

Multiplexed p53 Mutation Detection by Free-Solution Conjugate Microchannel Electrophoresis with Polyamide Drag-Tags

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We report a new, bioconjugate approach to performing highly multiplexed single-base extension (SBE) assays, which we demonstrate by genotyping a large panel of point mutants in exons 5–9 of the p53 gene. A series of monodisperse polyamide “drag-tags” was created using both chemical and biological synthesis and used to achieve the high-resolution separation of genotyping reaction products by microchannel electrophoresis without a polymeric sieving matrix. A highly multiplexed SBE reaction was performed in which 16 unique drag-tagged primers simultaneously probe 16 p53 gene loci, with an abbreviated thermal cycling protocol of only 9 min. The drag-tagged SBE products were rapidly separated by free-solution conjugate electrophoresis (FSCE) in both capillaries and microfluidic chips with genotyping accuracy in excess of 96%. The separation requires less than 70 s in a glass microfluidic chip, or about 20 min in a commercial capillary array sequencing instrument. Compared to gel electrophoresis, FSCE offers greater freedom in the design of SBE primers by essentially decoupling the length of the primer and the electrophoretic mobility of the genotyping products. FSCE also presents new possibilities for the facile implementation of SBE on integrated microfluidic electrophoresis devices for rapid, high-throughput genetic mutation detection or SNP scoring.

Although the sequencing of the first human genome was completed amidst much fanfare in 2003, a great need still exists for studying variability among different individual human genomes as well as among the genomes of other organisms. More than 90% of the genetic variability among humans is thought to consist of single-nucleotide polymorphisms (SNPs), and efforts are ongoing to map more than 300 000 SNPs.^{1,2} While many SNPs have no significant impact on protein expression or cell function,

specific SNPs have been found to predispose individuals to certain diseases, including sickle cell anemia and Alzheimer’s disease.^{3,4} For example, mutations in the p53 gene have been implicated in a wide variety of human cancers, with missense mutations comprising a large majority of deleterious p53 sequence alterations.^{5–9} Furthermore, sequence polymorphisms in a variety of interacting genes are suspected to be responsible for complex diseases such as cancer, heart disease, and psychiatric disorders; the results of multiplexed, multigene SNP analyses in large populations are expected to enable valuable insights into such conditions.^{1,10}

A wide variety of techniques have been proposed for SNP detection, and many of these methods have recently been reviewed.^{11,12} Most methods begin with PCR amplification of the gene region to be tested, typically followed by an enzymatic allele discrimination reaction, and then the detection and identification of the reaction products. Biomolecule detection schemes based on fluorescence or fluorescence resonance energy transfer, mass spectrometry, or microarrays can allow accurate identification of allele-specific products. Each method has its advantages and disadvantages with respect to simplicity, sensitivity, ease of multiplexing, throughput, and cost; the choice of SNP genotyping method varies, depending on the specific needs and resources of each laboratory.

One widely used technique for allele discrimination based on the synthesis activity of DNA polymerase is the single-base extension (SBE) assay, also known as mini-sequencing or primer-

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(1) Collins, F. S.; Brooks, L. D.; Chakravarti, A. *Genome Res.* **1998**, *8*, 1229–1231.

(2) Brookes, A. J. *Gene* **1999**, *234*, 177–186.

(3) Strittmatter, W. J.; Saunders, A. M.; Schmechel, D.; Pericakvance, M.; Enghild, J.; Salvesen, G. S.; Roses, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1977–1981.

(4) Strittmatter, W. J.; Roses, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4725–4727.

(5) Greenblatt, M. S.; Bennett, W. P.; Hollstein, M.; Harris, C. C. *Cancer Res.* **1994**, *54*, 4855–4878.

(6) Soussi, T.; Beroud, C. *Nat. Rev. Cancer* **2001**, *1*, 233–240.

(7) Soussi, T.; Lozano, G. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 834–842.

(8) Birch, J. M.; Alston, R. D.; McNally, R. J.; Evans, D. G.; Kelsey, A. M.; Harris, M.; Eden, O. B.; Varley, J. M. *Oncogene* **2001**, *20*, 4621–4628.

(9) Olivier, M.; Goldgar, D. E.; Sodha, N.; Ohgaki, H.; Kleihues, P.; Hainaut, P.; Eeles, R. A. *Cancer Res.* **2003**, *63*, 6643–6650.

(10) Kirk, B. W.; Feinsod, M.; Favis, R.; Kliman, R. M.; Barany, F. *Nucleic Acids Res.* **2002**, *30*, 3295–3311.

(11) Landegren, U.; Nilsson, M.; Kwok, P. Y. *Genome Res.* **1998**, *8*, 769–776.

(12) Chen, X.; Sullivan, P. F. *Pharmacogenomics J.* **2003**, *3*, 77–96.

guided nucleotide incorporation.^{13–15} In this technique, an oligonucleotide primer is hybridized with its 3'-end immediately upstream of the locus to be genotyped. The SBE reaction is analogous to Sanger cycle sequencing,¹⁶ except that only chain terminators (ddNTPs) are included in the reaction. The DNA polymerase incorporates the ddNTP complementary to the target base on the template, and further extension of the DNA chain does not occur. A variety of different detection schemes can be used to determine the identity of the ddNTP that was incorporated and the genotype of the target allele. SBE followed by MALDI-TOF mass spectrometry,^{17,18} solid-phase mediated fluorescence detection,¹⁹ or electrophoresis with 4-color laser-induced fluorescence (LIF) detection²⁰ are all capable of multiplexed allele discrimination and detection.

Electrophoretic separation is an attractive method for separating SBE reaction products because capillary array electrophoresis (CAE) instruments are widely available; however, it tends to be a relatively costly approach to SNP detection,¹² in part because CAE instruments are expensive to purchase and maintain. Increased throughput, either by higher-order multiplexing (more SNPs per capillary) or shorter analysis time, is required to make electrophoretic separation competitive for SNP detection. Both of these goals can be achieved by using free-solution conjugate electrophoresis (FSCE) in place of conventional electrophoresis with a gel or polymer sieving matrix. FSCE, which is sometimes called end-labeled free-solution electrophoresis (ELFSE),^{21–24} is also expected to simplify the transition to microfluidic electrophoresis devices, which promise to be both faster and much less expensive than the bulky, complex CAE instruments, and to greatly expand the range of potential users of this technology.

FSCE is a bioconjugate technique for separating charged biopolymers by microchannel electrophoresis in the absence of a gel or sieving matrix. Monodisperse, uncharged polyamide “drag-tags” can be appended to one or both ends of a collection of polydisperse, negatively charged DNA molecules to create DNA–polyamide bioconjugates that have size-dependent free-solution electrophoretic mobilities (Figure 1). For a given bioconjugate, the mobility is determined by both the size of the DNA “engine” (which experiences both an electrophoretic force and a hydrodynamic drag force that are each proportional to DNA size

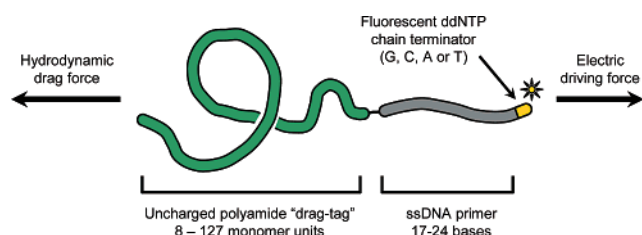


Figure 1. Schematic representation of a DNA–drag-tag conjugate. The DNA primer is designed to probe the template locus of interest by adding one dideoxynucleotide with an identifying fluorophore (shown in yellow) and ranges in size from 17 to 23 bases. The drag-tag, a synthetic, uncharged, unlabeled polyamide of precise length, modifies the electrophoretic mobility of the DNA, allowing for rapid electrophoretic separation, facilitating peak identification.

in an applied electric field), and by the hydrodynamic friction added by the drag-tag, which is proportional to the molar mass of the drag-tag.^{24–28} This approach has been demonstrated for the size-based separation of DNA sequencing fragments up to 110 bases in length,²³ denatured single-stranded PCR products,^{29,30} and double-stranded DNA restriction fragments,²² as well as the profiling of heparins³¹ and charged oligosaccharides.³² All of these studies used capillary electrophoresis (CE) in free solution, that is, without a gel or sieving matrix of any kind.

In 2002, we demonstrated the FSCE separation of the products of a 3-fold multiplexed SBE reaction by free-solution CE.³³ Using conventional SBE reaction protocols, 3 different oligonucleotide primers were used to interrogate 3 polymorphic loci in p53 exon 8. Each primer was conjugated to a monodisperse, synthetic polyamide drag-tag of unique length, allowing the SBE products to be separated by free-solution electrophoresis. Electrophoretic separation was performed in a MegaBACE CAE instrument; although the peaks were somewhat broad, there was sufficient resolution to allow accurate genotyping of each locus in several mutant samples.

In this study, we show that the SBE-FSCE technique can be modified to achieve a much higher degree of multiplexing, using 16 different oligonucleotide primers and 16 unique drag-tags to simultaneously genotype 16 p53 loci. An abbreviated thermal cycling protocol cuts the reaction time by over 90% from the 2002 study, and DNA–drag-tag peak resolution is greatly improved. We tested the method on numerous p53 samples with previously characterized point mutations in exons 5–9. Analyses were performed by both capillary and microfluidic chip electrophoresis. Throughput can be even further increased by the creation of a wider array of unique drag-tags. By contrast, multiplexed SBE genotyping by gel electrophoresis requires the solid-phase syn-

(13) Syvanen, A. C.; Aaltonen, K.; Harju, L.; Kontula, K.; Soderlund, H. *Genomics* **1990**, *8*, 684–692.

(14) Pastinen, T.; Partanen, J.; Syvanen, A. C. *Clin. Chem.* **1996**, *42*, 1391–1397.

(15) Pastinen, T.; Kurg, A.; Metspalu, A.; Peltonen, L.; Syvanen, A. C. *Genome Res.* **1997**, *7*, 606–614.

(16) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463–5467.

(17) Ross, P.; Hall, L.; Smirnov, I.; Haff, L. *Nat. Biotechnol.* **1998**, *16*, 1347–1351.

(18) Kim, S.; Ulz, M. E.; Nguyen, T.; Li, C. M.; Sato, T.; Tycko, B.; Ju, J. *Genomics* **2004**, *83*, 924–931.

(19) Bell, P. A.; Chaturvedi, S.; Gelfand, C. A.; Huang, C. Y.; Kochersperger, M.; Kopla, R.; Modica, F.; Pohl, M.; Varde, S.; Zhao, R. B.; Zhao, X. J.; Boyce-Jacino, M. T. *Biotechniques* **2002**, *32*, S70–S77.

(20) Makridakis, N. M.; Reichardt, J. K. V. *Biotechniques* **2001**, *31*, 1374–1380.

(21) Mayer, P.; Slater, G. W.; Drouin, G. *Anal. Chem.* **1994**, *66*, 1777–1780.

(22) Heller, C.; Slater, G. W.; Mayer, P.; Dovichi, N.; Pinto, D.; Viovy, J. L.; Drouin, G. *J. Chromatogr., A* **1998**, *806*, 113–121.

(23) Ren, H.; Karger, A. E.; Oaks, F.; Menchen, S.; Slater, G. W.; Drouin, G. *Electrophoresis* **1999**, *20*, 2501–2509.

(24) Meagher, R. J.; Won, J. I.; McCormick, L. C.; Nedelcu, S.; Bertrand, M. M.; Bertram, J. L.; Drouin, G.; Barron, A. E.; Slater, G. W. *Electrophoresis* **2005**, *26*, 331–350.

(25) Grossman, P. D. F.; Menchen, S. M.; Woo, S. L.; Winn-Deen, E. S. World Patent WO9320236AI, 1993.

(26) Noolandi, J. *Electrophoresis* **1992**, *13*, 394–395.

(27) Vreeland, W. N.; Desruisseaux, C.; Karger, A. E.; Drouin, G.; Slater, G. W.; Barron, A. E. *Anal. Chem.* **2001**, *73*, 1795–1803.

(28) Vreeland, W. N.; Slater, G. W.; Barron, A. E. *Bioconjugate Chem.* **2002**, *13*, 663–670.

(29) Won, J. I.; Meagher, R. J.; Barron, A. E. *Electrophoresis* **2005**, *26*, 2138–2148.

(30) Haynes, R. D.; Meagher, R. J.; Won, J. I.; Bogdan, F. M.; Barron, A. E. *Bioconjugate Chem.* **2005**, *16*, 929–938.

(31) Sudor, J.; Novotny, M. V. *Anal. Chem.* **1997**, *69*, 3199–3204.

(32) Sudor, J.; Novotny, M. V. *Anal. Chem.* **1995**, *67*, 4205–4209.

(33) Vreeland, W. N.; Meagher, R. J.; Barron, A. E. *Anal. Chem.* **2002**, *74*, 4328–4333.

Table 1. Design of Primers for Multiplexed SBE-FSCE^a

exon	locus	strand	sequence	drag-tag size	migration order	wild-type
9	328-1	–	AAG ACT TAG TAC CTG AAG GGT GA	8	1	A
5	128-1	+	CCT TCC TCT TCC TAC AGT ACT CC	12	2	C
9	330-2	–	GGT CCC AAG ACT TAG TAC CTG A	16	3	A
6	198-1	–	ACT CCA CAC GCA AAT TTC CTT	20	4	C
6	196-2	–	ACA CGC AAA TTT CCT TCC ACT	24	5	C
7	249-3	–	GTG ATG ATG GTG AGG ATG GG	28	6	C
6	196-1	+	CCC CTC CTC AGC ATC TTA TC	32	7	C
7	245-1	+	TAA CAG TTC CTG CAT GGG C	36	8	G
8	273-1	+	ACG GAA CAG CTT TGA GGT G	40	9	C
8	273-2	–	CCA GGA CAG GCA CAA ACA	44	10	C
5	173-1	+	CAG CAC ATG ACG GAG GTT	48	11	G
6	221-3	+	TGT GGT GGT GCC CTA TGA	52	12	G
5	149-1	+	GTG CAG CTG TGG GTT GAT	56	13	T
5	175-2	+	GAC GGA GGT TGT GAG GC	60	14	G
5	144-1	+	CCA AGA CCT GCC CTG TG	70 ^{*a}	15	C
5	173-2	+	AGC ACA TGA CGG AGG TTI	127 ^{**a}	16	T

^a The drag-tags are all linear poly-*N*-methoxyethylglycines made by solid-phase synthetic methods, except for a branched 70mer NMEG (*)³⁰ and a linear 127mer genetically engineered protein polymer (**).

thesis and purification of DNA primers with long non-hybridizing “tails” to enable good electrophoretic separations in gels,³⁴ which rapidly becomes difficult as the tail length increases. SBE-FSCE also offers an added degree of flexibility over conventional SBE with easily interchangeable primers and drag-tags, offering the potential for many different types of multiplexed assays with a single set of drag-tags.

MATERIALS AND METHODS

Synthesis of Polypeptoid Drag-Tags. A series of 14 linear polypeptoid drag-tags ranging in size from 8 to 60 *N*-methoxyethylglycine (NMEG) monomers was synthesized on an ABI 433A automated peptide synthesizer (Applied Biosystems, Foster City, CA) using the submonomer protocol.^{28,35} Aliquots of resin were removed every four cycles of peptoid synthesis beginning with the eighth cycle. Peptoid chains were capped at the *N*-terminus with 3-maleimidopropionic acid using diisopropylcarbodiimide (DIC) as a coupling reagent.²⁸ The maleimide-activated polypeptoids were cleaved from the resin with TFA and purified to near-total monodispersity by C18 reversed-phase HPLC. The monodispersity was assessed using FSCE by conjugating each polypeptoid to a fluorescently labeled, thiolated 20-base oligonucleotide, and analyzing by CE in free solution (an approach that provides a more rapid and sensitive characterization than typical RP-HPLC analysis).²⁸

The 15th drag-tag was a branched polypeptoid, consisting of a 30mer poly(NMEG) backbone derivatized with five 8mer oligo(NMEG) branches, activated at the *N*-terminus with sulfosuccinimidyl 4-(*N*-maleimidomethyl)-1-cyclohexane carboxylate (Sulfo-SMCC).³⁰ The 16th drag-tag was a linear protein polymer with the highly repetitive sequence (GAGTGSA)₄-GAGTGRA-(GAGTGSA)₇-GAGTGRA-(GAGTGSA)₅-G, with a total length of 127 amino acids. An artificial gene encoding this protein polymer was

constructed by the controlled cloning method,^{29,36,37} and the protein polymer was expressed in *Escherichia coli*. Following purification by affinity chromatography, the protein polymer was activated at the *N*-terminus with Sulfo-SMCC to yield a maleimide-activated drag-tag.

Primers for Single-Base Extension Reactions. A set of 16 oligonucleotide primers was synthesized by Integrated DNA Technologies (Coralville, IA) (Table 1). The primers range in length from 17 to 23 bases and include a 5'-thiol functionality to enable conjugation to maleimide-activated drag-tags. Each primer has a calculated T_m of 55 ± 1 °C and was designed to avoid stable hairpin structures or extendable homodimers. The forward (+) and reverse (–) strands of p53 were both considered for primer design, especially when probing for mutations at two adjacent loci.

We prepared 16 drag-tag–primer conjugates by reacting each thiolated primer with a different maleimide-activated drag-tag. The thiolated primers were reduced prior to conjugation by incubating 2 nmol of primer with a 20:1 molar excess of TCEP (Acros Organics, Morris Plains, NJ) for 90 min at 40 °C. We conjugated the reduced DNA to the drag-tags by mixing 90 pmol of reduced DNA with 2.5 nmol of drag-tag in a total volume of 10 μ L of pH 7.2 sodium phosphate buffer. The DNA–drag-tag mixture was left to react at room temperature overnight. The large excess of drag-tag relative to DNA ensured nearly complete conjugation of DNA. As shown in Table 1, the DNA primers were conjugated to drag-tags in reverse order of length; the longest DNA primers were paired with the shortest drag-tags to ensure an unambiguous migration order of the conjugates during free-solution electrophoresis. The 16 drag-tag–primer conjugates were pooled prior to the multiplexed SBE reactions, with a total primer concentration of 2 pmol/ μ L. We are not aware of any specific effect that the presence of the drag-tag may have on T_m for the primers, although it is possible that T_m for the drag-tag-labeled primers may be different from the T_m of 55 ± 1 °C calculated for the unmodified primers.

(34) Sanchez, J. J.; Phillips, C.; Borsting, C.; Balogh, K.; Bogus, M.; Fondevila, M.; Harrison, C. D.; Musgrave-Brown, E.; Salas, A.; Syndercombe-Court, D.; Schneider, P. M.; Carracedo, A.; Morling, N. *Electrophoresis* **2006**, *27*, 1713–1724.

(35) Zuckermann, R. N.; Kerr, J. M.; Kent, S. B. H.; Moos, W. H. *J. Am. Chem. Soc.* **1992**, *114*, 10646–10647.

(36) Won, J. I.; Barron, A. E. *Macromolecules* **2002**, *35*, 8281–8287.

(37) Won, J. I.; Meagher, R. J.; Barron, A. E. *Biomacromolecules* **2004**, *5*, 618–627.

Template DNA for SBE Reactions. Previously characterized p53 wild-type and mutant samples were a gift from the National Institute of Standards and Technology. Exons 5–9 of the p53 gene (including introns) were present as an insert of approximately 2 kbp in a plasmid cloning vector. A plasmid containing the wild-type p53 gene was available in large quantity, and it was used directly as a template for SBE reactions. Plasmid DNA containing variants of p53 exons 5–9 with point mutations were available in much lower quantities; the entire 2 kbp insert covering exons 5–9 was PCR-amplified prior to the SBE reaction, a common first step in SNP detection.¹² Residual nucleotides and PCR primers that could interfere with the subsequent SBE reaction were digested by treating the PCR product with Shrimp Alkaline Phosphatase (USB, Cleveland, OH) and Exonuclease I (USB) at 37 °C for 1 h, followed by deactivation of the enzymes at 75 °C for 15 min.

SBE Reactions. SBE reactions were carried out using the SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA), which includes a premix of sequencing polymerase, buffer concentrate, and ddNTP chain terminators labeled with four different dichlororhodamine (dRhodamine) dyes. The SBE reactions were prepared by mixing 2.5 μ L of the SNaPshot premix, 0.5 μ L of the pooled drag-tag–primer mix (1 pmol total primer), 0.025–0.10 pmol of template DNA, 0.5 μ L of 125 mM HCl, and water for a total volume of 5 μ L. The SBE reaction was carried out in five cycles: 96 °C for 2 s (denaturation), 51.5 °C for 5 s (annealing), and 60 °C for 10 s (extension). The complete thermal cycling procedure required approximately 9 min. Excess dye terminators and buffer salts were removed using Centri-Sep gel filtration spin columns (Princeton Separations, Princeton, NJ).

Capillary Electrophoresis Separations. High-throughput separations were performed in free solution in an Applied Biosystems Prism 3100 capillary array sequencing instrument, with an array of 16 capillaries (effective length 36 cm, total length 47 cm, inner diameter 50 μ m). The separations were conducted in 1X TTE buffer (89 mM Tris, 89 mM TAPS, 2 mM EDTA) with 7 M urea and 1:100 (v/v) aqueous dilution of the POP-6 polymer solution (ABI) as a wall-coating to suppress electroosmotic flow and prevent analyte adsorption. Samples were injected electrokinetically by applying a potential of 1–2 kV (22–44 V/cm) for 5–20 s. Electrophoresis was performed at 55 °C, with a potential of 15 kV (320 V/cm).

Microfluidic Chip Separations. Free-solution electrophoresis was performed in microfluidic chips, using a custom-built instrument.³⁸ Microfluidic separations were carried out in straight-channel, borosilicate glass microfluidic chips fabricated by Micronit (Enschede, The Netherlands). The microchannels were 50- μ m wide and 20- μ m deep with a standard 4-arm, “offset T” design.³⁹ Internal channel surfaces were coated (to eliminate electroosmotic flow) with an adsorbed layer of poly-*N*-hydroxyethylacrylamide by pretreating the channels with 1 M HCl for 10 min, and then flushing with a dilute solution of the polymer for 10 min.^{40,41} The glass found in these microchips (borofloat) has a significantly

different chemical composition than that of fused-silica capillaries. As a result of this chemical difference, the POP-6 polymer solution does not sufficiently coat the surface (from hydrophobic association) to decrease EOF and reduce non-specific binding. Poly-*N*-hydroxyethylacrylamide, on the other hand, was used to coat the microchannel because it binds to the surface by hydrogen bonding between the polymer and the glass-surface silanol groups.^{40,41}

Residual template DNA from the SBE reaction was removed by centrifugal ultrafiltration with a Microcon ultrafiltration device (Millipore, Bedford, MA) to achieve successful sample injection on the chip. Injection was accomplished by applying a potential between the sample and waste reservoir in the cross arm to fill the injection zone. After 30 s, the potentials were switched to separation mode, causing the material in the injection zone to migrate into the separation channel at a field strength of 530 V/cm. Pullback voltages were applied to prevent sample leakage into the separation channel. A custom-built temperature controller was used to maintain a temperature of 55 °C during the separation.

RESULTS AND DISCUSSION

Genotyping. The SBE-FSCE technique reported here allows the simultaneous genotyping of 16 mutation “hot-spots” in p53 exons 5–9, using the 16 different primers described in Table 1, which range in size from 17 to 23 bases. Each primer was conjugated to a monodisperse polyamide drag-tag of unique size, chosen from a set of drag-tags that included 14 different lengths of linear poly(*N*-methoxyethylglycine) (poly(NMEG)), one branched poly(NMEG), and one genetically engineered protein polymer. A multiplexed SBE reaction with fluorescent ddNTPs extends the primer–drag-tag conjugates by one base, and rapid, high-resolution separation of the bioconjugates by free-solution microchannel electrophoresis allows unambiguous determination of the genotypes simply by the observation of the color of each product peak.

Figure 2 shows a typical separation of the wild-type p53 SBE products, achieved using a commercial CE sequencing instrument. The CE separation gives 16 sharp, well-resolved peaks of different colors, each of which corresponds to the wild-type genotypes shown in Table 1. We confirmed the identity of each peak and the yield of the conjugation reaction by separate CE analysis of individual drag-tag–primer conjugates (data not shown).

In samples with a point mutation at one or more loci, the corresponding peak(s) change color from those observed for the wild-type sample. For example, in Figure 3A, the sixth peak is green rather than black, indicating a C-to-A substitution mutation at locus 249-3. Other templates displayed mixed genotypes at certain loci, as in Figure 3B, which illustrates peaks of 2 colors at 2 loci. This sample heterozygosity was confirmed by direct sequencing. Notably, these dual genotypes were typically a mixture of wild-type and the expected mutation, indicating that the original sample cell lines must contain mixed populations of wild-type and mutant cells.

Twenty-two different p53 templates were tested, and the resulting electropherograms are presented in a “pseudo-gel” format in Figure 3C, with blue, black, red, and green bands of varying intensity corresponding to the peak heights in the original electropherograms. This representation allows for the rapid comparison and identification of mutations in the different templates, although the original electropherograms (as in Figure 2 and Figure 3A,B) are also useful for identifying possible

(38) Chiesl, T. N.; Shi, W.; Barron, A. E. *Anal. Chem.* **2005**, *77*, 772–779.

(39) Shultz-Lockyear, L. L.; Colyer, C. L.; Fan, Z. H.; Roy, K. I.; Harrison, D. J. *Electrophoresis* **1999**, *20*, 529–538.

(40) Albarghouthi, M. N.; Buchholz, B. A.; Huiberts, P. J.; Stein, T. M.; Barron, A. E. *Electrophoresis* **2002**, *23*, 1429–1440.

(41) Albarghouthi, M. N.; Stein, T. M.; Barron, A. E. *Electrophoresis* **2003**, *24*, 1166–1175.

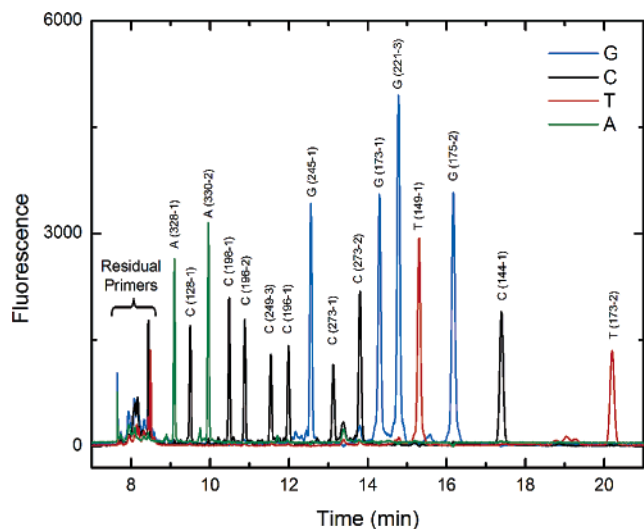


Figure 2. Four-color electropherogram showing the FSCE separation of the products of a 16plex SBE genotyping reaction with a wild-type p53 template. Separations were performed in free aqueous solution on an ABI 3100 CE instrument using a capillary array with an effective length of 36 cm. The buffer was 89 mM Tris, 89 mM TAPS, 2 mM EDTA with 7 M urea. The run temperature was 55 °C. Samples were injected electrokinetically at 44 V/cm for 20 s. The field strength for the separation was 312 V/cm, with a current of 11 μ A per capillary. Each peak is labeled with the corresponding p53 locus and genotype.

heterozygotes or low-level peaks that do not show up strongly in the pseudo-gel image.

Of 16 loci across 22 mutant templates (352 loci total), SBE-FSCE correctly and reproducibly genotyped 325 loci. Twenty-seven loci reproducibly gave genotypes that were different from those that we expected on the basis of direct sequencing that had been done by our collaborators at NIST, including 10 apparent heterozygotes. When the original NIST genetic samples were then re-sequenced at Northwestern University, 14 of the 27 unexpected genotypes were confirmed to be accurate, including 5 of the 10 apparent heterozygotes; hence, SBE-FSCE more accurately identified these heterozygotes than the original direct sequencing done at NIST. Ten of the remaining unexpected SBE-FSCE genotypes could not be confirmed because the scarce samples could not be sufficiently amplified for re-sequencing. We expect, however, on the basis of the other results, that many of these SBE-FSCE results are correct for these samples as well. Overall, 339 of the 352 loci could be confirmed to be correctly genotyped, representing a confirmed accuracy of 96.3% for SBE-FSCE. Accuracies in excess of 99% have been reported for other SBE-based assays,⁴² and the molecular biology of the SBE reaction is seemingly not affected by the drag-tag's presence.

Possible interaction or complementarity of the different primers used in a highly multiplexed SBE assay becomes more likely as the level of multiplexing increases; however, sophisticated software for multiplexed primer design can be used to analyze all possible combinations of primer–dimers for potential stable or

extendable structures.^{43,44} The issue of primer complementarity presents a difficulty for multiplexing any genotyping assay based on primer extension, and is not specific to the FSCE separation technique reported here, nor to CE separation and detection in general. For this study, the choice of primers was dictated by the loci of known mutations in the panel of available cell line samples. We used 7 M urea and elevated temperature during the electrophoretic analysis to ensure denaturation of any primer–dimers. We do suspect that some primer–dimers may have formed during the SBE reaction itself, as we occasionally saw some low-level peaks that did not correspond specifically to any of the individual drag-tagged primers—these can be seen, for example, as low-intensity bands in Figure 3C. However, the expected SBE genotyping peaks were the dominant products observed, indicating that the primers annealed preferentially with the template, rather than with each other.

The final primer listed in Table 1 for locus 173-2 included the “universal” base inosine (I) at the 3'-end to probe for mutations adjacent to a polymorphic site (locus 173-1), because chain extension following a 3'-mismatch is inefficient. In most cases, this inosine-containing primer gave the expected genotype (T) for locus 173-2, as can be observed by the topmost red band present for most of the samples depicted in Figure 3C. However, this strategy was found to be ineffective in the two templates tested with known mutations at locus 173-1 (mutants 1 and 20 in Figure 3C), with a low efficiency of chain extension and apparently incorrect genotyping results in both cases.

The SBE reaction conditions were modified slightly from the manufacturer's recommended protocol to give optimal performance with the drag-tag-labeled primers. The 51.5 °C annealing temperature was determined empirically as the annealing temperature that gave the most even peak heights across all of the loci in the wild-type sample. Only five cycles were used in an attempt to shorten the thermal cycling reaction; reactions with as few as two cycles gave sufficient signal in our ABI 3100 CE instrument. The shortened reaction time, along with the addition of a small amount of HCl to the SBE reaction mixture, also alleviated a side reaction which we believe to be the base-catalyzed ring-opening of the maleimidopropionic acid linker on the polypeptoid drag-tags.⁴⁵ Drag-tags prepared using a Sulfo-SMCC linker (including the branched polypeptoid and the linear protein polymer reported here) are much less prone to this side reaction. If signal strength were a limiting factor, a more conventional SBE reaction with 20–25 cycles could be performed using a set of drag-tags prepared with the more stable Sulfo-SMCC linker.

Microfluidic Chip Separations. Figure 4 illustrates the rapid separation of SBE reaction products in a microfluidic electrophoresis chip using the wild-type p53 template. The 16plex SBE-FSCE samples could be separated with high resolution in less than 70 s in a glass microfluidic device with an effective separation length of 8 cm, approximately 20 times faster than CE. Separations on microfluidic chips are achieved much faster because of the geometry and design of the injection scheme. By using isota-

(42) Hirschhorn, J. N.; Sklar, P.; Linbland-Toh, K.; Lim, Y.-M.; Ruiz-Gutierrez, M.; Bolk, S.; Langhorst, B.; Schaffner, S.; Winchester, E.; Lander, E. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12164–12169.

(43) Rachlin, J.; Ding, C. M.; Cantor, C.; Kasif, S. *Nucleic Acids Res.* **2005**, *33*, W544–W547.

(44) Kaderali, L.; Deshpande, A.; Nolan, J. P.; White, P. S. *Nucleic Acids Res.* **2003**, *31*, 1796–1802.

(45) Hermanson, G. *Bioconjugate Techniques*; Academic Press, Inc: San Diego, CA, 1996.

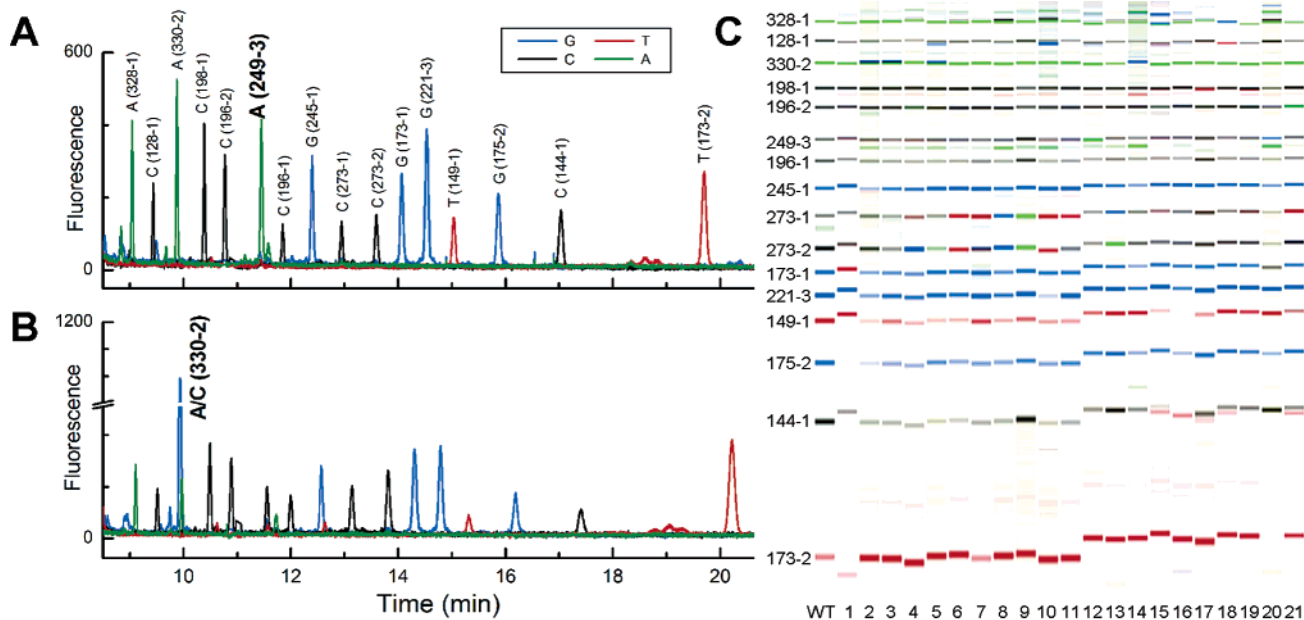


Figure 3. (A,B) Four-color FSCE electropherograms showing the analysis of 16plex SBE reactions using PCR amplicons of two different p53 variants as templates, with the mutated loci highlighted, including two heterozygotes confirmed by re-sequencing of the mutant template in (B). Separation conditions are the same as described for Figure 2. (C) Pseudo-gel representation of 22 separate SBE-FSCE analyses of p53 variants. The wild-type sample is the left-most column, with 21 different mutant samples shown.

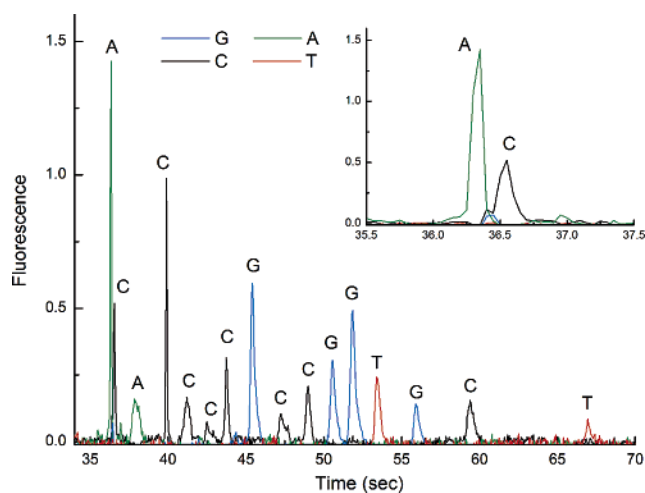


Figure 4. Separation of a 16plex wild-type p53 SBE sample by free-solution electrophoresis in a glass microfluidic chip, with an adsorbed layer of poly-*N*-hydroxyethylacrylamide used to coat the interior microchannel's surface to suppress electroosmotic flow and analyte adsorption. The buffer was 49 mM Tris, 49 mM TAPS, 2 mM EDTA with 7 M urea. The effective separation length was 8 cm, with an applied field of 530 V/cm and a temperature of 55 °C. The region between 35 and 38 s is magnified in the inset to show the distinct elution times of the first "A" and "C" peaks.

chophoretic injection on a chip with a double-T injection geometry,³⁹ the sample "stacks" into a very narrow, well-defined zone that is readily separated in the 8-cm separation channel of the microfluidic chip. The peak spacing is comparable to that observed with CE, except for the first two peaks that are more closely spaced. Further optimization of the chip-based separation (increased separation distance and higher electric field strengths) could enable faster and further multiplexed sample analysis, although a 70-s separation is probably fast enough for most users.

Flexibility of the SBE-FSCE Method. The polyamide drag-tags and oligonucleotide primers we created for this study are

interchangeable; any drag-tag can be paired with any primer to allow tailored conjugates for custom applications. Whereas the specific primer–drag-tag conjugates described in Table 1 allow for multiplexed mutation detection of 16 different loci from the same individual, other tests are possible. For example, many of the mutant samples have mutations at p53 loci 273-1 and 273-2. The genotypes of these 2 loci could be tracked across several different samples in parallel by creating a different multiplexed set of primers. To this end, the primer for locus 273-1 was conjugated to seven different poly-*N*-methoxyethylglycine (poly-*N*-MEG) drag-tags ranging in size from 8 to 32 monomers, and the primer for 273-2 was conjugated to larger drag-tags, 36–60 monomers in length. Seven separate SBE reactions were run in parallel with 7 different templates, each using a unique pair of the primer–drag-tag conjugates. The resulting SBE reaction products were then pooled and analyzed by CE in a single capillary (Figure 5). Since a unique pair of primer–drag-tag conjugates was used for each template, each peak in the electropherogram can easily be assigned to a specific template and locus. The wild-type is seen to have the genotype "CC", whereas mutant 7 has the genotype "TC", and mutant 8 has the genotype "GG". These results correlate well with the results determined by sequencing and also with the 16plex genotyping reaction result for each mutant sample.

This combination of drag-tags and primers would allow the 273-1 and 273-2 loci from 96 patient samples to be analyzed in 16 min with the 16-capillary ABI 3100, or in approximately 1 min by microfluidic chip electrophoresis. Any combination of primers can easily be paired with any combination of drag-tags. By contrast, conventional CE with polymer matrix-based separation of the SBE reaction products would require custom synthesis of primers with different lengths of DNA "tails" for each situation, so that SBE reaction products (which are very similar in size) could be

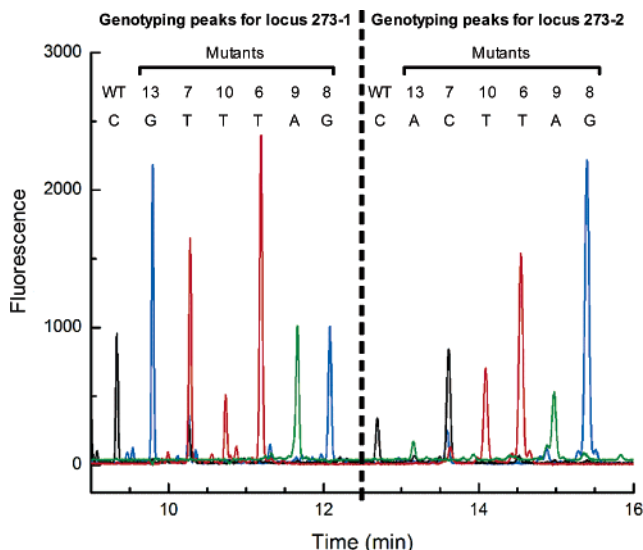


Figure 5. Parallel mutation detection of two p53 loci across seven templates (wild-type and six mutants), with a single electrophoretic analysis separating the pooled reaction products. The mutant sample numbers correspond to those in the pseudo-gel image shown in Figure 3C. CE separation conditions are as described in Figure 2.

separated by electrophoresis in a gel.³⁴ A thiolated primer of length 17–24 bases is of comparable cost to a standard primer with a long “tail” but can be used for multiple different applications using FSCE. Protein polymer and poly(NMEG) drag-tags can easily be synthesized on a lab-bench scale of tens of milligrams for a modest cost; these amounts would be sufficient to perform thousands of the reactions described here. Thus, the drag-tags themselves represent only a small added expense in the SBE-FSCE procedure.

The degree of multiplexing possible with SBE-FSCE depends primarily on the number of unique drag-tags available; however, no fundamental barrier prevents the synthesis of additional unique drag-tags to allow the use of additional primers. Given the possibility of creating an arbitrarily large number of unique drag-tags, the potential for further multiplexing of the SBE-FSCE technique is primarily limited by the ability to design a suitable set of compatible primers. Multiplexed SBE with simultaneous interrogation of 30 SNPs followed by MALDI-TOF mass spectrometry analysis (requiring primers of easily distinguished molecular weight) has been reported,¹⁸ so there is no reason to believe that the molecular biology of SBE itself would be incompatible with further multiplexing using the FSCE technique.

(46) Paegel, B. M.; Blazej, R. G.; Mathies, R. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 42–50.

(47) Paegel, B. M.; Yeung, S. H. I.; Mathies, R. A. *Anal. Chem.* **2002**, *74*, 5092–5098.

(48) Ferrance, J. P.; Wu, Q. R.; Giordano, B.; Hernandez, C.; Kwok, Y.; Snow, K.; Thibodeau, S.; Landers, J. P. *Anal. Chim. Acta* **2003**, *500*, 223–236.

CONCLUSIONS

A novel SBE-FSCE technique was used to simultaneously genotype 16 loci on p53 exons 5–9, with rapid electrophoretic analysis allowing separation of each of the genotyping products in free solution with excellent resolution. High-throughput analysis can be achieved with highly parallel, commercially available capillary array sequencing instruments or miniaturized microfluidic chips. The method was employed here for the detection of point mutations, but the same technique could also be employed as a high-throughput approach for SNP detection. The accuracy of the genotyping was in excess of 96.3%, which is typical for SBE techniques; a larger panel of well-characterized templates is desirable for further validation of this genotyping method.

The SBE-FSCE technique represents a “modular” genotyping approach, applicable to any gene, where the same oligonucleotide primer can be tailored to multiple uses by the facile attachment of different drag-tags. The molecular biology of SBE allows for a very high degree of multiplexing, while FSCE requires each genotyping product to be conjugated to a drag-tag of a unique size and hydrodynamic drag. Sixteen unique drag-tags were created for this study, but synthesis techniques for protein polymers and branched polypeptoids allow for a much larger number of possibilities. While designing an effective set of multiplexed primers becomes increasingly difficult as the number of primers grows, the use of computer algorithms for the future design of multiplexed primers can be helpful.

SBE-FSCE separation on microfluidic devices is promising; further optimization of sample cleanup and injection methods is underway and should improve performance further. The results we have obtained thus far indicate that extremely rapid (~1 min) separations of SBE-FSCE products are possible on microfluidic devices. This rapid, highly multiplexed assay, accompanied by efficient thermal cycling and sample purification protocols, is a promising candidate for implementation on a single integrated microfluidic device^{46–48} and could find many clinical and research applications.

ACKNOWLEDGMENT

We thank Dr. Catherine O’Connell and Dr. John P. Jakupciak from the National Institute of Standards and Technology in Gaithersburg, MD, for their invaluable help and the gift of the p53 mutant samples. This work was supported by the National Institutes of Health (NIH) (Grant No. NHGRI 5 R01 HG002918) and by a Northwestern University Henderson Fellowship for R.J.M.

Received for review October 9, 2006. Accepted December 7, 2006.

AC061903Z