Capillary and Microchip Electrophoresis for Rapid Detection of Known Mutations by Combining Allele-specific DNA Amplification with Heteroduplex Analysis

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Background: Detection of mutations by gel electrophoresis and allele-specific amplification by PCR (AS-PCR) is not easily scaled to accommodate a large number of samples. Alternative electrophoretic formats, such as capillary electrophoresis (CE) and microchip electrophoresis, may provide powerful platforms for simple, fast, automated, and high-throughput mutation detection after allele-specific amplification.

Methods: DNA samples heterozygous for four mutations (185delAG, 5382insC, 3867G→T, and 6174delT) in BRCA1 and BRCA2, and homozygous for one mutation (5382insC) in BRCA1 and two mutations (16delAA and 822delG) in PTEN were chosen as the model system to evaluate the capillary and microchip electrophoresis methods. To detect each mutation, three primers, of which one was labeled with the fluorescent dye 6-carboxyfluorescein and one was the allele-specific primer (mutation-specific primer), were used to amplify the DNA fragments in the range of 130–320 bp. AS-PCR was combined with heteroduplex (HD) analysis, where the DNA fragments obtained by AS-PCR were analyzed with the conditions developed for CE-based HD analysis (using a fluorocarbon-coated capillary and hydroxyethylcellulose). The CE conditions were transferred into the microchip electrophoresis format.

Results: Three genotypes, homozygous wild type, homozygous mutant, and heterozygous mutant, could be identified by CE-based AS-PCR-HD analysis after 10–25 min of analysis time. Using the conditions optimized with CE, we translated the AS-PCR-HD analysis mutation detection method to the microchip electrophoresis format. The detection of three heterozygous mutations (insertion, deletion, and substitution) in BRCA1 could be accomplished in 180 s or less.

Conclusions: It is possible to develop a CE-based method that exploits both AS-PCR and HD analysis for detecting specific mutations. Fast separation and the capacity for automated operation create the potential for developing a powerful electrophoresis-based mutation detection system. Fabrication of multichannel microchip platforms may enable mutation detection with high throughput.

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Germline (or inherited) mutations have been defined in several tumor susceptibility genes, including BRCA1, BRCA2, PTEN, p53, and APC (1–5). Detecting mutations in individuals who carry mutations in these genes can be important for early diagnosis, genetic counseling, and disease prevention (6). There is a need to establish a simple, fast, reliable, and cost-effective method to detect common mutations in individuals at risk. For example, three germline mutations, 185delAG and 5382insC in the BRCA1 gene and 6174delT in the BRCA2 gene, have been found in relatively high frequencies in Ashkenazi Jewish individuals (carrier frequency, ~1 in 50). Carriers of these mutations face an ~65% lifetime risk of developing breast cancer (7). Other genes have common alleles known to modestly modify a person’s lifetime risk of illness [e.g., methylenetetrahydrofolate reductase (MTHFR) variant in colon cancer, and APOE*4 in Alzheimer disease (5,8)].
Thus, efficient genotyping of a large number of samples is required. PCR has been used extensively in cancer research, molecular diagnostics, gene discovery, mRNA quantification, and gene expression profiling (9–11). A diverse array of techniques based on PCR have been developed in molecular biology and adapted in molecular diagnostics. Currently, heteroduplex (HD) analysis, single strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, and denaturing HPLC are used to screen unknown mutations, whereas direct DNA sequencing, restriction enzyme digestion, allele-specific amplification, oligonucleotide hybridization, primer extension, and oligonucleotide ligation are used for detecting known mutations [Refs. (12, 13) and references within]. Nearly all of these mutation detection methods rely on PCR to amplify the DNA fragment of interest for further analysis (12). Among these methods, HD analysis and allele-specific amplification are the simplest for detecting nonspecific and specific mutations, respectively, where PCR products are analyzed directly with little or no additional manipulation (7, 12, 14–16).

Amplification of heterozygous human DNA produces heteroduplexes (two mismatched duplexes) and homoduplexes (two complementary duplexes) in the PCR process, as originally described by Nagamine et al. (17). HD analysis relies on the fact that, under native conditions, homoduplexes (perfectly matched) and heteroduplexes [with a mismatched base pair(s)] formed during PCR amplification will have different electrophoretic mobilities (12, 13).

The general principle of allele-specific amplification by PCR (AS-PCR) is to design an oligonucleotide primer that will lead to the preferential amplification of one allele over another (11, 16). When applied to the detection of known mutations, a mutation-specific primer is designed to preferentially amplify a mutant allele. In the present study, one mutation-specific primer and two flanking primers were used to yield a shorter mutation-specific DNA fragment and a longer flanking DNA fragment. This strategy, illustrated in Fig. 1, is similar to the PCR primer design in slab gel-based AS-PCR. Wild-type, homozygous (containing known mutation), and heterozygous (containing a known or unknown mutation) alleles can be discriminated based on the number of peaks observed (i.e., the profile) under electrophoretic conditions conducive to AS-PCR-HD analysis.

Traditionally, gel electrophoresis is used for HD analysis (12) and for sizing PCR products in allele-specific amplification (7, 14–16). However, capillary electrophoresis (CE) and microchip electrophoresis offer several advantages over gel electrophoresis, the most important of which are high speed and high resolution as well as the small volume sample requirements, low reagent consumption, and the detection sensitivity across a wide range of sample concentrations [1~1000 relative fluorescence unit(s) (RFU)] (18). Whereas serial analysis in a single capillary (the standard configuration) is characteristic of most CE instrumentation, high-throughput parallel analysis can be achieved with capillary array instruments or multiple channels on a microchip (19–23).

In this report, we describe the combination of allele-specific amplification with HD analysis (AS-PCR-HD analysis) for detection of known mutations by CE and show that this method can be translated to microchip electrophoresis. For three BRCA1, one BRCA2, and two PTEN mutations used as model systems, analysis of allele-specific amplified PCR products required 10–24 min by CE and 180 s by microchip electrophoresis. With the method described, wild-type, homozygous, and heterozygous alleles could be rapidly identified based on the AS-PCR profiles under HD analysis conditions (13). Moreover, for DNA purified directly from whole blood or cancer cell lines by a silica-based microsolid-phase extraction method (24), each known mutation could be completely detected with a total analysis time <2.5 h, which included DNA purification (~10 min), DNA amplification by AS-PCR (1.5–2 h), and DNA fragment analysis by CE (10–24 min) or microchip electrophoresis (~180 s).

Materials and Methods

REAGENTS
GeneAmp thin-walled PCR tubes, 10× PCR buffer, 25 mM MgCl₂, 100 mM dNTP stock solutions, and Taq DNA polymerase (5 U/μL) were from Perkin-Elmer. Boric acid, EDTA, and Tris were from Sigma Chemical. Hydroxyethylcellulose (HEC; M₀ 250 000) was from Aldrich Chemical. PicoGreen and YO-PRO-1 were from Molecular Probes. μSil-fluorocarbon polymer-coated capillaries were from J & W Scientific. Ultrapure formamide and 2-mL disposable polystyrene cuvettes were from Fisher Scientific. The dRhodamine terminator cycle sequencing ready reaction reagent set was from PE Applied Biosystem. Polyvinylpyrrolidone (PVP; M₀ 1 000 000) was from Acros Organics.

GENOMIC DNA ISOLATION
Genomic DNA was isolated from lymphoblastoid cell lines obtained from the individuals heterozygous for the mutations in BRCA1 and BRCA2 (Coriell Cell Repositories). No personal identifiers were retained with these samples. Prostate cancer cells (LNCaP) and breast cancer cells (HCC1937, MCF7, and BT549) used for DNA extraction were obtained from the American Type Culture Collection, grown in DMEM, collected, washed, suspended in phosphate-buffered saline, then stored at

Nonstandard abbreviations: HD, heteroduplex; AS-PCR, allele-specific PCR; CE, capillary electrophoresis; RFU, relative fluorescence unit(s); HEC, hydroxyethylcellulose; PVP, polyvinylpyrrolidone; dsDNA, double-stranded DNA; FAM, 6-carboxyfluorescein; TBE, Tris-borate-EDTA; F:R:AS, ratio of forward, reverse, and allele-specific primers; and ssDNA, single-stranded DNA.
At 70 °C until use (25). For the purpose of our analyses, we refer to DNA isolated from the hemizygous cells of HCC1937, LNCaP, and BT549 as homozygous for 5382insC in BRCA1, 16delAA and 822delG in PTEN, respectively.

DNA was purified by the salt precipitation method (Purgene reagent set; Gentra Systems) and measured by the PicoGreen double-stranded DNA (dsDNA) quantification assay (26) before use. The presence of BRCA1, BRCA2, and PTEN mutations was confirmed by fluorescent dideoxy sequencing.

### ALLELE-SPECIFIC AMPLIFICATION BY PCR

Primers used to flank the mutations were mainly from published reports (7, 13, 27, 28). The primers were evaluated by the program on the following web site: http://www.williamstone.com/primers/calculator/ (December 1999). The annealing temperatures for all primers are listed as $T_m$ in Table 1. Unlabeled primers were used for sequencing PCR products; forward or reverse 6-carboxy-fluorescein (FAM)-tagged primers (Life Technologies) were used to obtain the AS-PCR-HD analysis profiles. The DNA fragment sizes amplified for detecting each muta-
<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
<th>Primer, mmol/L</th>
<th>$T_m$, °C</th>
<th>Size, bp</th>
<th>$Mg^{2+}$, mmol/L</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>185delAG (Exon 2, BRCA1)</td>
<td>Forward1: 5'-FAM-GAAGTTGGCATTTTATAAACCTT-3'</td>
<td>200</td>
<td>53</td>
<td>168 (AS)</td>
<td>1.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 30 cycles of 10 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C; 10 cycles of 10 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse1: 5'-TGACTTACAGATGGGACACTA-3' (AS)</td>
<td>100</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse2: 5'-TGCTTTTCTCCTAGTGTG-3'</td>
<td>200</td>
<td>53</td>
<td>258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5382insC (Exon 20, BRCA1)</td>
<td>Forward1: 5'-FAM-TGATGACGTGTCTGCTCCAC-3'</td>
<td>200</td>
<td>56</td>
<td>143 (AS)</td>
<td>1.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 30 cycles of 10 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; 10 cycles of 10 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse1: 5'-CCTTTCTGTCCTGGGGATT-3' (AS)</td>
<td>160</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Reverse2: 5'-AGTCTTACAAATGAAAGGGG-3'</td>
<td>200</td>
<td>55</td>
<td>257</td>
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<tr>
<td>3867 G→T (Exon 11, BRCA1)</td>
<td>Forward1: 5'-FAM-TATTCTAGCCATACACATTG-3'</td>
<td>200</td>
<td>53</td>
<td>189 (AS)</td>
<td>1.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 35 cycles of 10 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C</td>
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<td></td>
<td>Reverse1: 5'-CTCTGTGTTCTTAGACAGACACTA-3' (AS)</td>
<td>150</td>
<td>56</td>
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<td></td>
<td>Reverse2: 5'-CTTTTGCAATATTTACCTG-3'</td>
<td>200</td>
<td>53</td>
<td>260</td>
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<tr>
<td>6174delT (Exon 11, BRCA2)</td>
<td>Forward1: 5'-FAM-CACCTTGTGATGTTAGTTTGGA-3'</td>
<td>200</td>
<td>58</td>
<td>130 (AS)</td>
<td>1.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 30 cycles of 10 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C</td>
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<tr>
<td></td>
<td>Reverse1: 5'-TGATACCTGGACAGATTTTCCCT-3' (AS)</td>
<td>160</td>
<td>61</td>
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<tr>
<td></td>
<td>Reverse2: 5'-TGGAAAAGACTTGCTTGGTACT-3'</td>
<td>200</td>
<td>58</td>
<td>201</td>
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<tr>
<td>16delAA (Exon 1, PTEN)</td>
<td>Forward1: 5'-AGACATGACAGCCATCATCAG-3' (AS)</td>
<td>160</td>
<td>59</td>
<td>131 (AS)</td>
<td>2.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 30 cycles of 10 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C</td>
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<tr>
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<td>Reverse1: 5'-FAM-TCTAAGAGAGTGACAGAAAGGTA-3' (AS)</td>
<td>240</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward2: 5'-AGTCGCTGCAACCATCCA-3'</td>
<td>200</td>
<td>61</td>
<td>320</td>
<td></td>
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<tr>
<td>822delG (Exon 8, PTEN)</td>
<td>Forward1: 5'-AGGACAAAAATGTTCACCTTGGT-3' (AS)</td>
<td>160</td>
<td>60</td>
<td>138 (AS)</td>
<td>2.5</td>
<td>94 °C for 5 min; 10 cycles of 10 s at 94 °C, 40 s at 68 °C, and 30 s at 72 °C, decreasing the annealing temperature by 1.5 °C each cycle; 30 cycles of 10 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C</td>
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<tr>
<td></td>
<td>Reverse1: 5'-FAM-TGTCAATTACCTGCAGCCTG-3' (AS)</td>
<td>240</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward2: 5'-GCACATGTTACATAGGGA-3'</td>
<td>200</td>
<td>64</td>
<td>243</td>
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* Cycling conditions were modified from Struwing et al. (7).

b–d Primers from b Tian et al. (13); c Struwing et al. (7); and d Aveyard et al. (28).
tion are listed in Table 1. The reactions were carried out in a Progene thermocycler (Techne) with the following reagents in 25-µL reaction mixtures: 80–100 ng of genomic DNA, 1 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, and 2.5 U of AmpliTaq polymerase. For standard PCR or two-primer AS-PCR, the concentrations of the two primers were all 200 nM and the concentration of Mg²⁺ was 1.5 mM; for three-primer AS-PCR, the primer and MgCl₂ concentrations for detecting each mutation are listed in Table 1. PCR cycling conditions were modified from the protocols described by Struwing et al. (7) and are listed in Table 1.

**CE-BASED AS-PCR ANALYSIS**

To obtain AS-PCR profiles, we used a Beckman P/ACE 5510 system with a P/ACE LIF detector [excitation at 488 nm (argon ion laser) and emission at 520 nm]. CE conditions were as follows: The fluorocarbon-coated capillary (50 µm i.d.) was 27 cm long (effective length, 20 cm) for deletion and insertion mutations 185delAG, 5382insC, 6174delT, 16delAA, and 822delG, and 37 cm long (effective length, 30 cm) for the substitution mutation 3867G→T. The running buffer was 25 g/L HEC in 1× TBE buffer (89 mmol/L Tris, 89 mmol/L borate, 2 mmol/L EDTA, pH 8.6) containing 100 g/L glycerol for detecting the deletion and insertion mutations, and 45 g/L HEC in 1× TBE buffer containing 100 g/L glycerol and 150 g/L urea (a 500-µL syringe was used to push the buffer into the capillary) for detecting substitution mutations. The PCR products were introduced into the capillary by electrokinetic injection for 30 s at 10 kV. The separation was carried out at 370 V/cm (350 V/cm for the 3867G→T mutation) using reversed polarity (inlet as cathode and outlet as anode), and the capillary was maintained at 20 °C.

**MICROCHIP-BASED AS-PCR-HD ANALYSIS**

Single-channel glass microchips were purchased from Alberta Microelectronic Corporation. The channel on the microchip was coated by PVP according to previously published procedures (29, 30). After coating, the channel was rinsed with water and then with the running buffer [25 g/L HEC containing 100 g/L glycerol for deletions and insertions, or 45 g/L HEC containing 100 g/L glycerol and 150 g/L urea for substitutions]. Sample injection on the microchip was performed by applying a 400 V (333V/cm) potential across the sample and sample waste reservoirs, with the sample at ground. For separation, the sample and sample waste were grounded, and −400 V was applied to the inlet and 4300 V to the outlet (573V/cm). A fluorescence detection system, which has been described elsewhere (29), was used to detect the fluorescence intensity at 520 nm with the argon ion laser as the excitation source (at 488 nm). The data were collected by a LabView program at the rate of 15 Hz.

**Results**

Mg²⁺ and primer concentrations for allele-specific amplification by PCR

Several variables, including allele-specific primer design, nonspecific (flanking) primer design, Mg²⁺ concentration, primer concentration, DNA concentration, Taq polymerase concentration, dNTP concentration, number of amplification cycles, and annealing temperature, are important for successful allele-specific amplification (14–16). On the basis of previous reports defining the importance of Mg²⁺ concentration and primer ratios in the amplification of mutation-specific DNA fragments (7, 14–16), we explored the effect of varying the concentration of both of these components on AS-PCR amplification. A Mg²⁺ concentration titration was carried out for each mutation using a primer ratio of 1:1:0.5 [forward primer:reverse primer: reverse allele-specific primer (F:R:AS)]. An optimal concentration was chosen and is listed in Table 1. Detection of 5382insC was possible over a Mg²⁺ concentration range of 1.5–2.5 mmol/L (data not shown).

The primer ratio for detecting the 5382insC mutation was also important, as shown in Fig. 2 where the F:R:AS ratio was varied; the data associated with the 1:1:05, 1:1:2:0:5, 1:1:08, 1:1:2:0:65, 1:0:5, and 1:0:5:0:5 ratios are shown. The higher intensities of 143 bp and 257/258 bp in the mutant profile and the minimum nonspecific amplification of mutation-specific DNA fragments in the wild-type profile were found by using the F:R:AS ratios shown in Fig. 2 (electropherograms a–d).

**AS-PCR-HD ANALYSIS WITH CE**

Detection of the homozygous PTEN mutation 16delAA (LnCaP cell line) by AS-PCR-HD analysis. The presence of allele-specific products alone is insufficient to distinguish between samples homozygous or heterozygous for a mutant allele. Homozygous mutations are detectable by HD analysis via coamplification of the wild type and homozygous mutant DNA template by PCR, reannealing of the PCR products from the wild type with those from the mutant, or use of a 4-primer assay is necessary (12, 13). Fig. 3A demonstrates the detection of the homozygous mutation 16delAA in PTEN [from the prostate cancer cell line, LnCaP (31, 32)] with CE-based HD analysis. PCR products were obtained using a 1:1 ratio of the two primers flanking the mutation. A single peak in the duplex region (320-bp or 318-bp dsDNA fragment) was observed in the HD analysis profiles from the wild type and the homozygous mutant, shown in electropherograms a and b in Fig. 3A, whereas three peaks in the duplex region were obtained from coamplification and reannealing processes (shown in electropherograms c and d in Fig. 3A). The HD analysis profiles associated with coamplification/reannealing were not completely matched, which may be attributable to the concentration differences of the DNA fragments in the PCR products mixed in the reannealing process.
Detection of the homozygous mutation 16delAA by AS-PCR-HD analysis is illustrated in Fig. 3B, where the PCR products were obtained using three primers (the concentrations are specified in Table 1) and analyzed under the conditions for CE-based HD analysis. For the wild type, there was only a single PCR product peak (320 bp; RFU; migration time, 6.7 min) along with two very small nonspecific DNA fragments (RFU; migration time, 5.3 min; indicated by the arrow in electropherogram e in Fig. 3). In contrast, the homozygous mutation (16delAA) AS-PCR-HD analysis profile displayed two peaks: a mutation-specific DNA fragment, 131 bp (migration time, ~5.3 min); and a longer DNA fragment, 318 bp (migration time, ~6.7 min). When an artificial heterozygote was created by coamplification and reannealing, a dominant peak (related to the mutation-specific DNA fragment) and three peaks were observed in the duplex region of both (electropherograms g and h, respectively, in Fig. 3).

Detection of the homozygous PTEN mutation 822delG (BT549 cell line) by AS-PCR-HD analysis. The presence of multiple peaks in the duplex region of the AS-PCR-HD analysis profile may result from an unknown heterozygous muta-
tion in the DNA sequence flanking the known mutation (i.e., in the region of the longer DNA fragment in AS-PCR) as shown in electropherogram d in Fig. 1. It has previously been reported that the BT549 breast cancer cell line contains a homozygous mutation, 822delG, in exon 8 of the PTEN gene (32).

Fig. 3. Detection of the homozygous mutation 16delAA by CE-based HD analysis and AS-PCR-HD analysis.

Panels A and B show the HD analysis and AS-PCR-HD analysis results, respectively. Two primers (200 nmol/L forward2 and 200 nmol/L reverse1) were used for standard PCR amplification (A), whereas three primers (concentrations specified in Table 1) were used for AS-PCR-HD analysis (B). (a and e), wild type (wt). (b and f), homozygous mutant 16delAA (hm). (c and g), DNA template from wild type and mutant was mixed in a 1:1 ratio and amplified (co). (d and h), 4 μL of PCR products from the wild type and 8 μL of PCR products from the mutant were mixed, heated at 95 °C for 5 min, and cooled at 0 °C for 5 min before injection (re). Other conditions and symbols as in the legend for Fig. 2.
model for the homozygous mutation, but the difficulty in finding a wild-type control precluded this. The 15 samples tested (11 from the cell lines and 4 from blood samples) all displayed the same HD analysis profiles shown in electropherogram a in Fig. 4A. On the basis of the HD analysis profiles (using two primers), we could predict that there was a heterozygous mutation in both breast cancer cell lines: the control MCF7 cell line, which
contains wild-type PTEN, based on a previous report (32), and the BT549 cell line (shown in electropherograms b and d in Fig. 4). The peaks in the duplex region of the HD analysis profiles were almost identical to those obtained with AS-PCR-HD analysis, which utilized three primers for AS-PCR (shown in electropherograms e and f in Fig. 4C). DNA sequencing data confirmed a heterozygous mutation (delT) in the intronic region of exon 8. PTEN was found both in the control sample (MCF7, presumably containing wild-type PTEN) and the BT549 cell line (containing a homozygous mutation, 822delG, in PTEN). In two- or three-primer AS-PCR, the intensities of the mutation-specific DNA fragment (138 bp) in BT549 (indicated by the asterisk in electropherograms d and f in Fig. 4) were much stronger than those in the control (nonspecific PCR products; indicated by arrows in electropherograms c and e in Fig. 4), which indicated that BT549 contains the 822delG mutation as reported (32).

Detection of homozygous and heterozygous mutations in BRCA1 and BRCA2 by AS-PCR-HD analysis. Electrophoretic detection of one homozygous and four heterozygous mutations in BRCA1 and BRCA2 by CE-based AS-PCR-HD analysis is shown in Fig. 5. In general, little or no mutation-specific peak was seen in the homoyzgous wild type (indicated by arrow in electropherogram c in Fig. 5). For the wild type, only a single peak in the duplex region [indicated by a pound sign (#) in electropherograms a, c, f, and h in Fig. 5] was found, whereas there was an extra single-stranded DNA (ssDNA) fragment (257 bp) in the wild type under conditions for detecting 5382insC and 6174delT. For the homozygous mutant allele (5382insC), one mutation-specific peak (indicated by an asterisk) and a single peak (shaded) in the duplex region were found (electropherogram e in Fig. 5). For the heterozygous mutant alleles, all had a well-defined mutation-specific peak (indicated by an asterisk) and at least three peaks in their duplex regions of the profiles (bracketed and shaded; electropherograms b, d, g, and i in Fig. 5). It is noteworthy that the intensities of the peaks that represent ssDNA fragments were not enhanced when a dsDNA intercalator (1 μmol/L YO-PRO-1) was included in the separation buffer (data not shown). By examining the different patterns in the wild-type and mutant AS-PCR-HD analysis profiles, we could identify one homozygous mutation (5382insC) and four heterozygous mutations (185delAG, 5382insC, 6174delT, and 3867T→G) can be identified using the CE-based AS-PCR-HD analysis with an analysis time of <24 min.

![Electropherograms showing detection of mutations](image-url)
Fig. 6. Fast mutation detection via allele-specific amplification-HD analysis on a microfabricated electrophoretic chip.

Panels A, B, and C show the AS-PCR-HD analysis results for the wild-type (wt) and the heterozygous mutants (ht) specified. The separation buffer was 25 g/L HEC containing 100 g/L glycerol in 1× TBE (pH 8.6) for panels A and B and 45 g/L HEC containing 100 g/L glycerol and 150 g/L urea in 1× TBE (pH 8.6) for panel C. The microchannel on the chip was coated with PVP, and detection was mediated by laser-induced fluorescence (emission/excitation 520 nm/488 nm). The PCR products were injected into the channel for 100 s at 333 V/cm, and the separation voltage was 573 V/cm (effective microchannel length, 55 mm). Other symbols as in legend for Fig. 2.
The same buffer systems used for detecting the deletion, insertion, and substitution mutations by CE were translated to the microfabricated platform for microchip-based AS-PCR-HD analysis. Using a microchip with a single PVP-coated channel (depth, 20 μm; width, 50 μm; effective length, ~55 mm), we could identify each mutation in the same manner as with CE, except that analysis times were reduced to <170 s. For the deletion and insertion mutations, the resolution and the AS-PCR-HD analysis profiles obtained by microchip electrophoresis (Fig. 6, A and B) were almost identical to those obtained by CE (Fig. 5, A and B). For the 3867G→T mutation, the resolution obtained by microchip-based AS-PCR-HD analysis was slightly lower than that the resolution obtained by CE (compare Fig. 6C with Fig. 5D).

**Discussion**

**COMBINING ALLELE-SPECIFIC AMPLIFICATION WITH HD ANALYSIS (AS-PCR-HD ANALYSIS)**

In our study, one allele-specific primer and two flanking primers were used for a nested PCR amplification to yield a shorter mutation-specific DNA fragment and a longer DNA fragment (flanking the mutation), which is similar to slab gel-based AS-PCR. By combining HD analysis with AS-PCR (i.e., the AS-PCR products were analyzed using the conditions for HD analysis), we could identify homozygous wild-type, homozygous mutant, and heterozygous alleles based on the different patterns in the AS-PCR-HD analysis profile (shown in Fig. 1). Instead of four peaks according to the theoretical prediction, in most cases, three peaks were found in the duplex region of heterozygous mutations because of resolution limitations. In samples heterozygous for the mutation in the intronic region of PTEN (Fig. 4), more than four peaks were found in the profile, and the intensity pattern of the peaks differed from the other heterozygous mutations shown in Figs. 5 and 6. The reason for this may be related to the sequence of that particular DNA fragment, which is known to contain a T-rich region (in the intron of exon 8). Tsou et al. (33) reported problems with that particular intronic region and avoided it by detecting mutations via sequencing the genomic region flanking exons 8 and 9 in PTEN. The DNA sequencing data showed the alteration (delT) in the intronic region of exon 8 of PTEN in both MCF7 and PTEN cell lines. Therefore, it is possible that the heteroduplex pattern might be the result of replication slippage attributable to the mononucleotide runs (15 Ts in this case).

**OPTIMIZATION OF AS-PCR-HD ANALYSIS**

Several variables play a role in the effectiveness of AS-PCR (14–16). Our studies also suggested (15) that the concentration of Mg$^{2+}$ and the concentration ratios of the three primers used were critical to detecting the PTEN and BRCA1 mutations by this method. The experimental observation that high Mg$^{2+}$ concentration decreased the specificity in AS-PCR supports the previous findings for AS-PCR by Sommer et al. (15). However, the optimal Mg$^{2+}$ concentration and primer ratios (Table 1) for maximal specific amplification with minimal nonspecific amplification differed from those described by Struwing et al. (7) for the detection of the two common mutations in BRCA1 and BRCA2 by gel-based AS-PCR. Possible explanations may include the DNA fragment size and need for longer DNA fragments for effective AS-PCR-HD analysis in this study. Because DNA fragment size within a certain range is important for successful HD analysis by both CE and microchip electrophoresis (13), we chose primer sets that would amplify DNA fragments in the 200–320 bp range, a size that was optimal for both electrophoresis formats.

For analyzing the allele-specific amplified PCR products, we used two sets of conditions previously defined as optimal for HD analysis by CE and microchip electrophoresis (13). For deletion and insertion mutations, a short commercially coated capillary (27 cm) equilibrated with 25 g/L HEC containing 100 g/L glycerol was used, whereas a slightly higher HEC concentration and a longer capillary (37 cm) in the presence of urea was needed for substitution mutations. It is noteworthy that the latter conditions were also effective for detecting the deletions and insertions, but with longer analysis time. One obvious solution to lengthy analysis time is the use of microfabricated channels in planar glass substrates (microchips), which reduced the AS-PCR-HD analysis time by roughly one order of magnitude. There was no loss of resolution with the conditions for detecting deletion/insertion mutations, but there was a slight decrease in resolution (or sensitivity) with substitution mutation detection.

There are several ways to circumvent the loss-of-resolution problem accompanying detection of substitution mutations by microchip-based AS-PCR-HD analysis. The use of microchips with a longer effective length is justified not only in theory but based on AS-PCR-HD analysis experiments in shorter capillaries, which provided poorer resolution for substitution mutation detection (data not shown). A second approach would be to use three primers to produce two allele-specific DNA fragments labeled with different fluoros in AS-PCR; the two allele-specific primers can then be identified by two-color detection (11). The third and final approach would be to use four PCR primers to amplify three DNA fragments, i.e., a wild-type-specific fragment, a mutation-specific fragment, and the longer DNA fragment covering the two allele-specific DNA fragments. Although this latter method was used by van de Locht et al. (34) to detect a factor V mutation using allele-specific amplification and CE, two sets of four primers were explored, one of which was found to yield satisfactory results. According to Locht et al. (34), this approach requires the optimization of several variables, including having to add the mismatch bases into the allele-specific primers (at the 5’ end) for
AS-PCR amplification. In addition, internal loops or a strong similarity to other DNA sequences near the mutation site can complicate the analysis (34). Of the possibilities detailed above, optimizing the separation conditions to improve resolution (e.g., using a microchip with a longer channel) is the most economical and simplest modification to enhance detection of substitution mutations. This is currently under investigation in our laboratory.

As with any new method for mutation detection, comparison with existing techniques must and will be made for detecting specific mutations. Although all methods have advantages and disadvantages, it is clear that the AS-PCR-HD analysis method described here entails little sample manipulation. All that is required for detecting known mutation is DNA purification, allele-specific amplification of DNA, and DNA fragment analysis. Although optimization of the PCR protocol may be required, this is not unique to the AS-PCR-HD analysis method.

When used as a mutation screening tool, HD analysis is ~80% sensitive (12). In contrast to mutation screening, we have chosen to optimize HD analysis for the detection of known mutations in this study. In mutation detection, “sensitivity” and “specificity” are important issues, which involves measuring false-positive and false-negative rates for a large sample pool. The work described here is a concept-proof-type study; the next logical step is to carry out a large “blinded” study to determine the sensitivity and specificity of this assay.

In conclusion, we have demonstrated that allele-specific amplification can be combined with HD analysis for detecting homozygous wild-type, homozygous mutant, and heterozygous alleles using capillary and microchip electrophoresis formats. The method is simple, rapid, and does not require any post-PCR processing (such as restriction enzyme digestion). When three primers were used, analysis of AS-PCR products for detecting known mutations including deletions, insertions, and substitutions was achieved in 10–25 min by CE-based HD analysis conditions. Transfer to the microchip platform further decreased the analysis time by four- to eightfold. It is also possible to extend this assay to a four-color detection system (similar to DNA sequencing) to detect multiple mutations in the same run by using different dye-labeled primers or energy-transfer fluorescence-labeled primers (35) for each mutation. The benefits of transferring this AS-PCR-HD analysis method into a multicapillary electrophoresis system or a multichannel microchip electrophoresis system are obvious and have the potential to facilitate the detection of known mutations with high speed and high throughput in both the clinical and biomedical research arenas.

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