs from fully matched homoduplexes by continuously altering the temperature or chemical denaturant conditions in a gradient format during the migration of solutes through the separation channel, which affects the degree of dsDNA conformational stability throughout the separation process and as such, perturbs electrophoretic mobility. Here, denaturing of the dsDNA is induced either by heating (temperature, TGGE) or imposing linear increases in chemical denaturing agents within the sieving gel (denaturing, DGGE) to affect the separation. While it has been difficult to translate DGGE from the slab gel to micro-separation formats due to the inability to introduce heterogeneous denaturing conditions into the gels for the micro-separations, TGGE has proven to be more amenable to these separation platforms, especially microchip CE, because of the ability to fabricate heating elements along the length of the entire separation column.

Buch *et al.* used a 10 channel polycarbonate (PC) microfluidic device out-fitted with external bulk heaters or internally tapered micro-heaters to induce spatial and temporal temperature gradients along the length of the separation channels for gel-based sorting of G-C rich dsDNA fragments generated from a primary PCR. Comparing the two heating configurations, they found that the integrated micro-heaters provided better thermal response time and more accurate control, producing better TGGE separations [24]. This same group later introduced a mutation scanning network

consisting of an integrated two-dimensional micro-electrophoretic platform, which combined a standard size-based gel electrophoresis separation in the first dimension with TGGE in the second dimension [23]. This device, which is shown in Figure 2.1,

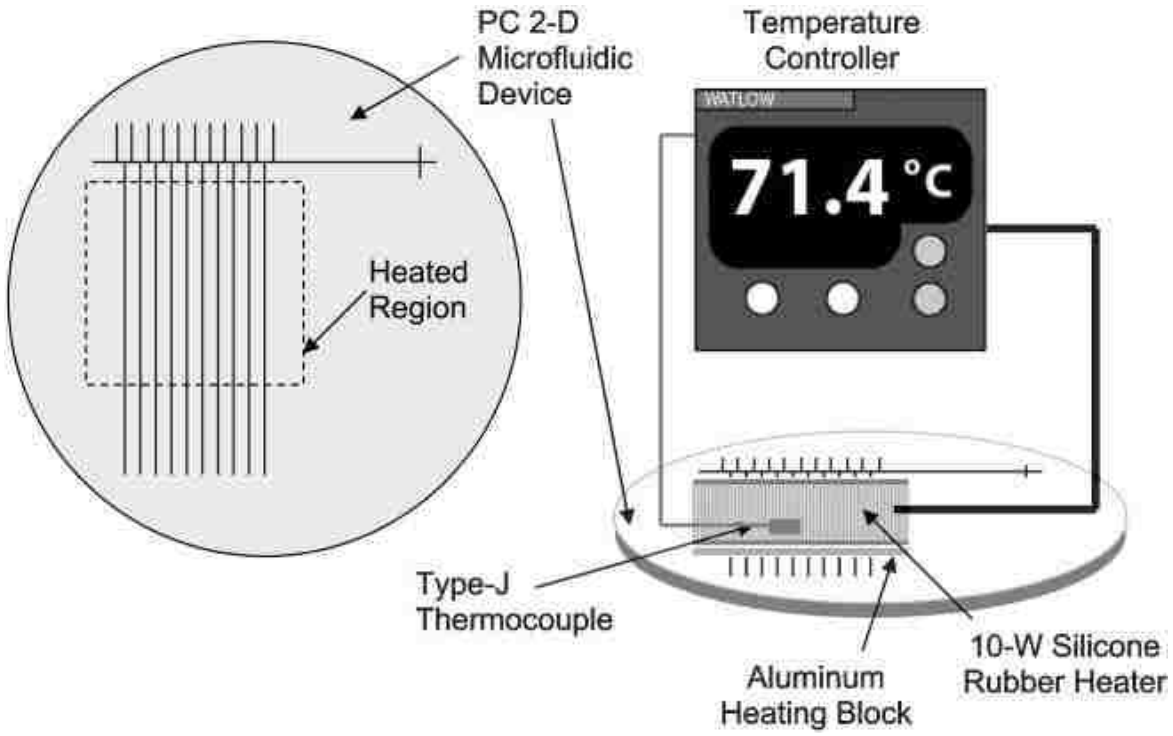

Figure 2.1. Illustration of the temporal temperature control setup used for microchip TGGE. The microchip in this case was made from polycarbonate (PC). Reprinted with permission from [23].

was made from PC with external bulk heaters used to establish the temperature gradient along the TGGE channels. An intermediate sample stacking strategy was implemented as well to reduce band broadening during sample transfer between the dimensions. The 2-D separations of multiplexed SNPs varying in size and sequence were conducted in a development time of less than 5 min with the prerequisite resolution to observe all of the SNPs [23].

2.3 Genotyping: Mutation Detection Applications

Unlike the mutation scanning techniques previously discussed, mutation detection methods are geared toward the identification and detection of particular genomic variations in which the locus is known within a certain gene. Most assays associated with this class of mutations involve the use of nucleotide recognition enzymes including endonucleases, exonucleases, and thermostable ligases, which specifically cleave or bond DNA sites that harbor the mutation being interrogated. Several of the mutation detection assays that have been utilized for the identification/detection of point mutations or SNPs include allele-specific PCR (AS-PCR), restriction fragment length polymorphism (RFLP), ligase detection reaction (LDR), ligase chain reaction (LCR) and single base extension assays (SBE), all of which depend on a high resolution electrophoretic step to sort the generated products in order to score the presence or absence of the mutation of interest. Most of these types of assays require DNA primers that hybridize to particular locations within the genome, which harbor the mutation being interrogated, prior to the enzymatic reaction and as such, require knowledge as to the specific location of the mutation in order to design the appropriate sequences in the primers.

2.3.1 Allele-Specific Polymerase Chain Reaction (AS-PCR)

While PCR is an attractive method to amplify target DNA sequences of interest prior to secondary treatments such as electrophoretic analyses, the successful generation of PCR amplicons can be directly interrogated for scoring the presence of SNPs based on the use of sequence-specific primers for the PCR that are generated for the locus or loci being interrogated. For example, if the PCR primers possess a mismatch with the target DNA, the amount of PCR product can be significantly reduced

or even eliminated by proper selection of the annealing temperature used in the 3-step thermal cycling process associated with PCR compared to fully matched primer/template duplexes. Therefore, the electrophoretic sorting of PCR amplicons can be used to detect the mutation of interest by preceding the electrophoresis with a PCR step. Challenges in AS-PCR include the need for tight control on the design of primers and understanding their annealing temperatures (T_m), the inability to highly multiplex the assay and the difficulty in securing quantitative information due to the non-quantitative nature of most PCR-dependent assays.

Huang *et al.* selected to separate gastric cancer-related SNPs using a microchip CE system to complement a simple-tube genotyping method, which combined a whole blood PCR with a tetra-PCR in which the PCR was conducted directly from 1 µL of unpurified whole blood or paper-dried blood [25]. Three typical genotypes of the two SNPs probed (IL-1B-31 and IL-1B-511) within the IL-1B gene were obtained in a 2 min analysis time using a commercial microchip system with its appropriate gel matrix (Agilent system).

Sung *et al.* demonstrated the use of PMMA microchips to perform fast exclusion tests via PCR product profiling of fragile X syndrome (FXS) alleles based on their repeating unit size, $(CGG)_n$, within the familial MR type 1 (FMR1) gene [26]. Using wire imprinted or hot embossed PMMA microchips filled with 1.3% or 1.8% hydroxyl propylmethyl cellulose (HPMC) containing an intercalating dye (TOPRO-3) to enhance detectability, they found that PCR bands with more than six unit repeat differences could be resolved in less than 3 min using a 6 cm electrophoretic separation length.

Low-density lipoprotein (LDL)-receptor gene microsatellite alleles of the D19S394 tetranucleotide repeat associated with familial hypercholesterolemia were sized using

an Agilent 2100 microchip system [27]; 17 alleles were easily distinguished using microchip CE, which varied from 0 – 17 repeats. Likewise, the same instrumentation was used by Sohni *et al.* to perform routine separations for a host of PCR-amplified variable number of tandem repeats (VNTRs) markers indicative of diabetic and cardiovascular complications stemming from irregularities in several genes [28]. A 12-lane microchip was used to detect spermatogenetic failure in a multiplexed format following an offline multiplexed PCR of three DNA sequence-tagged sites of interest on the Y chromosome [29]. With all of the required components resolved using a 1.5 cm effective separation channel, 36 samples could be processed within 180 s.

A PCR study of p16 tumor suppressor gene inactivation via hypermethylation was also evaluated on a microchip using AS-PCR. Comparing the ability of slab gel and an injection molded PMMA microchip to resolve methylation-specific PCR amplicons from 153 DNA plasma and tissue specimens including positive and negative controls, Zhou *et al.* found that the microchip separations yielded a 26.6% higher positive rate while maintaining specificity equal to that of slab gel electrophoresis [30].

While fluorescence detection schemes are primarily used for DNA genotyping analyses due to its high sensitivity and low limits-of-detection as well as the ease of labeling fragments using fluorescently-tagged primers required for the PCR step, an on-chip electrochemical detection strategy has also been introduced that was capable of detecting PCR amplicons [31]. By modifying a screen-printed carbon electrode, which effectively lowered the detection potential and reduced electrode fouling, unlabeled human heat shock factor (HSF1) gene amplicons were electrophoresed in a sieving matrix comprised of HEC at an optimal field strength of 200 V/cm and were detected with the screen-printed carbon electrodes. Some representative results from this study

are shown in Figure 2.2. The assay possessed a detection limit of 584.3 fg/µL (±1.3 fg/µL).

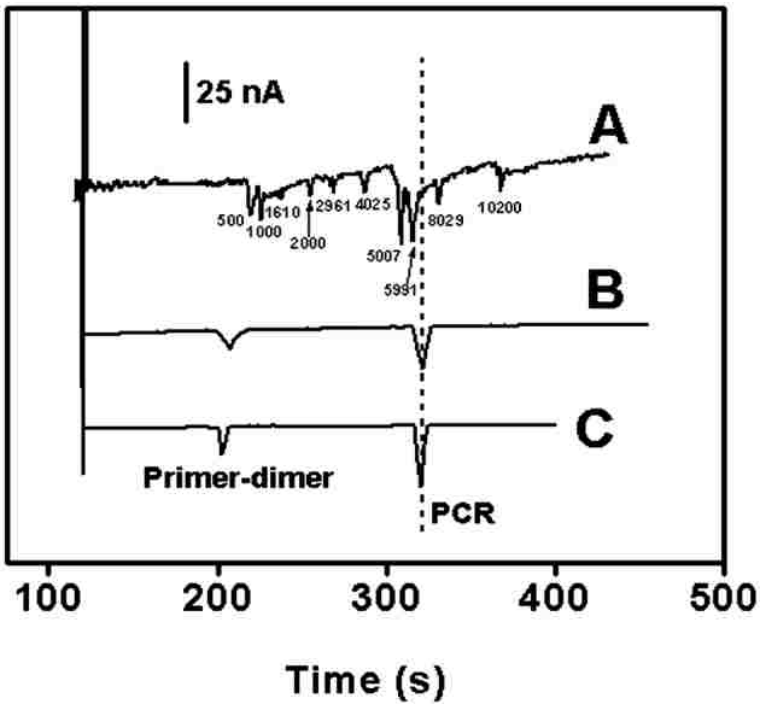

Figure 2.2. Elelctropherograms for the separation and detection of DNA fragments present in the BIONEER 1 kbp DNA standard marker (concentration = 130 ng/mL). The detector consisted of a modified screen-printed carbon electrode (A). The surfaces of the carbon electrodes were modified with poly-5,2'-5',2''-terthiophene-3'-carboxylic acid, which improved the analysis performance by lowering the detection potential. Electrophoretic results with electrochemical detection of 30-cycle PCR products at a bare (B), and modified (C) screen-printed carbon electrode. The standard marker consisted of 500, 1000, 1610, 2000, 2961, 4025, 5007, 5991, 8029, and 10 200 bp fragments. The detection potential used was 10.8 V (*vs*. Ag|AgCl reference electrode). Reprinted with permission from [31].

Besides routine testing for endogenous genetic conditions of individuals for clinical diagnostic/prognostic applications, PCR-based approaches for the detection of deleterious bacterial and viral infections affecting humans have also been subjected to electrophoretic analysis using microchip CE. For example, Kaigala *et al.* developed a hybrid microfluidic system for the production, detection and quantification of PCR products from the BK virus (BKV), a renal dysfunction highly associated with

transplanted kidney rejection [32]. Integrating PCR and separation processes onto PDMS-glass hybrid microdevices, they were able to detect as few as 1 – 2 viral copies as well as gauge the overall viral loads of clinical samples from renal transplant patients.

Chen *et al.* utilized wire-imprinted PMMA microchips to screen for hepatitis C present in blood serum of symptomatic patients and reported the completion of separations one order of magnitude faster than CGE [33]. In another study, fabricated PMMA microchips were also used to probe for SARS and hepatitis B virus (HBV) samples of known infected patients [34]. The device, containing trapezoidal imprinted channels transferred from a silicon master, reportedly yielded detection rates of 94.4% (17/18) and 80.6% (29/36) for multiplexed PCR SARS and HBV samples, respectively.

Karasawa *et al.* performed microchip CE separations to detect cariogenic (tooth decaying) bacteria in dental plaque [35]. In this case, AS-PCR was performed to amplify *S. mutans* and *S. sobrinus*, both known to promote tooth decay when they coexist in plaque. Comparing several mixtures of polyethylene oxide (PEO) and HPMC, they found 0.125% HPMC/0.6% PEO to provide the optimal mesh for the given amplicon sizes (202 and 226 bp, respectively). The separation of the two species was carried out in ~ 85 s on commercial Hitachi injection molded PMMA microchips.

2.3.2 PCR/Ligase Detection Reaction and Ligase Chain Reaction (LDR and LCR)

LDR is a mutation detection assay that involves the use of two complementary primers designed to flank a polymorphic site within the target DNA. One primer is called the discriminating primer and contains the polymorphic site on its 3’ end with the allelic content either matching or not matching the polymorphism on the target DNA. The other primer, called the common primer, is phosphorylated at its 5’ end. Upon

hybridization of these primers to the target DNA, a highly specific thermostable ligase seals them only if there is a complete match between bases on the 3' end of the discriminating primer and the polymorphic locus on the target DNA. If successful ligation does occur, it generates an elongated and fluorescently labeled oligonucleotide that can be distinguished from excess labeled unligated primers through differences in their sizes using electrophoretic sorting [36]. This assay is attractive because it decouples the PCR from the mutation detection reaction, which provides high specificity, it is amenable to multiplexing and it also performs exceptionally well in the presence of high levels of wild-type targets. The challenge associated with this technique rests not on the reaction itself, but with the ability to adequately resolve, via electrophoresis, several short single-base differentiated oligonucleotides (<100 bp) from the labeled and unligated primers that can potentially mask the positive signals indicative of the mutations due to their much higher concentration when the copy number of the mutated DNA is low compared to the wild-type sequences. Therefore, highly viscous polymers (8-10% polyacrylamide) are typically required to provide baseline resolution of all components generated from the LDR assay.

Thomas *et al.* demonstrated the ability to use a hot-embossed PMMA microchip filled with a 4% LPA or other commercial polymers for the analysis of a 12.2 *K-ras* LDR product (44 bp), indicative of a mutation that could be associated with the onset of colorectal cancer [37]. The results secured from the microchip CE result were compared to those using a conventional CGE format using a 4% LPA gel. CGE analysis of the LDR samples was incapable of providing reliable results due to electrokinetic injection biases; the higher concentration of the LDR primers as well as their higher mobility prohibited sufficient injection of the generated LDR products,

especially when the wild-type DNA was >100 fold higher in copy number compared to the mutant DNA. The microchip CE format performed much better under these conditions due to the absence of electrokinetic injection biases when adopting a cross-T injection format. The microchip CE separations of LDR products possessing a 100-fold molar excess of wild-type targets clearly showed the presence of the low copy number mutant alleles (well-resolved from the intense primer peaks) and required a development time of ~120 s in a separation channel length of only 3.5 cm [37]. In addition, the authors demonstrated that desalting prior to electrophoretic separation using microchip CE was not necessary as it was in the case for CGE.

The LDR assay, which is carried out with temperature cycling (denaturing and annealing) to linearly amplify the number of generated products, is often times coupled to a primary PCR in which the PCR is used to amplify the gene of interest, which can contain severe loci harboring the mutations of interest. PCR and LDR have been successfully integrated onto a polymeric microfluidic device, that can be used to generate products for subsequent interrogation using microchip CE [38]. The reaction time of the coupled PCR/LDR was reduced to 6.5 min as opposed to 1.5 h required for conventional benchtop thermal cycling instrumentation.

LCR is also a SNP detection method, which is based on the ligation of two pairs of oligonucleotide primers that hybridize to adjacent positions on complementary strands of a target dsDNA. Similar to PCR, LCR exponentially amplifies target sequences, however, LCR differs from PCR because it amplifies only the generated probe molecule (ligated primers). Moreover, LCR has been found to be more specific than PCR in some cases, which is prone to amplifying false positives occurring in early cycling events [39, 40]. The products of this application have also been detected and quantified

following electrophoresis on a microfluidic device. In a study highlighting the effect of polymers to dynamically passivate silicon-glass microchips, Lou *et al.* demonstrated the ability to resolve LCR products of NOD2/CARD15 genes related to inflammatory bowel disease using microchip CE [41].

2.3.3 Single Base Extension (SBE)

SBE, also known as minisequencing, has emerged as an effective mutation detection technique to determine the allelic composition at a particular locus; it is considered attractive due to its simplicity as only a few major components including a primer, polymerase and a nucleoside triphosphate substrate are required to conduct the assay [42]. Moreover, the total development time of reactions is considerably short compared to most typing methods in terms of those requiring thermal cycling [43]. SBE reactions entail annealing a primer one base removed from the locus containing the SNP to a particular DNA template. Following hybridization, fluorescently-labeled dideoxynucleotides are added to the reaction and the appropriate dideoxynucleotide is added based on the allelic composition of the locus being interrogated. The reaction is similar to conventional Sanger sequencing reactions except that deoxynucleotides are not included in the reaction cocktail and as such only a single base is added to the primer [44]. This method has been adapted to various microchip CE systems and the associated four-color multiplexing detection hardware associated with many CGE sequencing and genotyping equipment, which allows for spectral identification of the incorporated dideoxynucleotide. Although SBE in conjunction with conventional CGE has been extensively used and reported for multiplexed SNP genotyping, few reports discussing SBE/microchip CE combinations have surfaced, most likely due to the

stringent separation performance demanded for SBE (single base resolution of short, ~15-20 bp, DNAs).

Vreeland *et al.* illustrated the novel pairing of SBE with end-labeled free solution electrophoresis (ELFSE) for genotyping three p53 loci using a commercial CGE system and discussed the potential of transitioning this strategy to microfluidic platforms [45]. Recently, the Barron group demonstrated the microchip-based separation of multiplexed reactions to probe 16 p53 loci with 96% accuracy using ELFSE and microchip CE. With only a denaturing buffer containing a EOF suppressant as an electrophoretic medium, extension units harboring 16 unique, monodispersed, uncharged polyamide drag-tags facilitated the size dependent separation of species within 70 s using a commercial glass microchip [43].

Herbert *et al.* reported on an electrochemical approach for detecting mock SNP sites using SBE on a miniaturized platform. The microchip CE device was composed of a PDMS chip aligned and sealed with a cover slip containing working and reference electrodes positioned near the channel outlet. Sinusoidal voltammetry was used to distinguish electrochemically active extension products from unincorporated excess terminators following electrophoresis through agarose in less than 4 min [46].

2.3.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP relies on the use of various endonucleases, commonly referred to as restriction enzymes, that cleave recognition sequences usually 4 - 8 bp in length within dsDNAs. Upon treatment, DNA fragments having unique sizes corresponding to the distance between the restriction sites cut by the restriction enzymes are generated, and secondary treatments of the cleaved fragments with other restriction enzymes can provide further specific fragmentation patterns that can be subsequently subjected to

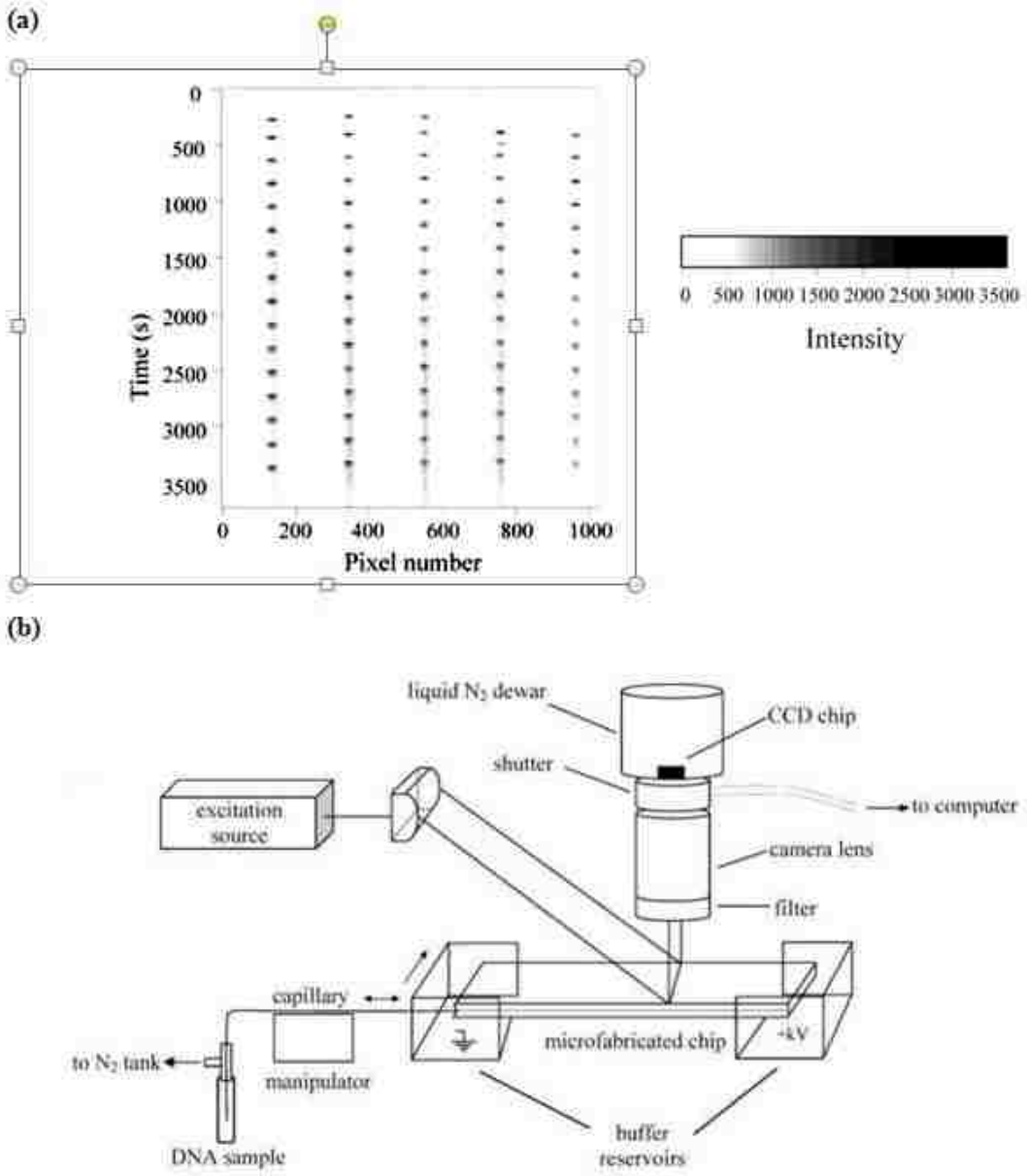

Figure 2.3 (a) Reconstructed plot of time versus pixel number of the CCD, which was monitoring restriction fragments generated across the multi-channel microchip. The shaded bar represents fluorescence intensity collected by the CCD. The sampling capillary moved from right to left depositing samples into the chip, so the lane farthest to the right is designated as lane 1. (b) Schematic of the chip setup with capillary sample introduction. Laser-induced fluorescence detection was achieved by focusing an argon ion laser into a line across the chip 5.5-6.5 cm from the injection point. A liquid nitrogen-cooled CCD was used to analyze the collected fluorescence photons. Adapted and reprinted with permission from [47].

electrophoretic sorting. If a mutation or SNP exists at a particular restriction site, it can change the restriction patterned generated, which can be deciphered using gel electrophoretic sorting. The limitation associated with this mutation detection strategy is that the mutation loci must be contained within a restriction site.

A high-throughput microfluidic design has been developed for the continuous assessment of restriction enzyme kinetics, which have been traditionally conducted using labor-intensive processing of millisecond-interval quenched enzymatic reactions and subsequently subjected to slab gel electrophoresis. The device for this application, as seen in Figure 2.3, used a capillary sampling device to dispense aliquots (1 injection/14 s) from a reaction mixture to a multi-channel (5 channels) glass chip with the collected fragments separated in the chip using an LPA sieving matrix and detected via laser-induced fluorescence with a CCD camera [47]. A 62 bp dsDNA containing a *Kpn*I restriction site was used for system evaluation and the effects of temperature and restriction enzyme concentration were also examined. The unique aspect of this setup was that the sampling device (a capillary) was not fixed to the separation platform (the microchip), and continuous intermittent injections could be processed in the pipeline without the need for completion of preceding separations, thus increasing throughput.

The ability of microchip electrophoresis to assess restriction digests of samples containing low percentages of mitochondrial DNA (mtDNA) mutations in blood samples from diabetic patients was investigated by Guttman *et al.* The assay included the generation of 250 and 251 bp products of a PCR-RFLP treatment designed to include a known *Apa*I site responsible for the mutation followed by the electrophoretic sorting of these products. In glass microchannels filled with a 2% agarose gel containing ethidium bromide, they were able to routinely discriminate 1 - 2% of A324G mtDNA point

mutations in mixtures containing 98 – 99% wild-type levels in less than 12 min [48]. In a related report, the same author introduced a high-throughput 96-lane format combining microfibrous membrane-mediated DNA digestion with subsequent microchip electrophoresis processing. An automated spotter was used to deliver submicroliter quantities of target DNA and digestion enzymes from 96 well plates onto a membrane, which served as both a reaction vessel as well as the microchip sample preconcentration/loading inlet. Results indicated that the digestions were completed within 1 – 10 min and subsequent separations in the agarose-filled channels (4 cm effective length) were completed in 5 – 10 min (20 min total analysis time) [49]. Recently, an on-chip treatment of mtDNA employing restriction enzymes was used by Taylor *et al.* to differentiate homogeneous and heterogeneous populations of mtDNAs. The procedure was carried out on a straight channel 8.0 cm commercial glass chip (Micralyne) and included excising a mtDNA sequence from plasmid DNA via enzymatic digestion with *Eco*RI to linearize the plasmid loops into dsDNA and denaturing/renaturing to form duplexes of the digested DNA all taking place in a single sample well. The generated products were labeled with an intercalating dye (sytox orange) that was present in the separation matrix (Genescan polymer containing 10% glycerol) during electrophoretic separation (7.6 cm effective separation channel length). According to the authors, the method could be performed in about 45 min, whereas conventional methods would require days to perform this same type of assay [50].

2.4 DNA Forensics

The identification of humans based on DNA profiling has become a powerful tool in the field of forensics. Usually, this profiling is achieved by the analysis of highly polymorphic variations presented in the form of short tandem repeats (STRs), which

consist of specific 2-7 base repeating patterns (e.g. $(CAGT)_n$) found in the genome. Primarily, STRs are distributed throughout non-coding regions of the genome known as junk DNA with the number of repeating units typically very specific for a particular individual, especially when occurring at several different loci.

Forensic DNA specimens are commonly matched to alleged criminal suspects in modern law enforcement using human identification systems validated according to the DNA Advisory Board's (DAB) Quality Assurance Standards. These DNA testing systems typically involve the amplification of highly polymorphic STRs by PCR. AmpF*l*STR® Profiler Plus and COfiler®, Powerplex®, Powerplex-Y, and Y-PLEX™ 12 are examples of commonly used multiplex systems for genotyping polymorphic STRs residing in the human nuclear (nDNA) and male Y-chromosomal DNA. These analyses are routinely performed using CGE and the genotyping capability of the ABI 3130 automated DNA Sequencer/Genotyper instrument within forensic laboratories. However, prior to gel sorting, the DNA sample must be extracted from the specimen, typically using precipitation-type techniques, purified and then, PCR amplified.

The resulting genetic "fingerprints" are then compared to profiles within the CODIS (combined DNA Index System) databank to identify a match. The advantage of these types of analyses lies within the very large number of STR alleles that provide a very high power of statistical discrimination. When DNA evidence is limited, analysis of human mtDNA targets is often employed because of the high copy number of mitochondria and mtDNA molecules in each cell [51].

Forensic DNA testing is also utilized to establish the paternity of children [52]. In investigative forensics, DNA fingerprinting is used to infer the geographic origin of unknown human DNA samples [53, 54], to identify potential microbial or viral pathogens

for bio-defense [55] or to identify species specific source(s) of unknown trace evidence [56] using PCR-based analysis of a variety of genetic systems including, STRs, single nucleotide polymorphisms (SNPs) and mobile element insertion polymorphisms.

The key elements of successful STR-typing include both high-resolution electrophoresis and precise sizing of DNA samples with respect to internal standards used to identify various alleles within a given locus. The core advantages of microchip CE in terms of DNA forensics includes the ability to integrate sample pre-processing steps into a monolithic wafer and the ultra-fast separations afforded by microchip CE, which could play a vital role in compiling more extensive forensic databases and also, relieving the backlog of DNA typing cases. For example, the cost, speed and lack of simple and user friendly equipment available for forensic DNA tests have contributed to the current backlog of over 350,000 DNA evidence samples from criminal trials.

Work was described by Schmalzing *et al.* in 1997 using a glass chip possessing a single channel (45 μm deep and 100 μm wide) to separate single-locus and four-loci PCR-amplified STRs spiked with allelic standard ladders with a CE development time of only 30 s and 2 min, respectively, with a 4% LPA matrix under denaturing conditions over a 2.6 cm effective separation distance [57]. This work was later extended upon by using a dual wavelength LIF detection approach to increase the multiplexing capacity of the system to process an eight-loci STR simultaneously in a single separation channel [58]. A micro-variant allele present in the sample requiring single base resolution was resolved in 10 min using an 11.5 cm effective channel length appeared in this same report.

In a subsequent study, the degree of multiplexing was further enhanced using a four-color excitation/detection system capable of examining 15 loci simultaneously

within 20 min with single base resolution ranging from 0.75 to 1 [59]. This work lead to the development of an STR analysis instrument designed around a 16-lane glass microchip CE device. The chip layout, as seen in Figure 2.4A, included double T cross injectors for electrokinetic injections in each separation channel and 20 cm effective separation lengths that converged near the detection point to fall within the range of an optical scanner. Peak accuracies of 0.4-0.9 bp were reported using the CODIS 13-locus multiplex established by the FBI [60].

More recently, plastic microchip CE devices have been assessed for their potential to accommodate high-resolution analysis of STR samples [61, 62]. In two related studies, polyolefin substrates having hot-embossed microchannels (4.5, 6.0, 10, and 18 cm long separation channels) filled with a replaceable 4% LPA matrix under denaturing conditions were used to electrophoretically sort common STR ladders. While the 4.5 cm channel was capable of providing single base resolution between micro-variants [61], higher quality performance was illustrated using the 10 and 18 cm length channels with a 3% LPA sieving matrix. However, it was noted that pressure tolerances associated with these devices dictated what sieving matrix concentrations could be used [62]. Exceeding these pressure tolerances by pumping high viscous gels through the plastic channel caused release of the cover plate from the chip substrate and subsequent device failure.

A high-throughput forensic study was demonstrated using a 96-lane microchip CE device and a 4-color radial scanning detection system. Profiled samples attained using conventional CGE were analyzed in parallel for comparison followed by the analysis of several (17) unexamined samples using the 96-lane microchip; microchip CE provided single-base resolution and runs were completed within 30 min [63]. The

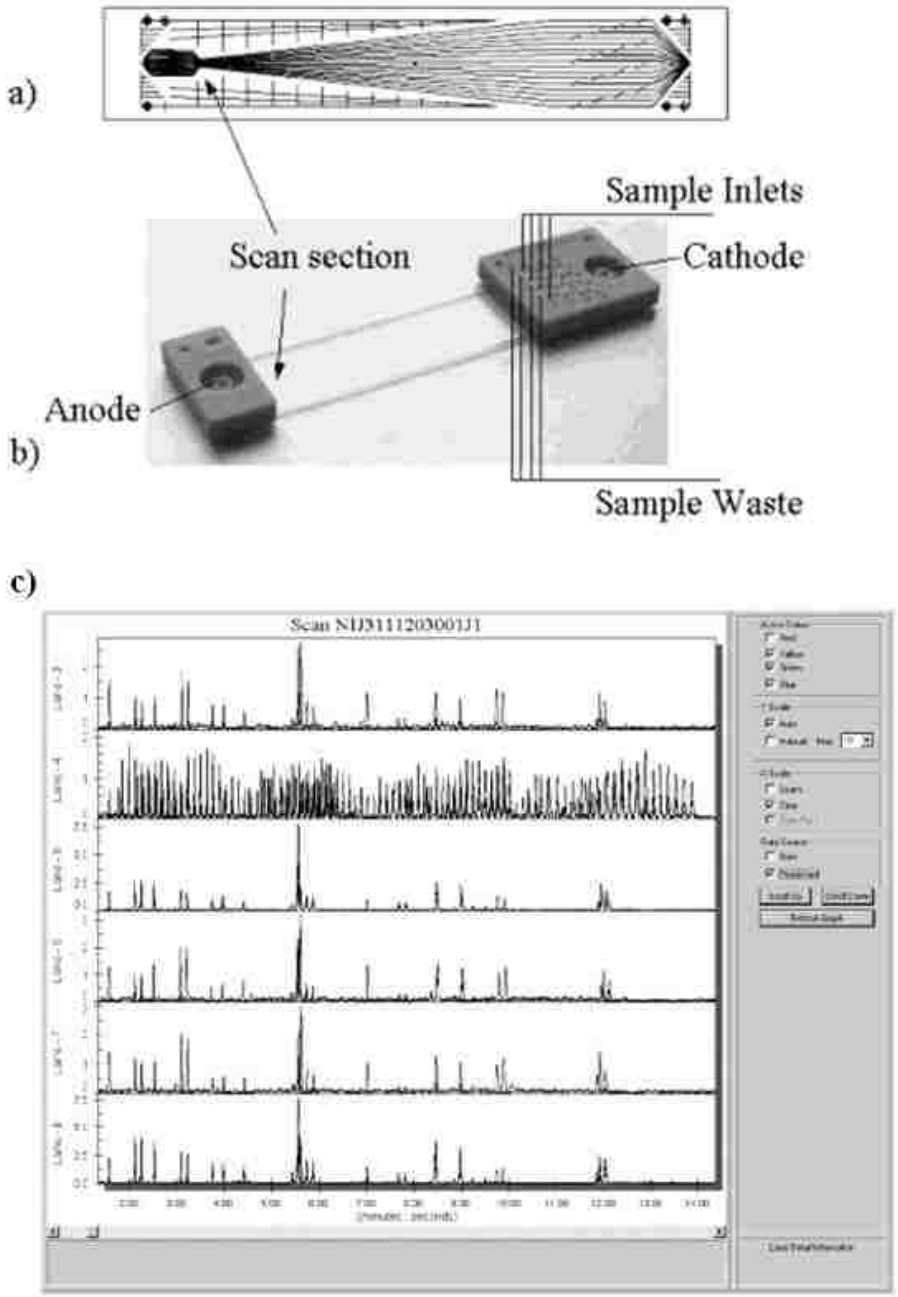

Figure 2.4.(a) CAD drawing of the chip layout. The design contained 16 lanes with 16 double-T injectors on the right for electrokinetic sample injection. It has a section where all channels are close together for scanning (arrow), the overall distance at this point is 3.2 mm from injection to detection. (b) Complete assembly with anode on the lower left and the cathode board on the top right corner; there is a vial for sample inlet and sample waste for each lane. The effective length of each lane is 20 cm. (c) Computer screen image of 6-channels of electrophoresis results for the chip system shown in (a). The electropherograms are from 5 data samples and one allelic ladder. Reprinted with permission from [60].

STR typing was accomplished using the PowerPlex 16® and AmpFlSTR® Profiler Plus® multiplexed PCR systems. Forty-eight previously analyzed single-source samples were accurately typed, as confirmed by an ABI Prism 310 and/or the Hitachi FMBIO II CGE instruments. Minor alleles in 3:1 mixture samples containing female and male DNA were reliably typed as well.

All of the aforementioned microchip-related forensic studies emphasized the use of STR specifically for human identification. However, in the field of anthropology the ability to differentiate human remains from those of animals by sampling hair and bone remnants for mtDNA is sufficient. A recent study has evaluated the usefulness of a microchip-processed PCR analysis using a commercial system and glass microchip for probing mtDNA of ancient sources [64]. Highly degraded human and non-human DNA samples were positively identified using a microchip CE separation by selectively amplifying target human-specific mtDNA genes (Cytochrome b and 16S ribosomal RNA).

2.5 DNA Sequencing

Sequencing remains the gold standard for the analysis of DNA in many applications such as genotyping or DNA forensics in spite of some of its current limitations, which mainly includes the labor intensive sample pre-processing, the slow development time and the high performance demands placed on the electrophoresis phase of sequencing. The quest to design microchip CE for demanding DNA sequencing applications as part of the HGP spawned the production of elaborate units featuring dispersion-limiting channel turn geometries to extend channels within a small footprint for increased DNA read lengths, offset injection crosses to increase sample

injection plug volumes to aid in detection and multi-lane formats for high throughput analyses.

The pioneering report for transitioning sequencing CGE separations to microfabricated CE chips was presented by Woolley *et al.* in 1995; the electrophoresis was conducted on a simple, straight-channel (50 µm wide, 8 µm deep) microchip channel etched into glass wafers filled with a polyacrylamide gel [65]. With an effective separation distance of 3.5 cm, four-color DNA sequencing traces of ~150 bp were performed with 97% base calling accuracy in only 540 s. Improvements upon this work followed refining subsequent platforms to improve both resolution and throughput. Ultimately, high density (96-lane) CE microchips with deeper (30 µm) and longer (15.9 cm) effective separation lengths that were radially-configured into a common anode were developed, which increased the average read length to 430 bases [66]. While this particular microfluidic device was initially intended for sequencing, it has been utilized in some of the previously mentioned high throughput genotyping and forensic applications as well [63].

After a stringent parametric study of micro-electrophoretic sequencing separations in terms of channel lengths and sieving gel concentrations [67], work by Aborn *et al.* introduced an automated microchip CE sequencing system employing 384-channel microdevices with channel lengths varying from 37 - 45 cm situated on large glass plates (25 cm x 50 cm). The device was capable of providing 800 base read lengths and called 1.72×10^5 bases per 384-lane run with 99% accuracy [68].

Although sparingly reported, plastic-based microfluidics has also been employed for DNA sequencing. Boone *et al.* reported four-color sequencing reads of 640 bases with 98% accuracy using an acrylic microchip bearing an 18 cm embossed channel

within 30 min [69]. Using polyolefin microchips with much shorter channels (4.5 cm), Shi *et al.* demonstrated the ability to read a sequence to 320 bases with 99.1% accuracy within 13 min [61]. In both studies, replaceable LPA matrices were used for sieving.

Along with the miniaturized sequencing separation platforms, auxiliary components have been incorporated into the microchip CE system to aid in the separation and detection. For example, Ueberfeld *et al.* used a solid support sample loading technique to minimize the obstacles associated with the processing of low concentrations of DNA sequencing fragments typically encountered using microfluidic devices (see Table 2.1). By directly injecting Sanger DNA sequencing samples reversibly adsorbed onto paramagnetic microspheres and extraction from solution with a magnetized wire, they were able to achieve fluorescence signal intensities that were equal to that of standard offset T injectors having >10 times the initial DNA sample content [70].

Also of interest are efforts in microchip CE for potentially simplifying the DNA separation phase of the sequencing processing pipeline by developing alternative electrophoretic separation mechanisms that could potentially eliminate the necessity of using sieving materials such as polymer gels and their associated high viscosity constraints. Using the previously mentioned ELFSE technology [72], ~110 base sequencing read lengths have been reported using conventional capillary electrophoresis in ~18 min [73].

Researchers are now pushing towards the production of highly integrated sequencing units to perform sample preparation and cleanup prior to the electrophoretic separation to minimize the labor and time overhead associated with DNA sequencing.

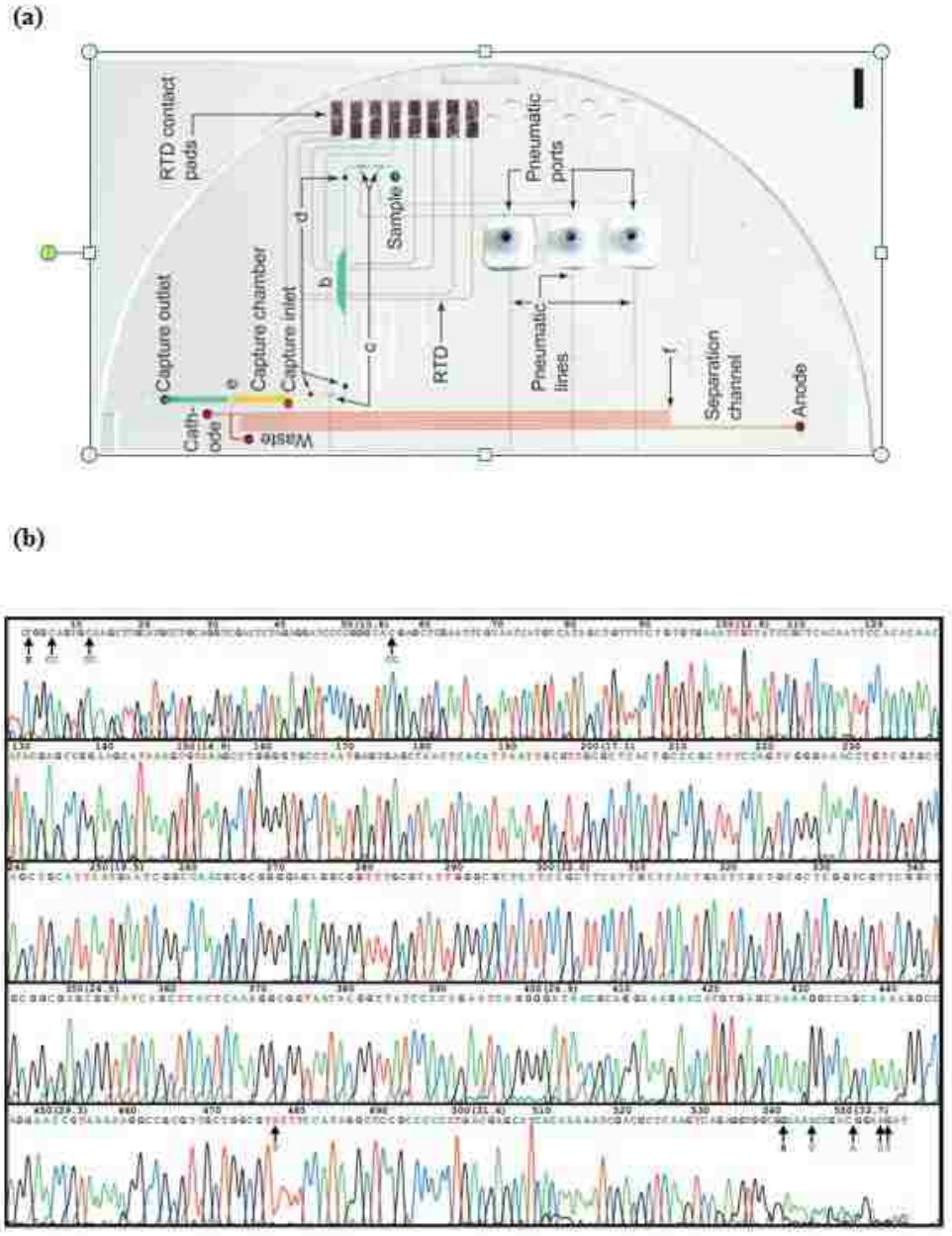

Figure 2.5. (a) Photograph of the integrated microchip device, showing one of two nucleic acid processing systems. Colors indicate the location of sequencing reagent (green), capture gel (yellow), separation gel (red), and pneumatic channels (blue). (b) High-quality sequencing data generated using the integrated bioprocessor shown in (a). Sanger sequencing extension fragments were generated from a 750-bp pUC18 PCR amplicon. Automatic base calls were secured by the program PHRED and base numbers are indicated above the electropherogram. Scale bar is 5 mm in (a). Reprinted with permission from [71].

Recently, a fully integrated microchip bio-processing unit combining temperature cycling, sample purification and electrophoretic separation has been fabricated, which contained nanoliter-scale reaction chambers. Shown in Figure 2.5 is the system, which consisted of a glass-PDMS hybrid device capable of performing complete Sanger sequencing on 1 fmol of DNA template with read lengths of up to 556 bases and a calling accuracy of 99% [71].

2.6 Conclusions

Significant strides have been made toward demonstrating the potential of planar microchip devices as viable miniaturized electrophoretic platforms for the separation of DNAs for a variety of different and important applications. Over the past decade, pioneering proof-of-principle separations of both single-stranded and double-stranded DNAs as well as enzymatically produced DNA products on primarily glass substrates have produced highly promising separations that offer the advantages of shorter electrophoretic development times compared to their slab gel and capillary counterparts without significant sacrifices in terms of separation resolution. While progress in microchip CE separations of DNA samples have been reported, significant advances and improvements in this technology platform still must be made to allow its permeation into clinical laboratories for diagnostics and forensic laboratories for DNA typing. For example, the development of lab-on-a-chip systems that incorporate all of the sample pre-processing steps into the system incorporating an electrophoretic separation would provide fully automated analyses with little manual or operator intervention or expertise, minimize sample contamination and allow permeation of this promising technology platform into a broader user community.

Another area that must evolve is significant reductions in the cost of producing microfluidic chips for CE-based separations and even those chips that consist of multiple processing steps to allow full automation of the DNA processing pipeline. This will be particularly important in forensic and diagnostic applications, where disposable fluidic components are demanded. Replication technologies of plastic microfluidic devices can provide an array of ideal fabrication techniques for producing low-cost electrophoretic and other DNA processing chips. Transitioning glass and fused silica-based techniques to polymeric materials will facilitate the evolution of disposable microfluidic cartridges for such applications.

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Chapter 3: Capillary and Micro-Electrophoretic Separations of Ligase Detection Reaction Products Produced from Low Abundant Point Mutations in Genomic DNA

3.1 Introduction

Electrophoresis can be utilized in screening assays for the diagnosis of presymptomatic disease states, such as the early detection of cancers, which can arise from the accumulation of mutations in certain gene fragments that may be inherited or somatic, caused either from exposure to environmental factors or from malfunctions in DNA replication or repair machinery. These mutations can then be used as markers in a variety of different electrophoresis formats, such as heteroduplex analysis, single strand conformational polymorphism analysis, denaturing gradient gel electrophoresis or DNA sequencing.

One example where electrophoresis can potentially provide diagnostic information is the early detection of colorectal cancers using the identification of point mutations in the human K-*ras* oncogene, which manifest themselves primarily as single base-substitutions located at two bases in codon 12, two bases in codon 13 and three in codon 61. These particular mutations occur in 35 – 50% of all colorectal adenomas and can manifest themselves early in the development of colorectal neoplasms [1-4]. Once acquired, these mutations are preserved throughout disease development and thus, have great utility for diagnostic testing.

Unfortunately, the detection of these mutations is complicated by the fact that often their presence is masked by significant amounts of wild-type sequences even at the primary tumor site, which can contain a majority of wild-type stroma. The frequency of the mutated allele at the primary tumor site can be as little as 15% for heterozygous samples and this number goes down precipitously if the sampling is done away from

this site. Therefore, improvements in early detection and treatment of cancer patients depends intimately on rapid and accurate high throughput methodologies capable of identifying low abundant mutations with high selectivity.

K-*ras* mutations may be detected by direct DNA sequencing, allele specific oligonucleotide hybridization or restriction digestion techniques [5-8]. Others have used high sensitivity techniques such as phage cloning, allele-specific PCR or repetitive restriction digestion to detect K-*ras* mutations in stool or lymph nodes of cancer patients as indicators of micrometastases [9-15]. Unfortunately, these techniques have intrinsic limitations, such as low sensitivity (DNA sequencing) or the inability to detect the full spectrum of K-*ras* mutations (restriction digestion or allele-specific PCR). Furthermore, allele-specific amplification techniques are prone to false positives generated from minute contamination or from the introduction of point mutations by polymerase errors during extension.

A slab gel-based electrophoretic assay has recently been reported for the detection of mutations associated with colorectal cancer using a ligase detection reaction (LDR) [16], a method that simultaneously amplifies DNA and identifies single base mutations in a multiplexed format [17]. LDR is based on an allele-specific ligation of two synthetic oligonucleotide primers, which flank the point mutation (a common and discriminating primer) that hybridize to one strand of the target DNA as shown in Figure 3.1. The junction point of these primers is selected so that the nucleotide at the 3' end of the discriminating primer coincides with the single base mutation site in the target sequence. The discriminating and common primers are covalently joined to form an LDR product by a thermostable ligase only if the nucleotide at the potential mutation site is complementary to the 3' end of the discriminating primer.

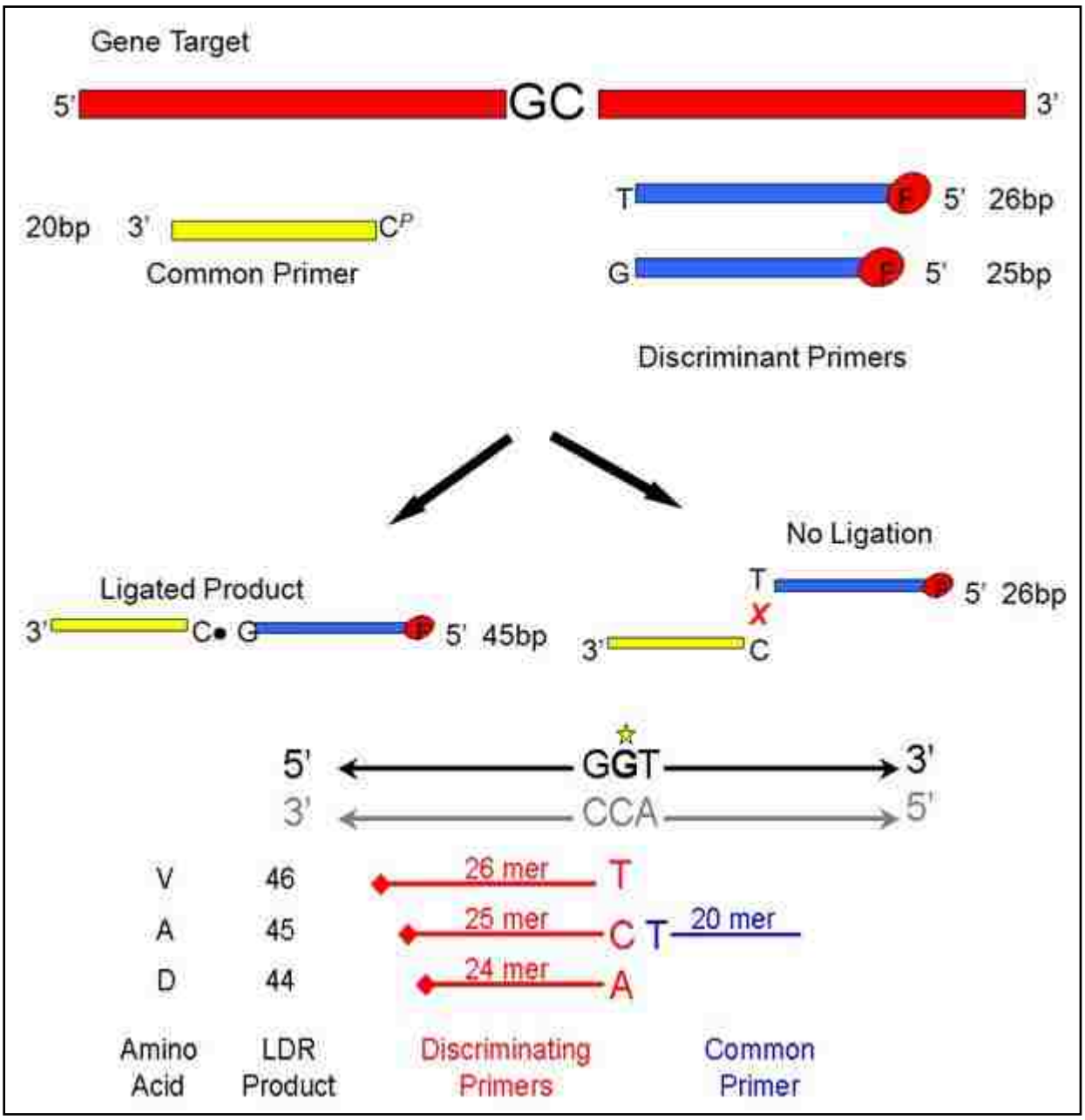

Figure 3.1. Diagram illustrating the ligase detection reaction and the LDR primer sizes used for human *K-ras* gene mutation discrimination at site 12.2 of codon 12 within exon 1. Discriminating primers were fluorescein-labeled on their 5' ends. The length of the discriminating primer and the corresponding amino acid abbreviations are indicated; V = valine, A = alanine, D = aspartic acid.

The process is multiplexed using electrophoresis to screen the full spectrum of point mutations associated with a particular disease via size discrimination by varying

the base numbers of the discriminating primer used for the LDR assay as shown in Figure 3.1 [16]. Generation of an LDR product indicates the presence of a mutation while the length of the LDR product designates which mutation was present (allelic composition or mutation location in the gene). In the example shown in Figure 3.1, the wild-type sequence contains a cytosine (C) nucleotide in the K-*ras* gene at position 12.2, which codes for the amino acid glycine during translation. Possible mutations at this site, the resulting amino acid substitution, and their respective abbreviations are adenine (A) / valine (V), guanine (G) / alanine (A) and thymine (T) / aspartic acid (D). Thus, a G12V mutation corresponds to a glycine-to-valine change at codon 12 resulting from a base substitution of G with A.

Using two discriminating primers and one common primer, a slab gel assay was able to detect one mutation in 4,000 wild type sequences at a 9 to 1 signal-to-noise ratio [18]. Using an eight-primer set (six discriminating primers and two common primers) designed to detect two possible mutations in codon 12, the assay was able to detect one mutation in 1,000 wild-type sequences at a signal to noise ratio of 3 to1. The same mutation was detected in 500 wild-type sequences using an entire 26 primer set (19 discriminating primers and 7 common primers) designed to probe for 19 possible mutations at codons 12, 13 and 61 simultaneously. Unfortunately, the slab gel assay required several hours of electrophoretic development time to adequately resolve the LDR products from excess unligated primers.

In this paper, we wish to report on the use of capillary and microchip electrophoresis formats for detecting single base mutations in selected gene fragments (K-*ras*) using LDR. Several issues must be considered when developing capillary or microchip electrophoresis platforms for LDR analyses. In the standard LDR assay, a

large excess of primer over the target template ensures efficient hybridization and ligation. Due to the large amount of primers contained within the reaction cocktail, the electrophoretic method must be able to resolve the LDR product from this excess, both of which contain a fluorescent label. In addition, single base resolution is required since the size of the product, as deduced from the migration time, is used to identify the particular mutation site and/or its allelic composition.

Capillary gel electrophoresis (CGE) can provide a method for the rapid analyses for LDR-based assays using a variety of different separation matrices to provide sufficient resolution to detect the LDR product, even at low abundance. However, a concern associated with this method is the well-characterized injection bias, in which high mobility species, such as short primers and salts, are loaded preferentially compared to slower migrating components. Therefore, high resolution must be achieved so as not to mask the LDR products in the large band generated from unligated primers when these are much higher in concentration [19]. In addition, injection biases can severely limit the loading of the LDR products, demanding high sensitivity detection as well. A final concern is the oligonucleotides that must be separated, which are single stranded DNAs (ssDNA) with a size range <100 bps and as such, special requirements are demanded for the sieving matrix to provide the necessary selectivity.

The microdevice format can potentially allow rapid analyses and reduced consumption of reagents, issues that are important for high throughput screening applications [20]. Compared to CGE, microelectrophoresis can provide more representative loading of all fragment sizes through unbiased volume-based injections

[21]. However, these devices also contain shorter separation lengths that have the potential to limit resolution unless high resolving power gels are used.

Using a K-*ras* mutation in codon 12 as a model, capillary and microdevice electrophoresis methods were developed to demonstrate the ability for analyzing point mutations in K-*ras* genes of low abundance with electrophoresis. Several ssDNA separation matrices in the capillary and microdevice format were evaluated in a modeling study using synthetic dye-labeled oligonucleotides for their ability to analyze low copy numbers of long oligonucleotides (LDR products) in an excess of shorter oligonucleotides (unligated primers). Using the optimized conditions and separation matrices in both electrophoretic formats, PCR products generated from cell lines of known K-*ras* genotype were used as templates for LDR to detect single base mutations in codon 12.

3.2 Methods and Materials

3.2.1 Capillary Electrophoresis Instrumentation

CGE separations were performed using a Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA). Data acquisition was performed using the Beckman P/ACE software. Detection was accomplished using the appropriate laser-induced fluorescence (LIF) module fitted into the CE instrument, which contained an Argon ion laser (λ_{ex} = 488 nm) and a 520 nm emission filter placed in front of the photomultiplier tube. In all cases, the electrophoresis was performed in a reverse mode, with the detection end anodic and the injection end cathodic.

3.2.1.1 Capillaries and Sieving Matrices

μPAGE-5 (5%T, 5%C) polyacrylamide gel-filled capillaries (referred to herein as 5T5C) and buffer were purchased from Agilent Technologies (Palo Alto, CA). The total

capillary length was 36 cm while the effective length was 30 cm; however, this length varied slightly from run to run due to clipping of the inlet end of the capillary prior to each run to restore column performance. The electric field strength was set to 250 V/cm at an operating temperature of 25°C. A water pre-injection of 10 s at 10 kV was used to increase sample loading. Typical injection times ranged from 10 s to several minutes as indicated in each electrophoretic trace.

The eCap ssDNA 100-R Gel (herein referred to as eCap), buffer and neutrally coated capillary (75 μm i.d.) were purchased from Beckman Coulter (Fullerton, CA). The optimized separation conditions for the eCap were found to be 308 V/cm at 30°C. The matrix was replaced in the capillary (30 cm effective length, 36 cm total length) prior to each run at approximately 800 psi using a pressure vessel designed in-house.

The Performance Optimized Polymer (herein referred to as POP5) and buffer were obtained from Applied Biosystems (ABI, Foster City, CA). The optimized field strength for this gel was determined to be 215 V/cm while the capillary temperature was set to 50°C. A bare fused silica capillary (75 μm i.d., 30 cm effective length, 36 cm total length) was filled with POP5 using approximately 1,000 psi pressure before each run.

3.2.2 Microdevice Fabrication and Assembly

The microdevice consisted of a 10 cm separation channel with 0.5 cm side channels to the sample, buffer and waste reservoirs as shown in Figure 3.2A. The microchannel was hot-embossed in poly (methyl methacrylate) (PMMA) (MSC, Melville, NY) using a Ni mold master containing microstructures 80 μm tall and 20 μm wide (channel dimensions) that were produced by a *LIGA* process as described elsewhere

[22]. The final device was assembled by annealing a PMMA cover plate to the open face of the device at 109°C in a circulating air oven for 15 min.

3.2.3 Laser-induced Fluorescence (LIF) Instrumentation

The LIF microdevice detection system was constructed in-house with an epillumination configuration, as shown in Figure 3.2B. The system consisted of a 488 nm air-cooled argon ion laser (Omnichrome, Model 532), which was directed to the focusing objective using a multimode fiber optic and a dichroic mirror. The excitation beam was focused using a 16X microscope objective (Melles Griot) onto the microdevice, which was situated on an X-Y-Z microtranslational stage in order to position the microchannel with respect to the focused laser beam. The fluorescence emission was filtered though a stack of optical filters and focused onto a single photon avalanche diode (SPAD, Model SPCM-AQ-141, EG&G Optoelectronics Canada, Vaudreuil, Canada). The filter stack consisted of a 520 nm bandpass filter (Oriel, Stratford, CT) and a 520 nm long-pass filter (Edmund Scientific, Barrington, NJ). The LIF signals were acquired on a personal computer using a 16-bit counter/timer board (Model AT-MIO-16XE-50, National Instruments, Austin, TX).

3.2.3.1 Microelectrophoresis Operation

Labview software and an electrophoretic “switch box” constructed in-house were used to control voltages during the microelectrophoresis. The device included three internal high voltage power supplies (EMCO) capable of receiving input of 0 or +5 V from DAC (digital to analog converter) outputs of a PCI-MIO-16XE-50 board (National Instruments). These power supplies delivered 0 to +2 kV to wells (A) and (B) (EMCO Model C20); and +0.3 to +5 kV to well (D) (EMCO Model G50) (see Figure 3.2A).

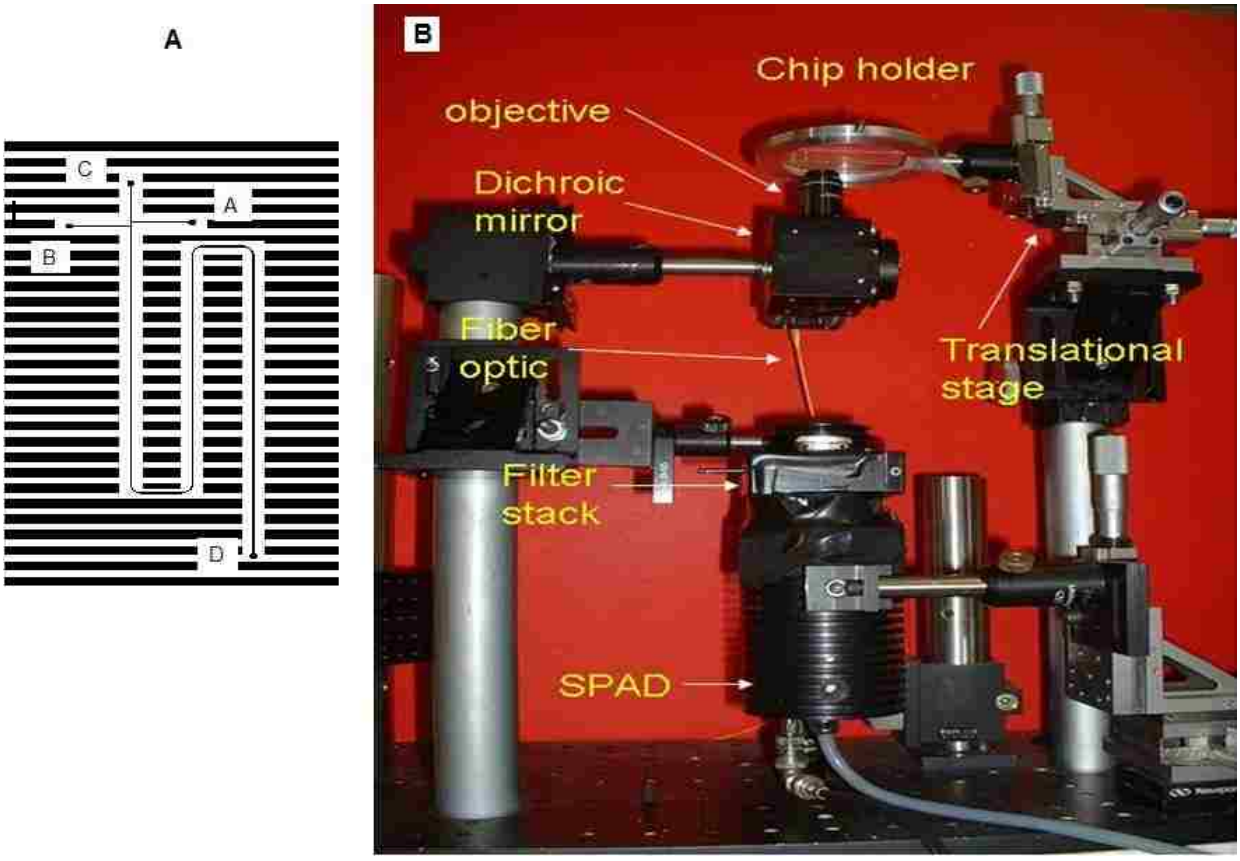

Figure 3.2. (A) The geometrical layout of the microelectrophoresis chip, which was fabricated using hot embossing from a Ni master in PMMA. The chip contains four reservoirs, A = sample; B = waste; C = buffer and D = buffer waste. The chip contained a separation channel that was 10 cm in total length with a channel width of 20 µm and depth of 80 µm. **(B)** Also shown is an annotated picture of the laser-induced fluorescence system used for detection on the microchips.

During injection, a positive voltage was applied to the waste well (B) (see Figure 3.2A) while the sample well (A) was grounded. During separation, a positive voltage was applied to well (D), (C) was grounded and "pullback voltages" were applied to the sample (A) and waste (B) reservoirs to prevent leakage of extraneous material into the separation channel. The microdevice was first manually filled with separation matrix. Following filling, the buffer and sample reservoirs were filled with the appropriate material. Once filled with the sieving matrix, the gel was pre-conditioned by electrophoresing for 5 min at 100 V/cm (voltage applied between reservoirs (C) and (D),

see Figure 3.2A). For the electrophoretic separation, the optimized field strength for the appropriate sieving gel was used as well as the appropriate pullback voltages on the sample and waste reservoirs. In all cases, the electrophoresis was performed in a reverse mode with the injection end cathodic and the detection end anodic.

3.2.4 Modeling Primers and DNA Sizing Ladder

All primers used in this work were obtained from Integrated DNA Technologies (IDT, Coralville, IA) with PAGE purification and fluorescein labeling of the discriminating primers at their 5' ends. The fragments used as models were constructed to mimic common and discriminating primers as well as LDR products and consisted of 25, 44 and 51 bp fragments of random sequence. Using the 25 base primer to represent the unligated primers, model samples representing 1:10, 1:100 and 1:1,000 molar ratios of LDR product to unligated primers were prepared. In each sample, the unligated primer (25 bp) was maintained at 1.0 μM and for the 1:10, 1:100 and 1:1,000 modeling samples, the 44 and 51 bp synthetic LDR products were 100.0 nM, 10.0 nm and 1.0 nM, respectively.

3.2.5 LDR Primers

Primers used for LDR were purchased from IDT with fluorescein labeling on their 5' ends (discriminating primer). LDR primers were designed for identification of single base substitutions in codon 12.2 by altering the length of each primer as shown in Figure 3.1. When analyzing the G12V allele, the discriminating primer for G12D was included in order to monitor potential misligations of the discriminating primer used to detect the G12D genotype, representing a C:A mismatch in wild-type DNA. The three-primer set consisted of two discriminating primers and one common primer as shown in Table 3.1.

Table 3.1. LDR discriminating and common primers. Primer size and sequence are indicated. * = common primer, which was phosphorylated at its 5' end to allow ligation.

Primers	Size	Sequence
Fluor-K-ras c12.2V	26	5' Fluor-CAA AAA CTT GTG GTA GTT GGA GCT GT 3'
Fluor-K-ras c12.2A	25	5' Fluor-CAA AAC TTG TGG TAG TTG GAG CTG C 3'
Fluor-K-ras c12.2D	24	5' Fluor-AAA ACT TGT GGT AGT TGG AGC TGA 3'
K-ras c12 Com-2*	20	5' pTGG CGT AGG CAA GAG TGC CT 3'

3.2.6 DNA Extraction from Cell Lines

PCR products were amplified from genomic DNA that was extracted from cell lines of known K-*ras* genotype (HT29, wild-type; G12V or G12D mutant alleles) [16]. Cell lines were grown in RPMI culture media with 10% bovine serum. Harvested cells (~1 x 10^7) were resuspended in DNA extraction buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0) containing 0.5% SDS and 200 µg/mL proteinase K and incubated at 37ºC for 4 hr. Thirty-percent (v/v) of 6 M NaCl was added to the mixture and the samples centrifuged. DNA was precipitated from the supernatant with 3 volumes of EtOH, washed with 70% EtOH and resuspended in TE buffer (10 mM Tris-HCl, pH 7.2, 2 mM EDTA, pH 8.0).

3.2.7 PCR Reagents and Conditions

PCR amplifications were carried out in 50 µL with 10 mM Tris-HCl buffer (pH 8.3) containing 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 as described above. The set of primers used were: Ex.1.3 forward =

5’ AAC CTT ATG TGT GAC ATG TTC TAA TAT AGT CAC 3’; Ex.1.4 reverse = 5’ AAA ATG GTC AGA GAA ACC TTT ATC TGT ATC 3’; Ex.2.9 forward = 5’ TCA GGA TTC CTA CAG GAA GCA AGT AGT 3’ and Ex.2.11 reverse = 5’ ATA CAC AAA GAA AGC CCT CCC CA 3’. After a 1 min denaturation step, 1.5 units of Amplitaq DNA polymerase (Perkin Elmer, Norwalk, CT) was added under hot start conditions and amplification was achieved by thermally cycling for 35 - 40 cycles at 95ºC for 30 s; 60ºC for 1 min; 72ºC for 1 min and 72ºC for 3 min for a final extension. PCR products were stored at –20ºC until required for use.

3.2.8 LDR Reagents and Conditions

LDRs were carried out using conditions similar to those published elsewhere [16]. Briefly, the PCR products were used as templates for the ligase reaction. The appropriate ratio of wild-type to mutant sequence was added to a solution containing 1X Tth ligase buffer (10 mM Tris-HCl (ph 7.6), 10 mM $MgCl_2$, 0.1 M KCl and 20 µg/mL bovine serum albumin), 10 mM DTT, 1.25 mM NAD^+ (nicotinic adenine dinucleotide, a cofactor for ligase enzyme) and 500 nM of each discriminating primer taken to a final volume of 20 µL. The mixture was heated to 94°C for 1.5 min prior to adding 1 nM Tth DNA ligase (received from Cornell). LDRs were thermally cycled 20 times at 94°C for 15 s and 65°C for 4 min. Reactions required cleanup prior to CGE using a cold ethanol precipitation to reduce the amount of salts. This was carried out by adding 7 µL of 7 M NaOAc and 100 µL of 100% cold ethanol to the LDR. The solution was vortexed and placed in a refrigerated centrifuge for 30 min at 10,000 rpm at 4°C. The supernatant was removed and the reactions thoroughly dried in a Centro-Vap (Brinkman Instruments, Westbury, NY, USA) for 1 hr. The DNA pellet was then reconstituted in ddH_2O (10 µL) and vortexed for 1 min prior to CGE.

3.3 Results and Discussion

3.3.1 LDR Modeling Samples Using CGE

Modeling samples were made using synthetically generated DNA fragments labeled on their 5' ends with fluorescein to mimic LDR- generated products in the presence of excess LDR primers. The fluor-K-*ras* C12.2A (25 bp) oligonucleotide was used at 10, 100 and 1,000 times molar excesses of the synthetic LDR products (44 and 51 bp oligonucleotides). Considering a sample of one mutated DNA for every 20 wild-type sequences and an LDR cocktail consisting of three discriminating primers (500 fmol each – 1,500 fmol total) and 20 thermocycles, the enzymatic reaction can

Table 3.2. Results from model studies of an LDR assay using 5T5C, eCap and POP5 matrices with CGE. Efficiency (plate numbers, N) was calculated for the 25 (unligated primer model), and the 44 and 51 bp fragments (ligation product models). Resolution was calculated between the 25 bp and 44 bp oligonucleotides as well as for the 44 and 51 bp LDR product models.

	N ($\times 10^6$) (plates/m)			R	
	Disc	LDR 44	LDR 51	Disc/44	LDR 44/51
5T5C					
1:10	0.54	0.97	1.01	18.04	10.03
1:100	0.35	1.12	1.21	16.29	12.06
1:1000	0.17	1.65	1.75	15.64	17.05
eCap					
1:10	0.58	0.56	0.43	18.78	4.56
1:100	0.45	0.69	0.92	13.06	7.40
POP5					
1:10	0.13	0.10	0.14	9.44	1.87
1:100	0.05	0.73	1.08	5.46	5.57

potentially yield 500 fmol of LDR product if 100% ligase efficiency is achieved using optimal conditions. Because 500 fmol of discriminating primer (one allele) are

consumed in the reaction, this results in a 1:2 ratio of LDR product to discriminating primer (500 fmol LDR product to 1,000 fmol discriminating primer). In an 1:1,000 (mutant:wild-type) sample, LDR should produce ~1:150 molar excess of discriminating primer. If the ligation efficiency per cycle is lower than 100%, the ratio of LDR product to unligated primers is much lower than that calculated here.

Modeling samples were analyzed under optimized conditions for CGE using the 5T5C capillary, the POP5 separation matrix and the eCap matrix. Efficiencies (plate numbers) were calculated for the 25 bp discriminating primer as well as the 44 and 51 bp synthetic LDR products. Resolution was calculated between the discriminating primer and the 44 bp product, as well as between the 44 and 51 bp products. These results are summarized in Table 3.2.

3.3.1.1 5T5C Analysis Using Synthetic Models

Using the 5T5C capillary, injection times required to load sufficient amounts of the 44 and 51 bp products were 20 s, 45 s and 75 s for the 1:10, 1:100 and 1:1,000 LDR product model to discriminating primer (25 bp) molar ratio, respectively. Baseline separation of all fragments was achieved in less than 45 min as shown in Figure 3.3 for the 1:100 modeling sample. In some cases, efficiencies were better than 1×10^6 m^{-1}, but in no case was it less than 1×10^5 m^{-1}. Inspection of the results shown in Table 3.2 indicated that the plate numbers decreased for the 25 bp oligonucleotide with decreasing amounts of the 44 and 51 bp ssDNAs due to the longer injection times used. This resulted in reduced resolution between the 25mer and 44mer. However, the opposite trend was generally observed for the 44mer and 51mer, with higher plate numbers observed as the molar ratio of 25mer to the 44/51-mers was increased.

A difficulty encountered when using this gel was the rapid rate of column degradation, which appeared to be a result of longer injection times required to load sufficient amounts of the longer oligonucleotides of lower concentration for detection. In addition, since the gel could not be removed from the capillary, the entire capillary needed to be replaced following column failure. Manufacturer supplied information reported lifetimes greater than 20 hours. However, in our case, we found that we could perform only 10 separations before the capillary displayed significant degradation in performance (~10 h of operation).

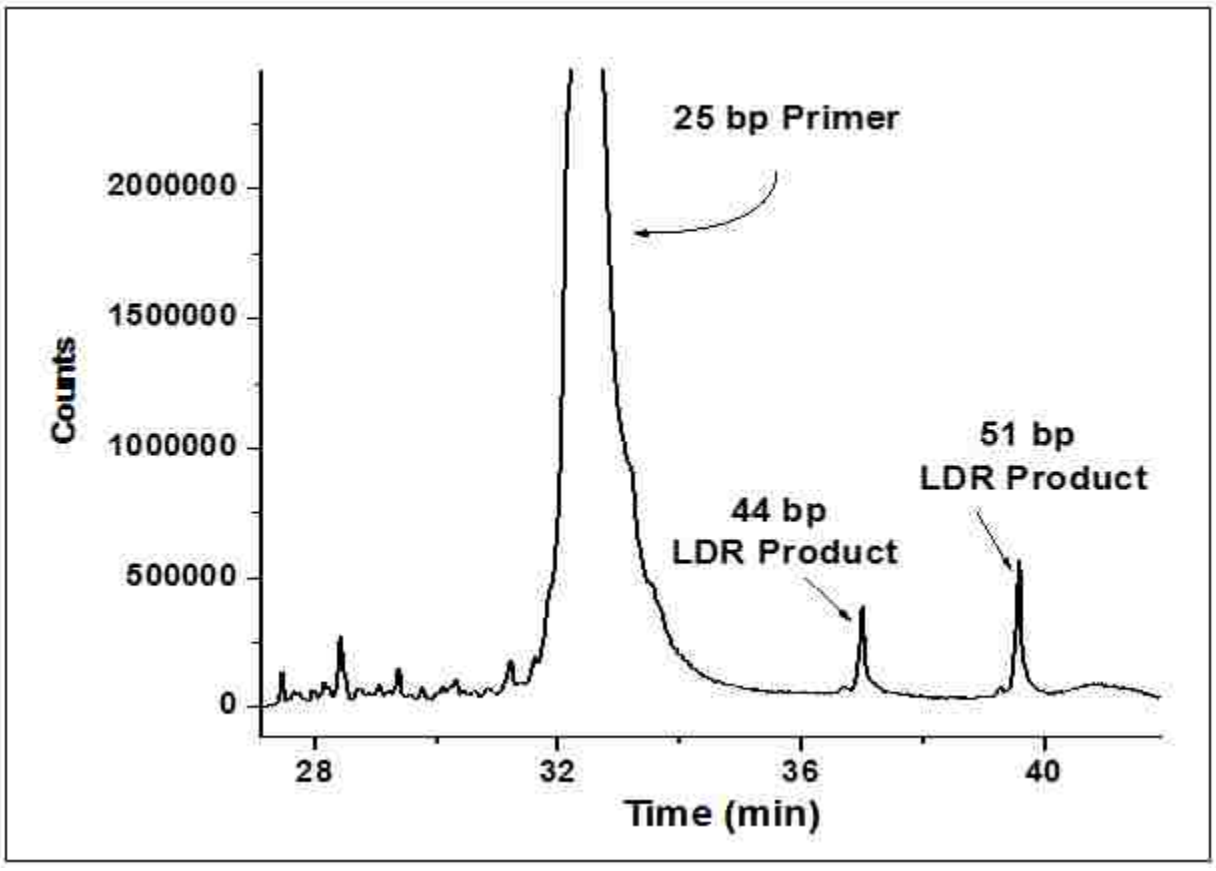

Figure 3.3. Analysis of a 1:100 modeling sample using the 5T5C gel. The capillary column was 36 cm in length (30 cm effective length) and it was maintained at 25ºC with a run voltage of 9 kV and the sample was injected for 45 s at 10 kV.

3.3.1.2 eCap Analysis

Analysis using the eCap gel column provided baseline resolution of all fragments in less than 30 min. Similar trends in plate numbers and resolution were observed for this gel matrix as that found with the 5T5C gel (see Table 3.2), with higher excesses of the 25mer producing reductions in the resolution between the 25mer and 44mer as well as smaller plate numbers for the 25mer. The model LDR products were well resolved in the 1:10 and 1:100 samples (R = 4.56 and 7.40, respectively) and discrimination was possible from the excess primer as shown in Figure 3.4 for the 1:100 sample.

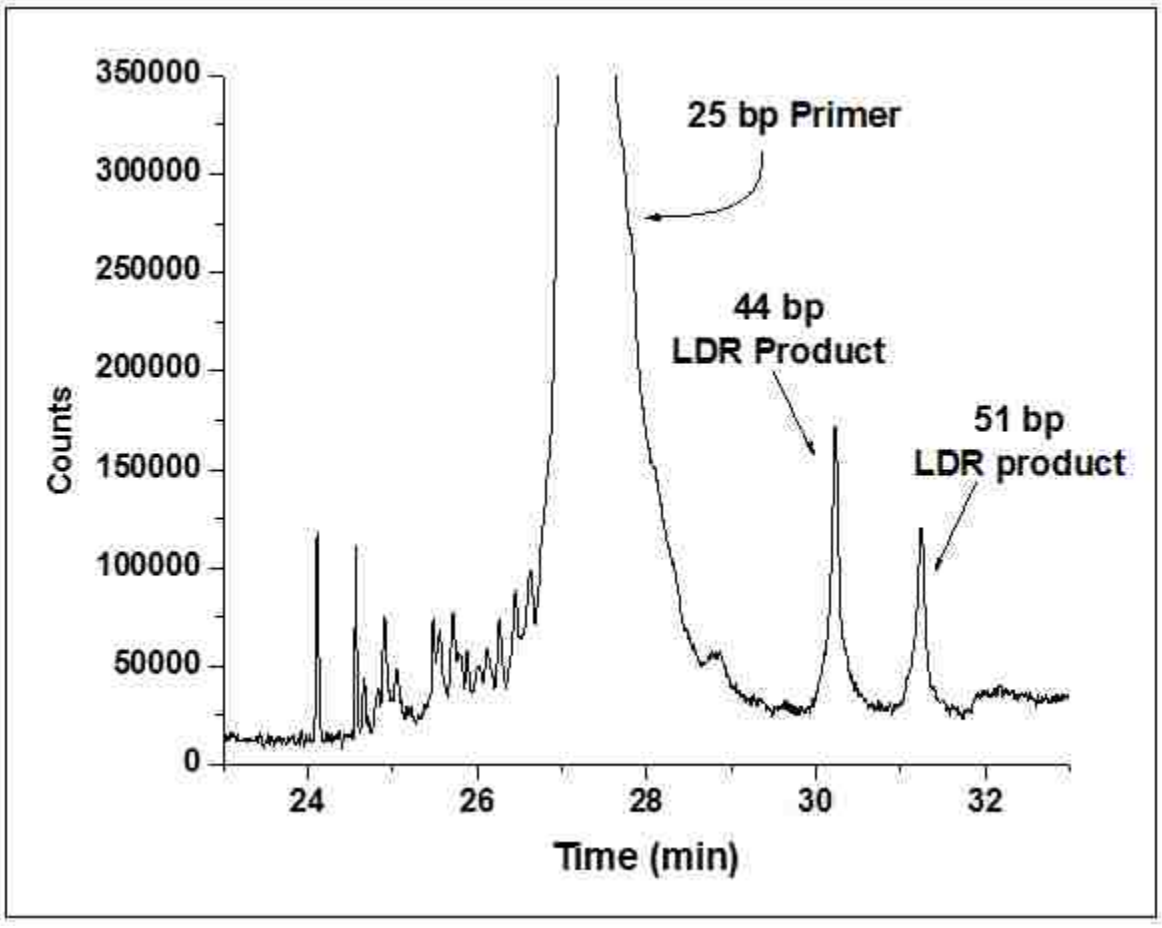

Figure 3.4. Analysis of a 1:100 modeling sample using the eCAP gel. The neutrally coated eCAP capillary was run at a voltage of 11.1 kV with the capillary maintained at 30 ºC. Electrokinetic injection was 20 s at 10 kV.

Interestingly, we were unable to load sufficient amounts of the 44 or 51 bp oligonucleotides when the 25mer was in 1,000-fold molar excess even when using electrokinetic injection times up to 5 min. This may indicate that severe injection biases occurred using this eCap matrix as compared to the 5T5C capillary. As such, analyzing samples with high molar ratios of short oligonucleotides cannot be accomplished using this linear polyacrylamide gel.

3.3.1.3 POP5 Analysis

Analysis using the POP5 separation matrix was also able to separate electrophoretically the LDR models and discriminating primer at the 1:10 and 1:100 molar ratios (see Figure 3.5 for the 1:100 sample). As can be seen from inspection of Table 3.2, the POP5 gel generally provided less plates and poorer resolution for these short ssDNAs compared to the eCAP and cross-linked gels. Severe loss in resolution and efficiency resulted in subsequent injections if the matrix was not replaced after each run. Injection times were 5 and 45 s for the 1:10 and 1:100 samples, respectively, while injections of up to 5 min were unable to load sufficient amounts of the LDR models for the 1:1,000 sample, similar to that observed for the eCAP gel.

3.3.2 CGE analysis of PCR/LDR Samples

Considering the severe injection biases encountered for injecting samples electrokinetically with high molar excesses of unligated primers using the eCap and POP5 separation matrices, LDR samples were analyzed using the 5T5C capillary, the results of which are shown in Figure 3.6 (1:100 G12V mutant to wild-type) and Figure 3.7 (1:1,000 G12V mutant to wild-type). In all cases, the LDRs were cold ethanol precipitated to remove excess salts prior to capillary injection to assist in loading the ssDNAs. The injection time for the 1:100 sample was 40 s while the injection time was

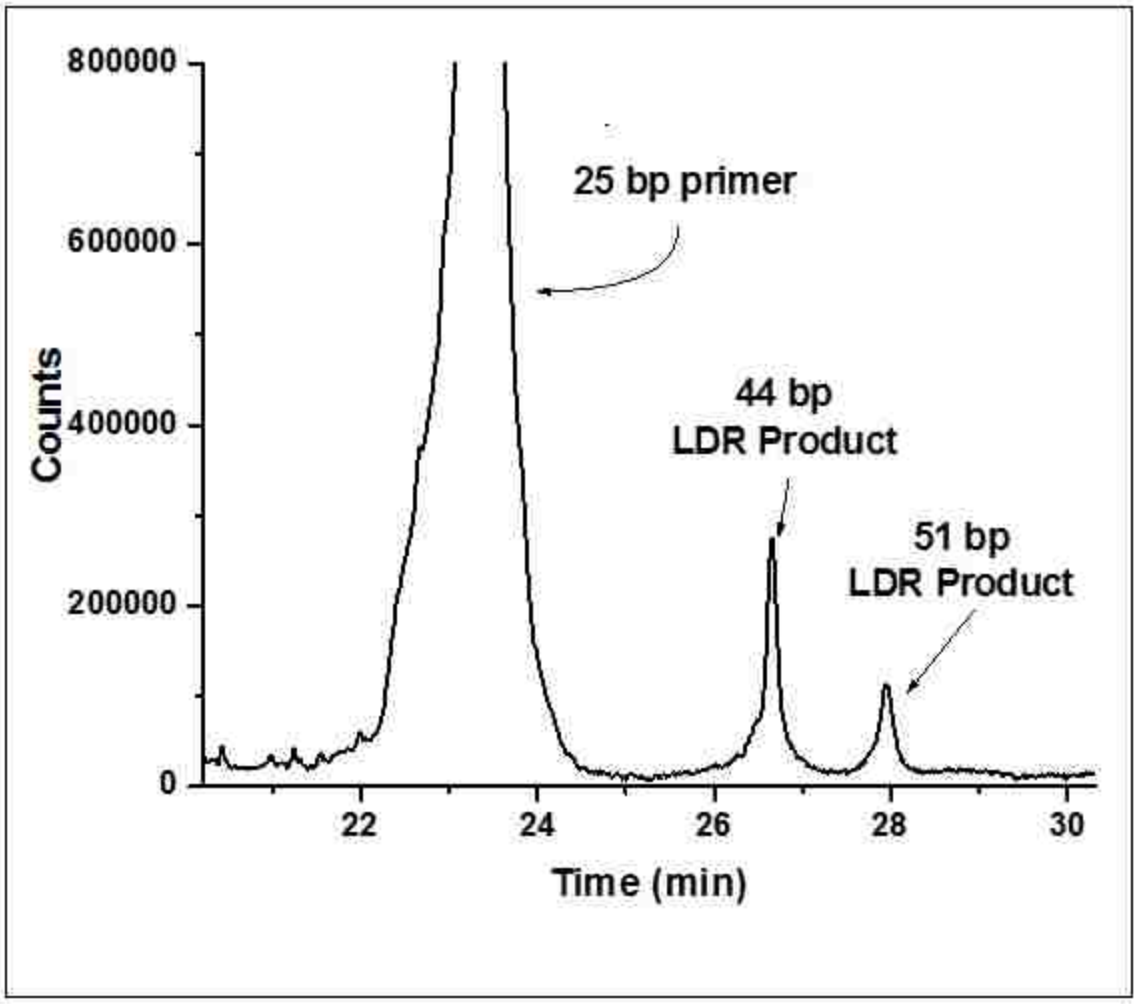

Figure 3.5. Analysis of a 1:100 modeling sample using the POP5 matrix. The capillary column was 36 cm in length (30 cm effective length). The POP5 analysis of 1:100 modeling sample consisted of a bare silica capillary with a run voltage of 7.7 kV and maintained at 50 ºC. The sample was injected for 45 s at 10 kV.

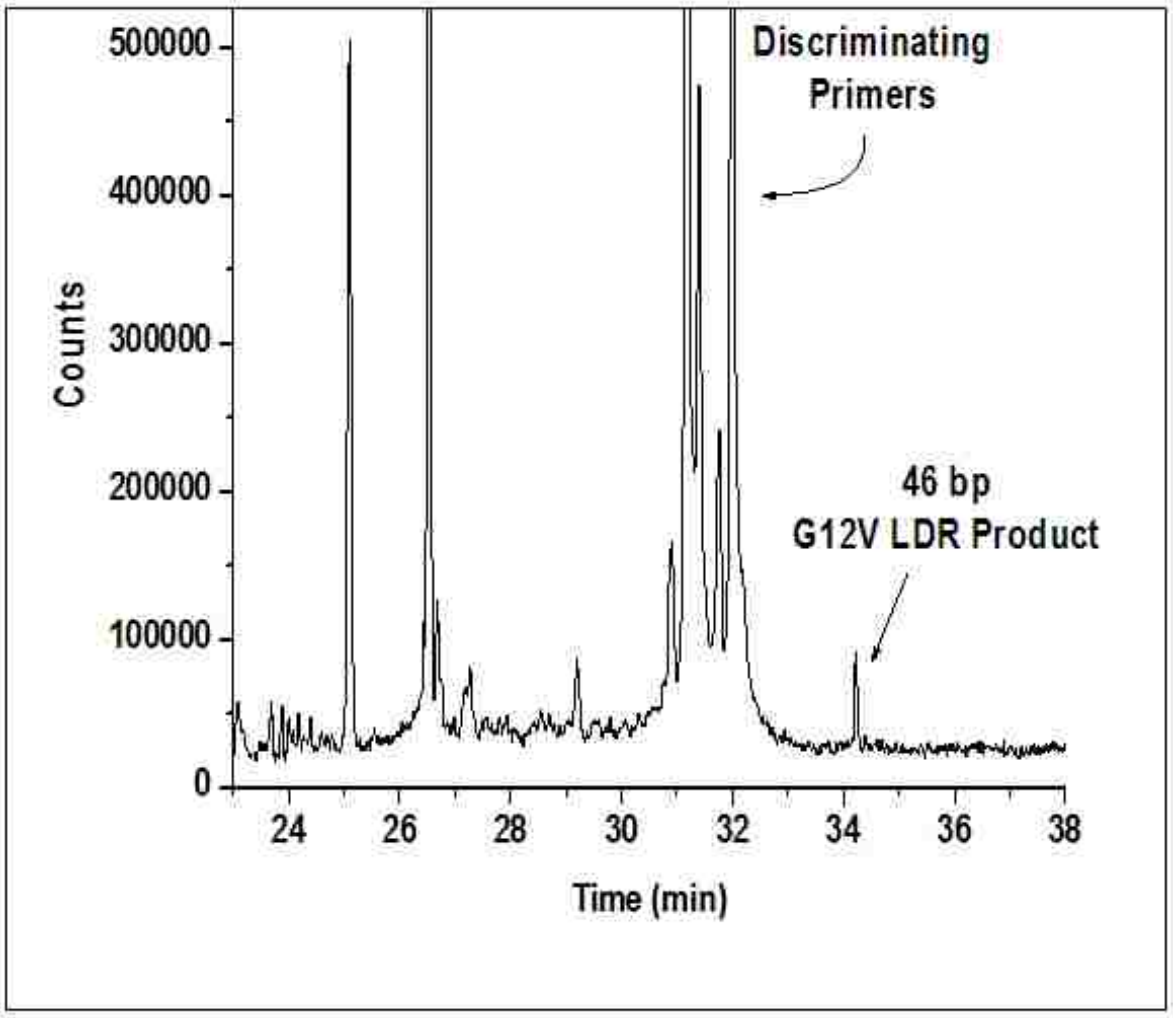

Figure 3.6. Analysis of a G12V 1:100 (mutant to wild-type) LDR sample. Electrophoretic conditions were the same as those listed in Figure 3.3 for the 5T5C column except that injection was for 40 s.

increased to 90 s for the 1:1,000 sample. With decreasing amounts of mutant template, resolution between the LDR product and unligated primers decreased similar to our model studies. The loss in resolution resulted from increased loading of the unligated primers due to the longer injection times required and the biased injections favoring loading of the shorter oligonucleotides. In both cases, the analysis required 36 min to generate sufficient plates to resolve the LDR product from unligated primers. For the 1:100 mutant to wild-type sample, the resolution between the unligated primers and

LDR product was calculated to be 3.14 and decreased to 1.51 in the 1:1,000 sample.

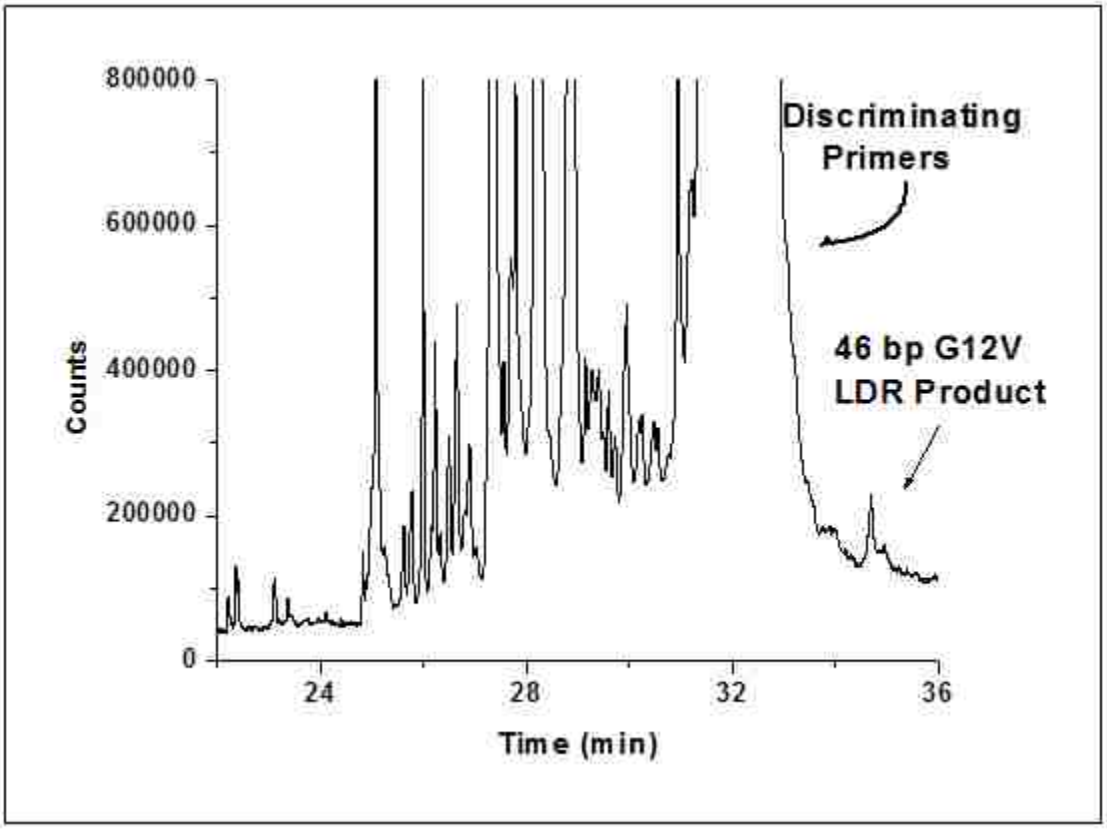

Figure 3.7. Analysis of a G12V 1:1,000 (mutant to wild-type) LDR sample. Electrophoretic conditions were similar to those for the 5T5C column in Figure 3.5 except injection was for 90 s.

3.3.2.1 Optimization of Electrophoretic Conditions Using Modeling Samples and Microelectrophoresis

The matrices (linear entangled polymers and cross-linked gels) were problematic for the detection of point mutations using LDR in the capillary format due to severe injection biases, which required desalting of the LDR prior to gel loading. These injection biases were particularly problematic when the mutant strands were significantly lower in abundance compared to the wild-types. Injection biases could potentially be minimized in a microelectrophoretic format by incorporating cross “T” injectors that

provide volume-based injections free from significant electrokinetic biasing effects. In addition, the smaller formats associated with microchip electrophoresis could allow higher sample throughput by reducing electrophoretic development time. Therefore, we evaluated the feasibility of using a polymer-based microelectrophoresis chip for analyzing LDR samples containing gene fragments of low abundance with point mutations of clinical significance.

We investigated three different gel matrices for use with the PMMA microchip; POP5, eCAP and a 4% linear polyacrylamide (LPA) containing 7 M urea as the denaturant. In the case of the eCAP and LPA matrices, the PMMA microchip was used without a coating to suppress its intrinsic electroosmotic flow, which has been measured to be 1.4 x 10^{-4} cm^2/Vs [22]. The POP5 gel contains a buffer, which suppresses the EOF in conventional capillaries (fused silica), but the ability of this buffer to suppress the EOF in polymer-based microchips has not been demonstrated. To test each matrix, an equal molar solution of the 25 and 51 bp synthetic oligonucleotides were electrophoresed in the microchip using an effective separation length of 3.5 cm. In all cases, the separations were performed at room temperature using an electric field strength of 113 V/cm. The 4% LPA matrix provided the best resolution between these synthetic oligonucleotides with R = 1.64, while the POP5 and eCAP matrices provided resolutions of 1.20 and 1.19, respectively. In all three cases, baseline resolution was achieved for these short single-stranded oligonucleotides, but the 4% LPA matrix provided the highest resolution, which should make it appropriate for analyzing the ligated products when the copy number of the mutants is significantly lower than the wild-types. The plate numbers for these gels were comparable (~3.5 x 10^5 m^{-1}) indicating that the enhanced resolution observed for the 4% LPA matrix was due

primarily to its higher selectivity for these short oligonucleotides. The injection conditions used in these examples was 35 s using 500 V applied between the sample (A) and waste (B) reservoirs (see Figure 3.2A).

For the eCAP and LPA matrices, the PMMA separations were performed in uncoated microchannels producing acceptable results in terms of plate numbers and migration times. Recently, it has been reported that successful DNA sequencing of short tandem repeats (read lengths < 400 bp) can be obtained in polymer-based microchips of 4.5 cm in length, but a coating procedure was required to secure favorable results [23]. The coating procedure, which was used specifically to suppress the EOF of the microchip, consisted of 2% w/v poly-dimethylacrylamide/diethylacrylamide in water, with a 2 h incubation time. Our results indicate that for the separation of short oligonucleotides as in the present case, no coatings for EOF suppression are required for PMMA microchips. However, inclusion of EOF suppression coatings may be necessary in these chips for longer oligonucleotides due to their smaller apparent electrophoretic mobilities. In addition, the results obtained by the eCAP and POP5 gel were similar in terms of their plate numbers and migration times indicating that the POP5 gel and buffer system may not possess dynamic coating capabilities in polymer microchips as they do for fused silica capillaries. This conclusion is not too surprising given the differences in surface chemistries between these materials.

Care was taken to optimize the injection conditions as well to prevent significant band broadening resulting from sample leakage into the separation channel while at the same time allowing complete filling of the fixed volume injector of the shorter oligonucleotide (*ie*, LDR product). It was found that injections less than 15 s (using a

500 V injection voltage) were insufficient for entirely filling the injection cross with the sample while injections longer than 25 s resulted in excessive band broadening as noticed by reductions in plate numbers. Using optimized injection conditions (t_{inj} = 20 s; V = 500 V) and an effective separation length of only 3.5 cm provided sufficient plate numbers (N = 1.51 x 10^6 and 1.67x 10^6, for the 25 and 51 bp oligonucleotides, respectively) to adequately resolve the generated LDR product from unligated primers even when the mutant allele was in low copy number compared to the wild-type.

3.3.3 Microchip Electrophoresis of LDR Samples

In Figure 3.8 (A) and (B) are shown the microelectrophoretic results of an LDR reaction containing the G12D mutant sequence only (A) and the wild-type only (B). Also, a 1:100 molar ratio of mutant to wild-type alleles are shown in Figure 3.9. In all cases, the LDR reaction was loaded directly into the appropriate reservoir of the chip without subjection it to a desalting procedure. As can be seen, in the case of the wild-type only one peak appears in the electropherogram most likely arising from unligated primers. In the case of the mutant allele, a new band appears at longer migration times compared to the primer peak, resulting from a successful ligation of the common and discriminating primers producing a 44 bp product in this case.

In Figure 3.9 is shown the results of a ligation assay where the wild-type allele was 100-fold higher in concentration compared to the mutant allele. Using these electrophoretic conditions, the LDR product could be resolved from unligated primers in under 120 s with a resolution of 1.3, approximately 17-times faster than that observed using CGE (see Figure 3.6) with only a slight reduction in resolution.

Microchip separations of two LDR mutations (G12D and G13D) generated in a single reaction at low abundance levels of 1:10 and 1:100 mutant to wild-type ratios

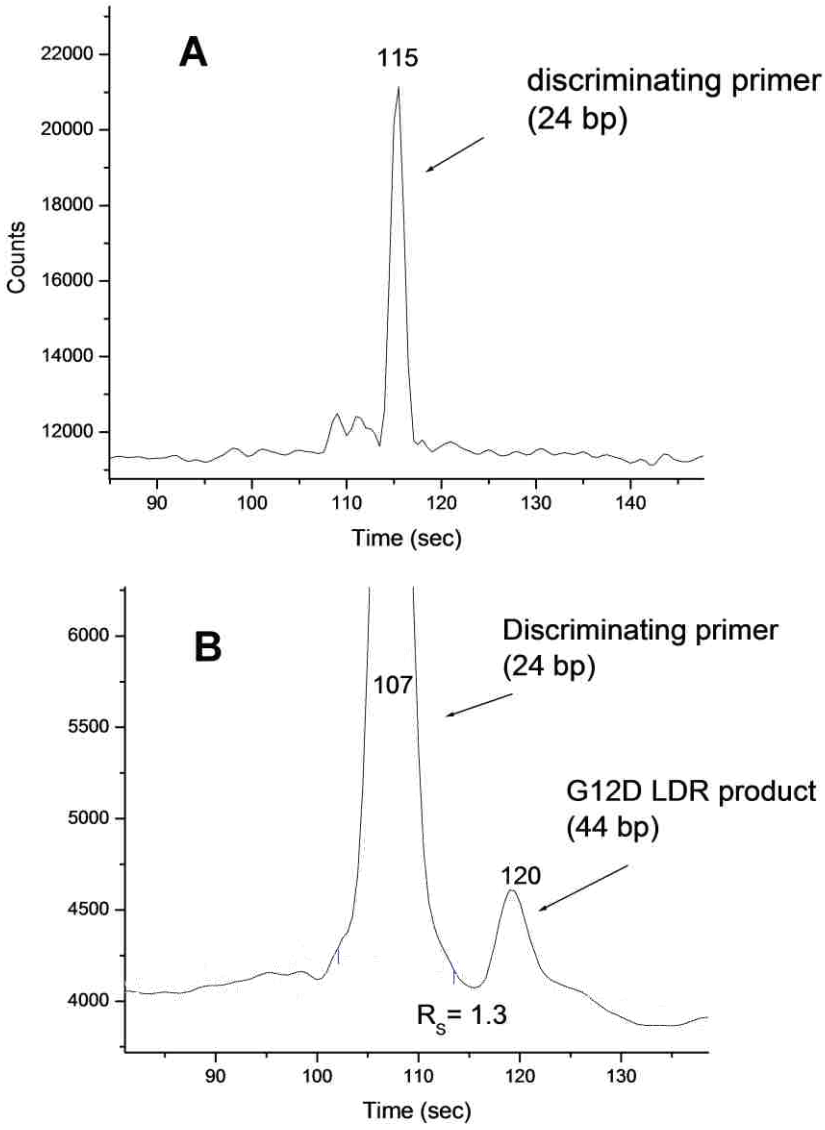

Figure 3.8. Microelectrophoresis analysis of the G12D LDR product. **(A)** G12D mutant LDR sample with no wild-type sequences, **(B)** the wild-type control sample (no mutant sequences). The electrophoresis was run using a 4% LPA sieving matrix with 7 M urea as the denaturant. The matrix was conditioned by pre-electrophoresis at 1 kV for 5 min. The sample was injected for 20 s at 500 V and electrophoresed at 113 V/cm using pullback voltages on the waste and sample reservoirs of 210 and 350 V, respectively. The effective channel length was 3.5 cm (total length 10 cm).

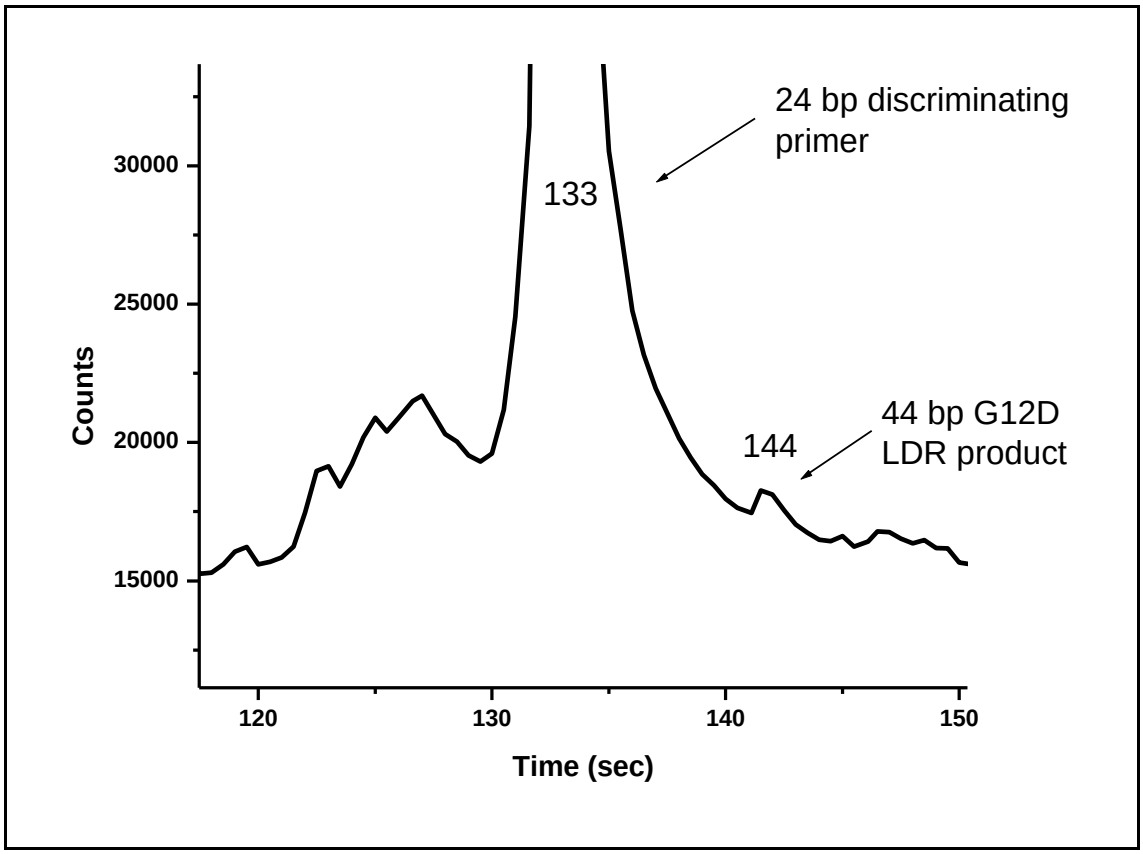

Figure 3.9. Microelectrophoresis analysis of a 1:100 mutant (G12D) to wild-type ratio. The electrophoresis was run using a 4% LPA sieving matrix with 7 M urea as the denaturant. The matrix was conditioned by pre-electrophoresis at 1 kV for 5 min. The sample was injected for 20 s at 500 V and electrophoresed at 113 V/cm using pullback voltages on the waste and sample reservoirs of 210 and 350 V, respectively. The effective channel length was 3.5 cm (total length 10 cm).

were later conducted with a custom, moderately viscous polymer solution composed sparsely cross-linked ultra-high molar mass polyacrylamide "nanogel" [24]. The polymer was reconstituted in 1xTTE buffer containing 7 M urea to a 2.75% (w/v) concentration. As seen in Figure 3.10, using a field strength of 125 V/cm and the same detection length of 3.5 cm, both LDR products for the 1:10 abundance ratio could be resolved within ~4.5 min. Similarly the PMMA microchip separation of the 1:100 ratio LDR sample, which is within range of biologically relevant levels, was successful.

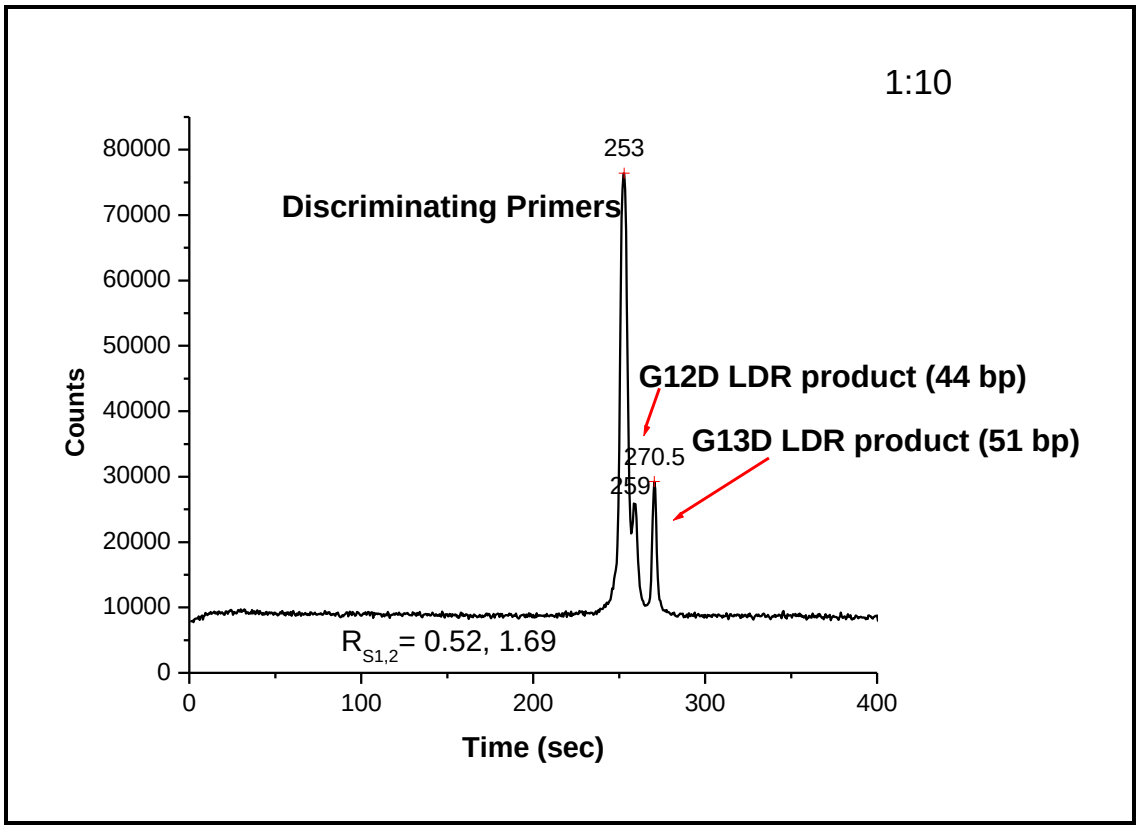

Figure 3.10. PMMA microchip separation using a 2.75% (w/v) nanogel for the sorting of multiplexed LDR of G12D and G13D products at an abundance ratio of 1:10 (mutant to wild-type) sample. The polymer was reconstituted in 1xTTE buffer containing 7 M urea. The separation was conducted at 125 V/cm after a 35 s injection at 350 V/cm with a detection length of 3.5 cm.

As seen in Figure 3.11, using the same parameters as listed for the microchip separation of the 1:10 LDR sample in Figure 3.7, both products were present after ~4.5 min of development time.

3.4 Conclusions

Several ssDNA separation matrices for CGE were evaluated for their ability to detect low abundant point mutations using LDR. The eCap and POP5 replaceable matrices provided successful separation of model LDR products at 1:10 and 1:100 molar ratios to the synthetic unligated model primers with sufficient resolution in less than 35 min, but with lower resolution compared to the 5T5C cross-linked gels. The eCap matrix

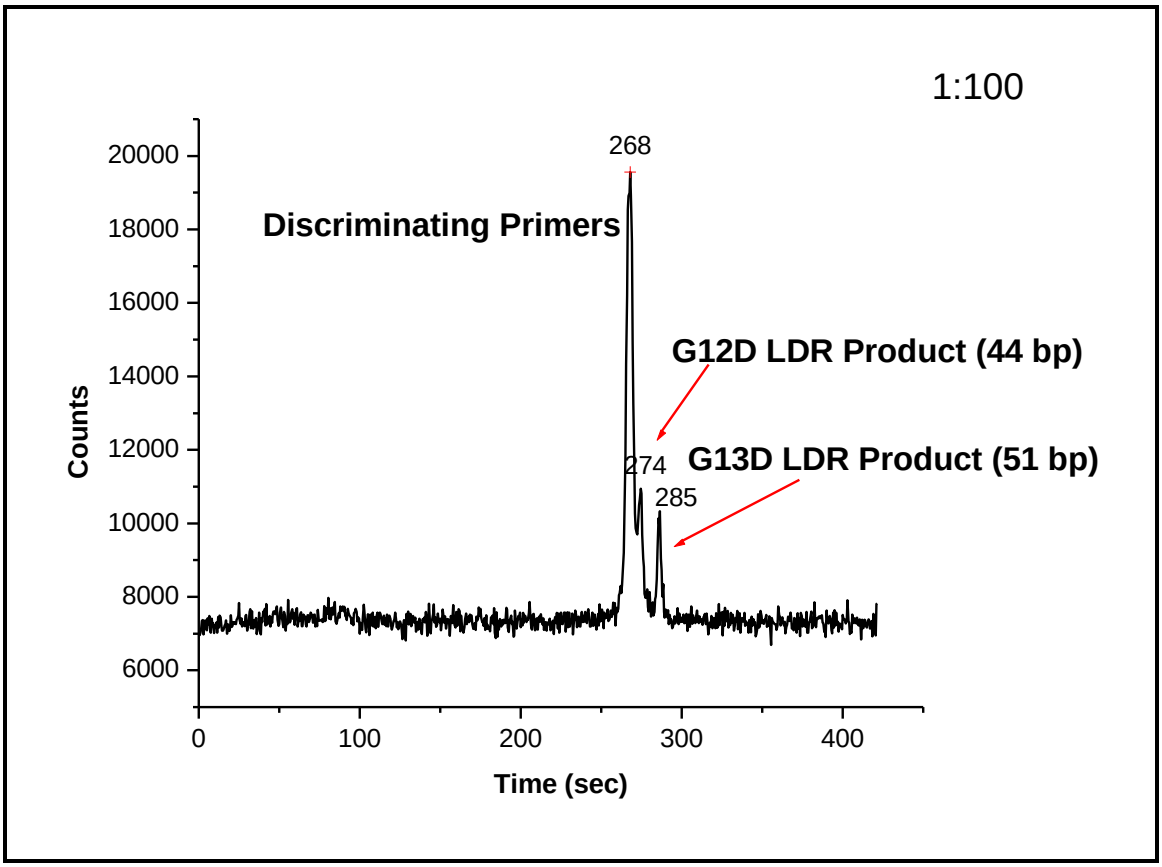

Figure 3.11. PMMA microchip separation using a 2.75% (w/v) nanogel for the sorting of a multiplexed LDR of G12D and G13D products at an abundance ratio of 1:100 (mutant to wild-type). Run conditions were the same as those listed in Figure 3.7.

required a neutrally coated capillary to suppress the EOF or prevent solute-wall interactions, which would adversely affect electrophoretic efficiency. The POP5 gel provided dynamic coating capabilities of the capillary that permitted use of a bare fused silica capillary with no pretreatment necessary. While these gels provided baseline resolution for these model LDR samples of relatively low molar excess of unligated model primers, they suffered from severe injection biases and appeared inadequate for the detection of samples containing an 1,000-fold molar excess of unligated primer. Under the conditions listed here, sufficient loading of the LDR products was not possible.

The 5T5C cross-linked capillary provided analysis of molar ratios up to 1:1,000 of LDR product to unligated primers with better resolution and efficiency as compared to the eCap and POP5 matrices; however, increased column deterioration resulted from unusually long injections required to sufficiently load LDR products. The covalent bonding of the polyacrylamide to the capillary wall prevented matrix replacement.

Microchip electrophoresis using uncoated polymer substrates demonstrated potential for providing rapid analyses of LDR products. The volume-based injection associated with these formats have been shown to provide representative loading of various sized DNA fragments where electrokinetic injection in capillaries are subject to biases favoring high mobility fragments and salts [21]. Initial microdevice separations using a 4% LPA separation matrix were favorable in providing high efficiency separations with adequate resolution in the size range of interest in as little as 120 s, nearly 17-times faster than capillary gel formats. In addition, sample processing prior to the electrophoresis was simplified by eliminating the need of desalting using ethanol precipitation or other similar techniques.

3.5 References

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Chapter 4: Ligase Detection Reaction Mutation Screening via Free Solution Electrophoresis in a Polymeric Microfluidic Device*

4.1 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 samples from CRC patients found only 0.01% to 1.7% of the 47,800 APC molecules collected per mL of plasma contained mutant alleles [11]. Even at the primary tumor site, the predominant cells are normal stromal cells (wild-type; WT) found at levels as high as 70% [12]. Successful and accurate genotyping for CRC therefore not only depends on the utilization of a multiplexed analysis format, but also the ability to detect low copy numbers of mutated sequences in a vast sea 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

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interest, the amplicon is mixed with two complementary primers (a common primer and 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 if and 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 this primer ligation-based scheme has allowed successful LDR implementation onto DNA microarrays as well as electrophoretic sorting platforms [12, 13, 21].

Typically, stringent measures have been required to sort products generated from LDRs, including cross-linked slab gels or capillary gel electrophoresis (CGE), which typically require >1 h to achieve separations with adequate resolution [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 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 their 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 DNAs 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 ~180 bases have been demonstrated and this electrophoretic approach has proven useful for sequencing and also single base extension (SBE) 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 poly(methyl methacrylate), 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. In 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 (~85 s) of LDR/drag-tag conjugates (LDR-dt) were achieved in PMMA microchips using only a Tris-based buffer containing an electroosmotic flow (EOF) suppression additive.

4.2 Methods and Materials

4.2.1 DNA Template Preparation

Genomic DNA was extracted from cell lines of known *K-ras* oncogenic expression associated with the onset of CRC (HT-29, wild-type; SW1116, G12A; SW620, G12V; LS180, G12D; and DLD1, G13D) (ATCC, Manassas, VA) using a Qiagen DNeasy kit (Valencia, CA). 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 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) was added under hot-start conditions and amplification was achieved by thermally cycling for 35 cycles at 95ºC for 30 s, 60ºC for 1 min, 72ºC for 1 min with a final extension at 72ºC for 3 min.

4.2.2 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 Figure 4.1) was achieved using a solid-phase submonomer synthetic protocol, which has previously been described in detail and was accomplished 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) and contained a C-6 spacer and thiol linker modifications on their 5' terminus to facilitate the attachment of the drag-tags (see Table 4.1). Common primers were synthesized bearing either FAM (excitation/emission = 492/517 nm; IDT) or IRD-800 (excitation/emission = 780/816 nm; LI-COR, Lincoln, NE) fluorescent labels (z) on their 3' ends with phosphorylation on their 5' termini (see Table 4.1). The thiol groups on the discriminating primers were reduced by incubating the primers with a 20x molar excess of TCEP, tris(2-carboxyethylphospine; Acros Organics, Morris Plains, NJ) in pH 7.2 100 mM sodium phosphate buffer at 40ºC 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 (21bp), and vice versa, to generate the greatest possible resolution between the LDR products (see Table 4.1) [35].

4.2.3 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 1x 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) and 25 nM of each discriminating and common primers taken to a final volume of

20 μL with ddH_2O. After an initial 2 min denaturation at 94ºC, 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 94ºC and 2 min at 65ºC. The reaction was then quenched by rapid cooling to 4ºC followed by the addition of 0.5 mM EDTA. Prior to electrophoretic analyses, samples were desalted using CentriSep spin columns (Princeton Separation, Adelphia, NJ).

Table 4.1. Design of Primers for LDR-ELFSE.

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

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

4.2.4 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) x 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) using an embossing system consisting of a PHI Precision Press model number TS-21-H-C (4A)-5 (City of Industry, CA) 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.

4.2.5 Laser-induced Fluorescence (LIF) and High Voltage Control System

A laser-induced fluorescence (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) was directed onto a dichroic mirror, which reflected the excitation beam through a 40x microscope objective (Newport, Fountain Valley, CA) 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) and an 800 nm long-pass filter (Edmund Scientific, Barrington, NJ). The LIF signals were acquired

on a personal computer using a 32-bit counter/timer board (Model PCI-6601, National Instruments, Austin, TX).

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

4.2.6 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 1x TTE (89 mM Tris, 89 mM TAPS, 2 mM EDTA) and 7M 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 1x TTE (50 mM Tris, 50 mM TAPS, 2 mM EDTA) and 7M urea buffer containing 0.05% (w/v) methyl hydroxyethyl cellulose, MHEC, (Sigma Aldrich, St. Louis, Mo) 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.

4.3 Results and Discussion

4.3.1 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 Figure 4.1 and Table 4.1, 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 a successful ligation event 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 drag-tag presence, 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

4.1) 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.

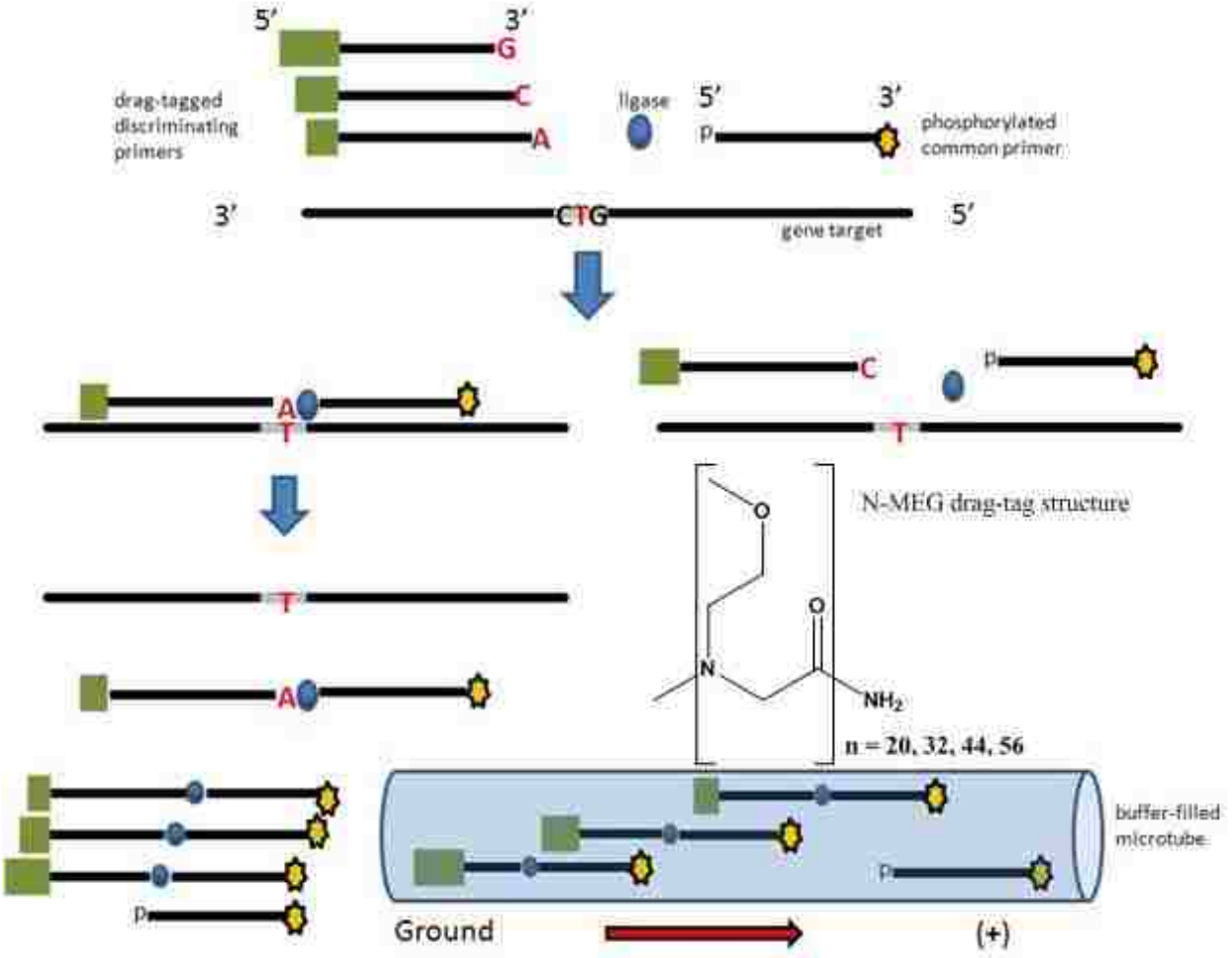

Figure 4.1. Diagram showing the generation of drag-tagged LDR products and their subsequent separation profile from a mixed population of these LDR-dt products. LDR primers having single base differences upon successful ligation are inversely paired with drag-tags of different sizes (largest drag-tag to smallest ligated-primer pair). Under an electric field, each LDR-dt conjugate 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 comprised of smaller oligonucleotides.

4.3.2 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 4.2 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 separation required ~11 min. Each product possessed a unique electrophoretic mobility yielding resolutions between the dye-labeled common primer and 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 conjugates 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 4.1).

In a direct comparison, the separation of the non-drag-tagged G12D LDR sample is shown in Figure 4.2. In this case, no LDR product was visible after the separation as the LDR product co-migrated with the unincorporated labeled common primers in free solution, thus illustrating the pronounced effects of 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 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 95ºC lead to partial hydrolysis of the maleimide linker between the primers and the drag-tags, which caused the formation of minor LDR product peaks with

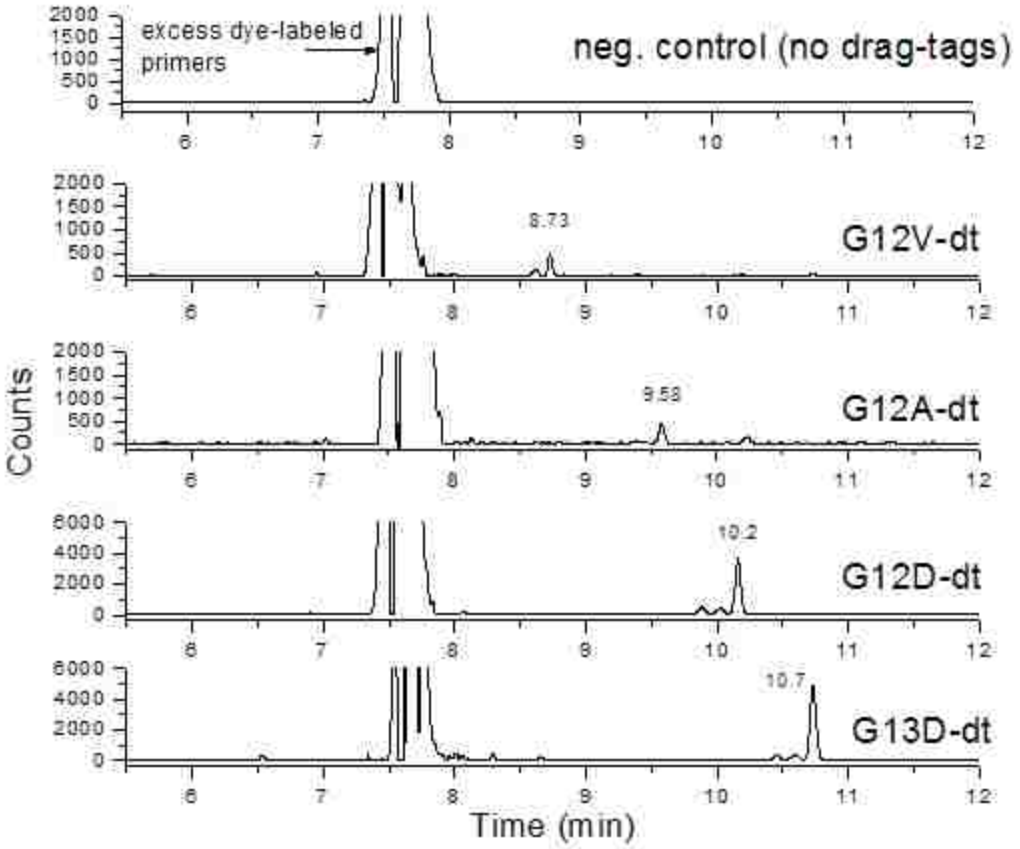

Figure 4.2. Free solution CAE electropherograms of positive control samples and multiplex LDR-dt samples that are probing four *K-ras* mutations. Four positive control LDR assays consisting of each mutagenic template paired with its respective discriminating (drag-tagged) and common primer. A negative control LDR assay excluding drag-tags was conducted and analyzed via FSCE for comparison. The reactions consisted of 1 nM of each mutagenic template with 25 nM each of the common and discriminating primers per reaction in 1x 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 1x TTE buffer containing 7 M urea and 0.5% POP6 (for EOF suppression) at E = 416 V/cm for 10 s for injection and E = 375 V/cm for the separation.

slight mobility shifts. 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 were enhanced by increasing their genomic template concentrations from 50 pmol to 100 pmol prior to PCR amplification. Additionally, subsequent reactions 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).

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 Figure 4.3 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 10-fold greater than the G12A and G12V mutant allele signals consistent with the relative abundance of these mutations.

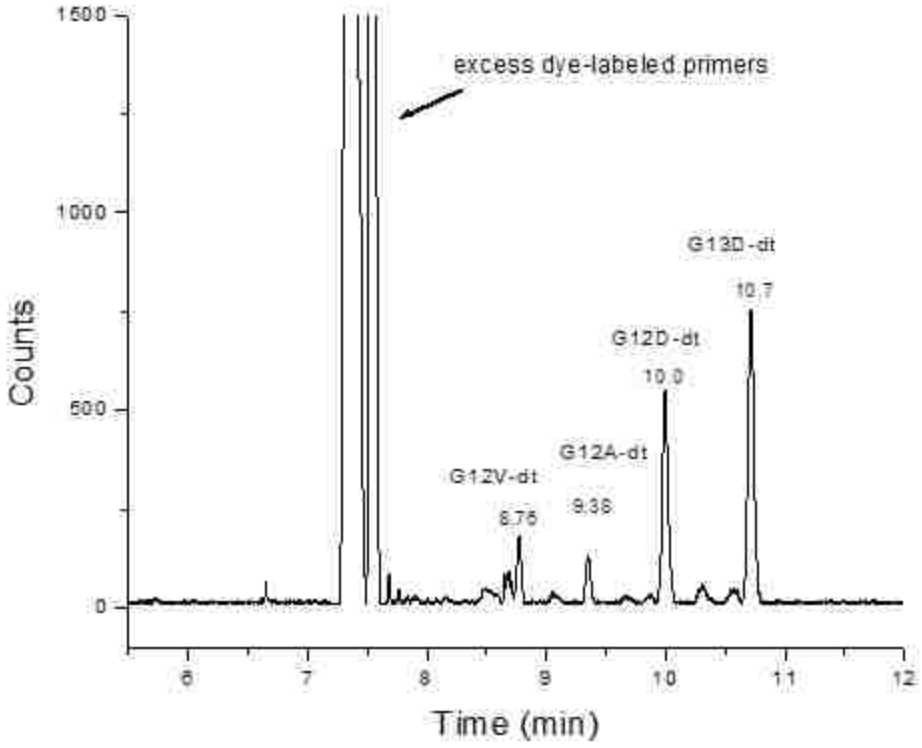

Figure 4.3. Free solution CAE electropherograms of positive control samples and multiplex LDR-dt samples that are probing four *K-ras* mutations. A FSCE separation of four *K-ras* LDR-dt products generated simultaneously in a single-tube reaction using the entire panel of mutant templates and LDR primers (see Table 4.1 for sequences). The reaction and run conditions were the same as listed in Figure 4.2.

4.3.3 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 4.4 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 ≤ 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 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 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 parameters, migration times were highly reproducible (e.g., T_m= 81 s ± 1.8 s for G12D-dt) over five successive runs as well as for chip-to-chip trial analyses.

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 Figure 4.4, except the genomic G12A

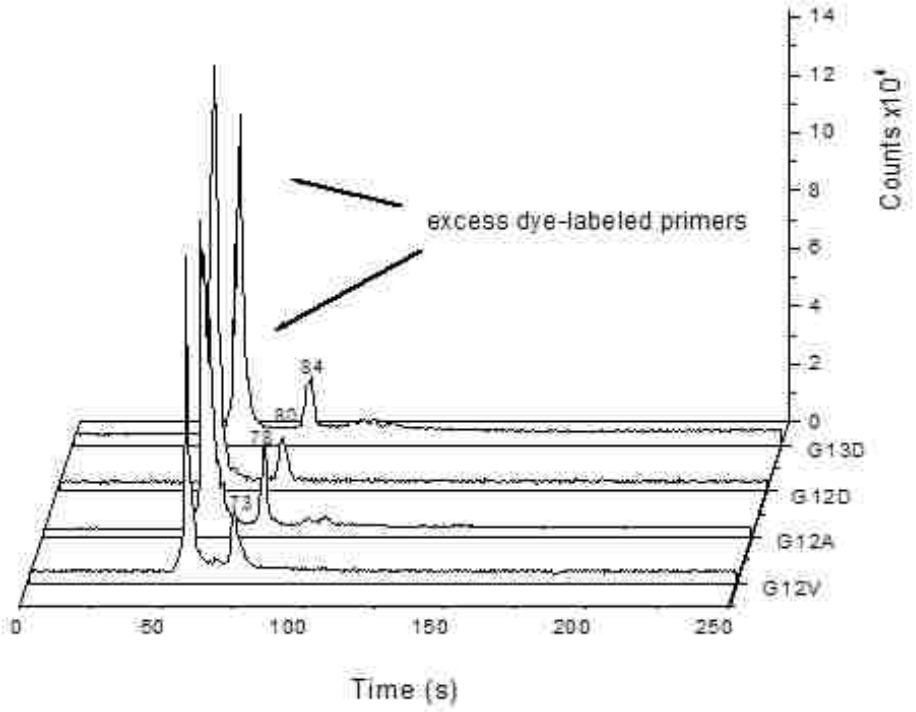

Figure 4.4. Microchip FSCE separations of positive control *K-ras* LDR-dt reactions. The custom-micro-milled PMMA microchip was hot embossed from a brass master (9.5 cm length, 30 µm width and 70 µm depth) utilized to separate the LDR-dt products generated using the same conditions given in Figure 4.2. The volume-defined cross injection (105 pL) and separation were performed in 1x 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.

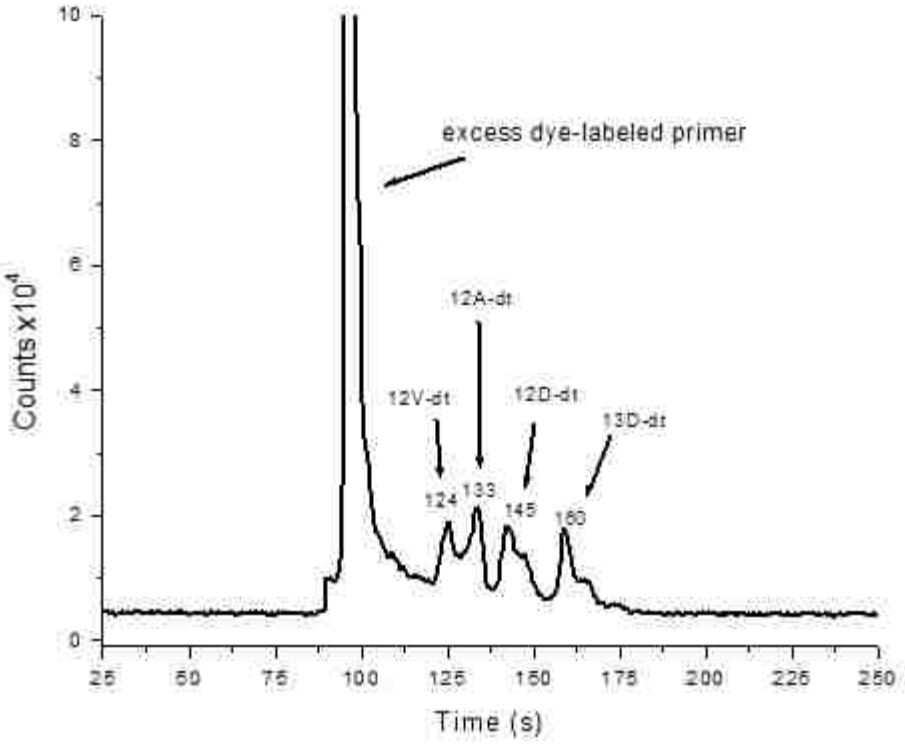

Figure 4.5. Microchip FSCE separation of a multiplexed LDR generating LDR-dt products. Following PCR of the mutagenic *K-ras* loci in a single-tube reaction, a multiplexed single-tube LDR was conducted using the conditions stated in Figure 4.2 with an increase in genomic DNA for G12A and G12V templates to 100 pmol prior to PCR. The effective channel separation length was 6 cm and E = 450 V/cm were selected to optimize the resolution of all LDR-dt products. The other separation conditions were indentical to those described in Figure 4.4.

and G12V template quantities were increased from 50 pmol to 100 pmol prior to PCR amplification in order to provide peak intensities similar to the predominant G12D and G13D alleles. As seen in Figure 4.5, 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 to 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 a SNR = 7.1 (see Figure 4.6). Attempts to detect LDR-dt products lower than 1:100 were not successful due to the detection limit 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 for 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 are discerned by a follow-up allele-specific ligation.

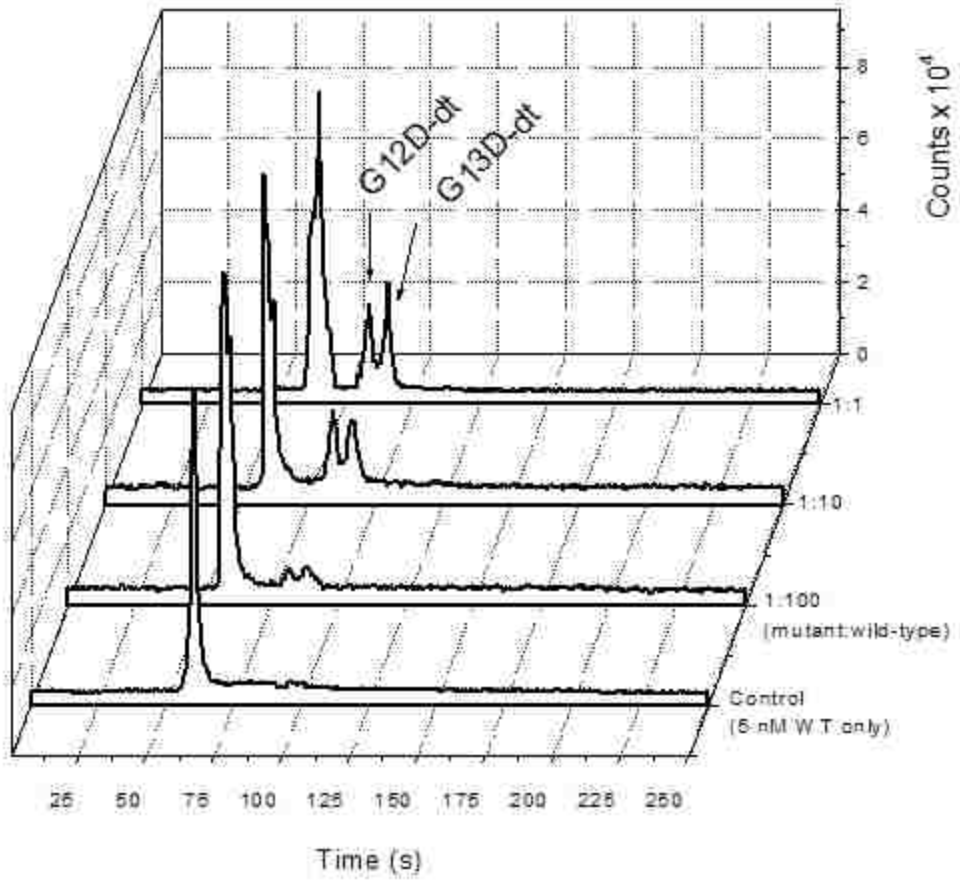

Figure 4.6. Microchip FSCE separations of multiplexed *K-ras* LDR-dt reactions probing the G12D and G13D mutant alleles with changes in the relative abundances of wild-type alleles with respect to the mutant alleles. The reaction compositions consisted of 5.0 nM WT template to 0.05, 0.5 and 5.0 nM of 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 Figure 4.4.

4.4 Conclusions

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 commercial CAE system and ~85 seconds 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 additions, 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.

4.5 References

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Chapter 5: Evaluating Low Frequency p53 Mutations Using an Endo V Mutation Scanning Assay and Microchip Electrophoresis

5.1 Introduction

An accumulation of genetic mutations stemming either from inherited or somatic alterations are ultimately responsible for spawning most cancer-related diseases. Overall, approximately 90% of all cancers are attributed to somatic mutations, ~20% are the result of germline mutations and ~10% involve both types [1]. The Human Genome Project (HGP) has resulted in a rapid progression of the identification and characterization of many deleterious genetic alterations that can be associated with a variety of diseases, including cancer [2]. This information has provided insights into the structure and function of oncogenes and tumor suppressor (TS) genes both of which play a pivotal role in tumorigenesis. Oncogenes produce growth and signal transduction factors that promote cell growth and proliferation [3]. Meanwhile, active TS genes, which are often found deactivated in my cancer-related diseases, regulate cell growth and initiate apoptosis in cells that cannot be repaired, but under certain circumstances, deactivated TS genes can be reactivated [4, 5].

The multiple and overlapping frequencies of many mutations associated with particular cancers must be accurately mapped and evaluated in clinical settings to realize diagnostic and prognostic metrics using these biomarkers. For example, *K-ras* mutations in codons 12 and 13 occur in 80 – 90% of pancreatic cancer and 35 –50% of colorectal cancers [6-8]; single nucleotide polymorphisms in BRCA1 and BRCA2 present at low frequencies (1 – 5%) are linked to significantly higher risks of developing breast, ovarian or prostate cancers for certain ethnic groups [9]. A locus in chromosome region 15q25 encompassing several genes - including three that encode

nicotinic acetylcholine receptor subunits (CHRNA3, CHRNA4, and CHRNA5) – have been found to account for 14% of 1,989 lung cancer cases, which were assessed in a recent study [10]. The location of these mutations are well known and thus, can be analyzed using mutation detection assays, such as allele specific ligation [11], allele specific PCR [12] or allele-specific hybridization [13].

The presence or likelihood of developing cancer-related diseases have also been related to the frequency or occurrences of sporadic p53 (TS gene) mutations [14]. The challenge of evaluating these mutations is that they are often inundated by excess wild-type DNA in clinical samples at early stages of development and also, their locus is not defined [15]. For instance, frequencies of sporadic p53 mutations in exons 5 – 9 can be as low as one mutant per 1,000 wild-type (WT) sequences [16] and over 22,000 p53 mutations in different human cancers have been recorded and compiled in an accessible database [17].

Several methods have been developed to detect sporadic mutations including hybridization analysis using high-density oligonucleotide arrays [18], denaturing high-performance liquid chromatography (DHPLC) [19, 20], single strand conformational polymorphism (SSCP) [21], denaturing gradient gel electrophoresis (DGGE) [22], heteroduplex analysis (HA) [23] and dideoxy-sequencing [24]. Unfortunately, none of these methods have the combined ability to provide low level detection when the mutated DNA is in a large excess of WT DNA and also, pinpoint the exact location of the sporadic mutations. Although dideoxy-sequencing can detect any single nucleotide polymorphism and determine its location, the procedure is often time-consuming and lacks the necessary sensitivity to detect low abundant mutant DNA in large excesses of WT DNA.

An approach for determining the approximate location of sporadic polymorphisms even in the presence of large excesses of WT DNA is the use of enzymes, such as T4 Endonuclease VII to cleave mutational sites within double-stranded DNA [25, 26]. However, these methods generally suffer from miscleavages leading to false signals, which limit their usefulness.

Recently, a one-step mutation scanning assay has been reported (see Figure 5.1), which employs *Thermotoga maritima* Endonuclease V (Endo V). Endo V is a mutation nicking enzyme that clips a duplexed DNA molecule containing mismatched base pairs (*i.e.,* heteroduplexes). Unfortunately, Endo V can nick dsDNA at matched sites as well that can be subsequently repaired using AK16D Taq ligase, which reduces background signals from incorrect DNA cleavage events [27]. This particular Endo V primarily recognizes and cleaves heteroduplexed DNA one base from the 3'-end with respect to the mismatch. Combined with a ligase detection reaction (LDR) [28, 29] using the *Thermus* species AK16D thermostable ligase, which displays up to a 5-fold better discriminatory power compared to other ligases [30-32], this Endo V/LDR strategy has been shown to provide sensitivity of up to 1:50 (Mutant to WT DNA) for the scanning of sporadic p53 mutations. Furthermore, this strategy employs universal PCR amplification steps making it amenable to multiplexing [27]. The terminal step required in this Endo V/LDR assay is a high-resolution electrophoresis step that sizes the DNAs in their single-stranded form to determine the origin of the nicks induced by Endo V.

The technological trend towards miniaturizing electrophoretic platforms for DNA separations initiated during the HGP has impacted genetic analyses for clinical diagnostics/prognostics as well and has led to devices that provide high resolution

separations that are potentially faster, possess simple operational characteristics and have lower cost compared to conventional capillary electrophoresis.

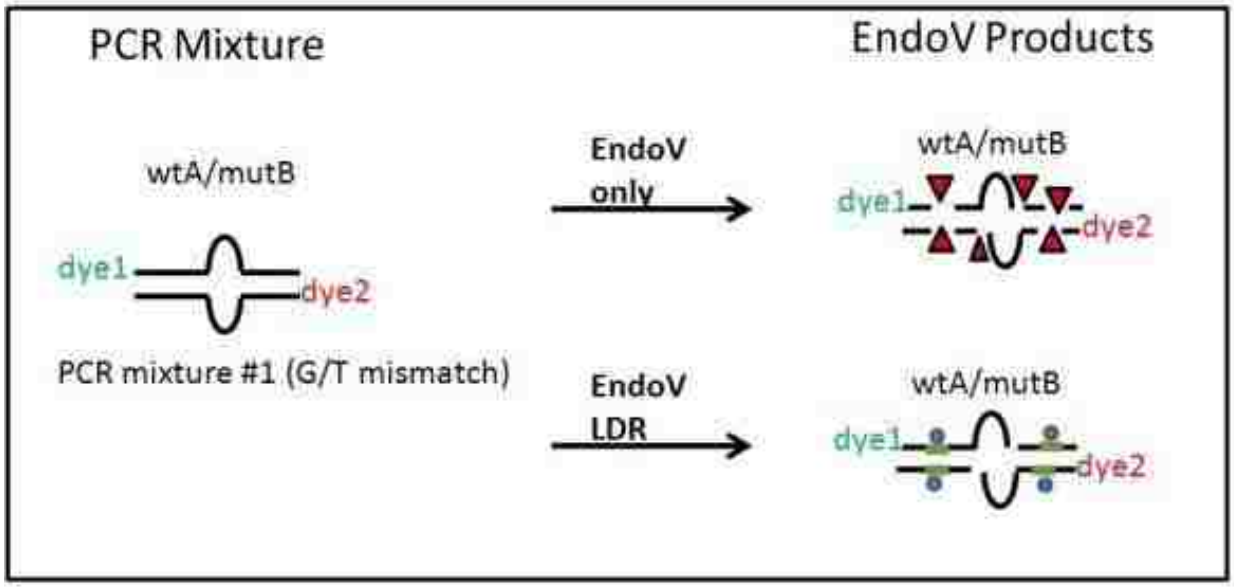

Figure 5.1. Diagram illustrating the outcome of a successful Endo V/LDR treatment of dye-labeled, heteroduplexed substrates. PCR amplicons of known wild-type cell lines and mutagenic cell lines having high levels of sporadic mutations are cross-paired and heteroduplexed to form Endo V targets. Endo V preferentially nicks DNA one base 3' to the mismatch, but also generates nonspecific nicks with minor activity. DNA ligase is used either subsequently or concurrently with Endo V to reseal these background nicks.

In addition, these microchip electrophoresis devices can be integrated to upstream sample pre-processing steps to create fully automated systems for mutation analysis. Efforts have prompted the engineering of various high-throughput and highly integrated electrophoretic devices in glass and, most recently, a host of polymeric substrates, which are relatively inexpensive and conducive to a variety of high production-mode fabrication techniques making them appropriate for clinical application, which require one-time use devices to prevent false-positive signals arising from sample carryover [33-36].

Recently, mutation detection and scanning assays, which depend on an electrophoresis step for reading the results of the molecular assay, have been demonstrated. For example, mutation scanning assays that have been transitioned to microchip platforms include SSCP and HA [37-39]. Many of these reports emphasized numerous operational parameters, such as electrophoresis temperature and polymer matrix/denaturing additive concentrations that must be optimized to provide highly sensitive detection for mutational analyses possessing adequate resolution for detecting the target mutations [40-42].

Here we present adaptation of the highly sensitive Endo V/LDR mutation scanning assay onto a poly(methyl methacrylate), PMMA, microchip for separation of the generated products using high-resolution electrophoresis for sorting the single-stranded products generated. Comparisons of the separation performance for identifying sporadic p53 mutations via Endo V/LDR performed using conventional capillary gel electrophoresis and with polymer-based microchip gel electrophoresis will be evaluated. The goal of this work is to assess the ability of this mutation scanning assay to be transitioned to a microfluidic platform and its potential for detecting low-abundant sporadic mutations using microchip-based scanning methods.

5.2 Methods and Materials

5.2.1 Protocol for DNA Template Preparation

Genomic DNA was extracted from HT-29 and LoVo cell lines having known K-ras oncogenic expressions associated with the onset of colorectal cancer (ATCC, Manassas, VA) using a Qaigen DNeasy kit (Valencia, CA); the LoVo cell line contains wild-type p53 gene mutations and HT-29 cell lines contain exon 8 R273H (G→A)

mutations. All of these epithelial cell lines were obtained from colorectal adenocarcinomas.

5.2.2 Protocol for Universal PCR Amplification

All primers used for Endo V/LDR were purchased from IDT (Coralville, IA) or LiCOR Biotechnologies (Lincoln, NE). Universal PCR reactions for amplification of p53 exon 8 gene fragments followed by universal primer amplification (50 mL) containing 20 mM Tricine, pH 8.7, 16 mM $(NH4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 mM of each universal primer, 0.02 mM of each gene-specific primer, 5 U of AmpliTaq Gold DNA polymerase and 150 ng of genomic DNA (see Table 1 for primer sets).

Table 5.1. PCR primers used for universal gene amplifications

Gene	Exon	Primer name	Primer sequence
Universal		**dye1**UniEV1F	5' dye1CGC**CGTCACGACACGAAAAC**-3'
Universal		**dye2**UniEV2R	5' dye2CGC**CGTCACGACACGAAACA**-3'
Universal		p-UniEV1F	5' P-CGC**CGTCACGACACGAAAAC**-3'
Universal		p-UniEV2R	5' P-CGC**CGTCACGACACGAAACA**-3'
p53	exon 8	F173	5' **CGTCACGACACGAAAAC**CAGGGTGGTTGGGAGTAGATG-3'
		R174	5' **CGTCACGACACGAAACA**GGTGATAAAAGTGAATCTGAGGCATAAC-3'

F=forward, R= reverse, Uni= universal, p=phosphorylation

The first thermocycling conditions in the bi-level amplification for the gene-specific template amplification were: 95ºC for 10 min to activate AmpliTaq Gold polymerase, followed by 20 cycles of 94ºC for 30 s, 65ºC for 1 min, 72ºC for 1 min. Secondly, the universal amplification cycling conditions were: 30 cycles of 94ºC for 30 s, 55ºC for 1 min, 72ºC for 1 min, followed by a final extension step at 72ºC for 7 min. In the universal PCR reaction, the universal primer pair consisted of a forward dye1-labeled primer and a reverse dye2-labeled primer for the standard

denaturation/renaturation procedure. The dye1 and dye2 designation represent different dye sets matching capillary array electrophoresis (CAE) and microchip detection systems; for CAE analyses, dye1 and dye2 were FAM (ex./em.= 492, 517 nm) and HEX (ex./em.= 538, 559 mn), while HEX and IRD-800 (ex./em.= 782, 812 nm) were used for the microchip system. In this procedure, PCR amplification used one labeled universal primer and one unlabeled universal primer (see Figure 5.2).

5.2.3 Preparation of Heteroduplexed DNA Substrates

For the denaturation/renaturation procedure required for heteroduplex formation for the Endo V phases of the assay, approximately equal ratios of dye1/dye2-labeled wild-type PCR amplicons were mixed with dye1/dye2-labeled mutant PCR amplicons in a 12 mL final volume (1500 ng total DNA). The wild-type control consisted of dye1/dye2-labeled wild-type DNA PCR products alone in a 12 mL final volume (1500 ng total DNA). The residual Taq DNA polymerase was inactivated by adding 1 mL of proteinase K (20 mg/mL) to each mixture and incubating at 65ºC for 30 min, followed by a 10 min incubation at 80ºC to deactivate the proteinase K. PCR mixtures were then heated at 95ºC for 2 min, and gradually cooled to room temperature with a 0.2ºC decrease in temperature every 15 s to 45ºC, and finally with a 10 min incubation at 25ºC to generate heteroduplexes as shown in Figure 5.2.

5.2.4 Endo V/ LDR Assay

A 6.5-µl volume of each denatured/renatured PCR mixture was incubated at 65˚C for 2 h in a 20 µl volume reaction containing a reaction cocktail (80 mM Tricine pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5% dimethyl sulfoxide (DMSO), 1.5 M betain, 2% glycerol, with 500 nM Endo V, 6 nM Taq ligase (LDR repair enzyme) and 5 mM nicotinamide adenine dinucleotide (NAD), pH 8.5). Then, reactions were terminated

with the addition of EDTA to a 10 mM final concentration to inhibit any further Endo V cleavage activity. Using the given buffer conditions, both Endo V cleavage and LDR DNA repair of miscleavages via thermostable ligation were carried out simultaneously.

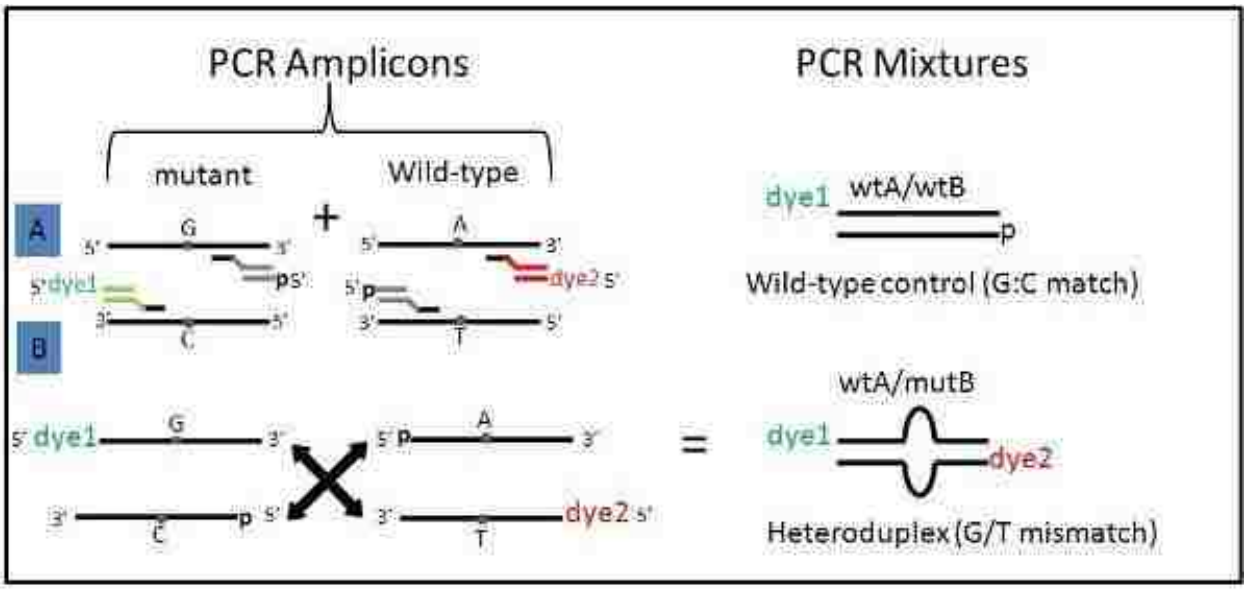

Figure 5.2. Schematic illustration of the universal PCR amplification, labeling and cross-pairing of wild-type LoVo and mutagenic HT-29 templates and the resulting homoduplex (control) and heteroduplex targets.

5.2.5 Microchip Fabrication

Microchips were fabricated using methods previously developed and reported in our group [33] . Briefly, the procedure involved machining a molding die by micro-milling raised microstructures onto a brass plate. These microstructures formed a separation channel with dimensions of 70 μm (depth) x 30 μm (width) that was 9.5 cm long 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 plates (MSC, Melville, NY) using an embossing system consisting of a PHI Precision Press model number TS-21-H-C (4A)-5 (City of Industry, CA) and a vacuum chamber

connected to the press to remove air (pressure, < 0.1 bar). The microchannel pattern was transferred into a PMMA plate at 155°C and 1000 lb for 4 min. After hot-embossing, the press was opened and the polymer 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 and heating to 107°C in a circulating air oven for 20 min.

5.2.6 Laser-induced Fluorescence (LIF) and High Voltage Control System

A laser-induced fluorescence (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) was directed onto a dichroic mirror, which reflected the excitation beam through a 40x microscope objective (Newport, Fountain Valley, CA) 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) and an 800 nm long-pass filter (Edmund Scientific, Barrington, NJ). The LIF signals were acquired on a personal computer using a 32-bit counter/timer board (Model PCI-6601, National Instruments, Austin, TX).

A custom Lab-View program was used to control applied voltages for the microchip electrophoresis. The unit included three internal high voltage power supplies (EMCO, Sutter Creek, CA) capable of receiving inputs of 0 or +5 V from a DAC (digital-to-analog converter) outputs of a CYDDA 04P board (CyberResearch, New Haven, CT).

These power supplies were capable of delivering 0 to +2 kV to sample and waste reservoirs (EMCO Model C20, Sutter Creek, CA) and +0.3 to +5 kV to the anode reservoir (EMCO Model G50), all of which could be dynamically altered throughout the separation.

5.2.7 Capillary and Microchip Conditions for Endo V/LDR

Separations of the Endo V/LDR products were performed on an ABI 3730 (Applied Biosystems) using 36 cm (31 cm effective separation length) capillaries filled with POP7 gel (Applied Biosystems), which was used in conjunction with POP7 buffer. Samples were diluted 1 to 10 with HiDi formamide and 0.4 μL of a Genescan size standard (Applied Biosystems), which were denatured at 95ºC for 2 min (to ensure all duplexes were converted to single strand form) and electrokinetically loaded into the capillaries by applying 277 V/cm for 10 s and electrophoresed at 416 V/cm. For the microchip analysis, separations were performed at room temperature using microchannels filled with a 2.75% w/v sparsely cross-linked polyacrylamide suspended in 1x TTE (50 mM Tris, 50 mM TAPS, 2 mM EDTA) buffer containing 7 M urea following a rinse with 0.05% (w/v) methyl hydroxyethyl cellulose, MHEC (Sigma Aldrich, St. Louis, Mo) to dynamically coat the PMMA channel walls for electroosmotic flow (EOF) suppression [43]. To generate a volume-defined injection plug, 347 V/cm was applied for 30 s from the sample reservoir (ground) to the waste reservoir (+ 3.5 kV). Electrophoresing of the sample was conducted using 125 V/cm, while adduction potentials of 345 and 276 V/cm were applied to sample and waste reservoirs, respectively, to prevent extraneous sample leakage into the separation channel.

5.3 Results and Discussion

5.3.1 Universal PCR Products

As seen in the Figure 5.2, these pre-processing steps overall yield DNA duplexes bearing unique fluorescent labels in order to ascertain whether mutation products present were derived from the top or bottom strands. This universal labeling strategy was implemented to eliminate the need for obtaining (expensive) fluorescently-labeled primers for high throughput scanning over several target loci. In order to verify successful universal PCR amplification, all amplicons were separated on a 2% agarose gel and compared to a size standard as seen in Figure 5.3. From this image, a band intensity measurement relative to the weighted size standard was determined to quantify the total concentrations for each template in order to determine the relative yields of the denaturation/renaturation processes. This was required as high DNA concentrations (125 ng/µL) and very small decreases in annealing temperature (- 0.2 °C/min) are critical the generation of heteroduplexes. The DNA concentrations of the amplicons in lanes 2 – 5 were were ~150 ng/µL compared to the negative control samples containing no universal primers for secondary amplification, which were ~8 ng/µL.

5.3.2 Capillary Separations of Endo V/LDR Products

Prior to microchip analyses, separations of the Endo V/LDR products were conducted using a commercial ABI 3730 CAE system to ensure the reactions were successful and to provide a comparison of the microchip electrophoresis to previous results using capillary array electrophoresis [27]. Figure 5.4 shows the presence of p53 mutations due to the observance of ssDNA bands appearing at 158 and 194 bases when referenced against the DNA sizing ladder (see Figure 5.4), which have been

confirmed via DNA sequencing [44]. As depicted in Figure 5.4, the panel on the right is a simplified band representation of all of the peaks generated from each of the six electropherograms, which was produced using customized software. As a simplified representation of the multi-channel output from the CAE system, a band representation of the CAE electropherogram is shown in the left panel of Figure 5.4 and it was used to

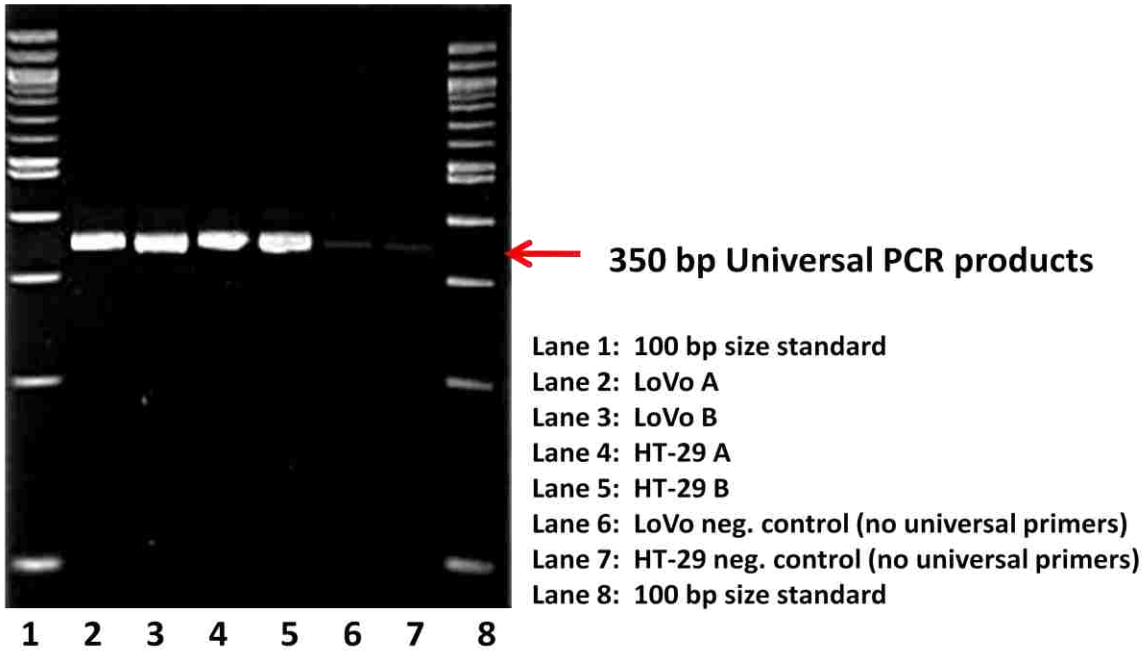

Figure 5.3. A 2% agarose gel/1x TBE running buffer separation verifying the generation of 350 bp universal amplicons of wild-type and mutagenic targets to be heteroduplexed. Separation was conducted with a 10 V/cm field strength and DNA was stained with ethidium bromide.

evaluate treated samples for the presence of mutations within the amplicons from either the top or bottom strands of the heteroduplexes according to their unique fluorescent labels. The third set of bands (orange) of relatively low intensity in each lane represents an internal size standard against which the DNA lengths present were referenced. The Endo V treated samples in lanes 1 – 3 showed elevated levels of sample cleavage

resulting in several bands that were <110 bp in size, which were a mixture of primers and miscleaved products. However, lanes 4 – 6, which were treated with both Endo V and an LDR, showed a significant reduction in false signal bands generated from Endo V cleavage events arising from cleavage at fully matched sites. Also, no product bands were present in the wild-types controls samples as expected (see lanes 1 and 4 in Figure 5.4).

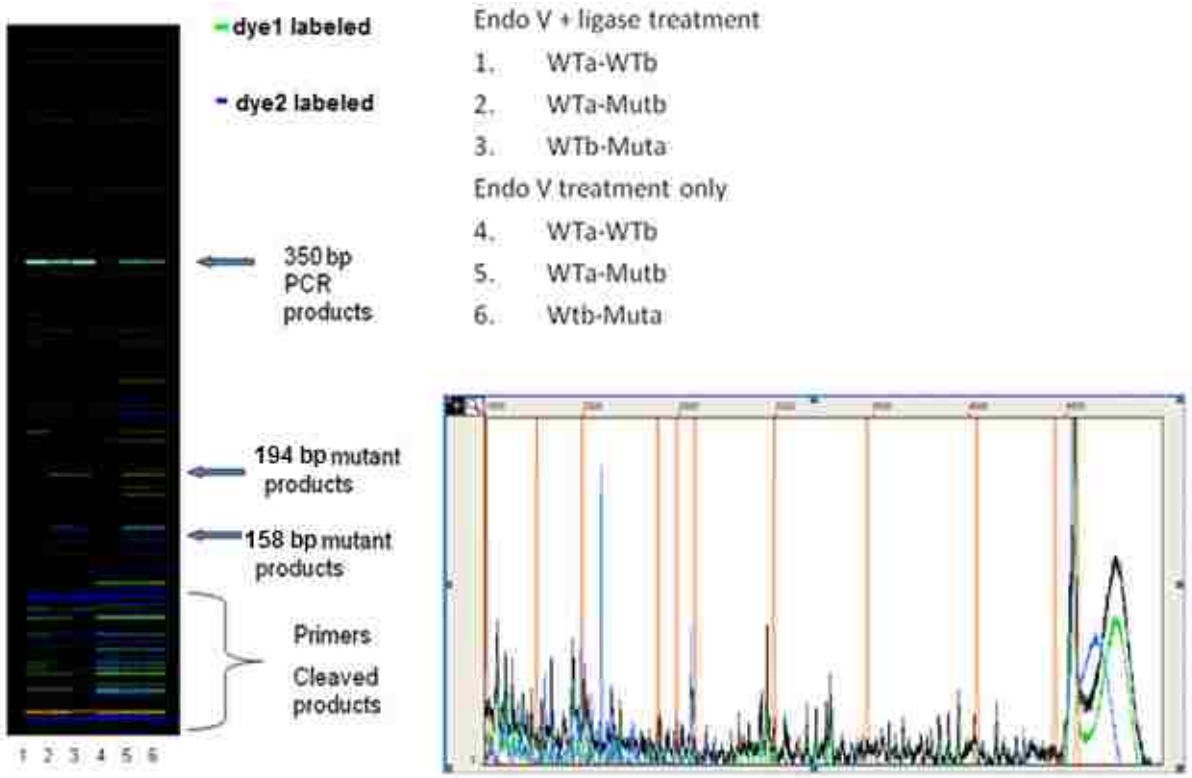

Figure 5.4. Capillary electrophoretic separations of Endo V and Endo V/LDR treated DNA samples. Heteroduplexed mixtures of wild-type and mutant DNA amplicons from LoVo and HT-29 cell lines, respectively, were incubated in the optimized reaction cocktail conditions, which included a 2 h Endo V reaction at 65˚C.

5.3.3 Microchip Separation of 32 Component Size Standard

In order to determine the ability to generate sufficient plate numbers and resolution for the Endo V/LDR products required for this mutation scanning assay, which range in size from 100 bp to 350 bp, we optimized the microchip separation

conditions using a 600 bp sizing ladder composed of 32 single stranded DNAs as a reference. As shown in the electropherogram in Figure 5.5, a PMMA microchip filled with a 2.75% (w/v) nanogel [45] suspended in 1x TTE and 7 M urea following a channel flush with 0.05% (w/v) MHEC (for EOF suppression) was capable of providing near baseline resolution for all 32 components of this 600 bp sizing ladder having 5, 10 and 20 bp differences in size. This sparsely cross-linked linear polyacrylamide (LPA) was

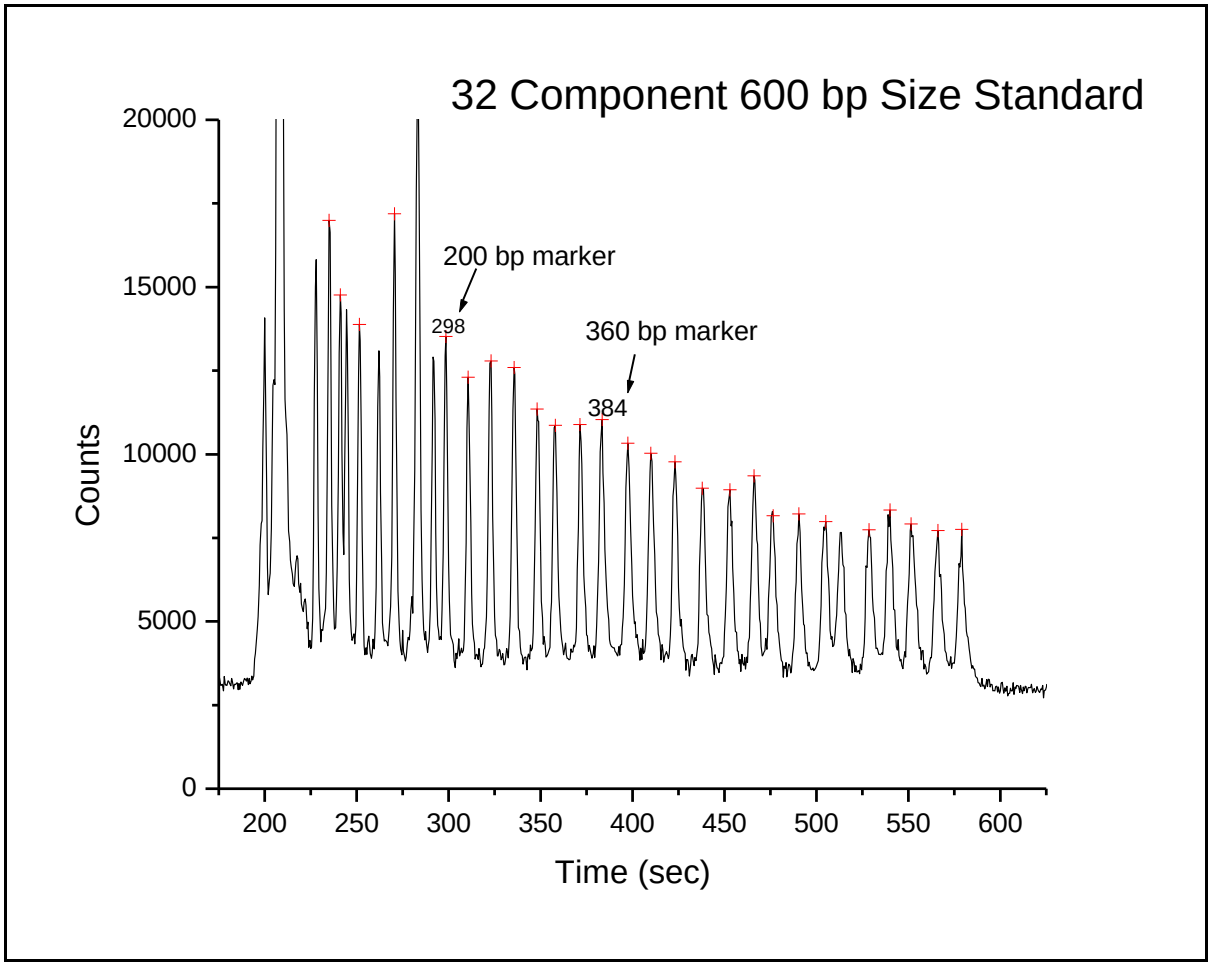

Figure 5.5. PMMA microchip separation of a 32 component Beckman 600 bp size standard. The separation was performed using a 2.75% w/v nanogel constituted in 1x TTE, 7 M urea buffer with a separation voltage of 125 V/cm at a detection length of 6.0 cm.

reportedly synthesized by incorporating a low percentage (~10^{-4} mol %) of *N, N*-methylene bisacrylamide (Bis) cross-linker in high-molar mass LPA, which generates localized cross-linking whereby ~75% of the polymer chains in the nanogel incorporate at least one point of cross-linking.and allows LPA to remain fluid [45]. We found this nanogel matrix provided ~18% greater resolution than a 4% LPA matrix prepared with the same buffer constituents, which was determined by the differences in the respective resolutions between 100/110, 300/320 and 580/600 bp markers of size standard separations. The PMMA microchip separations were conducted at field strength of 125 V/cm with sample adduction fields of 345 and 276 V/cm and an effective column length of 6.0 cm. The high resolution generated for this size standard separation using the PMMA microchip with the nanogel matrix was therefore considered to be a viable combination for the sorting of cleaved, miscleaved and uncleaved DNA fragments within the Endo V and Endo V/LDR treated samples.

5.3.4 Microchip Separation of Endo V/LDR Products

The described LIF system used in conjunction with the microfluidic chip was capable of only single color detection. Therefore, only one of the two labeled mutations (dye1-labeled 194 bp product) was analyzed in this assessment. Figure 5.6 shows the separation results of a negative control, which consisted of 1:5 mutant and wild-type p53 exon 8 sequences from HT-29 and LoVo cell lines, respectively. Here, no Endo V enzyme was added during the final treatment incubation. This microchip separation as well as the Endo V and Endo V/LDR separations were also performed using a 2.75% (w/v) nanogel constituted in 1x TTE, 7 M urea buffer with a separation voltage of 125 V/cm at a detection length of 6.0 cm. Upon separation development, the only visible components in the sample were residual fluorescently-labeled PCR primers remaining

after the PCR amplification and the 350 bp heteroduplexed PCR amplicons, which were in the single-strand form via denaturation with formamide prior to performing the separation. This Endo V control microchip and was completed within 6 min comparable to the same separation performed on the CAE system (data not shown). Thus, all other peak fragments present in the Endo V treated samples upon microchip sorting were attributed to Endo V activity.

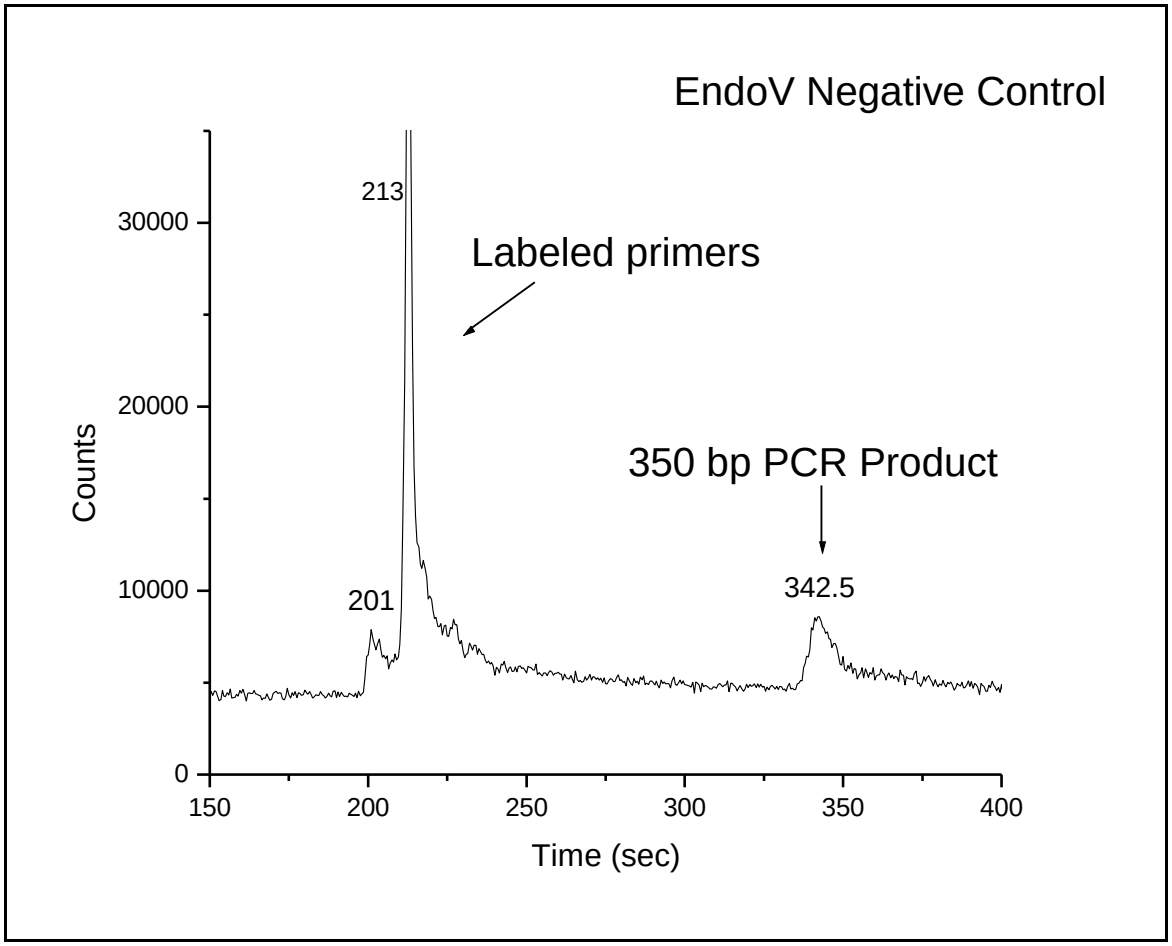

Figure 5.6. PMMA microchip separation of a negative control sample, which was not subjected to Endo V treatment. Electrophoretic run conditions were the same as those given in Figure 5.5.

As seen in Figure 5.7, the microchip separation of an Endo V treated 1:5 mutB/wtA sample using the same conditions given in Figure 5.5 reveals that several fragments of wtA/mutB heteroduplex, which were not present in the negative control PMMA microchip separation. Upon evaluation of the separations, most of the miscleavages resulted in several partially resolved fragments ranging from the excess primer peaks used for the universal amplicon generation (~20 bases) to approximately 110 bases. Therefore, these peaks represented false signals due to non-specific cleavage as measured against the size standard separation, which were similar to those observed in the CAE separations (see lanes 4 – 6 of Figure 5.4). As seen, the resulting microchip Endo V separations were in agreement with the elevated presence of amplicon fragmentation just prior to the 158 and 194 base mutation products of the Endo V separations performed on the CAE system. Interestingly, the microchip efficiencies of the Endo V separations were relatively low ($N = 1.42 \times 10^5$ plates/m) in comparison to that obtained in the microchip separation of the size standard ($N = 1.2 \times 10^6$ plates/m), which was determined by measuring a peak in both electropherograms located at approximately 300 s. This could possibly be attributed to high levels of miscleaved fragments of the same approximate size, which may have co-migrated as higher efficiencies of the Endo V sample peaks in the CAE separations (*i.e.*, $N \geq 7.0 \times 10^6$) were measured. The low sample recovery following the desalting sample prior to analysis on the microchip could also be a factor. The peak present at T_m= 304 s was determined to be the 194 base mutation product upon comparison to the size standard, which has a 200 bp marker at T_m= 298 s. This microchip separation was completed within 6 min compared to the CAE system, in which the analysis time was approximately 1 h.

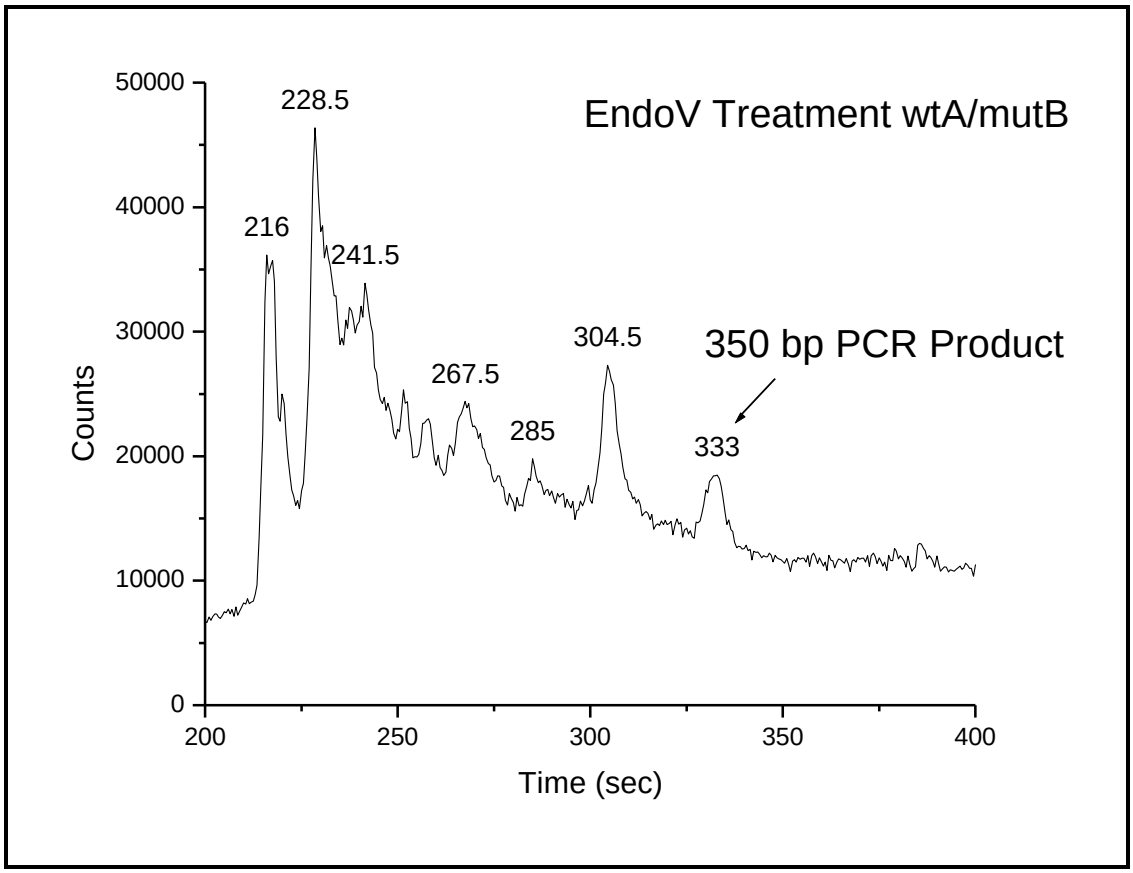

Figure 5.7. PMMA microchip separation of a wtA/mutB Endo V treated heteroduplex. Run conditions were the same as given in Figure 5.5.

The separation of a 1:5 mutB/wtA sample treated with both Endo V and a concurrent LDR is shown in Figure 5.8. As observed in the parallel CAE separations, fewer peaks generated via miscleavages from Endo V activity were present after the LDR repair treatment, which suppressed several spurious signals in the 100 – 110 bp range preceding the expected product location at 194 bp. The peak profile for this microchip analysis was also similar to the CAE separations of Endo V/LDR treated samples (see lanes 1 – 3 of Figure 5.4). Upon comparison to the 600 bp size standard, it was determined that the pronounced peak present following LDR resealing having a migration time of 290 s was the 194 bp mutation product as it was within close proximity (Δt_m= 8 s) to the 200 bp marker. The efficiency for the 194 bp product peak was 2.91 x

10^5 plates/m, which was slightly greater than that of the same product detected in the Endo V only treated sample. Upon the completion of a two-color LIF system, the size standard will be internalized in the same separation to further validate the Endo V product identities. The relative differences in overall signal intensities of these microchip electropherograms were also attributed in part to low sample recovery efficiencies following a desalting procedure.

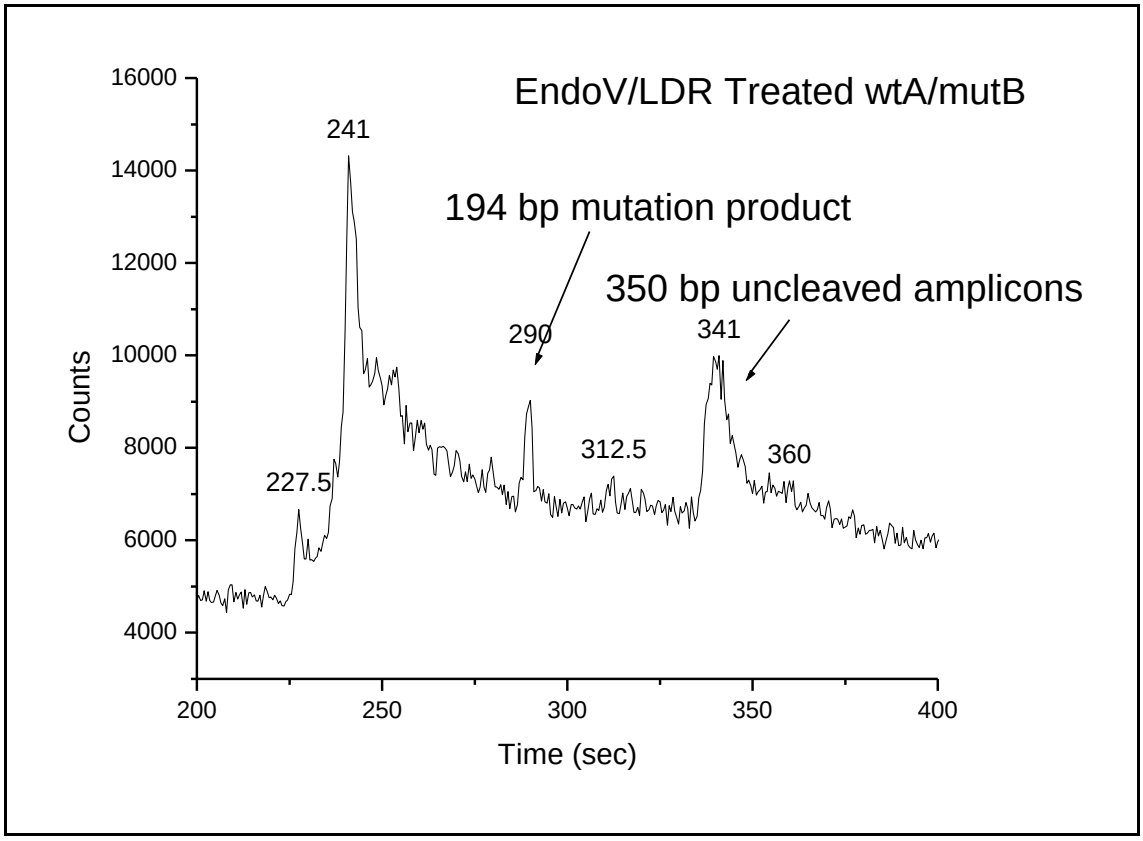

Figure 5.8. PMMA microchip separation of a wtA/mutB Endo V/LDR treated heteroduplex. Upon LDR treatment, the 194 bp p53 mutation product is observed. Run conditions were the same as given in Figure 5.5.

5.4 Conclusions

The PMMA microchip separation of sporadic p53 mutations interrogated using a novel, dual-enzyme Endo V/LDR assay provided some interesting results when

compared to CAE analysis of these sporadic mutations. The preliminary results suggest that Endo V/LDR products could be successfully separated and detected on the PMMA microfluidic filled with a sparsely cross-linked replaceable polyacrylamide in solution in less than 6 minutes, which was approximately a tenth of the development time of the 1 h CAE performance. Comparing the Endo V negative control, Endo V and Endo V/LDR sample separations, characteristics of Endo V cleavage and the subsequent clean-up of spurious false signals were observed in microchip separations, which were similar to that obtained with CAE separations. A two-color LIF system is currently being devised to allow the full two-color read-out of the existing multiplex assay. Also, front-end sample processing including the universal PCR and heteroduplexing of target DNAs could be integrated on-chip reduce the ~5 h sample preparation time presently required.

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Chapter 6: Conclusions and Future Work

6.1 Conclusions

The ever growing understanding of DNA and its linkage to disease development has demanded vast improvements in genotyping technology. The abundance of DNA processing necessary to further this base of knowledge has far out-paced antiquated slab gel systems and even the capillary array systems, which greatly aided in the completion of the sequencing of the human genome. Undoubtedly, the current miniaturization of electrophoretic platforms represents the future of DNA processing technology. It is expected that the development of polymer microchip separations for DNA analyses presented in the preceding chapters will be a significant contribution in the advancement of genotyping technology.

The evolution of genotyping platforms was alluded to in Chapter 1. The fundamentals of DNA structure/composition and processing including DNA replication (PCR) and genotyping methods as well as the electrophoresis and fluorescence detection of DNA were introduced.

The current and noteworthy genotyping accomplishments using microchips as electrophoretic platforms were discussed in Chapter 2. This chapter expanded in depth on the growing field of miniaturized separation devices for mutation screening and scanning, forensics and sequencing applications. Highlighted in this chapter were the myriad of genetic conditions currently being investigated using microfluidics and trends of the increasing utilization of polymer substrates for the development of planar microelectrophoretic platforms. In addition, microchip devices featuring high throughput

architectures and unprecedented levels of integration for front-end sample manipulation allowing complete lab-on-a-chip processing of DNA samples were reviewed.

Polymer microchip sorting of mutation products derived from a novel LDR assay was premiered in Chapter 3. Although widespread utilization of this Taq ligase-based genotyping technique was evident in the scientific literature since its inception in 1991, no microchip sorting adaptations of LDR products, including polymer and glass substrates, had previously been demonstrated. Recognizing the potential value of this genotyping method on a miniaturized electrophoretic platform, a productive collaboration with the research group of Francis Barany at Weill Medical College of Cornell University was forged leading to the initial polymer microchip separations of LDR products. Evaluating a host of polymer solutions, a 4% LPA solution was found favorable in providing high efficiency separations for an individual LDR product from its reaction constituents with adequate resolution in the small fragment size range of interest in as little as 120 s, nearly 17-times faster than capillary gel formats. In addition, sample processing prior to the electrophoresis was simplified by eliminating the need of desalting using ethanol precipitation or other similar techniques. Later, it was demonstrated that two simultaneously generated LDR products of a 1 to 100 low abundance mutation ratio could be effectively resolved on the same microchip using a sparsely cross-linked, low viscosity solution in approximately 4.5 min.

Directly stemming from the challenges presented in adapting the separation of relatively small LDR products onto a polymer microfluidic, a novel strategy of separating LDR primers in free solution was developed as described in Chapter 4. In collaboration with the Barron Research Group of Stanford University, LDR and ELFSE were merged

for the first time by redesigning LDR primers to allow attachment of molecularly-engineered set of polyamide drag-tags, which imparted unique frictional forces on DNAs having essentially the same size. Following the development of reaction conditions amenable for both drag-tag and LDR constituent fidelities, free solution electrophoresis cross injection and separation conditions were developed and optimized to sort LDR-dt products on PMMA microchips. Although the single base size differences of these LDR products were miniscule in terms of relative electrophoretic mobility for small DNA fragments, baseline resolutions were achieved for four clinically relevant colorectal cancer mutations. Low abundance mutation detection levels of 1 in 100 DNA molecules and simultaneous detection of multiple (four) mutations were achieved. Implementation of this free solution separation mechanism, LDR analysis times reached a new low of approximately 85 s using a PMMA microfluidic, improving upon the rapid 2 min analysis times achieved using the same PMMA microchip filled with a host of polymer solutions.

With the understanding gained from previous studies evaluating gel matrices for DNA separations on polymer microchips, the microchip separation adaptation for mutation products of a novel Endo V/LDR mutation scanning technique was initiated as presented in Chapter 6. Combined with LDR resealing of spurious DNA clipping, Endo V mutation cleavage provided an effective means of detecting unknown mutations occurring sporadically with high frequency within tumor suppressor genes. Given its effectiveness, it was found appealing to bolster the processing efficiency of the technique as the size of Endo V/LDR products fell within a suitable range (> 100 bp) favorable for microchip separations. Preliminary results indicate that microchip separations allow discrimination of Endo V-treated DNA samples from Endo V/LDR-

treated DNA samples as the latter shows fewer peaks, which could be directly attributed to Endo V cleavage and the subsequent resealing of DNA targets. Upon full optimization of the separation parameters in conjunction with the completion of a two-color LIF system, we expect to dramatically reduce the mutation product separation performance by at least a factor of ten as observed in the preliminary data.

6.2 Future Work

As alluded to throughout this dissertation, the next critical step necessary to realize the promise of transitioning DNA separations onto a miniaturized planar platform is the integration of DNA sample preparatory processes onto the same wafer. Such studies have already begun by coworkers within the Soper Research Group for front-end LDR thermal cycling using a miniaturized continuous flow device. To date, only on-chip microarray or off-line CE-LIF detection strategies have been implemented to complement this process. Up until now, the major bottleneck constraining the post reaction electrophoretic sorting of LDR products has been the incompatibility of viscous polymer solutions within the continuous flow system. With the development of LDR-FSCE in hand, seamless on-chip integrated post reaction separations of LDR-dt products should now be implemented with relative ease. In addition, high throughput format separations employing multichannel devices will be pursued in conjunction with LDR-FSCE since no viscous polymer solutions, which currently influence the choice of channel geometries, will be necessary for separations. Upon the receipt of a larger set of polyamide drag-tags, a multiplexed LDR assessment for the entire panel of 19 known *K-ras* mutations will be conducted. This strategy will be employed for other mutation groups having a high association with other forms of cancer developments as well. This

work will continue until μTAS units for the rapid and cost effective clinical testing of DNA samples are fully developed.

Polymer microchips separations of Endo V/LDR mutation products will continue to be investigated. Upon the completion of a two-color LIF system, which is currently being devised, Endo V/LDR products separations on this platform will be amenable to two-color read-out as intended with the design of the assay. Once Endo V/LDR separations are optimized on this microfluidic domain, front-end sample processing including the universal PCR and heteroduplexing of target DNAs could be integrated on-chip in a similar continuous flow setup as currently developed for LDR to reduce the ~5 h sample preparation time presently required.

Appendix A: References Displayed within Table 1.1

References

Ref. 12 [1]

Ref. 14 [2]

Ref. 39 [3]

Ref. 13 [4]

Ref. 10 [5]

Ref. 8 [6]

Ref. 11 [7]

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Vita

Rondedrick DeShaun Sinville was born in Shreveport, Louisiana, in May of 1978, the oldest child of Leroy and Delphine Sinville Taylor, both of whom were also natives of Shreveport. He has two younger siblings, Brandon and Shamya Taylor and was raised by both parents and his maternal grandmother, Escille Sinville. He attended Southwood High School in Shreveport from 1992 – 1996 where he developed a fondness for the subject of chemistry.

Rondedrick went on to attend Grambling State University in Grambling, Louisiana, where the motto is, “Where Everybody is Somebody”. While there, he earned a National Institutes of Health Fellowship and held two internships at the University of Minnesota and the University of North Texas Health Science Center. In the fall of 2000, he graduated *cum laude* and was awarded an accredited Bachelor of Science degree in chemistry.

In the fall of 2001, Rondedrick enrolled in the doctoral program at Louisiana State University in the Department of Chemistry and was the recipient of a Hewell D. Perkins Fellowship. The following spring, he joined the research group of Professor Steven A. Soper. During this time, his research focused on DNA genotyping separations using microfabricated polymer microfluidic devices. He was an active member of the NOBCChE Student Chapter at LSU serving as the Service Committee Chairman for several years. While at LSU, he presented his research findings at several national conferences and one international conference in Tokyo, Japan.

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