A Multiple Mutation Model System as a Test Development and Training Tool for Denaturing Gradient Gel Electrophoresis

Michael Hepburn and Glenn Miller, Molecular Profiling Laboratory, Genzyme, Framingham, MA

Introduction

The search for sequence variations in genomic DNA is important in the study of genes that play a role in the development of cancer and a variety of other single and multiple gene disorders. A wide variety of different methods to detect DNA sequence variations have been developed (Dianzani 1993). One of these methods, denaturing gradient gel electrophoresis (DGGE), has been shown to be a sensitive, reproducible, reliable technology for use in many research environments (Myers 1985a, 1985b, 1987).

DGGE involves electrophoresis of double-stranded DNA fragments through a polyacrylamide gel containing a linear gradient of denaturant. As the fragment migrates through the denaturant gradient, the strands begin to melt. The melting profile of a given DNA duplex is predominantly determined by its base sequence with greater GC content resulting in higher melting temperatures. The ability of DGGE to detect sequence alterations is based on the differential melting characteristics of homoduplex DNA vs. heteroduplex DNA. As heteroduplex DNA migrates through the denaturant gradient, the areas of nonhomology melt at a lower temperature than the comparable homoduplex region. This results in an area of decreased mobility within the fragment, retarding its progress through the gel. This reduction in mobility results in a separation of homoduplex from heteroduplex fragments, thereby identifying a region of sequence alteration.

The melting characteristics of a double-stranded DNA fragment can be predicted a priori, allowing one to predetermine the conditions under which all alterations within that fragment should theoretically be resolved. The juxtaposition of GC-rich and AT-rich regions commonly seen within the human genome often result in complex melting profiles with adjacent domains demonstrating widely divergent melting temperatures. Such multiple melting domain fragments do not permit the resolution of sequence alterations in the higher melting temperature domains due to the more rapid melting of the lower temperature domains. The addition of a GC-rich sequence (GC clamp) to the 3' or 5' end of an amplicon results in a single melting domain across the fragment, thus allowing the detection of sequence variation across the entirety of the sequence (Myers 1985c).

The prediction of melting domains within a DNA fragment of known sequence has been reduced to a computer algorithm by Lerman and coworkers (Lerman 1987). The melting profiles determined for the current work were performed using an adaptation of Lerman's MELT program created by Bio-Rad Laboratories, Inc., called MacMelt™ software.

This study presents our efforts within a model system to determine the feasibility of using DGGE in a clinical environment for the detection of mutations in the mismatch repair genes relevant to the development of hereditary nonpolyposis colon cancer. We selected exon 4 of the *hMSH2* gene as a model system for analysis. A series of mutations was created by site-directed mutagenesis in an exon 4-containing clone. The individual mutant-containing clones were then analyzed via DGGE to determine the limits of detection of the system.

Methods

Site-Directed Mutagenesis of *hMSH2* Exon 4

A series of mutations was designed across an amplified region of *hMSH2* exon 4. To accomplish the mutagenesis, a series of exon 4-specific PCR primers, each with a single-base change corresponding to the designed mutation, was synthesized. The incorporation of the single-base change within the primer resulted in the site-directed mutagenesis of the amplified product. As the mutagenic primers were internal to a larger exon 4 fragment, subsequent amplification of the larger product resulted in an amplified exon 4 containing a known mutation at a known site. The amplified, mutagenized fragment was then cloned into a pGEM-T vector, and selected clones were sequenced to verify the presence of the mutation. All subsequent DGGE model experiments were then completed using the cloned material.

Primer Design

The design of DGGE primers follows the general principles of PCR primer design with respect to sequence specificity, lack of internal homology, and minimal primer dimer formation. In addition, a GC-rich sequence or clamp is often appended to one of the primers. Such a clamp can be of various lengths and base composition as determined by its ability to produce a single melting domain for the sequence under study. For clinical laboratory use, additional criteria include: Uniform PCR conditions, amplification of exonic regions inclusive of exon/intron boundaries, and a minimum of different DGGE conditions.

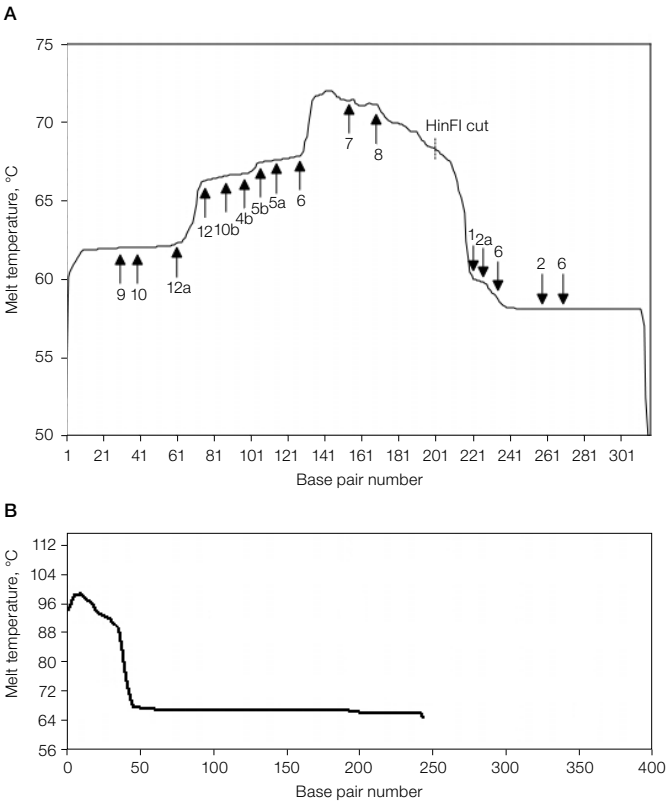

Fig. 1. General appearance of melt profiles. A, multiple melting domain profile of an unclamped *hMSH2* exon 4. Arrows locate the positions of mutations created in site-directed mutant clones; **B**, ideal single melting domain profile of an exon when one PCR primer contains a GC-rich clamp.

The exon 4 model used in this study is an exception to some of these criteria; its purpose was to test the limits of the DGGE assay. To illustrate the general appearance of melt profiles, Figure 1A shows the profile of amplicons with a high divergence from the ideal profile. Figure 1B shows the ideal melting profile achievable with a GC clamp where the amplicon melts within a single domain.

PCR

The polymerase chain reactions consisted of 0.6 µM of each primer pair, 250 ng of cloned DNA, 10 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 50 mM KCl, 200 µM NTPs, 2 U of *Taq* enzyme, and a 2.5 M final concentration of betaine in a volume of 50 µl. The cycling protocol consisted of 32 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min followed by a 5 min extension at 72°C.

DGGE

Denaturing gradient gels were prepared using a standard gradient mixer and stock gradient mixtures of 6% polyacrylamide-bis-acrylamide (37.5:1) containing denaturant concentrations of 0% urea-formamide in one mixing chamber and 80% urea-formamide in the second mixing chamber. The gradient conditions were calculated from the melting profile as determined by the MacMelt software. The gradient gels were cast and samples were electrophoresed for 5 hr at 130 V using the DCode™ universal mutation detection system.

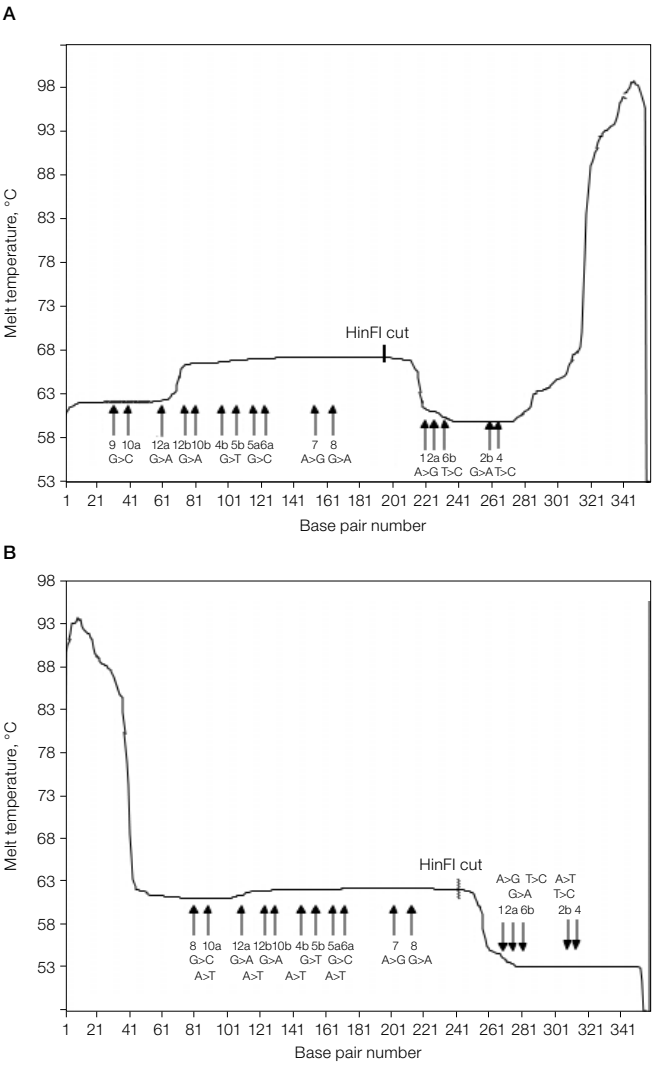

Fig. 2. Melt profiles of 3' and 5' clamped *hMSH2* exon 4. A, 3' clamped *hMSH2* exon 4 and locations of site-directed mutations; **B**, 5' clamped *hMSH2* exon 4. Note the location of the HinF1 site near the 3' end.

Results

The mutation content of each clone and the DGGE results are described in Table 1. The melting profiles of full-length amplicons regardless of the position of the GC clamp demonstrated extreme fluctuations in melting temperature. The 3' GC-clamped amplicon contained an 8° variation at its 3' end and a 6° variation at its 5' end, resulting in three melting domains (Figure 2A). The complexity of the melting profile using this clamped configuration resulted in no resolvable bands and thus an uninformative analysis. The 5' clamped amplicon contained three melting domains with a 10° difference at its 3' end and a 2° difference at the 5' end. DGGE detected 7 of the 16 mutations with the majority of the detected mutations located in the lowest melting domain (Figure 2B). The presence of a HinFI restriction enzyme site 5' to the lowest melting domain allowed the design of two new primers which excluded this lowest melting domain and yielded a more uniform melting profile. The first primer redesign used the original 5' GC-

Table 1. Mutation content and DGGE results of each clone.

Mutation	Base Position	Base Change	5' GC Clamp	3' GC Clamp	5' HinF1	3' HinF1	3' Overlap
9	34	G → C	-	-	+	+	N/A*
10A	42	A → T	-	-	-	+	N/A
12A	61	G → A	-	-	-	+	N/A
12B	76	A → T	-	-	-	+	N/A
10B	78	G → A	-	-	-	+	N/A
4B	99	A → T	+	-	+	+	N/A
5B	104	G → T	-	-	+	+	N/A
5A	116	A → T	-	-	+	+	N/A
6A	122	G → T	+	-	+	+	N/A
7	153	A → G	-	-	+	-	N/A
8	164	G → A	-	-	+	-	N/A
1	223	A → G	+	-	+	-	+
2A	226	G → A	+	-	N/A	N/A	+
6B	231	T → C	+	-	N/A	N/A	+
2B	261	T → C	+	-	N/A	N/A	+
4A	262	A → T	+	-	N/A	N/A	+

* Mutation not present in the amplicon.

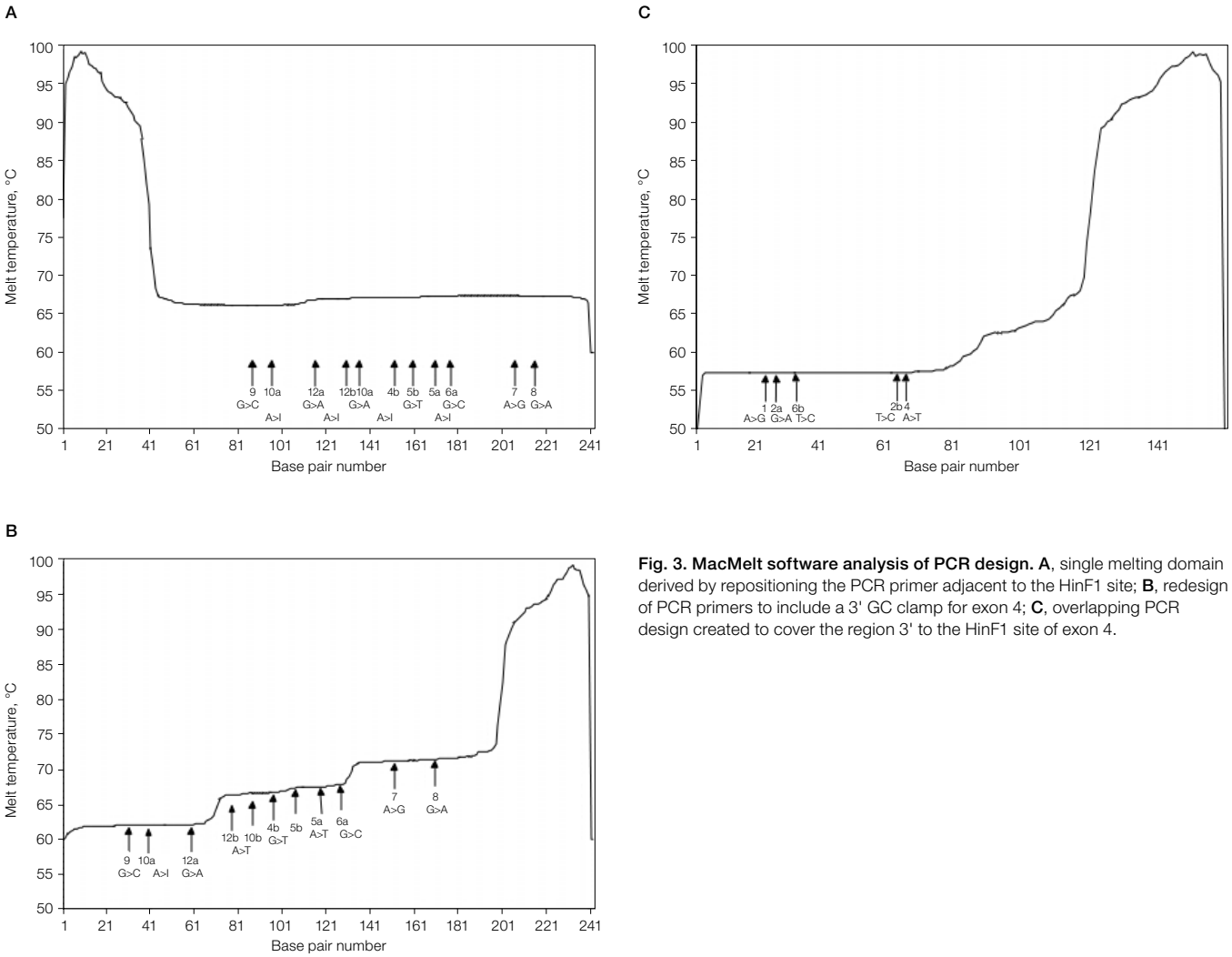

Fig. 3. MacMelt software analysis of PCR design. A, single melting domain derived by repositioning the PCR primer adjacent to the HinF1 site; **B**, redesign of PCR primers to include a 3' GC clamp for exon 4; **C**, overlapping PCR design created to cover the region 3' to the HinF1 site of exon 4.

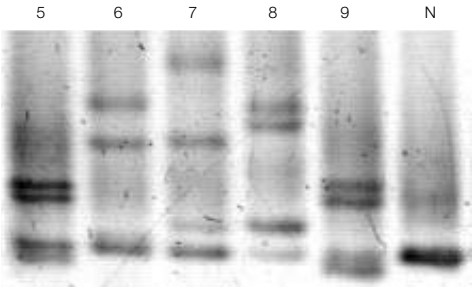

Fig. 4. Typical DGGE results obtained in the site-directed mutant model system. Mutation numbers are listed above each lane. See previous figures for location of mutations by number. N, normal.

clamped primer and a new nonclamped primer overlapping the HinFI site (termed the 5' HinFI amplicon) (Figure 3A). The second redesign used a nonclamped version of the 5' primer and a 3' GC-clamped primer proximal to the HinFI site (termed the 3' HinFI amplicon) (Figure 3B). A third primer pair was synthesized to include the region of exon 4 excluded by the other primer designs (termed the 3' overlap amplicon) (Figure 3C). DGGE of the 5' HinFI amplicon detected 8 of the 12 mutants known to be present in the amplified product. DGGE of the 3' HinFI amplicon detected 9 of the 12 mutants known to be present. DGGE of the 3' overlap amplicon detected all 5 of the mutations known to be present in the amplicon. Figure 4 depicts a typical gel result following electrophoresis of exon 4 fragments through a DGGE gel.

Discussion

DGGE compares favorably to other gene scanning techniques with respect to detection rate, robustness, and size of analyzed fragment. An important advantage of DGGE is the possibility to design primers and gel conditions to maximize the detection of alterations in advance. This is in contrast to other methods of detection that rely on empirical evidence of the discovery of mutations in a fragment. The disadvantages of DGGE include the specialized equipment necessary to run the gels, the consistency of pouring gradient gels in a high-throughput environment, and the expense of synthesizing clamped primers. In the current model, there was no correlation between the position of the mutation and its detection. It is currently accepted, however, that mutations less than 50 bp from the GC-clamped primer are less detectable than those located at a greater distance. The model system demonstrated the importance of melting domains in DGGE design. The 5' GC-clamped amplicon contained three domains with two differing by 10°. The lower temperature domain prevented mutations being detected in the higher temperature domain. The 3' HinFI amplicon also had three domains but two differed by only 3°. All mutations were detected in the lower two domains. The third domain, which differed by 10° from the lowest melting temperature domain, contained two mutations which were not detected in this amplicon. These results demonstrate the importance of designing amplicons with one or a small number of melting domains which differ by less than 5°. The observed requirement for at least two overlapping amplicons being clamped at opposing ends in these experiments demonstrates an important aspect of DGGE design for the clinical laboratory. To detect the greatest number of mutations, it is often necessary to analyze samples with amplicons clamped in either the 5' or 3' orientations. In this manner, it is then possible to obtain scanning data covering an entire amplicon in a single assay. Since this work was completed, a redesigned set of clamped primers is now used to analyze exon 4, reducing the total number of primer pairs from the three used in this work to two sets of GC-clamped primer pairs. Through the use of GC clamps of various lengths and GC content, it is possible to reduce the number of melting domains in virtually all fragments to a single uniform temperature. With careful planning and design of primers and gel conditions, it is possible to analyze large genes in a relatively high-throughput manner while maintaining a high degree of sequence alteration detection. Sequencing to determine the precise character of a mutation is then reduced to a single easily analyzed region rather than an entire gene. In summary, DGGE is a robust, reliable technique applicable to both research and clinical endeavors.

References

Dianzani I et al., Dilemmas and progress in mutation detection, Trends Genet 9, 403–405 (1993)

Lerman LS and Silverstein K, Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis, Methods Enzymol 155, 482–501 (1987)

Myers RM et al., Detection of single base substitutions in total genomic DNA, Nature 313, 495–498 (1985a)

Myers RM et al., Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis, Nucleic Acids Res 13, 3111–3129 (1985b)

Myers RM et al., Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis, Nucleic Acids Res 13, 3131–3145 (1985c)

Myers RM et al., Detection and localization of single base changes by denaturing gradient gel electrophoresis, Methods Enzymol 155, 501–527 (1987)

Information in this tech note was current as of the date of writing (1998) and not necessarily the date this version (rev B, 2008) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *61 02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 364 3435* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*