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Original Research

Use of GC Clamps in DHPLC Mutation Scanning

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ABSTRACT

OBJECTIVE

Development of a systematic mutation detection assay strategy for denaturing high performance liquid chromatography (DHPLC).

DESIGN

Adaptation of Guanine and Cytosine (GC)-clamping from denaturing gradient gel electrophoresis (DGGE) to DHPLC.

METHODS

Three target sequences harboring known allelic variants were studied to develop a general DHPLC assay design strategy. These were exon 10 of the human *RET* (**RE**arranged during **Tr**ansfection) gene, exon 52 of the mouse *Colla2* gene, and exon 9 of the human *FAS* (APO-1, CD-95) gene. Available software was used to analyze melting curves and determine assay conditions. GC clamps of 20 bp or 36 bp were added to polymerase chain reaction (PCR) primers to introduce a high melting temperature (T_m) domain to each of the target molecules. DHPLC was performed under partially denaturing conditions.

RESULTS

DHPLC assays of PCR-amplified sequences can be developed using a personal computer. The following three steps allowed for mutation detection in all three targets.

1) The target sequence should have a uniform T_m
2) GC clamps of length sufficient to introduce a second melting domain with a $T_m \geq 8^{\circ}$ above that of the target sequence should be appended to one of the primers.
3) The DHPLC assay should be performed at the highest temperature at which the target sequence is predicted to be ≥ 90% double stranded

CONCLUSION

Addition of GC-clamps to primers facilitates mutation detection by DHPLC.
The theoretical basis for this observation is identical to that underlying the utility of GC-clamps in DGGE.

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INTRODUCTION

Efficient, robust mutation detection methods can potentially have a major impact on the diagnosis of genetic disorders and the identification of genetic contributions to multifactorial disorders. Many investigations depend on relating genotypic variations to specific phenotypes. Such assays should ideally possess several widely recognized performance characteristics.[1,2]

This report describes a simplified strategy for designing mutation detection assays by denaturing high performance liquid chromatography (DHPLC), based on DHPLC's ability to separate partially denatured double stranded DNA molecules. Particular attention is devoted to the use of GC clamps with this system, allowing assay design to be carried out in a nearly algorithmic fashion. This approach allows investigators to shift their efforts from mutation detection assay design to interpretation of the biological consequences of detected sequence variation.

MATERIALS AND METHODS

DNA preparation

Mouse and human DNA were prepared from tail and peripheral blood mononuclear cells using the Invitrogen (Carlsbad, CA) and Puregene (Minneapolis, MN) DNA extraction kits, respectively. Human subjects provided written informed consent under protocols approved by the Memorial Sloan-Kettering Cancer Center's IRB[3,4] or the Hospital for Special Surgery's IRB.[5-7]

PCR

All amplifications were performed in 50 μL reactions including 1 U of Red Taq DNA Polymerase (Sigma, St. Louis, MO), 0.2 mM dNTP mix (Amersham Pharmacia, Piscataway, NJ), and 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, and 20 to 50 ng of genomic DNA. Primers were designed using Primer 0.5 software.[8] Reactions were performed in a PE Biosystems (Norwalk, CT) 2400 thermal cycler. Amplifications were carried out by an initial denaturation of 3 minutes at 95°C, followed by 35 cycles of 94°C x 30 seconds, $T_{annealing}$ x 30 seconds, and 72°C x 30 seconds, and concluded by a final denaturation at 95°C x 5 minutes followed by slow cooling to room temperature. Primer sequences and annealing temperatures are as follows:
clamp 20 = GCGGCCCGCCGCCCCCGCCG
clamp 36 = CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCG[9], FOIM = GAAATGGCTTTCCTAGACCCCG, ROIM = AATGATTGTCTTGCCCCATTCA with $T_{annealing}$ = 60°C, primers HFAS47 and HFAS49[5] and primers FRET10 and RRET10[10] and their annealing temperatures were previously published.

DHPLC conditions

Loading, elution and washing of the DHPLC column was carried out with varying combinations of three buffers injected at a flow rate of 0.9 ml/min: Buffer A contains 100 mM triethylamine acetate (TEAA), pH 7.0 and 0.025% acetonitrile, Buffer B contains 25% acetonitrile, 100 mM TEAA, pH 7.0, and 0.1 mM EDTA, and Buffer D contains 75% acetonitrile. Loading and elution buffers are combinations of buffers A and B, whose relative proportions form a gradient over a specified time interval. Buffer D is used to wash the column. DHPLC elution buffer gradients were generated by WAVEMAKER version 3.3.3 software (Transgenomic, Omaha, NE) and are reported as % buffer B at specified times. Assays were performed using the WAVE DNA fragment analysis system (Transgenomic, Omaha, NE). Oven temperature was determined from inspection of the melting profile, choosing the highest temperature at which the target sequence was predicted to be >90% duplex. Following the gradient elution, all remaining bound material was washed from the column for 36 seconds with buffer D and the column was re-equilibrated with the loading buffer for 156 seconds. Sample elution was monitored by absorbance at 260 nm.

DNA Sequencing

PCR products were purified by passage through Microcon microconcentrating centrifugal filter columns (Millipore, Bedford, MA) prior to sequencing. Sequencing reactions were performed using the Applied Biosystems Dye-Terminator Kit and analyzed on a ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

RESULTS

First, an established denaturing gradient gel electrophoresis (DGGE) assay to DHPLC was adapted, since both DGGE and DHPLC-based mutation detection operate on the principle of detecting differences in melting behavior among individual species in a mixed population of DNA molecules. Previously a GC-clamped DGGE assay was developed to detect pathogenic mutations in exon 10 of the *RET* protooncogene,[3,4] this was used as the starting point in adapting GC-clamping to DHPLC. Without a GC clamp, the target sequence has a single, uniform melting domain, with a predicted T_m between 66°C (>90% double stranded) and 67°C (>80% single stranded). Addition of a 36 bp GC clamp introduces a second higher T_m melting domain (figures 1A and 1B). The target sequence therefore denatures under conditions in which the GC clamp remains double-stranded. The WAVEMAKER-generated elution profile was loading in 44% buffer B, 49% buffer B at 30 seconds, and 58% buffer B at 300 seconds. The software recommended 67°C for assay performance. *RET* exon 10 was amplified from subjects harboring the C620F and C620R mutations, suffering from familial medullary carcinoma of the thyroid and MEN 2A, respectively, and from unaffected family members.[4] Mutations were detected at 66°C (figure 1C) and 67°C (not shown), but not at 65°C or 68°C (not shown) when the GC clamp was included. At 65°C a single sharp peak was seen, regardless of genotype, while at 68° all genotypes eluted as a broad, low intensity peak. Resolution of heteroduplex peaks was clearer at 66°C than at 67°C. These mutations were resolved at 60°C using a 20% to 60% gradient by DGGE.[3] Mutations were not detected either by DHPLC or

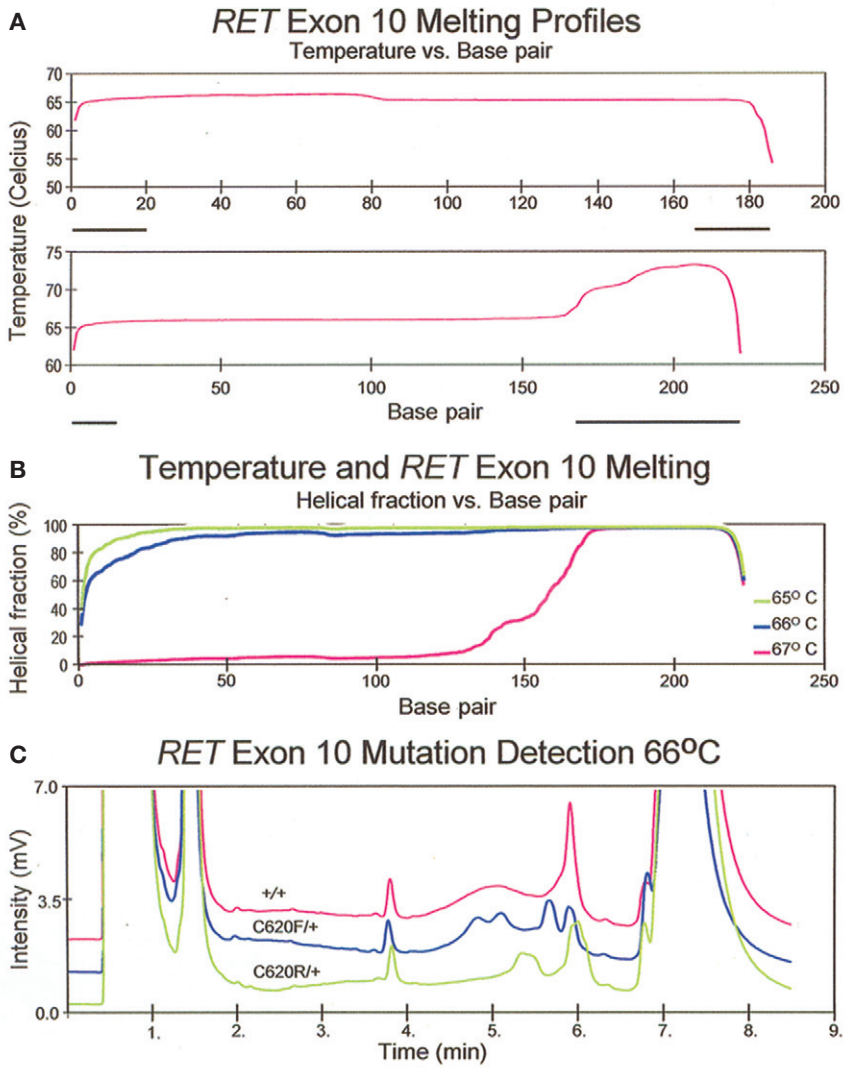

Figure 1.
A. Wavemaker-generated melting profiles for *RET* exon 10 without (top) and with (bottom) inclusion of a 36 base GC clamp on the 3′ amplification primer. The bold lines under the base pair axis indicate the extent of the primer sequences.
B. Predicted melting behavior of GC clamped *RET10* amplicons at 65°C, 66°C, and 67°C. The GC clamp remains double stranded at all 3 temperatures. The target sequence is ≥ 90% double stranded at 65°C and 66°C, but not at 67°C.
C. CDHPLC elution profiles for 3 *RET* genotypes at 66°. Heterozygotes harboring the C620F and C620R mutations are readily distinguished from normals and from each other.

DGGE when the GC clamp was not used (not shown). At 65°C and 66°C, unclamped samples elute as a single sharp peak regardless of genotype, while at 67°C and 68°C they elute as a broad, low-amplitude peak regardless of genotype.

Next we designed a mutation detection assay for the mouse *Col1a2*^{oim} mutation; a one-base pair deletion of a G residue in exon 52, encoding the c-terminal propeptide of the α2 chain of type 1 procollagen.[11,12] The melting profile was determined to be uniform in the target region (figure 2A). The target sequence is >90% double stranded at 58°C but not at 59°C, so assays were conducted at 58°C (figure 2B). Gradient conditions were injection in 43% buffer B, 48% buffer B at 30 seconds, and 57% buffer B at 300 seconds. Heterozygotes were readily distinguished from either wild-type or mutant homozygotes when a 36 bp GC clamp was included (figure 2C), but not when it was omitted (not shown). As in the case of *RET* exon 10, the unclamped products all elute as single sharp peaks at 58°C, a temperature at which the molecule is predicted to be double stranded. We did not attempt to resolve this target at other temperatures. A 50:50 mix of wild-type and mutant homozygous samples eluted as a single sharp peak when unheated, but eluted as a pair of peaks when heated to 95°C and cooled slowly (figure 2D). This demonstrates that assay conditions are robust for detecting heterozygosity, but are not adequate for determining the allele present in homozygous samples. Moreover, the behavior of the unheated mixture reveals that there is no difference in the elution times of the wild-type and mutant products, as the peak is not broadened relative to the peak

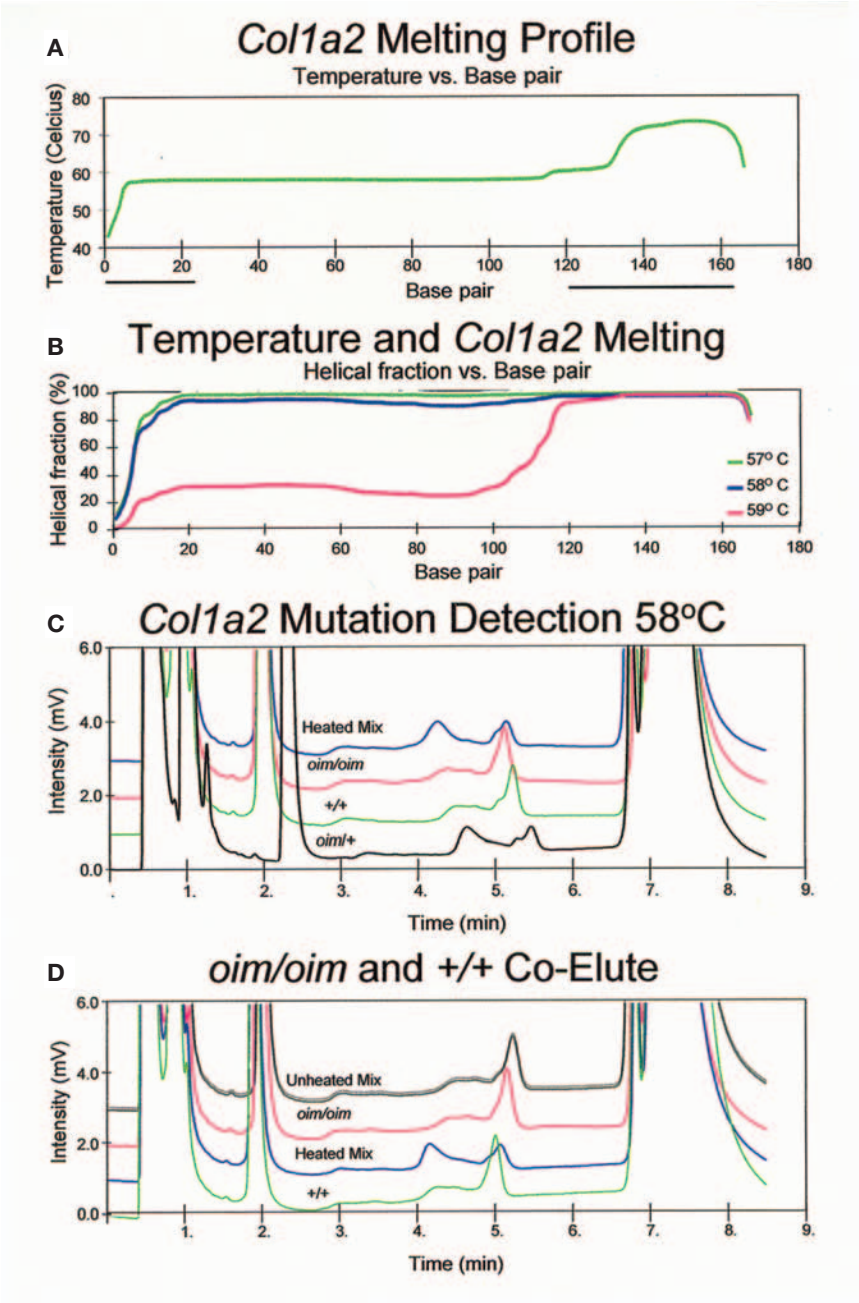

Figure 2.

A. Wavemaker-generated *Col1a2* GC clamped melting profile. The bold lines under the base pair axis indicate the extent of the primer sequences.

B. The predicted melting behavior of *Col1a2* amplicons at 57°C, 58°C, and 59°C.

C. Resolution of *Col1a2* heterozygotes from homozygotes. A *Col1a2*$^{oim/+}$ heterozygote is separated from either wild type or *oim/oim* homozygotes. A denatured and slowly reannealed mixture of the wild type and *oim/oim* PCR products produces an elution profile identical to that of the *oim/+* heterozygote.

D. Inability to resolve +/+ homozygotes from *oim/oim* homozygotes. Both homozygotes and an unheated mixture of *oim/oim* and *+/+* PCR products result in single elution peaks. However, heating the mixture to denature the DNA and allowing it to reanneal slowly allows heteroduplex formation and results in the appearance of a second elution peak, as for *oim/+* heterozygotes.

generated by homozygous samples. This finding indicates that decreased T_m at the site of mismatch is a critical element of mutation detection by DHPLC.

Homozygous samples can be genotyped in a two-step assay.

1) The assay is performed on a pure sample
2) Samples found to be homozygous are mixed with a reference wild-type sample, denatured, reannealed and reanalyzed

Mutant homozygotes will yield a heterozygous pattern on mixing, while wild-type homozygotes will retain a homozygous pattern on mixing.

The same strategy was applied for the detection of the D244G and R234P missense mutations in exon 9 of the human *FAS* (*APO-1, CD95*) gene, which cause the Canale-Smith Syndrome[5,7]. The melting profile predicts that 59°C is the highest temperature at which the target sequence is >90% double stranded (figures 3A and 3B). DHPLC analysis at 59°C with injection at 45% buffer B and elution with a gradient profile of 50% buffer B at 30 seconds, and 59% at 300 seconds is shown in figure 3C. The mutations were readily detected with a 20 bp GC clamp, while omission of the GC clamp did not allow us to detect the mutations under these conditions, with all genotypes eluting as a single sharp peak (not shown).

DISCUSSION

Sheffield and colleagues demonstrated the utility of GC clamps in designing DGGE assays,[9] showing that the inclusion of a clamp allowed detection of mutations in the murine β^{major} globin gene that were undetectable without clamping. The additional sensitivity arose from detection of mutations in the highest T_m domain of the target sequence. Subsequent sensitivity analyses of DGGE have consistently found that analyses performed with GC clamps have sensitivities of ~95% and specificities approaching unity.[13-16] The theoretical basis for this increase in sensitivity is that resolution of the various molecular species occurs primarily when individual molecules are partially denatured. Inclusion of a GC clamp provides an artificial, high T_m domain in the molecules being analyzed, allowing the target sequence to occur in the context of a low T_m domain. The data suggest

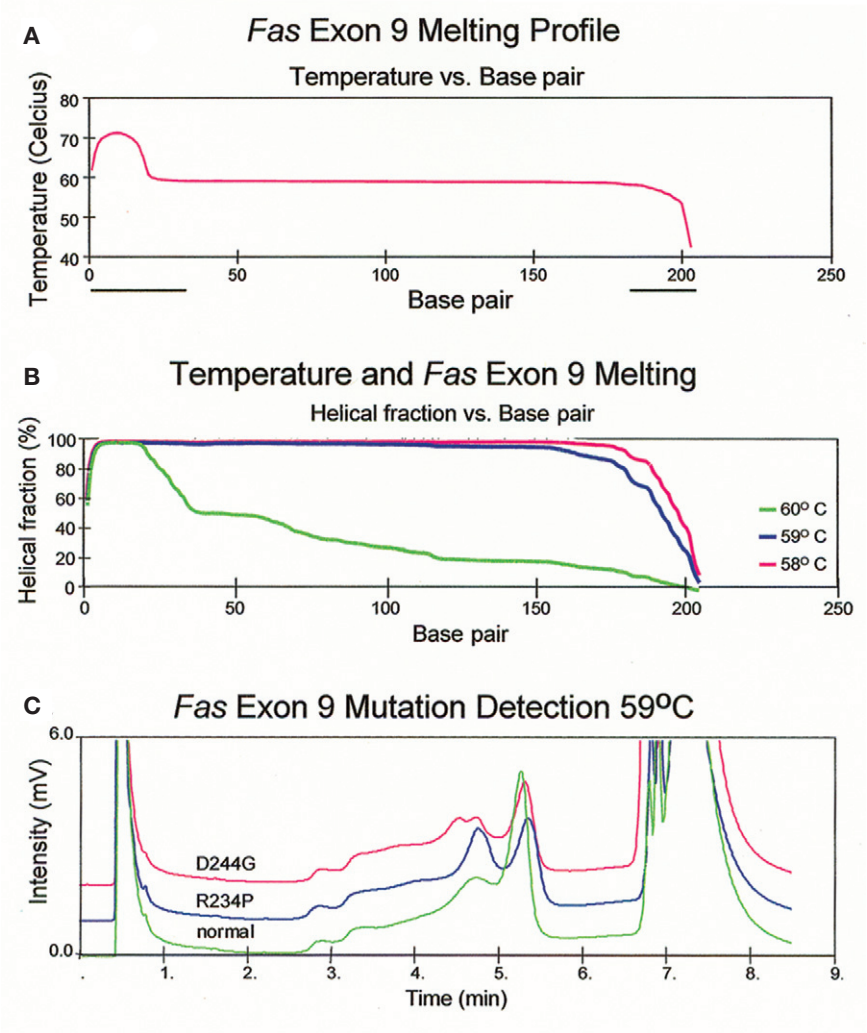

Figure 3.
A. Wavemaker-generated *FAS* exon 9 GC clamped melting profile.
B. Temperature and *FAS* Exon 9 Melting. The predicted melting behavior of *FAS* amplicons at 58°C, 59°C and 60°C.
C. Resolution of R234P and D244G heterozygotes from a normal homozygote.

that resolution of the various molecular species by DHPLC also occurs primarily when these have partially denatured, so that the principles guiding design of DGGE assays can be applied to DHPLC mutation detection.

Heteroduplexes include mismatches of one or more base pairs, resulting in early melting of the mismatched region. DHPLC allows resolution of heteroduplex from homoduplex DNA molecules based on differences in their retention time in the column under partially denaturing conditions, with heteroduplex molecules eluting earlier. This behavior mirrors heteroduplexes' lower T_m's and is similar to retardation of electrophoretic transport at different points along a denaturing gradient gel. In DGGE, mutations in the highest T_m domain are resolved poorly, if at all. It was determined that while the analytical technology differs in DHPLC, the principle of heteroduplex detection should be the same as in DGGE and that inclusion of GC clamps would facilitate mutation detection. The inability to detect sequence variants in the highest T_m domain may explain the anecdotally poor ability of DHPLC to detect mutations in short amplicons (K. Hecker, personal communication), which often include only a single melting domain.

A basic and general approach to designing new mutation screening assays by DHPLC was established. First, the target region's sequence is evaluated for uniformity of T_m. If this condition is not satisfied, alternative primers are chosen in order to generate a target sequence with a uniform T_m. Second, a GC clamp of sufficient length to create a high-T_m domain is appended to one of the primers. Once again, the predicted melting curve is inspected, to ensure that the clamp produces the desired high T_m domain, as illustrated in the "A" panels of the figures. Melting curves of the PCR product are calculated and the highest temperature at which the target sequence is >90% duplex is chosen for assay performance, as illustrated in the "B" panels of the figures. WAVEMAKER software, native to our DHPLC instrument, allowed both the calculation of melting profiles and generation of elution profiles. When using this software, inspection of the melting profiles is necessary. WAVEMAKER sometimes suggests an assay temperature that is too high for optimal resolution of sequence variation. Freely accessible programs perform equivalent calculations and give equivalent results.[17-20]

The trio of assays described here demonstrate that inclusion of a GC clamp allows detection of mutations by DHPLC under conditions that can be fully established prior to assay performance, using predicted melting curves. The experiments presented above do not exclude the development of assays without GC clamps, but rather show that the conditions identified *in silico* work well without further optimization when GC clamps are included. Unclamped primers for *RET* exon 10 were tested at four temperatures. The other two assays were tested at a single temperature. We did not attempt to optimize gradient conditions. It was expected that such optimization would likely have allowed mutation detection, even with unclamped molecules.

In the assays described here, the GC clamp had a melting point 8°C to 13°C higher than the target sequence, while the target sequence varied in T_m by no more than 3°C. Therefore, investigators should include GC clamp sequences sufficiently long to provide an 8°C increment in T_m relative to the target. The required length will vary according to the T_m of the target sequence, but it is straightforward to determine the minimum necessary length with melting profile software prior to ordering modified primers.

Theoretically, psoralen clamping provides an alternative to GC clamping by generating a covalent interstrand bond rather than by raising the T_m of a region of the target molecule.[21] The covalent bond introduced by psoralenation and subsequent UV light exposure effectively clamps one end of the molecule regardless of temperature. The advantage of psoralen compared to GC clamping is that the clamp need not be changed from assay to assay; the disadvantage is that an additional UV crosslinking step must be added prior to analysis. However, experiments with psoralen clamps were not conducted, as a result it is not known if this approach would perform as well in practice as in theory.

Electrophoretic mutation detection assays are difficult to perform on a large scale, since casting and loading gels are tasks that are difficult to automate. Because DGGE and Single Strand Conformation Polymorphism (SSCP) suffer from these limitations, the DHPLC-based assay is more readily adaptable to even a medium-throughput setting. The WAVE system can load samples automatically from a 96-sample block. Each sample is processed in approximately 6 to 9 minutes. The automation provided by the WAVE system vs. the gel-based methods, allows investigators to perform mutation screens at higher throughput and with less hands-on time. Further improvements in throughput might be achieved by pooling samples prior to DHPLC analysis, as has been done for gel-based methods.[22] These scale issues are most relevant to research laboratories engaged in mutation screening and genotype-phenotype correlation, and may become applicable to diagnostic clinical laboratories as well.

The assays we describe here scan relatively short target sequences and therefore cannot estimate the maximum length over which DHPLC based methods might work. High sensitivity and specificity have been reported for DHPLC in a growing body of analyses for sequences up to 400 bp in length.[23-32] Our approach to primer design has a shorter practical limit of about 250 bp, imposed by our criterion that target sequences have a uniform T_m.

Other approaches may lead to further improvements in mutation scanning methodology. For example, two groups have recently described a purely optical assay strategy that obviates the need for physical separation of homoduplex and heteroduplex molecules.[33,34] This approach may prove applicable to large-scale genotyping applications, but has yet to be widely validated.

The mutation detection strategy presented in this article leads to higher primer costs than other approaches, because of the need for either GC-clamped or psoralen-clamped primers, and shorter target sequences. At an estimated incremental cost of $0.70/base x 30 bases for "ordinary" scale primer synthesis, the extra primer costs for a typical length GC clamp is about $21. Psoralen clamps are more expensive than GC-clamps, requiring both an arbitrary primer adaptor arm and the psoralenation, thus adding about $40 to the cost of each clamped primer. Theoretically, psoralen primers could be reserved for the most GC-rich targets, in which GC clamps of practical length would not impact T_m. Costs arising from extra primer pairs needed as a result of studying shorter target sequences are less easily quantified and depend in part on the scope of the length of DNA to be scanned for mutations and the nature of its sequence. At an average cost of $50/GC clamped primer pair, addition of 10 additional primer pairs leads to a relatively small incremental cost. These are trivial costs compared with the time of technical personnel, whose salaries will typically exceed $10/hour. Therefore, even modest reductions in assay development time lead to net cost savings.

The greatest limitation on mutation scanning throughput, however, remains the time necessary to design mutation detection assays. Several previous reports addressed DHPLC assay design optimization.[18,30,31,35,36] These authors all recommend performing assays at various temperatures to maximize sensitivity and some narrow the buffer gradient ranges, requiring a still greater number of temperatures. Another report[37] suggests that a gradual decrease in T_m from the clamp to the free end of a molecule is the optimal configuration for mutation detection by heteroduplex-based methods. We have demonstrated that inclusion of GC clamps obviates the need for tedious assay optimization. GC clamps of sufficient length to create a domain with T_m 8°C greater than that of the target sequence allows an experimenter to develop a working assay in less than one hour. As genomic analysis moves from determination of additional sequence data to understanding the biological consequences of sequence variation, the ability to perform mutation screening efficiently will become ever more valuable. The algorithm for establishing DHPLC conditions described here will facilitate this.

CONCLUSION

Assay design principles adapted from those previously established for DGGE facilitate development of DHPLC mutation scanning strategies, as both methods resolve sequence variants by virtue of differences in the melting behavior of partially denatured DNA molecules. Use of GC clamps in DHPLC obviates the need for empirical optimization of new assays. The use of clamps is preferable to evaluation of a greater number of assay conditions for two reasons. First, unlike the situation with model assays such as those we have used for illustration, investigators performing mutation screens will not have positive controls available to test conditions. Second, the alternative strategy of varying conditions to increase sensitivity depends primarily on varying assay temperature. Heating and cooling the instrument oven is slow, and the time needed for temperature equilibration eliminates much of the time advantage arising from DHPLC.

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