Technical Advance

Solution-Based Scanning for Single-Base Alterations Using a Double-Stranded DNA Binding Dye and Fluorescence-Melting Profiles

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DNA molecules differing by as little as a single-base substitution have traditionally been distinguished by gel electrophoresis-based methodologies that exploit differences in the sequence-specific properties of double-stranded DNA (dsDNA) such as melting temperature and secondary conformational configuration. By comparison, solution-based fluorescence methods using sequence-specific probes are limited to detecting mutations restricted to very short segments of DNA (~20 bp). We describe a solution-based fluorescence method that discriminates between wild-type and mutant sequences using a dsDNA binding dye, and interrogates a region of >200 nucleotides. This method is based on melting theory and entails fluorescence monitoring of the melting temperatures of GC-clamped amplicons subjected to gradual and progressive thermal denaturation in the presence of a constant concentration of urea. Heterozygous samples are easily identified by the lower melting temperatures of the less thermodynamically stable heteroduplex mismatches from the wild-type:mutant DNA hybrids as compared to the more stable wild-type Watson-Crick duplexes. All of the four possible sets of mismatches (A·G/T·C, T·G/A·C, G·G/C·C, and T·T/A·A) represented in 17 heterozygous mutations distributed throughout the length of 20 different amplicons (104 to 212 bp), were distinguished from the wild-type by their altered melting profiles. This methodology is advantageous in that it obviates gel electrophoresis or labeled oligonucleotide probes. Significantly, it expands the region of interrogation for detection of single-base changes using fluorescence-based methods in solution, and is amenable for automation and adaptation to high-throughput systems. (*Am J Pathol* 2001, 159:845–853)

Methodologies that permit the detection of single-base changes in specific regions of the genome have enjoyed tremendous utility in the field of genetics by facilitating genetic linkage analysis,^{1,2} and the identification of mutations with specific disease associations.^{3–5} These single-base alterations have been detected using a variety of methods including those relying on the abolition or creation of novel restriction enzyme sites (eg, restriction fragment length polymorphism analysis),^{2,5,6} the polymerase chain reaction (PCR),^{7,8} and subsequent distinction of base mismatches by oligonucleotide hybridization,⁹ or differences in the conformational or melting temperature (T_m) characteristics of the mutated and wild-type (WT) sequences (eg, single-strand conformation polymorphism analysis¹⁰ and denaturation gradient gel electrophoresis¹¹). All of these methods require multiple steps including gel electrophoretic separation and/or radioisotopic detection of the sequence variants.

Recent trends favor fluorescence-based technologies for the detection of specific nucleic acid sequences.¹² These assays have typically exploited any one of several fluorescence chemistries.^{13,14} The nonspecific methods incorporate a double-stranded DNA dsDNA binding dye such as SYBR Green I into the amplification reaction, and the fidelity of product detection is dependent on the inherent specificity of the PCR amplification conditions.¹⁴ The sequence-specific probe-based methods incorporate oligonucleotides that hybridize to a sequence within the amplicon, thereby providing an additional parameter for verification of product identity. The specificity of this hybridization interaction has been further exploited for the identification of single-base changes by virtue of the fact that the single-base mismatches within the hybridization probe to DNA target hybrids exhibit lower T_ms

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Table 1. Primers Utilized for Synthesis of Mutant *N-RAS* Exon 2 Templates and Amplification of *N-RAS* Sequences

Primer	Sequence (5' to 3')	GenBank accession no.	Paired with
Codon-56-1A	CCTGTTTGTGGACATAATGGATAC	L00041	Exon2-R
Codon-61	CCTGTTTGTGGACATACTGGATACAGCTGGAXYZGAAGAGTAC	L00041	Exon2-R
Codon-70-2T	GATGGCAAATACACAGAGGAAGCCTTCGCCTGTCCTCATGTAT ^u GGT	L00041	Exon2-F
	CTCTCATGGC		
Codon-79-3A	GATGGCAAATACACATAGGAAGCC	L00041	Exon2-F
Exon 2-F	CCTGTTTGTGGACATACTG	L00041	Exon2-Rt, Exon2-R
Exon2F (GC)	*CCTGTTTGTGGACATACTG	L00041	Exon2-R
Exon2-R	GATGGCAAATACACAGAGGA	L00041	Exon2F (GC), Exon2-F, Codon-56-1A, Codon 61
Exon2-Ft [†]	*CCTGTTTGTGGACA	L00041	Exon2-R
Exon2-Rt [‡]	*GATGGCAAATACA	L00041	Exon2-F

*Position of GC clamp (5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCGCCCCG-3').

[†]For amplification of codon 56 mutant sequences, a truncated forward primer containing the GC clamp was used.

[‡]For amplification of codon 79 mutant sequences, the reverse primer contained the GC clamp rather than forward primer.

^uUnderlined letters indicate position of base substitutions in codons 56, 70, and 79.

X, Y, and Z represent the nucleotide positions in codon 61 at which a base substitution was introduced. Only one base was substituted in each primer. The wild-type sequence in codon 61 is CAA. Thus, mutational insertions at position X were A, G, T; in position Y were C, G, T; and in position Z were C, G, T.

than perfectly complementary strands.¹⁴ However, the detection of single-base changes using probe-based methods is limited to very short segments of DNA (<20 bp) and would require several probes in multiple separate reactions for screening larger regions.

It is desirable to detect single-base changes in long DNA fragments in solution because such methods dramatically reduce assay times and are easier to automate. In this study, we describe a strategy, designated "mutational scanning by temperature ramping and chemical denaturation" (MUSTERD) that permits the detection of single-base alterations in solution using SYBR Green I and fluorescence analysis of PCR product T_m. After amplification, the sample to be investigated is rapidly cooled to effect annealing of DNA strands and subjected to a gradual and uniform temperature gradient augmented by a constant concentration of urea. These denaturing conditions convert the dsDNA into single strands as the T_ms of the respective domains are reached. Attachment of a high-melting domain (GC clamp) to the DNA fragment of interest converts the fragment into a lower melting domain and renders its entire length accessible for detection of point mutations. Using synthetic mutant, genomic WT, and heterozygous cell line DNAs each containing 1 of 17 single-base substitutions in a total of 20 different amplicons, we show that this method detects single-base alterations in longer PCR products than has been feasible with solution-based fluorescence methods.

Materials and Methods

DNA Templates

WT Sequences

DNA was extracted from peripheral blood lymphocytes of healthy individuals and human placenta using standard methods. All samples were confirmed by direct sequencing using the ABI PRISM 377 (Perkin Elmer Ap-

plied Biosystems, Emeryville, CA) to contain the WT *N-RAS* and Factor V genes.

Mutant Sequences

We synthesized nine 104-bp DNA fragments each containing one of the possible base substitutions in codon 61 of the *N-RAS* gene (GenBank Accession no. L00041; nucleotides 38 to 141) by PCR site-directed mutagenesis as previously described.^{15,16} Three additional PCR products with single-base substitutions at nucleotide 18 (CTG to ATG transversion at codon 56), nucleotide 61 (CAA to CTA transversion at codon 70), and nucleotide 89 (CTC to CTA transversion at codon 79). Segments (160 and 212 bp) of the Factor V gene (GenBank Accession no. Z99572, nucleotides 62,832 to 62,991 and 62,783 to 62,994, respectively) were also synthesized containing base substitutions at positions 1689, 1691, and 1692¹⁷ (Tables 1 and 2). The heterozygous state for each mutation was simulated by mixing equivalent quantities of synthesized homozygous mutant DNA and WT genomic DNA. Four cell lines harboring mutations in codon 61 of the *N-RAS* gene were also investigated (HL-60, SK-N-SH, SW1271, and HT1080 that harbor heterozygous CAA→CTA, CAA→AAA, CAA→CGA, and CAA→AAA mutations, respectively).¹⁶ Patient samples previously determined to contain heterozygous mutations of the Factor V Leiden locus were also evaluated using MUSTERD. All samples were confirmed by direct sequencing using the ABI PRISM 377 (Perkin Elmer Applied Biosystems) to contain the WT and the desired mutant sequences of the *N-RAS* and Factor V genes.

Computational Prediction of Melting Domains

Computer algorithms that predict the melting behavior of a DNA fragment based on its nucleotide sequence have been previously described.¹⁸ We assessed the 104-bp

Table 2. Primers Utilized for Synthesis of Mutant Factor V Templates and Amplification of Factor V Sequences

Primer	Sequence (5' to 3')	GenBank accession no.	Paired with
FacV-F(m)	GAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAA GAGCAGATCCCTGGACAGXCYZGGAATACAGGTATT	Z99572	FacV-R
FacV-R	TGTTATCACACTGGTGCTAA	Z99572	FacV-F(m)
FV-160(GC) [†]	*GCCCCATTATTAGCCAGGA	Z99572	FV-160
FV-160 [†]	AGACATCGCCTCTGGGCTAA	Z99572	FV-160(GC)
FV-212(GC) [‡]	*CTGGTGCTAAAAAGGACTAC	Z99572	FV-212
FV-212 [‡]	GAGAGACATCGCCTCTGGGCTA	Z99572	FV-212(GC)

*Position of GC clamp (5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCGCCCCG-3').

[†]For amplification of 160 bp Factor V product.

[‡]For amplification of 212 bp Factor V product.

X, Y, and Z represent the nucleotide positions in the Factor V gene at which a base substitution was introduced. Only one base was substituted in each primer. The wild-type sequence for those bases is G, G, A. Thus, mutational insertions at position X were A, T; in position Y were A, T; and in position Z the insertion was G.

N-RAS and 160-bp and 212-bp Factor V gene sequences with and without the attached 40-bp GC clamp using the WINMELT DNA analysis program (MedProbe AS, Oslo, Norway), which predicts the thermodynamic stability of DNA duplexes based on the DNA sequence.

PCR Amplification of Target Sequences

Rapid-cycle PCR amplification was performed in a micro-volume fluorimeter (LightCycler; Roche Molecular Biochemicals, Indianapolis, IN)¹⁹ using primers directed at the *N-RAS* and Factor V genes as summarized in Tables 1 and 2. Briefly, a 100-ng aliquot of template DNA was amplified in a 20- μ l reaction containing 1 $\times$ PCR buffer (50 mmol/L Tris, pH 8.5, 3.0 mmol/L MgCl₂, 500 μ g/ml bovine serum albumin), four deoxynucleotide triphosphates at 200 μ mol/L each, 0.2 μ mol/L of each primer, 10% dimethyl sulfoxide (by volume), and 0.8 U of Promega DNA polymerase (Promega Corporation, Madison, WI) with 176 ng of TaqStart antibody (ClonTech, Palo Alto, CA). PCR entailed a denaturation step at 94°C (0 seconds), annealing at 50°C (0 seconds), and extension at 72°C (10 seconds) for 45 cycles. Each reaction included the dsDNA-binding dye SYBR Green I (1:30,000 dilution; Molecular Probes, Eugene, OR). In the LightCycler, filtered excitation light (450 to 490 nm) from a blue light-emitting diode is reflected from a 505-nm dichroic filter and focused on the capillary tip. The samples are interrogated by paraxial epi-illumination. A portion of the excitation light is passed up the capillary tube by total internal reflection at the glass/air interface. Similarly, emitted light is passed down the capillary tube and exits out at the tip. The light is then reflected off a 505-nm dichroic filter, passed through a 520- to 560-nm interference filter, and focused onto silicon photodiodes for detection. Fluorescence signals were obtained once in each cycle by sequential fluorescence monitoring of each tube at the end of extension.

Standard Fluorescence-Melting Curve Analysis

Standard fluorescence-melting curve analysis entailing postamplification denaturation of non-GC-clamped am-

plified DNA segments in the absence of urea was performed as previously described.^{20,21}

MUSTERD

The optimal conditions for MUSTERD were empirically determined by varying the length of the GC clamp, SYBR Green I concentration, temperature ramping rate, and urea concentration, both individually and in combination. Once established, no further manipulation of the conditions was necessary as different amplicons were evaluated. The optimal conditions were applied to all amplicons as detailed below. After amplification, the amplicon was removed from the capillary tubes by reverse centrifugation. Ten μ l of the PCR products were added to 10 μ l of 26.6 mol/L urea and mixed vigorously to produce a final urea concentration of 13.3 mol/L. Ten μ l of this amplicon/urea mixture were subjected to MUSTERD analysis. The procedure entailed sample heating to 95°C (10 seconds), cooling to 33°C (2 minutes), followed by continuous acquisition of fluorescence during gradual temperature elevation from 33 to 85°C at 0.02°C/second in the presence of a constant concentration of urea (13.3 mol/L). The ramp rate of 0.02°C/second was achieved using custom software kindly provided by Dr. Carl T. Wittwer (University of Utah School of Medicine). SYBR Green I fluorescence signals were continuously monitored during the postamplification denaturation process. For easier visualization of the T_m, the fluorescence *versus* temperature curves (*F versus T*) were converted into fluorescence-melting peaks by plotting the negative derivative of fluorescence over temperature *versus* temperature ($-dF/dT$ *versus* *T*). Up to six replicates per sample were assessed by MUSTERD analysis, and the melting profiles were analyzed in a blinded manner and scored for the presence of mutations (Table 3).

Statistical Analysis

The within-run standard deviations and *P* values (paired two-tailed Student's *t*-test) for the experimentally derived T_m values from replicate samples assessed by MUS-

Table 3. Summary of Heterozygous Mutations Analyzed by MUSTERD

Gene target	Mutation	Fragment length (bp)	Location of mutation from 5' end (bp)	Base change	Mismatch pair	Sequence context (sense strand)	Detected using MUSTERD	Tm shift (°C)	SD	n	P value for n = 3
N-RAS exon 2	WT	104	NA	NA	NA	GGACAAGAA	NA	NA	0.13	6	NA
N-RAS exon 2	C181A	104	33	C to A	C-T/A-G	GGAAAAGAA	+	1.88	0.08	3	0.003
N-RAS exon 2	C181G	104	33	C to G	C-C/G-G	GGAGAAGAA	+	1.73	0.10	3	0.000
N-RAS exon 2	C181T	104	33	C to T	C-A/T-G	GGATAAGAA	+	1.21	0.26	3	0.007
N-RAS exon 2	A182C	104	34	A to C	A-G/C-T	GGACCAGAA	+	0.95	0.25	3	0.043
N-RAS exon 2	A182G	104	34	A to G	A-C/G-T	GGACGAGAA	+	0.51	0.05	3	0.026
N-RAS exon 2	A182T	104	34	A to T	A-A/T-T	GGACTAGAA	+	1.04	0.24	3	0.035
N-RAS exon 2	A183C	104	35	A to C	A-G/C-T	GGACACGAA	+	0.98	0.17	3	0.023
N-RAS exon 2	A183G	104	35	A to G	A-C/G-T	GGACAAGAA	+	1.29	0.10	3	0.003
N-RAS exon 2	A183T	104	35	A to T	A-A/T-T	GGACATGAA	+	0.61	0.38	3	0.061
N-RAS exon 2	WT	104	NA	NA	NA	CATCTGGA	NA	NA	0.04	3	NA
N-RAS exon 2	C166A	104	18	C to A	C-T/A-G	CATAATGGA	+	0.99	0.04	3	0.000
N-RAS exon 2	WT	104	NA	NA	NA	GACCAATAC	NA	NA	0.04	3	NA
N-RAS exon 2	A209C	104	61	A to T	A-A/T-T	GACCTATAC	+	1.166	0.06	3	0.000
N-RAS exon 2	WT	104	NA	NA	NA	TCCTCTGTG	NA	NA	0.04	3	NA
N-RAS exon 2	C240A	104	89	C to A	C-T/A-G	TCCTATGTG	+	0.5866	0.06	3	0.002
Factor V	WT	160	NA	NA	NA	CAGGCGAGGA	NA	NA	0.12	3	NA
Factor V	G1689A	160	96	G to A	G-T/A-C	CAGACGAGGA	+	0.57	0.13	3	0.001
Factor V	G1689T	160	96	G to T	G-A/T-C	CAGTCGAGGA	-	0.4	0.12	3	0.240
Factor V	G1691A	160	98	G to A	G-T/A-C	CAGGCAAGGA	+	0.78	0.02	3	0.008
Factor V	G1691T	160	98	G to T	G-A/T-C	CAGGCTAGGA	+	0.71	0.10	3	0.005
Factor V	A1692G	160	99	A to G	A-C/G-T	CAGGCGGGGA	+	0.53	0.05	3	0.005
Factor V	WT	212	NA	NA	NA	CAGGCGAGGA	NA	NA	0.13	4	NA
Factor V	G1689A	212	96	G to A	G-T/A-C	CAGACGAGGA	+	0.46	NA	1	NA
Factor V	G1689T	212	96	G to T	G-A/T-C	CAGTCGAGGA	-	0.09	NA	1	NA
Factor V	G1691T	212	98	G to T	G-A/T-C	CAGGCTAGGA	+	0.56	NA	1	NA

All samples analyzed were heterozygous for the mutations indicated.

The bolded and underlined bases indicate the site of the base substitution.

Tm shift, the difference between the peak Tms of the wild-type (WT) and heterozygous mutant samples; +, indicates that the mutation was detected; -, indicates that the mutation was not detected; bp, base pairs; Tm, melting temperature; SD, standard deviation; n, number of samples/replicate peaks analyzed; NA, not applicable.

P value was determined using the two-tailed Student's *t*-test comparing Tms from replicates of the WT and mutant sequences within the same run.

TERD were determined using the Microsoft Excel 2000 statistical tool (Microsoft, Redmond, WA).

Dilutional Analyses

Dilutional assays were performed using serial dilutions of HT1080 cell line DNA into WT genomic DNA.

Results

Sequence-Based Computational Prediction of Melting Profiles

The 104-bp *N-RAS* amplicon showed two distinct melting domains (Figure 1A). Similar analysis of the 160-bp Factor V DNA fragment showed three distinct melting domains with a different distribution from that observed for the *N-RAS* amplicon (data not shown). The 212-bp Factor V fragment yielded a profile with three melting domains showing Tms differing by only 7°C (data not shown). Importantly, the GC-clamped counterparts for all amplicons showed only two melting domains comprising a higher melting domain corresponding to the 40-bp GC clamp and a lower melting domain corresponding to the entire length of the DNA fragment of interest. Figure 1A illustrates the predicted melting profiles of the 104-bp *N-RAS* product, with and without the 40-bp clamp.

Standard Fluorescence-Melting Curve Analysis

Standard postamplification-melting curve analysis was performed in the absence of urea using non-GC-clamped amplicons created from WT DNA sequences, heterozygous cell line DNA, and artificial heterozygotes (50/50 mixtures of homozygous mutant and WT DNA mixtures). The three targets (104-bp *N-RAS*, 160-bp and 212-bp Factor V) showed an abrupt decline in fluorescence corresponding to their experimental Tms. Figure 1B, inset, shows the WT 104-bp *N-RAS* amplicon with an abrupt decline in fluorescence at 80°C on the *F* versus *T* curves. A corresponding melting peak was evident at 80°C on the *-dF/dT* versus *T* plots (Figure 1B). In our experiments, standard fluorescence-melting curve analysis failed to distinguish between WT and heterozygous mutant samples in products of ≥ 104 bp in length. Figure 1B illustrates identical fluorescence-melting profiles (same Tm) for a 104-bp segment of *N-RAS* exon 2 for both the WT sample and the HT1080 cell line, which harbors a heterozygous CAA→AAA point mutation at codon 61 of the *N-RAS* gene.

MUSTERD

The incorporation of a GC clamp to a primer generates amplicons with a high-melting domain (GC clamp) attached to a lower melting domain (fragment of interest).²²

Figure 1. A: Predicted melting domains for the 104-bp *N-RAS* exon 2 amplicon. T_m is plotted against the nucleotide sequence in the WINMELT computational constructs. The **dashed black line** represents the 104-bp *N-RAS* exon 2 fragment, and the **solid black line** represents the 104-bp fragment with attached 40-bp GC clamp at its 5' end (nucleotide no. -40). Thus the total length of the entire amplified fragment is 144 bp. The 104-bp amplicon without the GC clamp shows a lower domain spanning nucleotides 1 to 67 (T_m ~ 72°C) and a second domain with a slightly higher T_m (~76°C) spanning nucleotides 68 to 104. The attachment of the 40-bp GC clamp results in a 144-bp fragment with two discrete domains consisting of a higher melting domain (40-bp GC clamp) and the entire 104 DNA fragment of interest that is converted into a single lower melting domain (~74°C). This renders the detection of point mutations possible throughout the length of the fragment of interest. **B:** Standard fluorescence melting curve analysis without urea or GC clamps. Non-GC-clamped amplicons from the 104-bp *N-RAS* exon 2 amplicon were subjected to melting curve analysis entailing fluorescence acquisition during gradual heating of the PCR products from 33 to 95°C at a rate of 0.02°C/second. The **inset** shows a *F* versus *T* plot with a sharp decline in fluorescence at ~80°C corresponding to the T_m of the PCR product. The $-dF/dT$ versus *T* plots depict the same data as melting peaks for easier visualization. An identical melting profile is observed for both the WT sample and the HT1080 cell line harboring a heterozygous CAA→AAA mutation at codon 61 of the *N-RAS* gene.

To create GC-clamped amplicons for MUSTERD, 40-bp clamps were attached to one primer to generate PCR amplicons with a high-melting domain juxtaposed to the DNA segment of interest. The amplified fragment maintains a double-stranded configuration until it reaches its T_m.^{11,22} Because the disparity in T_ms is most evident in the lower melting domain of the fragment, single-base changes are distinguished in the lower melting domain whereas the higher melting domains of both WT and mutant samples show identical T_ms.²²

Wild-Type Sequences

MUSTERD analysis performed on GC-clamped amplicons derived from the 104-bp *N-RAS* and 160- and 212-bp Factor V amplicons showed two distinct melting domains for all products (Figures 2 to 4). Acquisition of fluorescence was performed during temperature ramping from 33 to 85°C at 0.02°C/second. *F* versus *T* plots

Figure 2. Melting profiles generated by MUSTERD analysis. All assays were repeated (≥ 3 replicates) with reproducible results. Although the absolute T_m of the replicate samples assessed was consistent within the same run (SD = 0.015 to 0.37), slight variations were observed between runs. Nevertheless, the T_m shift between the WT and mutant peaks remained relatively constant. Only one profile of each heterozygous sample type is illustrated for clarity. **A:** Reproducibility of melting profiles generated by MUSTERD. The amplicons to be assessed are GC-clamped at one end and subjected to gradual temperature denaturation at a rate of 0.02°C/second in the presence of 13.3 mol/L urea. Quintuplicate MUSTERD analyses of the GC-clamped WT *N-RAS* exon 2 product reveals two major melting domains. Melting peak I represents the 40-bp GC clamp with a higher T_m (~79°C). Melting peak II represents the 104-bp segment of interest (T_m = 70°C). All five samples are WT and show an identical melting profile with the same T_m in both melting domains. The H₂O control (**blue line**) shows fluorescence at levels below background. **B:** MUSTERD detection of heterozygous *N-RAS* codon 61 CAA→AAA mutation. The HT1080 cell line (**green line**) and a 50/50 mixture of the *in vitro* generated homozygous mutant and WT DNA (**red line**) represent heterozygous mutations harboring the CAA→AAA mutation. Both heterozygous mutants show virtually identical melting profiles with an anomalous peak (T_m ~ 68°C), and a prominent shoulder (T_m ~ 70°C). This profile is readily distinguishable from that of the WT sequence (**black line**) with a single melting peak at 70°C. The higher melting domains (GC clamp) in both WT and heterozygous samples show smaller peaks at 80°C. The H₂O control (**blue line**) shows fluorescence at levels below background. **C:** MUSTERD detection of heterozygous *N-RAS* codon 61 CAA→CTA mutation. The artificial CAA→CTA heterozygote shows an additional peak in the lower melting domain (T_m ~ 65°C) distinct from the WT peak at 68.5°C. The higher melting domain (GC clamp) in both the WT (**black line**) and heterozygous sample (**red line**) show smaller peaks at 77.9°C. The H₂O control (**blue line**) shows fluorescence at levels below background.

Figure 3. MUSTERD detection of heterozygous mutations throughout the length of the *N-RAS* 104-bp amplicon. **A:** Detection of heterozygous *N-RAS* codon 56 CTG→ATG transversion. An anomalous melting profile easily distinguishable from the WT profile (solid black line) is observed for the artificial heterozygous mutant (dashed black line). The H_2O control (dotted black line) shows fluorescence at levels below background. **B:** Detection of heterozygous *N-RAS* codon 61 CAA→CCA transversion. An anomalous melting profile with an additional melting peak ($T_m \sim 67^{\circ}\text{C}$) distinct from the WT peak ($T_m \sim 69^{\circ}\text{C}$) is observed in the heterozygous mutant (dashed black line). The WT (solid black line) sample shows a single peak at 69°C . The dotted black line represents the no template or H_2O control. **C:** Detection of heterozygous *N-RAS* codon 70 CAA→CTA transversion. An anomalous melting profile for the artificial heterozygous mutant (dashed black line) with a prominent melting shoulder ($T_m \sim 68^{\circ}\text{C}$) is distinguishable from the WT sample (solid black line) that exhibits a single peak ($T_m \sim 70^{\circ}\text{C}$). The H_2O control (dotted black line) shows fluorescence at levels below background. **D:** Detection of heterozygous *N-RAS* codon 79 CTC→CTA transversion. An anomalous melting profile for the artificial heterozygous mutant (dashed black line) with a prominent melting shoulder ($T_m \sim 66^{\circ}\text{C}$) is distinguishable from the WT sample (solid black line) that exhibits a single peak ($T_m \sim 70^{\circ}\text{C}$). The H_2O control (dotted black line) shows fluorescence at levels below background.

revealed similar profiles consisting of a lower melting domain corresponding to the amplified DNA segment of interest, and a higher melting domain corresponding to the 40-bp GC clamp. The T_m s of these two melting domains were better visualized by assessing the fluorescence-melting peaks derived from the negative derivative of fluorescence over temperature ($-dF/dT$) versus temperature (T) plots (Figure 2A, peaks I and II). WT samples assessed in quintuplicate yielded consistent melting profiles with identical T_m s.

Mutant Sequences

We assessed a total of 17 different heterozygous single-base substitutions in 20 amplicons using MUSTERD. As detailed in Table 3, the mutations included all nine possible single-base substitutions in codon 61 of a 104-bp *N-RAS* exon 2 amplicon and three single-base changes distributed throughout the length of the 104-bp

N-RAS amplicon (Figure 3). In addition, we studied five single-base substitutions in a 160-bp Factor V gene segment encompassing the Leiden locus (Table 3). Three of these mutations were also evaluated in the context of a 212-bp amplicon (Table 3).

Using the 104-bp *N-RAS* amplicon, all nine heterozygous mutations in *N-RAS* codon 61 were distinguishable from the WT sequence by virtue of their variant melting profiles. In contrast to the WT sequence that displayed only one melting transition in the lower melting domain, the heterozygous mutants exhibited either one or two melting subtransitions with lower T_m s than the WT in the lower melting domain (Figure 2, B and C).

Analysis of artificially synthesized single-base substitutions distributed throughout the length of the 104-bp *N-RAS* DNA sequence (within the lower melting domain of the GC-clamped amplicon) was also performed using artificial heterozygotes composed of a 50/50 mixtures of WT genomic DNA and homozygous mutants. These mu-

Figure 4. MUSTERD detection of heterozygous G1691A mutation (Leiden) in a 160-bp fragment of the Factor V gene. A patient sample with a known G1691A heterozygous Leiden mutation (**dashed black line**) shows a melting peak at 66°C that is easily distinguishable from the melting peak for the WT sample (**solid black line**; $T_m \sim 67^\circ\text{C}$). The higher melting domain (GC clamp) in both the WT and heterozygous samples show smaller peaks at 79°C. The H_2O control (**dotted black line**) shows fluorescence at levels below background.

tants consisted of a C to A substitution at nucleotide 18 (codon 56), an A to T substitution at nucleotide 61 (codon 70), and a C to A substitution at nucleotide 89 (codon 79). All of these heterozygous mutations were easily distinguished from the WT sequence by their variant melting profiles (Figure 3). Although melting curve analysis of non-GC-clamped amplicons in the absence of urea failed to distinguish mutant from WT sequences (Figure 1B), melting curve analysis of non-GC-clamped amplicons in a 13.3 mol/L urea solution discriminated between WT and heterozygous mutant sequences, provided the mutation was located in the lower melting domain (data not shown).

To assess the functionality of MUSTERD in a different sequence context, we evaluated five different single-base substitutions in a 160-bp Factor V gene amplicon (Table 3). The heterozygous G1689A, G1691A, G1691T, and A1692G substitutions were readily distinguished from the WT sequence by the variant melting peaks with lower T_m s distinct from that of the WT sample (Figure 4). However, the heterozygous G1689T transversion was essentially indistinguishable from the WT sequence (Table 3). In like manner, the G1689A transition and the G1691T transversion in the 212-bp Factor V product were distinguished from the WT sequence (Figure 4).

Dilutional Analysis

Serial dilutions were performed using DNA extracted from the *N-RAS* mutation-bearing cell line (HT1080) into WT genomic DNA. The assay sensitivity was 25% using our MUSTERD method (data not shown).

Discussion

Unstacking of long helical DNA fragments has been shown in denaturing gel-based systems to occur in a succession of segments or discrete cooperative units referred to as "domains."²³ The T_m s of these domains are principally determined by the base composition and pre-

cise DNA sequence. Single-base differences in otherwise homologous DNA fragments can be discriminated provided that the differences are located within the lowest melting domain,¹¹ and this domain is clearly separated from the onset of duplex to strands dissociation.²⁴ Both of these conditions can be satisfactorily achieved by the attachment of a higher melting section or a GC-rich sequence to the DNA fragment of interest, thus rendering the detection of virtually all single-base changes possible.²⁴ Indeed, the melting properties of dsDNA have been widely exploited for the detection of point mutations in such gel-based approaches as denaturation gradient gel electrophoresis and related methods,^{11,22,25} as well as in elution-based methods such as high performance liquid chromatography.²⁶

Fluorescence detection of specific PCR products may use a nonspecific DNA dye or sequence-specific fluorescently labeled probes.^{20,21} dsDNA binding dyes such as ethidium bromide and SYBR Green I are relatively inexpensive and easy to use. For our experiments, we have used SYBR Green I that binds to the minor groove of dsDNA and exhibits an excitation maximum (497 nm) similar to that of fluorescein. SYBR Green I fluorescence correlates with the quantity of dsDNA in a sample such that continuous monitoring of the dye fluorescence during PCR has been used for quantitative PCR analysis in real-time.²⁷ Because dsDNA denaturation is accompanied by loss of binding of the dsDNA dye, the T_m of the DNA duplex is observed as a dramatic decrease in SYBR Green I fluorescence in the *F* versus *T* curves, or alternatively as fluorescence melting peaks in the derivative ($-dF/dT$ versus *T*) plots. Because the T_m of a DNA fragment is determined by its length, GC content, and specific DNA sequence, important information can be deduced from the melting characteristics of long PCR products. In this regard, we have previously shown that products of identical size, but with dissimilar GC content and sequence can be distinguished by their T_m s.^{20,21} Other investigators have demonstrated the feasibility of single nucleotide polymorphism genotyping by T_m analysis, but these assays have required previous knowledge of the precise site of the base substitution for effective design of the appropriate allele-specific PCR primer.²⁸ More recently, Lipsky and colleagues²⁹ have also described a solution-based approach for the detection of single nucleotide polymorphisms, but the majority of the base changes were located within the middle third of the fragment of interest. Thus suitability for detection of mutations throughout the length of >100-bp fragments was not systematically addressed.

Our current study demonstrates another approach that takes advantage of the melting characteristics of dsDNA for the detection of single-base substitutions. Amplified mutant and WT DNA from the genomic region of interest are subjected to a gradual and uniform linear temperature gradient augmented by a constant concentration of urea. These denaturing conditions convert the double-stranded molecule into single strands as the T_m s of the respective domains are reached. Fluorescence monitoring of the melting profiles during application of the temperature gradient yielded melting transitions correspond-

ing to the GC clamp and the DNA fragment of interest, confirming the existence of the two distinct DNA-melting domains in solution.

The cyclical reiteration of denaturation and annealing during PCR generates heteroduplex molecules composed of WT and mutant DNA sequences in heterozygous samples. As predicted by melting theory and experimental data,¹¹ our method identified single-base substitutions by the presence of one or two subtransitions within the lower melting domain with lower T_m s than the WT. These results can be explained by the fact that heterozygous samples contain both the WT and mutant sequences that when annealed give rise to homoduplexes containing WT fragments and heteroduplexes consisting of mismatched mutant:WT hybrids. Because the Watson-Crick homoduplexes are more thermodynamically stable than the heteroduplexes, they exhibit higher T_m s than the less stable heteroduplex mismatches. Thus the heterozygous mutants were identifiable by the presence of an additional subtransition with a lower T_m than was observed for the WT sample. The different heterozygous single-base substitutions yielded T_m shifts of varying magnitudes depending on the destabilizing ability of the resulting heteroduplex mismatch (Table 3). Nevertheless, no false-positives were recorded and 18 of the heterozygous mutations in 20 amplicons analyzed were detected (sensitivity, ~90%) (Table 3). Our inability to detect all mutations may result from the relatively stable mismatches present in these samples and the sequence contexts in which they appear. Overall GC content as well as nearest neighbor sequences may affect the applicability of this method to some targets. However, we also observed that there was a diminution in the magnitude of T_m shifts associated with specific mutations as the length of the interrogated DNA segment increased (Table 3), suggesting that this method may be most applicable to amplicons of the order of 200 bp. In addition, our dilution studies suggest that this method is better suited to the detection of polymorphisms or germline mutations, rather than somatic/acquired mutations.

In summary, we have shown here that screening for heterozygous mutations is feasible in solution using SYBR Green I generated fluorescence-melting peak analysis during gradual thermal denaturation of GC-clamped amplicons. This methodology is advantageous in that it is simple and rapid requiring only 40 minutes of postamplification analysis for mutational screening of single nucleotide polymorphisms or point mutations. Furthermore, gel electrophoresis is not required, neither is there a need for radioactive, biotinylated, or sequence-specific fluorescently labeled probes. Although other solution-based fluorescent methodologies used for detection of point mutations are limited to short segments, our current method is particularly applicable when the precise site of the mutation or polymorphism is unknown in advance, or spans a length of more than a few nucleotides. We anticipate that this method will find utility in the screening for single nucleotide polymorphisms or point mutations that may subsequently be confirmed by DNA sequencing or other methods of genotyping.

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