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Articles

Multiplexed, High-Throughput Genotyping by Single-Base Extension and End-Labeled Free-Solution Electrophoresis

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Technologies that allow for high-throughput, economical, and accurate single nucleotide polymorphism (SNP) genotyping are becoming crucial for modern genomic efforts. Here, we present a method for multiplexed single-base extension (SBE) genotyping that takes advantage of the unique separation modalities made possible via end-labeled free-solution electrophoresis (ELFSE). Three unique SBE oligonucleotide primers, which probe for mutations of clinical importance in the human p53 gene, were covalently conjugated to three unique polypeptoid frictional end labels and mixed together. This primer−polypeptoid conjugate cocktail was then used in a multiplexed SBE reaction followed by free-solution separation in a 96-capillary array electrophoresis (CAE) instrument. The study was designed to demonstrate multiplexed SNP genotyping of several loci in a single reaction and a single subsequent analysis. Further, the electrophoretic analysis was conducted without any viscous polymeric separation media, was complete in less than 10 min, and can be implemented in any capillary or microfluidic electrophoretic system with four-color fluorescent detection capabilities. Multiplexed SBE-ELFSE genotyping analysis resulted in the simultaneous and accurate genotyping of three p53 loci on five different DNA templates in a single reaction set and single CAE analysis. With the implementation of this method in 96 or more capillaries in parallel, high-throughput screening of SNPs will be accessible to a large number of laboratories.

When the human genome has been completely sequenced with high accuracy, one of the next major challenges to the genomics community will be the determination of the importance and impact of a myriad of single nucleotide polymorphisms (SNPs) found in subsets of the population.[1,2] To take full advantage of the genetic knowledge gained by the Human Genome Project, it will be necessary to genotype a large number of SNPs in different individuals in order to link complex genetic identities to phenotypic traits, including many human diseases.[3,4] Central to this goal will be the development of technologies that allow for robust, accurate, and high-throughput determination of a large number of genotypes for many different samples.

Currently, a number of techniques are being used for SNP genotyping. These can be divided into two different classes according to their operation: those based on DNA hybridization alone and those based on enzymatic nucleotide recognition. Hybridization relies on small differences in the thermal stability of DNA duplexes between perfectly matched and slightly mismatched hybrids and is typically employed using DNA microarrays.[5−9] This technology readily scales to high parallelization; with multiplexed PCR, multiple SNP genotypes in a single sample can be screened in one analysis. However, this technique, with its unparalleled utility to screen for the presence of a particular gene or gene product, lacks high specificity, because single-base mismatches between the target and the surface-bound DNA "probe" oligomer can be difficult to distinguish on the basis of differences in DNA melting temperature alone.[10] Additionally, a DNA microarray can be used for only one genotyping analysis before it must be replaced in order to avoid sample-to-sample contamination that may occur if a single chip is used for multiple analyses.

The second class of general SNP genotyping methods functions through an enzymatic recognition event that catalyzes a particular reaction. Products of this reaction are then analyzed to yield information on the SNP genotype. This general category of

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enzymatic reactions may be further subdivided into three categories on the basis of the particular enzymatic reaction utilized in the analysis. The first of these subcategories has been commercialized by Third Wave Technologies (Madison, WI) under the trade name Invader assay, and relies on cleavage of an overlapping probe DNA oligomer by a flap endonuclease.[11] The second of these subclasses takes advantage of the 5′-exonuclease activity of DNA polymerase enzymes and includes the Taqman assay (Applied Biosystems, Inc., Foster City, CA),[12] eTag chemistry (ACLARA BioSciences, Moutain View, CA),[13] oligonucleotide ligation,[14] differential hybridization, and molecular beacons.[15] The third of these subcategories relies on DNA polymerase to incorporate specific nucleotides onto a growing DNA oligomer. Techniques in this subcategory include single-base extension[16,17] (originally called primer-guided nucleotide incorporation) and pyrosequencing.[18−21] Because of the difficulty in scaling these techniques to screen several loci on a single sample in one reaction, enzymatic techniques have been primarily used to screen one SNP sample per reaction and analysis. For this reason, these techniques are not readily amenable to a high-throughput genotyping reaction, where multiple SNPs in multiple samples are scored, ideally with a minimal experimental effort.

Here, we present a methodology that incorporates the high accuracy of DNA polymerase nucleotide incorporation using single-base extension reactions (SBE) with a novel multiplexing strategy enabled by end-labeled free-solution electrophoresis (ELFSE).[22] This creates a high-throughput assay that can be run in any standard capillary or microfluidic electrophoretic device. Multiplexing SBE methodology in order to screen multiple loci requires several locus-specific SBE reactions, followed by separation and detection of the reaction products. To date, this separation has been performed via gel electrophoresis[23] or by DNA microarray hybridization.[7] The implementation of ELFSE with SBE allows for the high-resolution electrophoretic separation of single-base extension products in free solution. Thus, this technique allows for SNP genotyping of multiple loci of a sample in a single reaction and single subsequent analysis. We believe that this technique will be especially applicable in a clinical screening environment in which a certain region of the genome is screened for particular SNP genotypes that have a high correlation with a particular disease state or its etiology. As a demonstration of our multiplexed SBE-ELFSE technique, we have chosen to genotype exon 7 of the p53 gene for a set of three single-base substitution mutations that have a high correlation with metastatic cancer. Although these sequence alterations represent somatic mutations and hence are not strictly "SNPs", the analysis of germline SNP sequence alterations could be done in exactly the same manner.

Single-Base Extension. Single-base extension is a robust technique that allows for straightforward genotyping of a particular locus. In this technique, a DNA oligomeric primer is designed that anneals one base 5′ of the locus that is to be genotyped. A DNA polymerase, in the presence of only ddNTP chain-terminating monomers, then appends a single DNA base to the 3′ end of the DNA primer. The identity of this appended base is then indicative of the genotype of the original template molecule. Using fluorescently labeled ddNTP's in the reaction mix allows for facile determination of the appended base identity through fluorescence detection.

Multiplexing SBE to genotype multiple SNPs in a single reaction can allow for decreased reagent consumption and thus lower cost of analysis but requires the separation of the different SBE reaction products. Standard "gel", or matrix-based, electrophoresis separates DNA oligomers on the basis of chain length, thus for multiplexed SBE SNP genotyping using a standard microfluidic or capillary electrophoresis platform, one must design SBE primers having different chain lengths. Additionally, to perform optimal multiplexed SBE reactions, one must design multiple DNA primers that anneal to unique loci with sufficiently similar annealing temperatures to allow for efficient nucleotide incorporation. Because the annealing temperature (often called the melting temperature) of DNA oligomers is largely a function of chain length, the design of DNA oligomers with similar annealing temperatures but different chain lengths is limited to a relatively small number of possibilities. Thus, one must seek a balance between matching the annealing temperatures (which requires similar DNA oligomer lengths) and maximizing the difference in electrophoretic mobility (which requires different DNA oligomer lengths). Additionally, the separation of short-chain DNA oligomers via matrix-based electrophoresis is still challenging in modern microchannel instrumentation using entangled polymer solutions. Very concentrated and high-viscosity polymer networks are generally required and can be difficult to load into capillary microchannels.

In 1992, end-labeled free-solution electrophoresis was proposed as a methodology to allow size-based electrophoretic separation of DNA fragments in buffer alone (i.e., without any polymeric matrix).[24] This technique requires that all DNA fragments be derivatized with a monodisperse frictional end-label (or "drag-tag") that alters the charge-to-friction ratio of the DNA and allows for size-based electrophoretic separation in free solution.[25] In this report, we have extended this technique to enable multiplexed single-base extension reactions, the products of which are subsequently separated and analyzed via free-solution microchan-

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Table 1. Summary of SBE Primers Used in This Study Showing the Genotyping Locus on the P53 Gene, DNA Sequence, Primer Length, and Melting Temperature

primer no.	genotyping locus	sequence (3′ → 5′)	length (bases)	mp, °C
1	14059	CGT ACT TGG CCT CCG GGT A	19	71.9
2	14060	GTA CTT GGCCTC CGG GTA G	19	73.5
3	14073	GGG TAG GAG TGG TAG TAG TGT GA	24	71.9

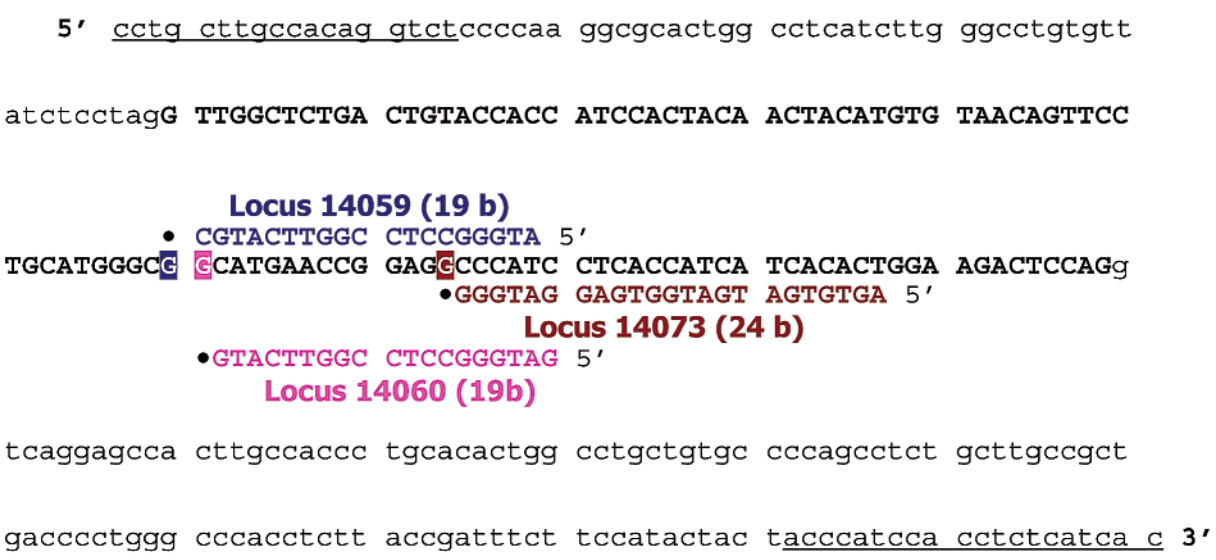

Figure 1. Schematic diagram showing the annealing location on the p53 Exon 7 template amplicon of each of the primers used in this study. Bold capital text is exonic sequence, lowercase text is intronic sequence, underlined lowercase text is the primer sequence used to amplify from genomic DNA samples. Bold, capitalized text is primer sequence used for SBE analysis in this work.

nel electrophoresis. This embodiment has entailed the creation of a set of differently sized, monodisperse, uncharged oligomeric drag-tag molecules and a set of SBE primers that each anneal to a unique locus to be genotyped. Each of these SBE primers is then conjugated to a unique drag-tag molecule; thus, each resulting conjugate molecule has a unique electrophoretic mobility and can be easily separated by free-solution microchannel electrophoresis.

This methodology, in effect, decouples the annealing temperature of the SBE primer from its electrophoretic mobility and allows for multiplexed SNP genotyping in an electrophoretic platform. Further, this technique is applicable in any microchannel electrophoretic device with four-color fluorescent detection capabilities. Implementation of the method by capillary array electrophoresis will allow this SBE-ELFSE technique to be used to simultaneously genotype multiple loci of clinical interest in many samples, using a single reaction set and a single subsequent analysis.

MATERIALS AND METHODS

Sample Identity. PCR amplicons isolated from immortalized ATCC cell lines containing mutations in exon 7 of the p53 gene were a generous gift from Catherine O'Connell at the National Institutes of Standards and Technology. The identities of the mutations were verified by fluorescent DNA sequencing (MegaBACE system, Molecular Dynamics, Sunnyvale CA).

Sample Preparation. The PCR reaction mix used to generate the amplicons contains components that are detrimental to an SBE reaction, which need to be eliminated. This was accomplished by incubating 10 μL of PCR reaction mix (containing the amplicons) with 32 U of exonuclease I (Sigma, St. Louis MO) to digest any residual ssDNA primers and 16 U of shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech, Piscataway NJ) to neutralize any residual dNTPS. The incubation was conducted simultaneously with both enzymes at 37 °C for 45 min in the buffer provided with the SAP enzyme, followed by incubation at 95 °C for 20 min to heat-inactivate the enzymes.

SBE Primer Design. Each SBE primer (of three total) was designed to have an annealing temperature of ~73 °C and to anneal one base 5′ on the template DNA of a given mutation. The particular mutations that we chose for this demonstration are in exon 7 of the p53 gene and are "hot-spot" or high-frequency mutations in cancer. Table 1 summarizes the design of the SBE primers. Figure 1 shows the annealing locus on the p53 exon 7 template DNA amplicon. Two of the primers were 19 bases in length, and the third was composed of 24 bases. SBE primers were synthesized with a C6-thiol linker on the 5′ terminus to allow for conjugation with polypeptoid drag-tag oligomers (Oligos, Etc., Wilsonville, OR).

Polypeptoid Drag-Tag Oligomer Synthesis. Uncharged poly(*N*-methoxyethyl)glycine oligomers (or "polypeptoids") of 10, 20, and 30 monomers in length were synthesized on a 433A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified to monodispersity by reversed-phase HPLC as described previously.[26,27] The polypeptoid structure is shown in Scheme 1.

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Scheme 1. Synthetic Scheme for the Conjugation of a Thiol-Terminated SBE Primer to a Polypeptoid Frictional End Label for Use in an SBE-ELFSE Assay

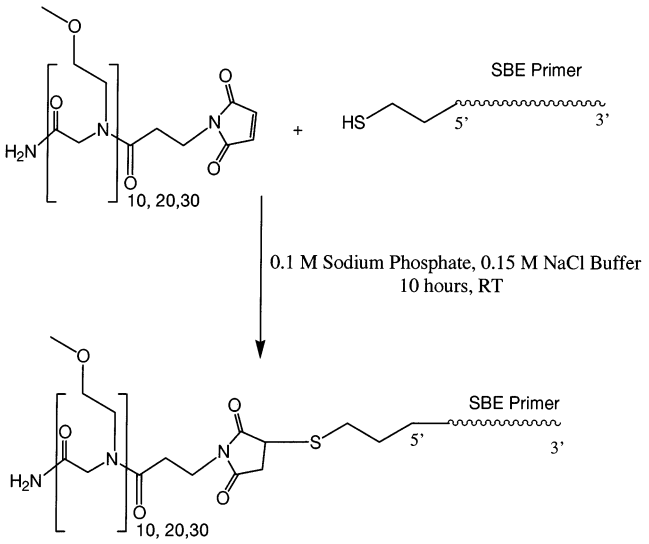

Table 2. Summary of the Conjugates Used in This Study, Showing Primer Size, Polypeptoid Frictional Drag-Tag Length, and Expected Elution Order When Separated by Free-Solution Capillary Electrophoresis

conjugate no.	primer no. (in Table 1)	primer size (no. bases)	drag-tag length (no. monomers)	expected elution order
1	1	19	30	3
2	2	19	20	2
3	3	24	10	1

Molecular mass of the purified polypeptoid drag-tags was verified by MALDI-TOF mass spectrometry and closely matched the expected values of 1333 (10-mer), 2484 (20-mer), and 3636 g/mol (30-mer) (data not shown).

Conjugation. Each of the polypeptoid drag-tags was conjugated to a unique SBE primer as described previously.[26] The reaction is shown in Scheme 1. Table 2 details the identities of the polypeptoid and primer constituents of each conjugate molecule.

SBE Reaction. The SBE reaction was conducted by mixing 3.2 pmol of each SBE primer−drag-tag conjugate, 0.5 μL of the template DNA to be genotyped, 1 μL of each dye-labeled ddNTP chain terminator, and 0.1 U of ThermoSequenase in 20 μL of reaction buffer, all from the dRhodamine terminator core kit (Amersham Pharmacia, Piscataway NJ). The mixture was then thermocycled for 25 cycles of 96 °C for 30 s (denaturation), 54 °C for 30 s (annealing), and 60 °C for 4 min (extension). Samples were then held at 4 °C until cleanup and analysis.

Sample Cleanup. The SBE reaction mixture was passed through Centri-Sep gel filtration columns (Princeton Separation, Aldelphia, NJ) according to the manufacturer's directions to remove any unincorporated dye-labeled ddNTP terminators as well as buffer salts. The eluent from these columns was then speed-vacuumed down to minimal volume and resuspended in 10 μL of deionized formamide. Immediately before analysis, the samples were heated to 95 °C for 1 min.

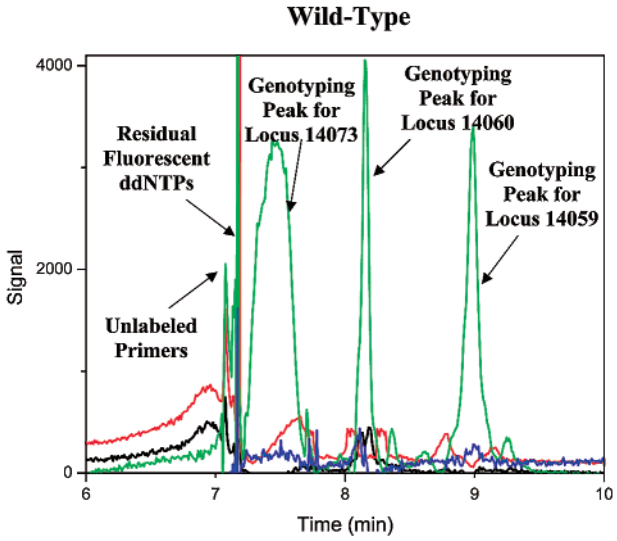

Figure 2. Four-color electropherogram showing the results of an ELFSE-SBE analysis of an amplicon of p53 exon 7 with a wild-type genotype. All four genotyping peaks are green in color, showing that the genotype in each of the loci screened in this analysis is G. Channels: green = G channel, red = C channel, black = T channel, and blue = A channel. Conditions: buffer 1× TTE, 7 M urea. Capillary: 64-cm total length (40-cm effective), 75 μm i.d., LPA coated. Temperature: 30 °C. Electric field: 312 V/cm, 10 μA. Injection at 156 V/cm, 30 s.

Electrophoresis Conditions. Capillary array electrophoresis (CAE) was conducted using a MegaBACE 1000 96-capillary DNA sequencing system with 75-μm-i.d. fused-silica capillaries (total length, 64 cm; effective length, 40 cm) (Molecular Dynamics, Sunnyvale, CA). The capillaries had a covalently bound linear polyacrylamide coating to minimize electroosmotic flow and analyte interactions with the inner capillary wall. Uncoated capillaries were not used, because these analytes give irreproducible electropherograms when analyzed in uncoated capillaries, presumably as a result of nonspecific adsorption of sample elements to the bare fused-silica surface. The capillaries were filled with 1X TTE, 7 M urea buffer (50 mM Tris, 50 mM TAPS, 2 mM EDTA, 7 M urea). Samples were introduced into the capillary using a voltage of 156 V/cm for 30 s, followed by separation at 312 V/cm for 20 min. The capillaries were thermostated at 30 °C during the analysis. Samples were detected by PMTs using the standard rhodamine-terminator optical filter set. Electropherograms were produced from the raw data with a standard spectral deconvolution matrix to minimize the appearance of a single dye in multiple channels.

RESULTS AND DISCUSSION

In this study, our SBE-ELFSE SNP analysis technique allowed genotyping of three separate loci in a single reaction and single subsequent electrophoretic analysis. In all cases, the electropherograms contained three well-separated SNP genotyping peaks that clearly show the base identity of each of the loci probed for by the SBE primers. The visual analysis of these clearly separated peaks allows for facile SNP scoring of each of the three loci in each sample.

Electropherograms. Figure 2 presents a typical electropherogram resulting from an SBE-ELFSE genotyping analysis, in this

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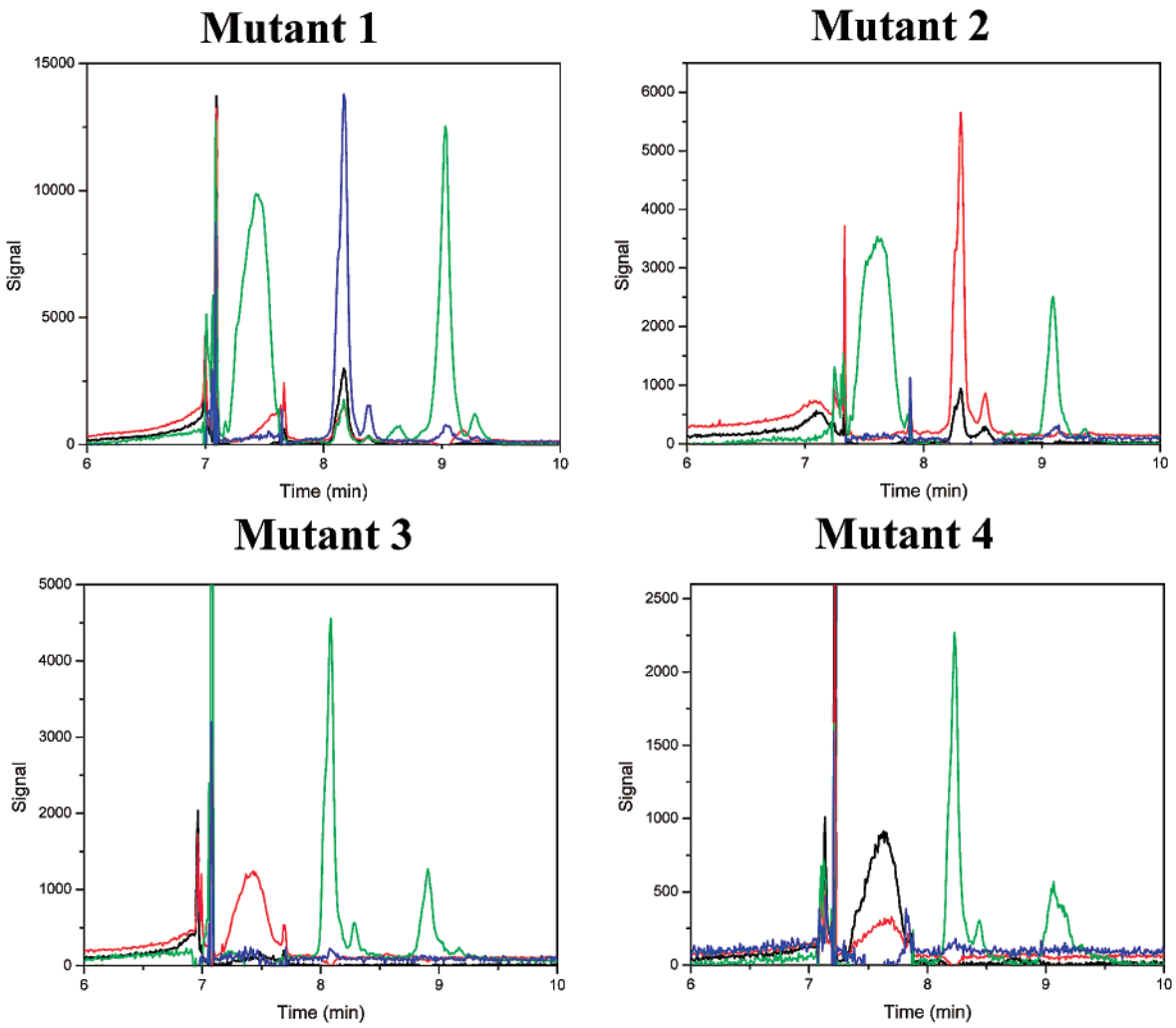

Figure 3. Four-color electropherograms showing the results of an ELFSE-SBE analysis of amplicons of the p53 exon 7 of four different mutant genotypes. Conditions as in Figure 2.

case of a wild-type p53 exon 7 amplicon. In this analysis, there are five clear peaks. In all analyses, electroosmotic flow was suppressed to negligible levels; thus, the analytes elute strictly according to their electrophoretic mobilities (see Table 2 for expected elution order). The first of these peaks, at ~7.1 min, is produced from a small concentration of SBE-DNA probe oligomers that are not derivatized with polypeptoid drag-tags. These underivatized SBE primers, nevertheless, anneal to the locus they are designed to probe and the Thermosequenase enzyme appends a dye-labeled ddNTP to the 3′ terminus. However, because these primers do not carry an uncharged polypeptoid drag-tag, they have a higher electrophoretic mobility and, thus, elute earlier than the drag-tag-labeled SBE primers. Additionally, because all three primers used in this study, when not derivatized with a polypeptoid drag-tag, have essentially the same electrophoretic mobility under the electrophoretic conditions used here, the color of this first peak changes as the average sum of SNP genotypes of the three loci screened in the sample changes. This color change can be seen in the analysis of samples with different genotypes. A small amount of unincorporated dye-labeled ddNTP chain terminators that were not removed by the gel filtration sample cleanup procedure produce the second sharp peak at ~7.2 min. The last three peaks, which are broader, represent the SBE-ELFSE genotyping peaks. The peaks resulting from unlabeled primers and unincorporated ddNTP's were identified by spiking these components into the analysis and noting which peaks were of increased amplitude. All genotyping peaks were identified by removing that particular component from the SBE analysis and observing which peak was absent in the electropherogram.

The first SBE-ELFSE peak, centered at ~7.5 min, is produced by the locus 14073 primer which carries a 10-mer polypeptoid drag-tag label. This conjugate has the largest DNA chain length (24 bases) and smallest polypeptoid component and, thus, has the highest mobility of the conjugate molecules. In this color scheme, the green color of the first peak is indicative of a "G" genotype in the original sample at locus 14073; hence, the result of this analysis is in agreement with sequencing data. The increased width of this peak is likely due to combination of an electrodispersive effect[28] and the increased diffusion coefficient of this conjugate molecule. The second peak at 8.2 min is produced by the SBE primer for locus 14060. This SBE-ELFSE primer has a DNA chain length of 19 bases and a 20-mer polypeptoid frictional label and, thus, has an electrophoretic mobility between those of the other two SBE-ELFSE primer conjugates. The peak's signal

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Table 3. Summary of ELFSE-SBE Genotyping Results for Five Samples with Characterized Mutation in P53 Exon 7

	Expected Genotype			ELFSE − SBE Genotype		
sample	locus 14059	locus 14060	locus 14073	locus 14059	locus 14060	locus 14073
wild-type	G	G	G	G	G	G
mutant 1	G	A	G	G	A	G
mutant 2	G	C	G	G	C	G
mutant 3	G	G	C	G	G	C
mutant 4	G	G	T	G	G	T

is greatest in the green channel and is indicative of the SNP genotype G at locus 14060. The final peak results from the primer for locus 14059, which carries a 30-mer polypeptoid drag-tag. This conjugate, after SBE reaction, produces a signal primarily in the green channel, indicative of a G genotype at locus 14059.

In all cases, the SBE-ELFSE genotyping analysis of this wild-type sample agrees with the results obtained with standard fluorescence sequencing. However, SBE-ELFSE has the experimental advantages that it can be accomplished in an analysis time of less than 10 min, is less complicated to "base-call" than a standard sequencing reaction, and does not require viscous polymeric sieving matrixes.

Electropherograms of SBE-ELFSE analyses for the other four p53 Exon 7 genotypes present in our characterized sample set are presented in Figure 3. SNP genotyping results, as well as genotypes of the cell lines from which these DNA templates were produced, are presented in Table 3. In each case, the SBE-ELFSE genotyping analysis agrees with the genotype predicted for the cell line from which the DNA amplicon was produced. A visual inspection of these 4 panels, as well as Figure 2, shows the ease with which SNP genotypes can be determined. In Figure 3, the panel for mutant 1 shows the second genotyping peak in the blue channel, indicative of an A genotype at locus 14060, whereas the other two loci have the wild-type G genotype (green). In the analysis of mutant 2, the second peak is most strongly seen in the red channel showing a C genotype at locus 14060, with the other loci showing the green G genotype. In the panel for mutant 3, the first genotyping peak, although lower in amplitude, is strongest in the red channel showing a C genotype at locus 14073, with the other loci produce green wild-type G genotyping peaks. Finally, in the panel for mutant 4, the first genotyping peak is predominantly black, indicative of a T genotype, and all other loci are wild-type G.

Additionally, the presence of each of the genotyping peaks shows that the DNA portion of the polypeptoid−DNA conjugate molecule retains its biological activity in the SBE reaction. That is, the presence of the polymeric drag-tag molecule on the 5′ terminus does not impact the molecular biological activity of the DNA SBE primer. This is an important and novel conclusion of this work.

We believe that with further development, the SBE-ELFSE technique could be used to analyze an arbitrarily large number of genetic loci in a single reaction and analysis. Since it is possible to make and purify polypeptoid frictional labels of any length up to ~60 monomers in length,[27] and given that a difference of 10 monomers (and perhaps less) in frictional label size is sufficient to engender a mobility difference large enough to allow baseline CE resolution of SBE primer + drag-tag conjugates, the methodology as we have shown it would readily allow for a "6-plex" genotyping reaction. However, with microchannel electrophoresis equipment that offers longer separation distances, a smaller capillary inner diameter, or more efficient injections, which will allow for higher resolution ELFSE separations, the difference in drag-tag chain length could be lowered to five or fewer monomers while still maintaining sufficient resolution.

We believe that this method will be particularly useful for SNP genotyping of particular loci believed to be implicated in a disease state. The use of capillary array electrophoresis (and later, multilane chip electrophoresis) will allow this process to be highly parallelized. Typical capillary array instrumentation will allow for the analysis of 96 samples in less than 10 min, which would allow for 288 samples to be genotyped in approximately 10 min of analysis time. A 6-plex primer set would allow for 576 genotypes in a single analysis, making this analysis technique competitive with presently available commercial systems in terms of throughput and speed of analysis.

CONCLUSIONS

We have presented a novel SBE-ELFSE technique that allows for rapid, multiplexed SNP genotyping in a single reaction and subsequent analysis. Specifically, conjugation of each of three unique SBE probe primers with a unique frictional polypeptoid end label allows for facile separation of SBE reaction products by free-solution electrophoresis. SBE-ELFSE was used to genotype three loci of the human p53 gene in each sample with one reaction per sample and a single subsequent CAE analysis. In all cases, results from this method agreed with results from fluorescent sequencing, with the SBE-ELFSE analysis being complete in under 10 min. Further, this method requires no viscous polymeric separation matrix and is, thus, ideally suited for miniaturized electrophoresis equipment for which the introduction of viscous material can be challenging. Given that parallel separation systems, such as capillary array electrophoresis, can be operated in a high-throughput mode, we believe this system will be ideally suited for eventual use in molecular diagnostic clinics, where multiple loci in many samples are screened for clinically relevant SNP genotypes. Finally, this technique can be used in any microchannel system with four-color fluorescent detection capabilities.

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