Christa N. Hestekin
Annelise E. Barron

Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA

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Review

The potential of electrophoretic mobility shift assays for clinical mutation detection

As the understanding of the links between genetic mutations and diseases continues to grow, there is an increasing need for techniques that can rapidly, inexpensively, and sensitively detect DNA sequence alterations. Typically, such analyses are performed on PCR-amplified gene regions. Automated DNA sequencing by capillary array electrophoresis can be used, but is expensive to apply to large numbers of patient samples and/or large genes, and may not always reveal low-abundance mutations in heterozygous samples. Many different types of genetic differences need to be detected, including single-base substitutions and larger sequence alterations such as insertions, deletions, and inversions. Electrophoretic mobility shift assays seem well suited to this purpose and could be used for the efficient screening of patient samples for sequence alterations, effectively reducing the number of samples that must be subjected to full and careful sequencing. While there is much promise, many of the mobility shift assays presently under development have yet to be demonstrated to have the high sensitivity and specificity of mutation detection required for routine clinical application. Hence, further studies and optimization are required, in particular the application of these methods not only to particular genes but also to large numbers of patient samples in blinded studies aimed at the rigorous determination of sensitivity and specificity. This review examines the state-of-the-art of the most commonly used mobility shift assays for mutation detection, including denaturing gradient gel electrophoresis, TGGE, SSCP, heteroduplex analysis, and denaturing HPLC.

Keywords: Denaturing gradient gel electrophoresis / Denaturing HPLC / Heteroduplex analysis / Mutation detection / SSCP

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1 Introduction

With the sequencing of the human genome and the future sequencing of the cancer genome, a wealth of information about the link between genetic alterations and disease is being collected. However, in order for this information to be translated to clinical care, there must be a rapid, sensitive, and inexpensive method to examine the genes of importance within the 3 billion base pairs of the human genome (considering both sets of chromosomes, it is in fact 6 billion). A variety of mutation detection methods exist, including automated sequencing by capillary array electrophoresis (CAE), DNA microarrays, and other PCR-based methods. One class of mutation detection methods, electrophoretic mobility shift assays, have the potential to be used as screening methods to allow for the analysis of multiple genes or gene regions for sequence differences at a reasonable cost.

The quality of a mutation detection method for clinical use is typically measured in terms of its sensitivity and specificity of mutation detection. Sensitivity is a reflection of the ability of the method to detect all mutations present (*i.e.*, not to give false negatives). Specificity is a measure of the ability of a mutation detection method to determine accurately when mutations are not present (*i.e.*, not to give false positives). Both numbers should ideally be as close to 100% as possible. Typically, the sensitivity and specificity of the mutation detection method are determined by comparing the results the method gives to what is determined by careful DNA sequencing. For DNA-

Correspondence: Professor Annelise E. Barron, Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA
E-mail: a-barron@northwestern.edu
Fax: +1-847-491-3728

Abbreviations: CAE, capillary array electrophoresis; **DGGE**, denaturing gradient gel electrophoresis; **DHPLC**, denaturing HPLC; **HA**, heteroduplex analysis; **SGE**, slab gel electrophoresis

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based mutation detection, sequencing is considered the "gold standard" and in principle should always be able to determine the exact location and type of mutation that exists.

Although approximately 60% of humans will be affected by a mutation in their genes [1], there are very few human DNA-based genetic tests approved by the United States' Food and Drug Administration (FDA). The first DNA-based genetic test to be approved by the FDA was a test for cystic fibrosis, in May 2005. Obviously, there is still a need for a wide range of genetic tests for other diseases, including the ultimate genetic disease, cancer. As cancer is in fact many different diseases, and is highly "individual" in terms of the correlation between DNA sequence differences and patient outcome, this is perhaps the most important disease model and will be the most complex to understand.

In order to evaluate a large number of patient samples, genes, or gene regions in a cost-effective manner, DNA sequence alteration screening methods, such as electrophoretic mobility shift assays, need to be sensitive and specific enough for routine clinical use (both the sensitivity and specificity should exceed 97% for the best clinical applicability). We consider here five different types of mobility shift assays that have been studied in some detail and also applied to the screening of patient samples, including denaturing gradient gel electrophoresis (DGGE), TGGE, SSCP, heteroduplex analysis (HA), and denaturing HPLC (DHPLC).

2 DGGE

DGGE uses a gradient of a denaturant, such as urea or formamide, to detect mutations by separating homoduplexes from heteroduplexes. Homoduplexes form between complete Watson–Crick base pair matches, including mutant-to-mutant ssDNAs or wild-type to wild-type ssDNAs. A heteroduplex occurs when a mutant single strand of DNA anneals to a wild-type single strand of DNA, creating a region at the point of sequence mismatch in which there is incomplete Watson–Crick base-pairing. The presence of this unpaired region decreases the overall stability of the dsDNA and therefore allows it to be denatured at a lower percentage of denaturant. As dsDNA begins to denature, its mobility will decrease. As the heteroduplexes have a less stable structure, they denature first, decreasing their mobility and separating them from the homoduplexes. Hence, the presence of a mutation can be detected by the observance of additional peaks on the gel or in the electropherogram, for slab gels and microchannel electrophoresis systems, respectively.

Interestingly, there are only a small number of studies that have examined the sensitivity and/or specificity of DGGE. The sensitivity of DGGE was reported to be ~82% for samples from the MEN1 gene [2]. In a study of TP53 exons 5–8, Holmila and Husgafvek-Pursiainen [3] reported the sensitivity of slab gel DGGE to be 88%. However, they noted that DGGE has a more difficult time detecting mutations in the GC-rich exon 5 because the melting profiles are less resolved, possibly due to a lesser degree of DNA denaturing. This raises an important point: the applicability and usefulness of mobility shift methods can be highly dependent on the particular sequence characteristics of the gene region of interest. Hence, even if a method gives very high-sensitivity mutation detection for one gene region, it may not work well for another, and the converse is also true. The apparent "quirkiness" of mobility shift methods is one aspect of these workhorse technologies that has so far slowed their introduction into the clinical laboratory, but there is growing evidence to suggest that once optimized for particular gene regions, these methods can be very reliable.

Although DGGE has been commonly used in slab gel electrophoresis (SGE), it is difficult to translate to capillary or microchip platforms where generating the denaturant gradient is more complex. As will be shown in many of the techniques below, the implementation of mobility shift assays originally developed for slab gels on capillary and microchip electrophoresis platforms will typically increase the sensitivity of a method, as well as increasing the potential for automation and easy multiplexing. Currently, the sensitivity of DGGE is too low to be used in a routine clinical setting. Perhaps, if DGGE can be cleverly translated into a capillary or microchip format, with careful optimization for particular PCR amplicons, it will have the potential to be used in the clinical setting.

3 TGGE

TGGE is similar to DGGE, except that a temperature gradient rather than a denaturant gradient is used to separate the heteroduplexes from the homoduplexes. TGGE determines the presence of a mutation by differences in DNA mobility due to the melting of homoduplexes and heteroduplexes by denaturing with increased temperature. As in DGGE, the heteroduplexes should denature first due to their decreased thermodynamic stability, which will in turn increase their migration time.

In a study by Biyani and Nishigaki [4], a new format for TGGE called micro-TGGE (μTGGE) was introduced, which is similar to conventional TGGE except for its greatly reduced dimensions. They compared the performance of conventional TGGE to μTGGE and found that

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μTGGE requires significantly lower sample volumes (5 μL or less *vs.* 90 μL) and also requires significantly less electrophoresis analysis time (< 10 *vs.* 90 min). They found that μTGGE gave highly reproducible results that were 99% similar to conventional TGGE by PaSS, the peak pattern similarity scores. Overall, they found that the μTGGE system was 5× to 10× smaller, required 10× less electrophoresis time, produced a 5× reduction in cost, and achieved a 100× higher performance than conventional TGGE. This is a very good example of the typical advantages of miniaturization of electrophoretic DNA assays. Here, the performance of the slab-gel system and the miniaturized system were essentially equivalent.

A study by Salimullah *et al.* [5] examined μTGGE/HA on human *c-Ki-ras* and rat p53 samples. By using a parallel temperature gradient and an electrophoresis time of < 10 min, they were able to separate both homoduplexes and heteroduplexes. In HA alone, homoduplexes with different sequences but very similar or the same molecular sizes are typically not separated from each other because they have very similar mobilities. By applying a temperature gradient, these authors could separate the homoduplexes according to the specific mutation change, which is a very impressive result. For example, if the mutation was an A/T to G/C, the thermal stability of the mutant homoduplex was increased and therefore the mobility was also increased.

Although μTGGE appears to be a powerful method for the discrimination of DNA sequence differences and also shows a clear potential to decrease both the sample requirements and the analysis time, to our knowledge there have not yet been any large-scale studies to determine its sensitivity and/or specificity, which will be necessary before it can be translated for use in a clinical setting.

4 SSCP

SSCP detects mutations based on the observable electrophoretic mobility shifts for different conformations formed by single strands of DNA that have different sequences. The different conformers typically are formed by starting from double-stranded PCR products, and denaturing a dilute mixture of wild-type and mutant dsDNA at 95°C followed by "snap-cooling" of the sample on ice to cause the single-stranded conformers to fold upon themselves in such a way as to maximize the number of base-pairs that are formed. Often a denaturant, such as formamide or NaOH, is added to sharpen the bands or to prevent background smearing, although the exact mechanism for these improvements is not known [6]. The use of a low concentration of denaturants, as well as glycerol in some cases, has been more or less routine when SSCP is used in the slab gel format. However, in CE-based studies, Kourkine *et al.* [7] and Kozlowski and Krzyzosiak [8] have both reported a decrease in the efficiency of the electrokinetic injection of DNA and poorer performance of the method with the addition of denaturants such as formamide. Figure 1 schematically presents a typical analysis by CE – SSCP.

SSCP is a useful method of mutation detection because it is simple and relatively inexpensive. The concept behind it is really quite brilliant. Since its invention and development by Orita *et al.* in 1989 [9], it has been used in thousands of research articles, and discussed in several excellent reviews [10–12]. However, the varying reports of its sensitivity and specificity have so far hindered it from becoming a routinely used clinical screening method [10].

A variety of studies have looked at the sensitivity of SGE – SSCP. A study by Berggren *et al.* [13] looked at 34 mutants from p53 exons 5–8 and determined a sensitivity of 91% and a specificity of 88%. Semprini *et al.* [14] examined 56 carriers and 20 control individuals for muta-

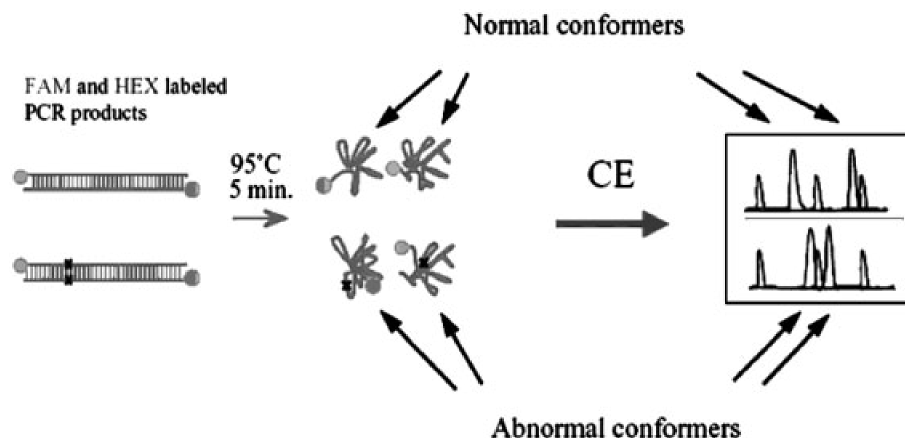

Figure 1. For CE-SSCP, fluorescently labeled PCR fragments are denatured at 95°C and snap-cooled on ice to produce both wild-type and mutant ssDNA conformers. These conformers can then be separated by CE as shown on the right-hand side of the figure. Reprinted with permission from [42].

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tions in the SMN1 gene and found a sensitivity of 96.4% and a specificity of 98%. A method of highly sensitive SGE-SSCP mutation detection called "detection of virtually all mutations" (or "DOVAM") – SSCP uses many different conditions, including different temperatures and buffers, to detect close to 100% of the sequence alterations in a given sample set. A study by Liu *et al.* [15] using DOVAM-SSCP determined that they could achieve 100% mutation detection by using five different conditions to detect 84 single-base substitution mutations in the Factor IX gene. Although these results are very impressive, and demonstrate the potential of SSCP for highly sensitive and specific mutation detection, slab gel-based electrophoresis methods have a number of disadvantages that make them less desirable for clinical use. Compared with CE, slab gel technologies are labor-intensive and time-consuming, require relatively long electrophoresis times, and may require multiple different analysis conditions for high sensitivity. If DOVAM-SSCP can be automated to a larger degree, it will be an excellent screening method. The open question is whether, if SSCP is translated to a different format than slab gels, there will be the same requirement for different buffers and different electrophoresis temperatures.

Several technological improvements are moving SSCP closer to potential use in the clinical setting. The introduction of fluorescent DNA labeling rather than radiolabeling enabled the use of automated detection systems and also created the potential for multiplexing to allow higher throughput systems. The development of capillary and CAE systems as part of the Human Genome Project has also generally improved the peak resolution and throughput capabilities of genotyping systems over previous slab gel systems. In addition, many groups have looked at optimization of SSCP variables and their effect on resolution and mutation detection. One important variable is sample preparation, which has been explored in several recent papers [7, 8]. The sensitivity of SSCP is in particular improved when both the forward and reverse strands are labeled with different fluorescent dyes, typically *via* the use of fluorescently labeled primers. This increases cost (since the primers are expensive), but makes the electropherograms easier to interpret and also increases the chance of seeing a mobility shift in one strand or the other. dsDNA is also easily distinguished in this case since the two "colors" overlap, since both forward and reverse strands are present in the duplex DNA.

CE – SSCP offers the advantages of being automated, high-throughput, and allowing the use of fluorescence detection. In addition, several automated CE sequencing instruments have also been reported to be useful for SSCP, with some minor adjustments [3, 16–18]. This would enable clinical laboratories to reduce their capital equipment expenses by being able to use a single instrument for both the initial screening and the detailed confirmation and identification of mutations by sequencing.

The sensitivity of CE-SSCP has often been reported to be $>90\%$ for fragments with sizes of 250 bp or smaller [17]. A study by Larsen *et al.* [18] found that by using three different temperatures (14, 20, and 35°C) for CE-SSCP, they could achieve 100% sensitivity of mutation detection, whereas each individual temperature produced a sensitivity of between 47 and 84%. Mogensen *et al.* [19] used CE-SSCP for detection of mutations in the hypertrophic cardiomyopathy (HCM) gene and obtained 95% sensitivity and 97% specificity for the 78 mutants they tested. Andersen *et al.* [10] looked at 185 mutations from the MYH7, MYL2, MYL3, TNNI3, and KCNQ1 genes and determined that CAE-SSCP had a sensitivity of 98% and a specificity of 100% when it was performed at two temperatures (18 and 30°C) [10]. Sasaki *et al.* [20] used "postlabeled" (typically with fluorescent dyes) automated CE (PLACE)-SSCP to obtain 90% sensitivity and 100% specificity for the detection of SNPs. This was the first time that CE-based SSCP was shown to be directly applicable to SNP screening, and this should increase interest in the method, since many other prospective methods for SNPs are much more expensive and/or complicated than CAE-SSCP.

A study by Holmila and Husgafvek-Pursiainen [3] compared CE-SSCP, DGGE, and direct sequencing for the analysis of 20 lung cancer patient samples for TP53 mutations. Interestingly, they found that CE-SSCP had the highest mutation detection rate at 94% and that direct, automated sequencing with the ABI Prism 310 CE system had the lowest mutation detection rate at 71%. This highlights an important point: automated sequencing by CAE does not necessarily deserve its present status as the gold standard for comparison, since patient samples are often heterozygous for a mutation, and sequencing electropherograms with representation from two different bases in the same position (one is typically present in lower abundance) are often miscalled by the base-calling software. In the same study, DGGE gave a mutation detection rate of 88%, better than automated sequencing. Importantly, for the CE-SSCP analysis, they found that all the mutations were detectable at 30°C; hence, it was not necessary to use more than one electrophoresis condition, at least for this set of samples. Ellis *et al.* [21] compared SGE-SSCP, CE-SSCP, and DHPLC for 50 DNA fragments from the ABCC7 gene. At 20°C, the SGE-SSCP system (using a special "mutation detection enhance-

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ment" (MDE) gel from Cambrex) had a sensitivity of 94% whereas the SGE-SSCP using a standard polyacrylamide gel had a sensitivity of 96%. At the lowest CE temperature available on the instrument (35°C), CE-SSCP had a sensitivity of 72%, which was comparable to SGE-SSCP at a similar temperature (74% MDE, 66% acrylamide gel). This highlights the importance of using relatively low temperatures – close to 20°C seems to be best – for SSCP analyses. DHPLC, on the other hand, demonstrated a sensitivity of 90% for the same sample set.

Electrophoresis on microfluidic chips offers the potential for rapid and integrated "lab-on-a-chip" analytical abilities, as has been reported in many studies in the literature. Tian *et al.* [22] have demonstrated the ability of microchip electrophoresis-SSCP to analyze 3 BRCA1 and BRCA2 mutant samples in less than 120 s [22].

While capillary and microchip electrophoresis-SSCP clearly have significant potential as clinical mutation detection assays, there are still several important drawbacks. SSCP's sensitivity is optimal when DNA amplicon fragment sizes are less than 300 bp [18, 22, 23], restricting its applicability to relatively small gene regions. In addition, the optimization of SSCP is an empirical process because there are currently no computer programs that can accurately predict the ssDNA conformation or conformer migration order during electrophoresis. However, there has been some work in this direction. Glavac *et al.* [24] compared the single-stranded secondary structures predicted by the program MFOLD with the mobility shifts produced by DNA fragments of 52, 77, and 193 bp. For the shorter DNA (52 and 77 bp) they found a promising correlation between the mobility shifts and the predicted conformers, but for the larger fragment (193 bp), they saw much less correlation. It is important to note that they only looked at 10 mutations for the 52 and 77 bp fragments, while they considered 46 different mutations for the 193 bp fragment. Hence, the better correlation for the smaller fragments may simply be due to some bias in their smaller sample set rather than a true correlation. Another study by Atha *et al.* [25] used a modified version of MFOLD and was able to obtain good correlations, but again the sample size was very limited (only 3 mutations in this case). Although SSCP is a broadly used method, it seems that there is a glaring lack of understanding of how the SSCP conformers fold and behave during electrophoresis in a cross-linked gel (in the slab format) or an uncross-linked polymer solution (in capillary or microchip systems). In-depth studies aimed at increasing this understanding could help to decrease the number of experimental conditions required for highly sensitive mutation detection by SSCP. The development of this deeper understanding would also be likely to increase the general acceptance of the method, by creating objective explanations for the apparently "quirky" character of SSCP in detecting mobility-shifted conformers when different DNA samples and/or different electrophoresis systems or conditions are used.

5 HA

HA, also sometimes called duplex analysis (DA), separates heteroduplexes from homoduplexes based on differences in their electrophoretic mobilities that result from incomplete Watson–Crick base-pairing. Figure 2 demonstrates the concepts of HA on a CE system. A type of HA

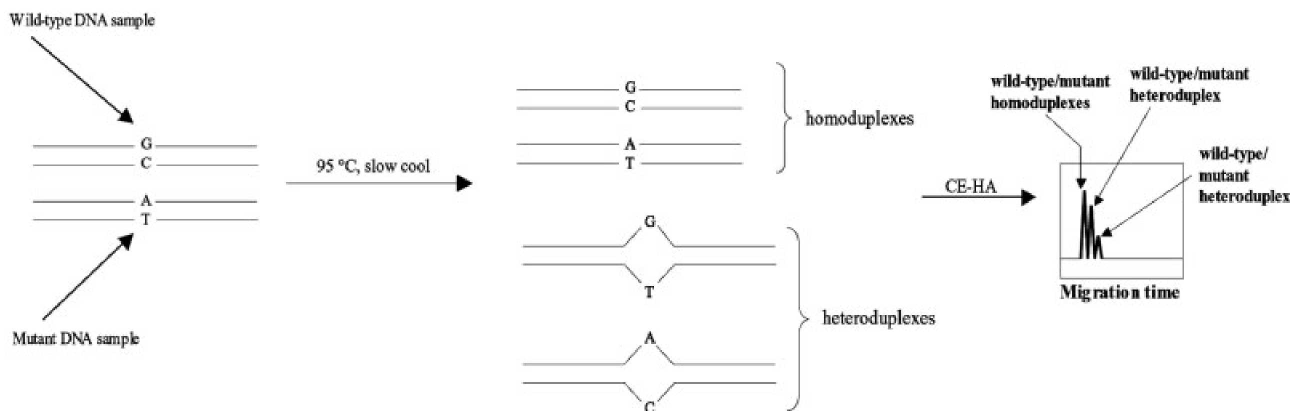

Figure 2. For CE-HA, PCR fragments are denatured at 95°C and slowly cooled to produce homoduplexes and heteroduplexes. Often, the wild-type to wild-type and mutant to mutant homoduplexes cannot be separated because of the similarity of their mobilities. However, the heteroduplexes can typically be separated from the homoduplexes and often from each other as well.

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called conformation-sensitive gel electrophoresis (CSGE) has also been developed, which has been reported to increase the sensitivity [26]. HA has been reported to have a sensitivity of ~90% or less [27].

A study by Valasco *et al.* [28] examined the potential of HA–CAE on the ABI 3100 system for detecting BRCA1 and BRCA2 mutations. They were able to detect 114 mutations in BRCA1 and BRCA2 in a large set of 598 samples. In addition, they looked at some regions of the MLH1 and MSH2 genes and were able to detect six different DNA sequence alterations. Although exact values of sensitivity and specificity were not given in this paper, they reported their method to be highly sensitive and identified several important parameters for highly sensitive and specific mutation detection. Interestingly, they recommended that the concentration of ABI's Genescan™ polymer used should be at least 5% w/v, that glycerol should be used in both the electrophoresis buffer and separation matrix, that 50 cm long capillaries be used instead of 36 cm long capillaries, and that the number of HA runs on the capillaries be limited to < 150 for the highest resolution and to prevent false positives. Hence, as on a slab-gel system, a very "dense" separation matrix and a higher viscosity buffer gives higher resolution HA conformer separations, and a long electrophoresis channel is also better. The last recommendation, for limited sequential use of the same capillary array, probably reflects the fact that DNA conformers can adsorb on the capillary walls and lead to peak broadening over time.

HA has often been reported to have difficulty in detecting substitution (or single-base) genetic mutations [26]. Valasco *et al.* [28] were able to detect 100 single-nucleotide substitutions in their study, which makes the method seem extremely interesting. They also found HA-CAE to be highly specific, with a false-positive rate of just 0.24% (31/13 651). In addition, they estimated the cost of HA-CAE to be ten times lower than direct sequencing on the same CAE system. Esteban-Cardenosa *et al.* [29] found CAE-HA was able to detect all 57 mutations that they examined from the BRCA1 and BRCA2 genes. Similar to CE-SSCP, CE-HA can also be performed on instruments designed for sequencing and therefore would decrease the capital costs incurred for a clinical laboratory [28, 29].

HA also has the potential to be used on the microchip electrophoresis platform. Tian *et al.* [30] have used microchip electrophoresis-HA to detect 6 mutations in the BRCA1 and BRCA2 genes in < 130 s [30]. Footz *et al.* [31] used glass microchips with the POP-6™ separation matrix (Applied Biosystems, Foster City, CA) and found that they were able to detect all eight sequence variants they examined from the BRCA1 and BRCA2 genes, without any optimization of either the PCR or of the microchip electrophoresis-HA conditions [31]. The same sensitivity of mutation detection was provided by a DHPLC method that had been significantly optimized.

While there is clear promise for the clinical use of capillary and microchip electrophoresis-HA, there are several important shortcomings of the method at present. Typically, HA's sensitivity on slab gels has been reported to be optimal when sample fragment sizes are less than 500–600 bp [2, 26]; hence, this is a longer range method than SSCP, but still, the gene regions that can be probed in one run are relatively small. More recently, Tian *et al.* [30] reported that their upper size limit for CE-HA was 200–300 bp and for microchip electrophoresis-HA was 150–260 bp, which may be an interesting difference between the slab gel and miniaturized electrophoresis systems. Microchip electrophoresis-HA seems to offer the best potential in terms of cost-effectiveness, rapid analysis, and small requirement of sample volumes; however there is still significant work to be done to confirm that the sensitivity and specificity of HA in that platform is useable for routine clinical screening.

6 Combined or tandem SSCP/HA

The SSCP and HA methods have similar sample preparation protocols and complementary mutation detection abilities, which make combining these two techniques an attractive approach to increase the overall sensitivity and specificity of mutation detection. Many studies have shown that the combination of the two methods is extremely powerful. Axton *et al.* [32] demonstrated that SGE-SSCP/HA could detect 100% of 12 mutations in the PAX6 gene, while SSCP and HA alone could detect only 83 and 50%, respectively. A study by Kozlowski and Krzyzosiak [23] demonstrated that CE-SSCP/HA could detect 31 mutations in the BRCA1 and BRCA2 genes with 100% sensitivity of mutation detection, whereas SSCP and HA alone provided 90 and 81% sensitivity, respectively. In a previous study, we demonstrated that 32 mutations in exons 7 and 8 of the p53 gene could be detected by CE-SSCP/HA with 100% sensitivity, whereas SSCP and HA alone had sensitivities of 93 and 75%, respectively [33].

Microchip electrophoresis-SSCP/HA was first demonstrated by Vahedi *et al.* [34], in which they used formamide to denature their samples on chip and analyzed two BRCA1 mutant samples and two mutant samples from the HFE gene. Figure 3 shows representative electropherograms of their BRCA1 mutation detection analyses, which were completed in less than 3.5 min. Both BRCA1 mutations are clearly detected, both by HA and

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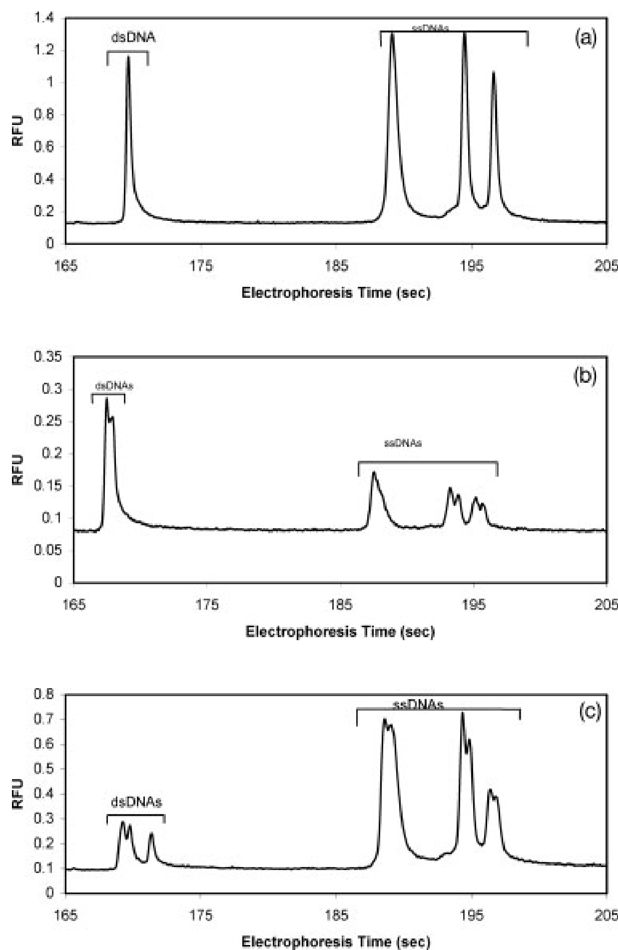

Figure 3. Mutation detection in BRCA1 exon 20 by microchip electrophoresis-SSCP/HA of (a) wild-type sample, (b) heterozygous G to A, and (c) heterozygous insertion of a C. SSCP and HA conformers were formed on chip by use of formamide. Homoduplexes and heteroduplexes (dsDNA) are on the left-hand side of the electropherogram and SSCP conformers (ssDNA) are on the right. Reprinted with permission from [34].

SSCP. Manage *et al.* [35] were able to detect the three most common mutations in the HFE gene by microchip electrophoresis-SSCP/HA in around 4 min on glass microchips. In a recent study, we performed a blinded analysis of 106 samples from human p53 exons 5–9 and found that we were able to obtain an overall 98% sensitivity and specificity using microchip electrophoresis-SSCP/HA with analysis times of <10 min (Hestekin, C. N., Senderowicz, L., Jakupciak, J. P., O'Connell, C. D. *et al.*, submitted, 2006). This result demonstrates the promise of this tandem method to offer very high sensitivity and specificity, which could allow for its eventual use in the clinic to screen for the presence of sequence alterations.

Compared to CE systems, microchip electrophoresis systems offer the potential for more rapid analyses in shorter separation distances (thanks to the well-defined sample plugs that are obtained in offset-T chip sample injectors). Chip systems will probably enable smaller sample amounts to be used, and also offer the potential to allow users to perform all of the analysis steps (PCR, sample purification, and sample analysis) on one integrated microchip platform. However, the sensitivity and specificity of these mobility shift methods by microchip electrophoresis, with large sets of real patient samples and preferably in blinded studies, will still need to be determined before they can be implemented in a clinical setting.

7 DHPLC

DHPLC is a type of "mobility shift" assay that does not involve electrophoresis, but instead detects mutations based on the decreased retention time of heteroduplexes in an HPLC column. We discuss it here because it is seen as a similar type of method to many of those we have discussed above. While HPLC is typically not a parallel method (there is only one HPLC column), each DHPLC separation is quite fast. Before DHPLC analysis, DNA homoduplexes and heteroduplexes are formed in a manner similar to that for HA. Typically, the DNA is then mixed with an ion-pairing agent, such as triethylammonium acetate (TEAA) [36]. TEAA is able to bind to DNA because of its positively charged ammonium group and is able to bind to the DHPLC column because of its hydrophobic ethyl groups. A gradient of organic solvent, such as ACN, can then be used to elute the DNA molecules from the column. Heteroduplexes are separated from homoduplexes in DHPLC because their decreased thermal stability causes them to be denatured first, decreasing their retention time on the column. A schematic illustration of this method is provided in Fig. 4.

DHPLC can be run in three different modes: nondenaturing, partially denaturing, and fully denaturing [36]. Nondenaturing DHPLC is typically used for the determination of PCR fragment size and/or purity. Partially denaturing DHPLC is the most commonly used method for mutation detection. Fully denaturing DHPLC is typically used only to analyze small (50–100 bp) DNA fragments or ssDNA [36, 37].

A study by Mogensen *et al.* [19] looked at 78 mutations for HCM and determined that DHPLC had a sensitivity and specificity of 100%. Crepin *et al.* [38] examined 160 patients for mutations in the MEN1 gene and found that DHPLC had a sensitivity of 100% for 213 mutations and a specificity of 98.6%. It is important to note that

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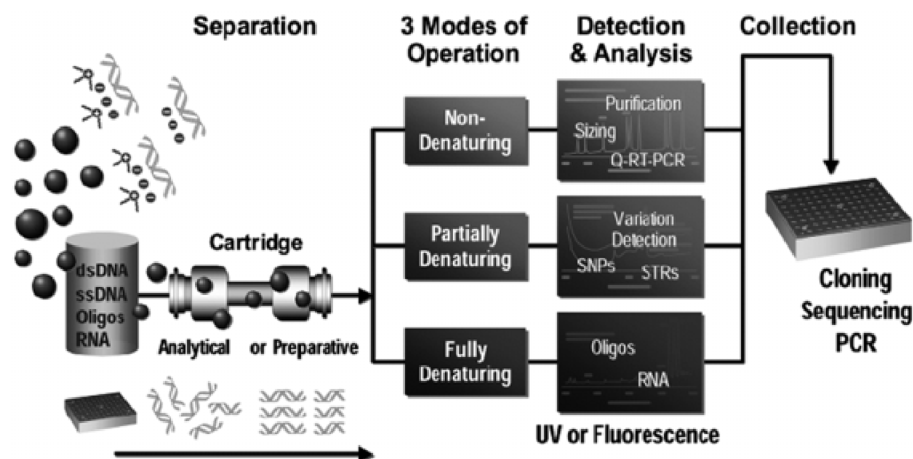

Figure 4. The alkyl groups of TEAA interact with the hydrophobic surface of the alkylated column matrix (represented by black dots on the left-hand side of the figure). The three modes of the DHPLC column (nondenaturing, partially denaturing, and fully denaturing) are shown. Samples can be detected with either UV or fluorescence detection. Fractions can be collected at the end of the run for further analysis, such as sequencing or cloning. Reprinted with permission from [36].

these high sensitivities and specificities were obtained after significant optimization of the analysis temperatures for all the fragments around the program-predicted temperature values. In addition, six of the ten fragments that they analyzed had to be analyzed at multiple temperatures to ensure sensitive mutation detection. Fasano *et al.* [39] used DHPLC to detect 30 mutations in the ABCA1 gene with 97% sensitivity. Several of their fragments also had to be analyzed at multiple (2–5) temperatures to ensure sensitive mutation detection, and they found that this increased their analysis time *per* sample from 7–8 min to 60–70 min. Holinski-Feder *et al.* [40] examined 74 mutations in the hMLH1 and hMSH2 genes and determined that DHPLC had a sensitivity of 97% and a specificity of 100%.

A study by Eng *et al.* compared SSCP, CSGE, 2-D gene scanning (TDGS), and DHPLC by sending out a panel of 58 mutations to different laboratories and comparing the results. Interestingly, they found that only DHPLC was able to achieve 100% sensitivity of mutation detection. TDGS had a sensitivity of 91%. SSCP provided a sensitivity of 72%, with only 65% confirmed by sequencing, and HA had a sensitivity of 76% with only 60% confirmed. Yamanoshita *et al.* [41] compared DHPLC to SGE-SSCP for analysis of mutations in p53 exons 5–8 for 207 esophageal cancer patients. DHPLC detected mutations with 97% sensitivity and 85% specificity, while SGE-SSCP detected mutations with 81% sensitivity and 97% specificity. Although they concluded that DHPLC was the superior method and should be used for mutation detection, its specificity seems to be too low to be used in a routine clinical setting. Similarly, the low sensitivity of SGE-SSCP in this study would not be clinically usable, unless it was combined with another method that reliably gave high specificity to allow crosschecking.

DHPLC has several significant drawbacks that presently make it impractical for a clinical mutation detection method. The instrument is relatively expensive and cannot be used for sequencing, and therefore requires a larger investment in capital equipment [2]. In addition, DHPLC has a low throughput because typically only one sample can be run at a time through the column. Although computer programs can be used to calculate the denaturation temperature for known sequences, there is still often a need for empirical determination of the optimum temperature(s) for detection of unknown mutations. In addition, Premstaller and Oefner [37] point out that the temperature of the columns must be carefully monitored for inaccuracies, which can occur over time (months), after power outages, or even in different columns. The relatively expensive DHPLC columns can also be easily contaminated, have a finite lifetime, and require the careful selection of primers for each gene region of interest, to prevent multiple melting domains in the samples [31].

Recently, there has been some work to increase the throughput of DHPLC. Monolithic poly(styrene-divinylbenzene) columns have been developed that are smaller (200 μm id *vs.* 4.6 mm id for conventional) and therefore require less sample volume. These columns can be bundled together, similar to capillary arrays, to greatly increase the number of samples that can be analyzed at a given time. In addition, the smaller columns also allow for more sensitive LIF detection, which allows for multiplexing with different dyes [37]. These advances make the DHPLC method more clinically desirable because of the increase in throughput, decrease in required sample volume, and high sensitivity, but they still do not overcome the high cost of the equipment and the need for significant temperature optimization. Nonetheless, given the

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Table 1. Summary of mobility shift assay characteristics for use as routine clinical screening methods

Assay	Sensitivity	Specificity	Throughput	Advantages	Disadvantages	Clinical potential
DGGE	~80–90%	N/D	Medium to high in SGE format	Inexpensive	Difficult to implement on capillary or microchip electrophoresis platforms	Low
TGGE	N/D	N/D	Medium to high	μTGGE shows great potential over conventional TGGE	Sensitivity and specificity for μTGGE need to be determined	Low to medium
SSCP	>90% (often multiple conditions required)	>80%	High when implemented in CAE format, high potential for μCE format	Simple, relatively inexpensive, allows CE and μCE platforms, CE and μCE instruments can also be used for sequencing to decrease capital expenses	Optimization highly empirical, fragment size limit of <300 bp	Medium
HA	<90%	~100%	High when implemented in CAE format, high potential for μCE format	Simple, relatively inexpensive, allows CE and μCE platforms, CE and μCE instruments can also be used for sequencing to decrease capital expenses	Difficulty detecting substitution mutations, size limits of ~600 bp for CE and ~300 bp for μCE	Medium
Tandem SSCP/HA	~100% (CE) 98% (μCE)	N/D (CE) 98% (μCE)	High when implemented in CAE format, high potential for μCE format	Simple, relatively inexpensive, allows CE and μCE platforms, CE and μCE instruments can also be used for sequencing to decrease capital expenses	Same size limitations as SSCP and HA alone	High
DHPLC	~100%	~100%	Typically low	High sensitivity	High equipment cost, even with computer programs, often still requires significant optimization, often low throughput	Medium to high

N/D = not determined or unable to be found in literature.

high sensitivities that have been observed with patient samples, it is likely that this method will continue to be developed, and perhaps in future, its miniaturized incarnations will meet all of the requirements for a clinically applicable mutation screening method.

8 Conclusions

The preponderance of evidence suggests that electrophoretic mobility shift assays have a real potential to serve as sensitive, specific, rapid, and cost-effective

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methods for screening PCR-amplified gene regions for genetic mutations or SNPs, reducing the need for full sequencing of patient samples to discover the presence of these sequence differences. A summary of some of the important characteristics of the different assays we have discussed is provided in Table 1. Although the DGGE and TGGE methods have been widely used in SGE, the complexity of translating them onto capillary and microchip platforms, especially in the case of DGGE, may hinder them from achieving the rapidity, sensitivity, and specificity required for routine clinical testing. Currently, DHPLC may be the method that has the consistently highest sensitivity and specificity with patient samples. However, the high cost of the equipment and the need for significant optimization for each sample to find the right conditions of analysis may hinder its clinical usability. SSCP and HA seem to perform best in tandem and have potential for high throughput, low cost, and rapid analysis, but need their sensitivity and specificity to be proven with large sets of patient samples, for various gene regions of medical significance, before they can be implemented clinically.

SSCP and HA also seem to offer the best potential to be implemented in complete lab-on-a-chip electrophoresis systems. Moreover, the ability to perform all necessary steps (*e.g.*, PCR, purification, and analysis) on one disposable device is highly desirable in a clinical setting. However, this is probably at least several years in the future, and will require a new instrument and software to be developed and offered commercially. In the mean time, tandem SSCP/HA is ripe for clinical application on CAE systems such as the ABI 3100. As a greater understanding of the important variables for highly sensitive and specific mutation detection by SSCP and HA become clear, they have the greatest potential to be used as clinical screening methods. In the end, the combination of a couple of complementary methods, such as DHPLC and tandem SSCP/HA, may be the preferred way to screen for genetic mutations; this combination of methods would no doubt easily provide 100% sensitivity and specificity once all were optimized. This is important since, at the present time, no such technology is available to clinical researchers or physicians, and could fulfill a growing need for personalized genetic information at a relatively low cost.

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