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METHODS

Masking Selected Sequence Variation by Incorporating Mismatches Into Melting Analysis Probes

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Hybridization probe melting analysis can be complicated by the presence of sequence variation (benign polymorphisms or other mutations) near the targeted mutation. We investigated the use of "masking" probes to differentiate alleles with similar probe melting temperatures. Selected sequence variation was masked by incorporating mismatches (deletion, unmatched nucleotide, or universal base) into hybridization probes at the polymorphic location. Such masking probes create a probe/target mismatch with all possible alleles at the selected polymorphic location. Any allele with additional variation at another site is identified by a lower probe melting temperature than alleles that vary only at the masked position. This "masking technique" was applied to *RET* protooncogene and HPA6 mutation detection using unlabeled hybridization probes, a saturating dsDNA dye, and high-resolution melting analysis. Masking probes clearly distinguished all targeted mutations from polymorphisms when at least 1 base pair (bp) separated the mutation from the masked variation. We were able to mask polymorphisms immediately adjacent to mutations, except in certain cases, such as those involving single-base deletion probes when both adjacent positions had the same polymorphic nucleotides. The masking probes can also localize mutations to specific codons or nucleotide positions. Masking probes can simplify melting analysis of complex regions and eliminate the need for sequencing. Hum Mutat 27(3), 269–278, 2006.

KEY WORDS: polymorphism; mutation; high-resolution melting analysis; masking; *RET*; MEN2; probe hybridization; unlabeled probe; SNP

INTRODUCTION

There are many methods for identifying point mutations, including gel analysis after restriction enzyme digestion, allele-specific PCR, microarrays, sequencing, and probe-based methods that generate allele-specific melting curves [Bugalho et al., 2002; de la Fuente et al., 2001; Kim et al., 2002; Kruckeberg and Thibodeau, 2004; Roque et al., 2002; Ruiz et al., 2001b; Xue et al., 1994]. The latter method uses hybridization probes, often of wild-type sequence, to detect any sequence variation under the probe by melting temperature (T_m). Mutation detection by hybridization probe melting analysis is a rapid, closed-tube assay that does not require any post-PCR sample processing. However, the need for expensive fluorescently-labeled probes is a disadvantage of this method.

High-resolution melting of PCR products in the presence of saturating dsDNA binding dyes can detect and genotype most sequence variation by differences in the shape and position of the melting curve [Gundry et al., 2003; Liew et al., 2004; Wittwer et al., 2003]. However, it is commonly believed that the extra specificity of a probe is necessary for clinical diagnostic assays. To provide probe specificity without the cost of fluorescently-labeled probes, an unlabeled probe method was recently introduced [Zhou et al., 2004]. This method requires three oligonucleotides: two PCR primers and one internal probe, which is 3′ phosphorylated to prevent extension. The melting transition of the unlabeled probe is monitored by the fluorescence of LCGreen® PLUS dye as the probe/target duplex melts.

When wild-type hybridization probes are used for genotyping, melting analysis does not always distinguish the possible sequence variations [Schutz et al., 2000; von Ahsen et al., 1999a; Warshawsky et al., 2002; Zhou et al., 2004]. For example, the wild-type hybridization probe used to detect the prothrombin 20210G>A mutation could not differentiate the sequence variants of 20209C>T and 20221C>T [Warshawsky et al., 2002]. Sequence variation under the detection probe can include benign polymorphisms, sequence variants of undetermined significance, and pathogenic mutations. One can sometimes eliminate polymorphisms from analysis by changing the size or location of the probe. In other cases this may not be possible, depending on the position and number of polymorphisms relative to the targeted mutation. Thermodynamic predictions suggest that

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32% of all single-base mismatches with wild-type probes have a T_m within $\pm 1°C$ of a targeted mutation [Schutz et al., 2000]. Since unique alleles can have nearly the same T_m with detection probes [Lyon et al., 1998; Schutz et al., 2000; von Ahsen et al., 1999a,b; Warshawsky et al., 2002; Zhou et al., 2004], there is a risk of interpreting polymorphisms as mutations or mutations as polymorphisms. Polymorphisms may occur near targeted mutations across the entire genome, since about 90% of human sequence variation is due to SNPs, with one SNP per 100–300 base pairs (bp) [Collins et al., 1998; Orban et al., 2000].

In this study we systematically evaluated techniques for masking nontargeted sequence variation in melting curve analysis using hybridization probes. The probes were designed with a "mask" over the nontargeted sequence variation to create an artificial mismatch with all possible alleles. Wild-type and masked variant alleles have a single mismatch with the probe and a similar T_m. In contrast, the targeted mutation results in an additional mismatch with the probe and a lower T_m than the wild-type and masked alleles. This report demonstrates the use of three types of masking probes: probes with deletions (1–3 bp), probes that incorporate a universal base, and probes with an unmatched nucleotide that does not complement the possible nucleotides at the masked, polymorphic location. This "masking technique" was demonstrated with the *RET* proto-oncogene (MIM# 164761) and human platelet antigen type 6 (HPA6), where polymorphisms are near or immediately adjacent to targeted mutations [Ceccherini et al., 1994; Eng and Mulligan, 1997; Stenson et al., 2003; Wang et al., 1993]. This technique was also used to identify the location of sequence variation under the probe.

TABLE 1. **Sequence Variation in the *RET* Protooncogene**

RET exon	Codon	Protein change[a]	Nucleotide change[b]
10	**609**	**p.C609Y**	**c.1826G>A**
		p.C609S	**c.1826G>C**
	611	**p.C611R**	**c.1831T>C**
		p.C611Y	**c.1832G>A**
		p.C611F	**c.1832G>T**
	618	**p.C618R**	**c.1852T>C**
		p.C618G	**c.1852T>G**
		p.C618Y	**c.1853G>A**
		p.C618S	**c.1853G>C**
		p.C618F	**c.1853G>T**
	620	**p.C620S**	**c.1858T>A**
		p.C620Y	**c.1859G>A**
		p.C620S	**c.1859G>C**
		p.C620F	**c.1859G>T**
		p.C620W	**c.1860C>G**
11	**631**	**p.D631D**	**c.1893C>T**
	634	**p.C634S**	**c.1900T>A**
		p.C634R	**c.1900T>C**
		p.C634G	**c.1900T>G**
		p.C634Y	**c.1901G>A**
		p.C634S	**c.1901G>C**
		p.C634F	**c.1901G>T**
		p.C634W	**c.1902C>G**
13	**768**	**p.E768D**	**c.2304G>C**
	769	**p.L769L**	**c.2307T>G**

[a]For all cysteines listed, the codon wild-type DNA sequence is "TGC."
[b]Nucleotide position of sequence variation using *RET* cDNA sequence derived from *RET* mRNA GenBank NM_020630.3, where nucleotide +1 is the "A" of the ATG start codon. Sequence variation is heterozygous unless otherwise stated in the text. Pathogenic mutations are in bold, while benign polymorphisms are underlined.

MATERIALS AND METHODS

Samples

Mutations in *RET* exons 10, 11, 13, 14, 15, and 16 cause multiple endocrine neoplasia type 2 (MEN2) disorders: MEN2A, FMTC (MIM# 171400), and MEN2B (MIM# 162300). The most common mutations for MEN2 are in exon 10 (codons 609, 611, 618, and 620) and exon 11 (codons 630 and 634), which accounts for 95% of MEN2A and ~85% of FMTC. The rest of the MEN2 mutations are spread over exons 13, 14, 15, and 16, with the exon 16 mutation at codon 918 resulting in 98% of MEN2B [Hansford and Mulligan, 2000] (Wiesner and Snow-Bailey, GeneReviews for MEN2, www.geneclinics.org, accessed February 2004). Only the *RET* exons 10, 11, and 13 are used in this report.

De-identified wild-type and *RET* variant genomic DNA samples were obtained from the Mayo Clinic (Rochester, MN) with institutional review board approval, and were amplified by the GenomiPhi™ protocol (Amersham Biosciences, Piscataway, NJ). All *RET* genotypes were confirmed by sequence analysis and comparison with the *RET* genomic sequence in GenBank AJ243297.1. The generated PCR products were sequenced with BigDye terminator chemistry (Applied Biosystems, Foster City, CA).

All of the *RET* sequence variation tested in this study resulted from a single nucleotide change (Table 1). The *RET* variants that alter RET function to cause MEN2 syndromes are mutations, while benign variants that are not causative of MEN2 syndromes are called polymorphisms [Borrego et al., 1999; Ceccherini et al., 1994; Elisei et al., 2004; Eng and Mulligan, 1997; Ruiz et al., 2001a; Stenson et al., 2003]. In this report, a *RET* mutant nucleotide change is indicated in bold, while a nucleotide change that results in a benign polymorphism is underlined. All variant *RET* samples were heterozygous for mutations or polymorphisms unless otherwise stated. The cDNA nucleotide position for *RET* sequence variation was derived using *RET* mRNA from GenBank accession number NM_020630.3 (nucleotide +1 is the "A" of the ATG start codon) as listed in Table 1. *RET* exon 13 has a reported mutation at codon 768 (c.2304G>**C**) and a common benign polymorphism within codon 769 (c.2307T>G) [Ceccherini et al., 1994; Elisei et al., 2004; Eng and Mulligan, 1997; Stenson et al., 2003]. *RET* exons 10 and 11 have possible mutations at codons 609, 611, 618, 620, 630, and 634, which include all possible nucleotide changes from the wild-type codon nucleotide sequence "TGC" for the amino acid cysteine (Table 1) [Ceccherini et al., 1994; Stenson et al., 2003]. Exon 11 has a polymorphism within codon 631 (c.1893C>T) [Kruckeberg and Thibodeau, 2004].

The HPA6 locus is found in the *beta-3 integrin* gene, which is also called the *glycoprotein IIIa* gene (MIM# 173470 and Genbank accession number M57489.1). The HPA6 targeted sequence variation (c.1544G>**A**) is immediately adjacent to the polymorphic nucleotide c.1545G>A or C, with 63%, 37%, and <1% allelic frequencies, respectively [Wang et al., 1993; Zimrin et al., 1990]. Homozygous HPA6 templates were engineered from de-identified patient genomic DNA [Barik, 1998] and included all possible combinations of wild-type, mutant, and neighboring polymorphic sequences [Liew et al., in press]. The HPA6 allelic sequences are listed by the c.1544 nucleotide followed by the c.1545 nucleotide (GG, GC, GA, AA, AC, and AG) for simplicity in the text and figure. These alleles correspond to wild-type sequence, c.1545G>C, c.1545G>A, c.[1544G>**A**; 1545G>A], c.[1544G>**A**; 1545G>C], and c.1544G>**A**, respectively. All homozygous artificial templates were sequenced to confirm the genotype.

TABLE 2. Primers and Probes

RET exon	Primers[a]	Amplicon (base pair)	Codon of variation[b]	Probes (base pairs)[c]	Probe sequences[d]
10	GGGCAGCATTGTTGGGGGAC	146	609,611	WT 609/611 (30 bp)	GGCTATGGCACC**TGC**AAC**TGC**TTCCCTGAG
	TGGTGGTCCCGGCCGCCA		618,620	WT 618/620 (31 bp)	GGAGAAG**TGC**TTC**TGC**GAGCCCGAAGACATC
11	TGCCAAGCCTCACACCAC	109	630,631	WT exon 11 (27 bp)	CGTGCG**GCA**CAGCTCGTC**GCA**CAGTGG
	GACAGCAGCACCGAGAC		634	WT 634 (31 bp)	TGCGATCACCGTGCG**GCA**CAGCTCGTC**GCA**C
13	ACTTGGGCAAGGCGATGCAG	274	768,769	WT exon 13 (30 bp)	CCCGAGTGA**G**CTTCGAGACCTGCTGTCAGA
	GAACAGGGCTGTATGGAGC				
HPA6	TGGGATCCCAGTGTGAGTGCTCA	180		WT HPA6 (31 bp)	CTGCAGACGGGCTGACCCTCTCGGGGGCTGC
	AGAAGTCGTCACACTCGCAGTAC				

[a]Primers are listed 5′ to 3′, with the forward primer above the reverse primer.
[b]The underlined codons contain a polymorphism, while the other codons contain pathogenic mutations.
[c]WT, wild type.
[d]Probe sequences are wild type and listed 5′ to 3′. *RET* exon 10 and 13 are forward probes, while *RET* exon 11 and HPA6 are reverse probes. The possible mutation locations are highlighted in bold and the polymorphism locations are underlined. The masking probes have the same sequence as the wild type probes, except at the incorporated masking mismatch(es) displayed in each figure. The universal base and unmatched nucleotide masking probes were the same size (base pair) as the wild type probes, while the masking deletion probes were reduced in size by the number of deleted nucleotides from the wild type probe sequence.

Primers and Probes

The primers and probes were synthesized at Integrated DNA Technology (IDT, Coralville, IA). The primer locations for the different *RET* exons were chosen to create amplicons that included all known pathogenic mutations and, if possible, to exclude any benign polymorphisms from analysis [Ceccherini et al., 1994; Eng and Mulligan, 1997; Stenson et al., 2003]. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed March 2004).

The probes were 3′ phosphorylated to prevent extension during PCR. The universal base masking probes contained a 5-nitroindole at the masking site [Loakes, 2001]. The primer and wild-type probe sequences are listed in Table 2. The masking probe type, masking position, and location of the polymorphic sequences are diagrammed in each figure.

PCR

Sample DNA was amplified by asymmetric PCR to increase the amount of single-stranded DNA product for hybridization with the unlabeled probe [Zhou et al., 2004]. Asymmetric PCR used the LightCycler FastStart DNA master hybridization probe kit (Roche Diagnostics Corp., Indianapolis, IN) and 1 μL of purified DNA (50–100 ng) with a final reaction volume of 10 μL. PCR included 1 × FastStart master hybridization mix, 2 mM of $MgCl_2$, 0.5 μM excess primer, 0.05 μM limiting primer, 0.01 U/μL Uracil-DNA glycosylase (Roche Molecular, Indianapolis, IN), 0.5 μM unlabeled probe and 1 × LCGreen® PLUS (Idaho Technology, Salt Lake City, UT). The reverse primer was in excess for exons 10 and 13, while the forward primer was in excess for exon 11.

Thermocycling for the *RET* exons was performed on a LightCycler® (Roche) and included a uracil-DNA glycosylase step at 50°C for 10 min, polymerase activation at 95°C for 10 min, and 60 PCR cycles consisting of denaturation at 95°C for 1 sec, annealing at 62°C for 1 sec, and extension at 72°C for 10 sec. HPA6 was amplified with the same protocol, except that a 68°C annealing temperature was used for 55 PCR cycles with a 9:1 (forward to reverse) asymmetric primer ratio. After PCR, unlabeled probes were hybridized to the target single-stranded DNA by heating to 95°C followed by rapid cooling of the samples to 40°C.

High-Resolution Melting

Analysis was performed on a high-resolution melting instrument, the HR-1™ (Idaho Technology, Salt Lake City, UT). The LightCycler capillaries were transferred into the HR-1 and heated at 0.3°C/sec. Melting data for *RET* exons were acquired between 55°C and 95°C, and for HPA6 between 65°C and 95°C. The melting data was directly converted to a derivative plot (–dF/dT vs. temperature) with the HR-1 software. The temperature at the highest –dF/dT value of the derivative melting peak was used as the T_m. The T_m difference (ΔT_m) is the difference in T_m between the wild-type allele and the variant allele [Lyon, 2001].

Although only one patient sample per genotype (Table 1) was available for this study, there were multiple wild-type samples. For each of the wild-type or masking probes tested, the multiple wild-type samples had nearly identical derivative melting curves with T_ms that were within 0.25°C of each other. This is demonstrated in Figure 3 (three panels on the right), in which five individual wild-type samples are shown with the wild-type and masking probes. For simplicity, only one wild-type sample trace is shown in the other figures.

RESULTS

Masking Polymorphisms Near Targeted Mutations

RET exon 13 has a common benign polymorphism of 0.26 allelic frequency separated by 2 bp from a rare pathogenic mutation (Fig. 1A) [Ceccherini et al., 1994; Elisei et al., 2004; Eng and Mulligan, 1997; Stenson et al., 2003]. Both the polymorphism and the targeted mutation have one mismatch with the wild-type probe, which resulted in similar probe melting temperatures (Fig. 1B). Masking probes that incorporated a universal base, deletion, or unmatched nucleotide over the nontargeted polymorphism location within codon 769 (c.2307T>G) were evaluated for *RET* exon 13 (Fig. 1C–F). The unmatched nucleotides used for the masking probes did not complement the possible nucleotides at the polymorphism location (noncoding strand: A or C). Each masking probe reduced all possible alleles to one mismatch status with the probe, creating a nearly identical T_m for the masked polymorphism allele as for the wild-type allele. The targeted mutant allele had an additional mismatch with the masking probes, and was clearly distinguished by a 4°C lower T_m than the wild-type or masked polymorphism alleles.

Since the codon 769 nonpathogenic polymorphism is commonly found in the general population, 16 random non-MEN2 patient samples were tested with the Exon 13 wild-type and masking probe (data not shown). With the wild-type probe, eight samples were wild-type and eight had one or both alleles with a lower T_m than the wild-type allele. With the masking probe, all 16 samples had similar T_ms as the wild-type control and nearly identical derivative melting curves, indicating wild-type or masked polymorphism alleles. With only the wild-type probe data, 50% of the unknown samples would have been sequenced due to the detected sequence variations; however sequencing was not necessary because the masking probe identified all sequence variations as heterozygous/homozygous for the common exon 13 polymorphism.

RET exon 11 has two codons of possible pathogenic mutations at 630 and 634, and a polymorphism within codon 631 [Kruckeberg and Thibodeau, 2004]. To analyze the two pathogenic codons but not the codon 631 polymorphism, we tested a masking deletion and two unmatched nucleotide probes (Fig. 2). With all three probes, the codon 631 polymorphism allele was masked from analysis with a nearly identical T_m as the wild-type allele, which allowed the lower T_m mutant alleles to be clearly detected.

Locating the Position of Sequence Variation Under Probes

Masking probes can locate the position of a mutation to a particular sequence location. *RET* exon 10 mutations are mainly

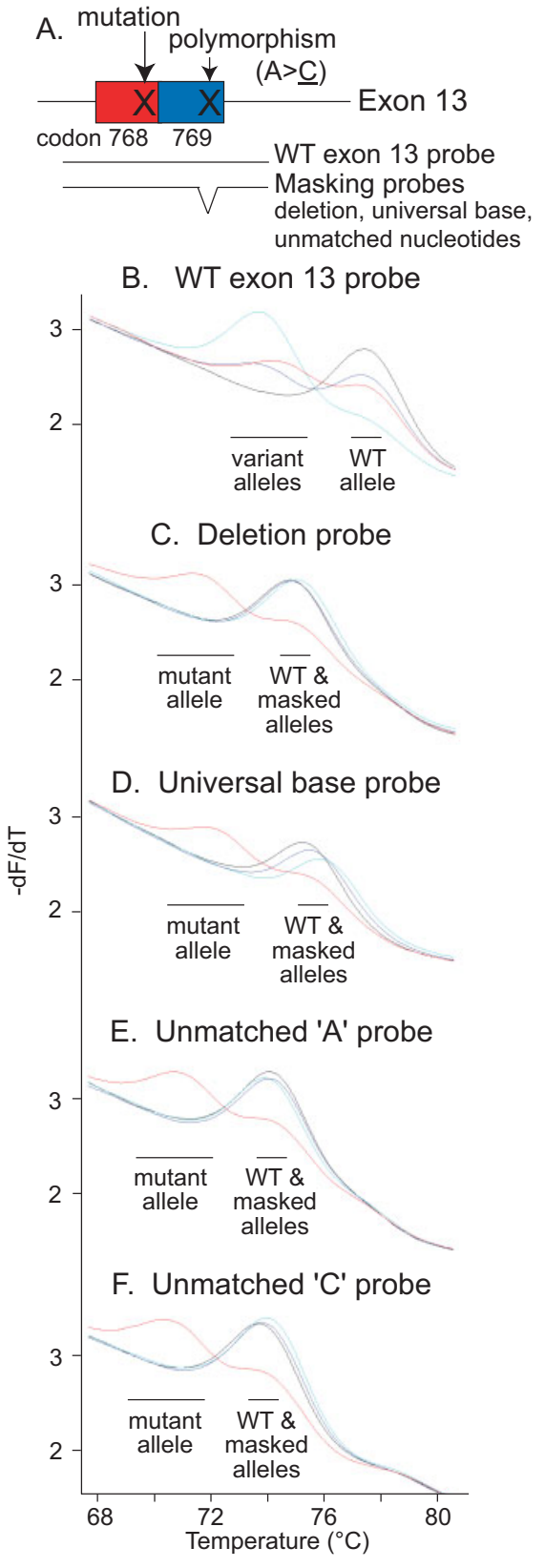
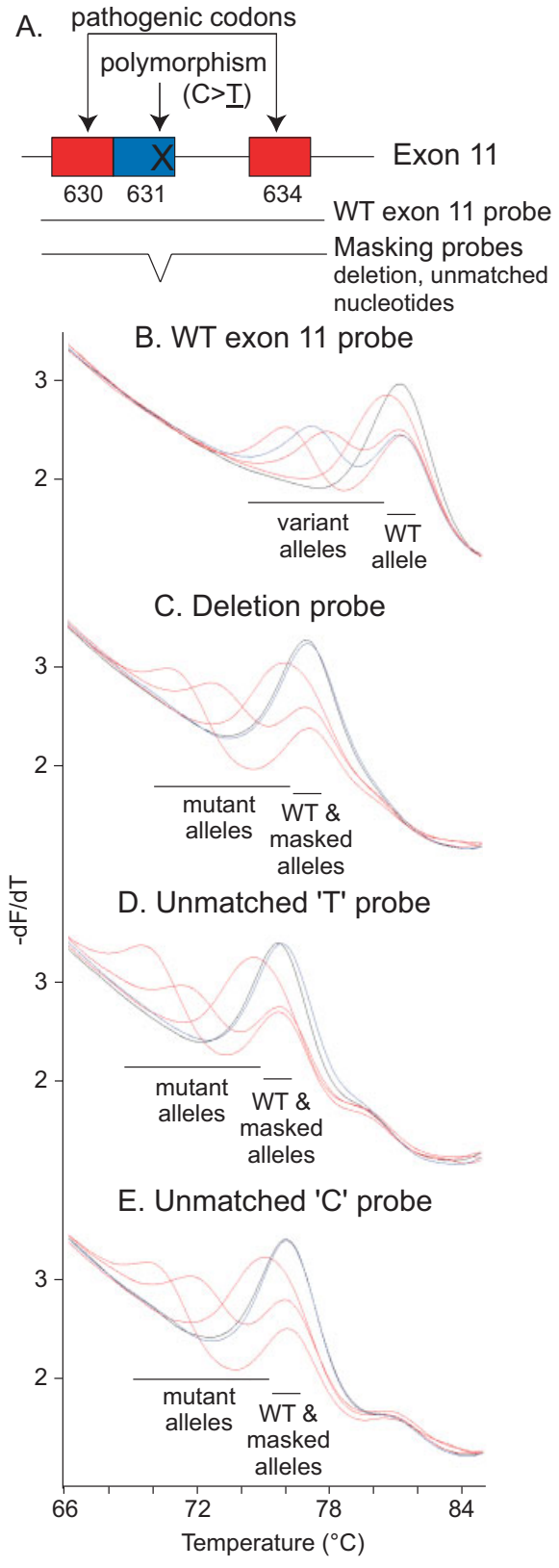


FIGURE 1. **Masking a polymorphism near the targeted mutation. A:** The diagram illustrates *RET* exon 13 with the pathogenic codon 768 in red and the polymorphism codon 769 in blue. The **X** represents the location of the sequence variation within the codons. For the codon 769 polymorphism (c.2307T>G), the nucleotide sequence for the complementary strand is displayed (n.A>C). The masking probes have a wild-type sequence with an incorporated mismatch of a universal base, unmatched nucleotide, or deletion at the polymorphism location, represented by the V. The graphs (**B–F**) are derivative plots of high-resolution melting analysis data using unlabeled probes. In each graph the black curve is homozygous wild-type, the red curve is the heterozygous mutation within codon 768 (c.2304G>**C**), the dark blue curve is heterozygous for the codon 769 polymorphism, and the light blue curve is homozygous for the codon 769 polymorphism. Two T_m ranges are underlined on each panel, listing which alleles melted at each T_m range. **B:** The wild-type probe (WT exon 13 probe) was used over codons 768 and 769. Heterozygous samples have two derivative melting peaks, while homozygous samples have only one peak. The codon 769 polymorphism was masked by four different masking probes: (C) deletion probe, (D) universal base probe (5-nitroindole), (E) unmatched nucleotide "A" probe, or (F) unmatched nucleotide "C" probe.

restricted to four pathogenic codon locations: 609, 611, 618, and 620 [Ceccherini et al., 1994; Eng and Mulligan, 1997; Stenson et al., 2003]. Both wild-type probes (over two pathogenic codons each) can detect mutations but cannot identify which codon contains the mutation due to nearly identical T_ms (Fig. 3B and E). Figure 3 demonstrates how the codon of exon 10 mutations can be located. Masking probes were designed with a 3-bp deletion over one of the codons. An allele with a mutation within the masked codon will have the same T_m as the wild-type allele. However, any mutation outside of the masked location will have an additional mismatch with the codon deletion probe and result in a lower T_m. In each case, mutations within the masked codons were as stable as the wild-type allele, whereas alleles with the mutation outside of the masked codon were clearly identified by lower T_ms.



Masking Sequence Variation Immediately Adjacent to the Targeted Mutation

The positional effects of single-base masking deletions in the probe relative to targeted mutations are shown in Figure 4. Five different single-base deletion probes were designed across *RET* exon 11 codon 634 of wild-type nucleotide sequence "TGC." Alleles with mutations in the second position of codon 634 ("blue mutations," c.1901G>**A**, **C** and **T**) had a T_m 3–4°C below the wild-type allele with all probes (Fig. 4B–E, and G), except with one of the deletion probes (probe 4, Fig. 4F). This probe deletion was over the mutation site, which masked all the codon 634 second position mutations (at c.1901) and resulted in a similar T_m as the wild-type allele. Similarly, a mutation at the third position of codon 634 ("light blue mutation," c.1902C>**G**) had a T_m 2–3°C below the wild-type allele with all probes (Fig. 4B–F), except when the deletion was over the mutation site, masking only this mutation (Fig. 4G). Mutations at the first position of codon 634 ("red mutations," c. 1900T>**A**, **C**, and **G**) were also masked by probes with a deletion over the mutation site (Fig. 4E). However, when the deleted base was immediately adjacent to these mutations (Fig. 4D and 4F), the T_ms of the mutations were very similar (within 0.8°C) to the wild-type T_m.

The HPA6 c.1544G>**A** mutation is immediately adjacent to the polymorphism c.1545G>A or C. Deletion probes, rather than universal base or unmatched probes, resulted in the greatest T_m separation of mutant and wild-type sequences (Fig. 5 and data not shown). Although the wild-type probe separated all wild-type and mutant alleles by 1°C, use of the masking deletion probe increased this separation to 3°C, with one exception (Fig. 5). The wild-type "GA" and the mutant "**A**G" allelic nucleotide sequences resulted in very similar T_ms (Fig. 5B), and both these alleles are predicted to have only a single "A" bulge with the masking deletion probe (Fig. 5C).

FIGURE 2. **Masking a polymorphism between two pathogenic codons. A: The diagram illustrates *RET* exon 11 with pathogenic codons 630 and 634 shown in red. Codon 631 is blue with an X to represent the location of polymorphism. The masking probes have a wild-type sequence that incorporates either a deletion or an unmatched nucleotide at the polymorphism location, represented by the V. The graphs (B–E) are derivative plots of high-resolution melting analysis data using unlabeled probes. In each graph the black curve is homozygous wild-type, the red curves are three unique heterozygous mutations within codon 634 (c.1900T>C, c.1900T>G, and c.1901G>C), and the blue curve is heterozygous for the codon 631 polymorphism (c.1893C>T). Two T_m ranges are underlined on each graph panel, listing which alleles melted at each T_m range. B: The wild-type probe (WT exon 11 probe), over codons 630, 631, and 634. The codon 631 polymorphism was masked by three different masking probes: (C) deletion probe, (D) unmatched nucleotide "T," and (E) unmatched nucleotide "C" probe.**

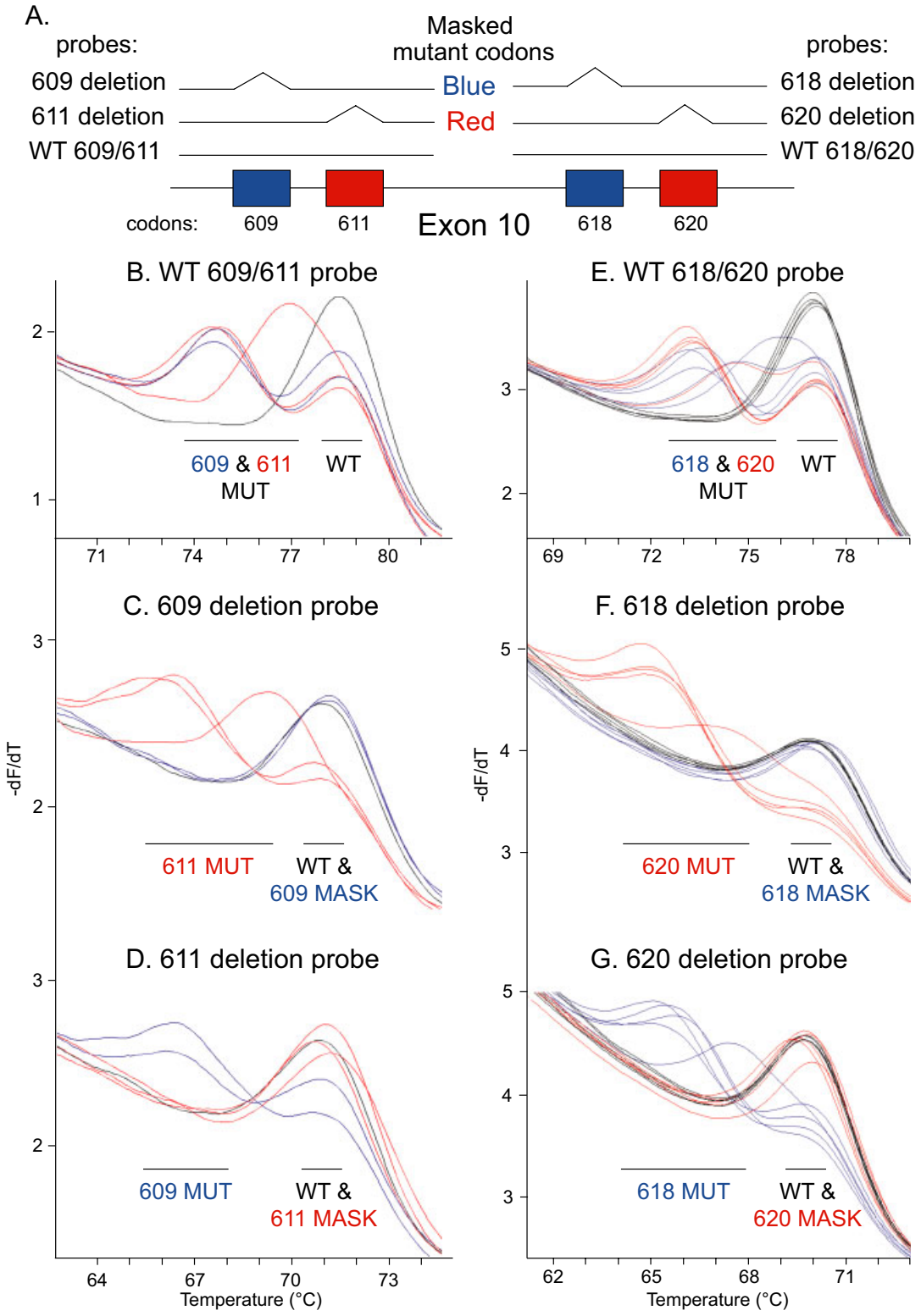


FIGURE 3. **Locating mutations by codon position with masking deletion probes. A:** The diagram illustrates *RET* exon 10, where pathogenic mutations can be any nucleotide change within codons 609, 611, 618, and 620 (all of wild-type nucleotide sequence TGC). Codons 609 and 618 are shown in blue, while codons 611 and 620 are in red. Each masking probe has a 3-bp deletion of the wild-type probe sequence over one pathogenic codon, as illustrated. The codons predicted to be masked by each probe are listed by codon color (blue or red) under "masked mutant codons." In the graphs (**B–G**) heterozygous mutations at codons 609 and 618 are the blue traces, heterozygous mutations at codons 611 and 620 are the red traces, and the black traces are homozygous wild-type samples. Codons 609 and 611 data are displayed in the left panels, while codons 618 and 620 data are displayed in the right panels. Two T_m ranges are underlined for each graph with codon mutant alleles (MUT), wild-type alleles (WT), and masked codon mutant alleles (MASK) noted in each panel. **B**: The wild-type probe (WT 609/611 probe), over the codons 609 and 611. **C**: The masking 609 deletion probe. **D**: The masking 611 deletion probe. **E**: The wild-type probe (WT 618/620 probe), over the codons 618 and 620. **F**: The masking 618 deletion probe. **G**: The masking 620 deletion probe. The 15 *RET* exon 10 heterozygous mutations tested in this figure are listed in Table 1.

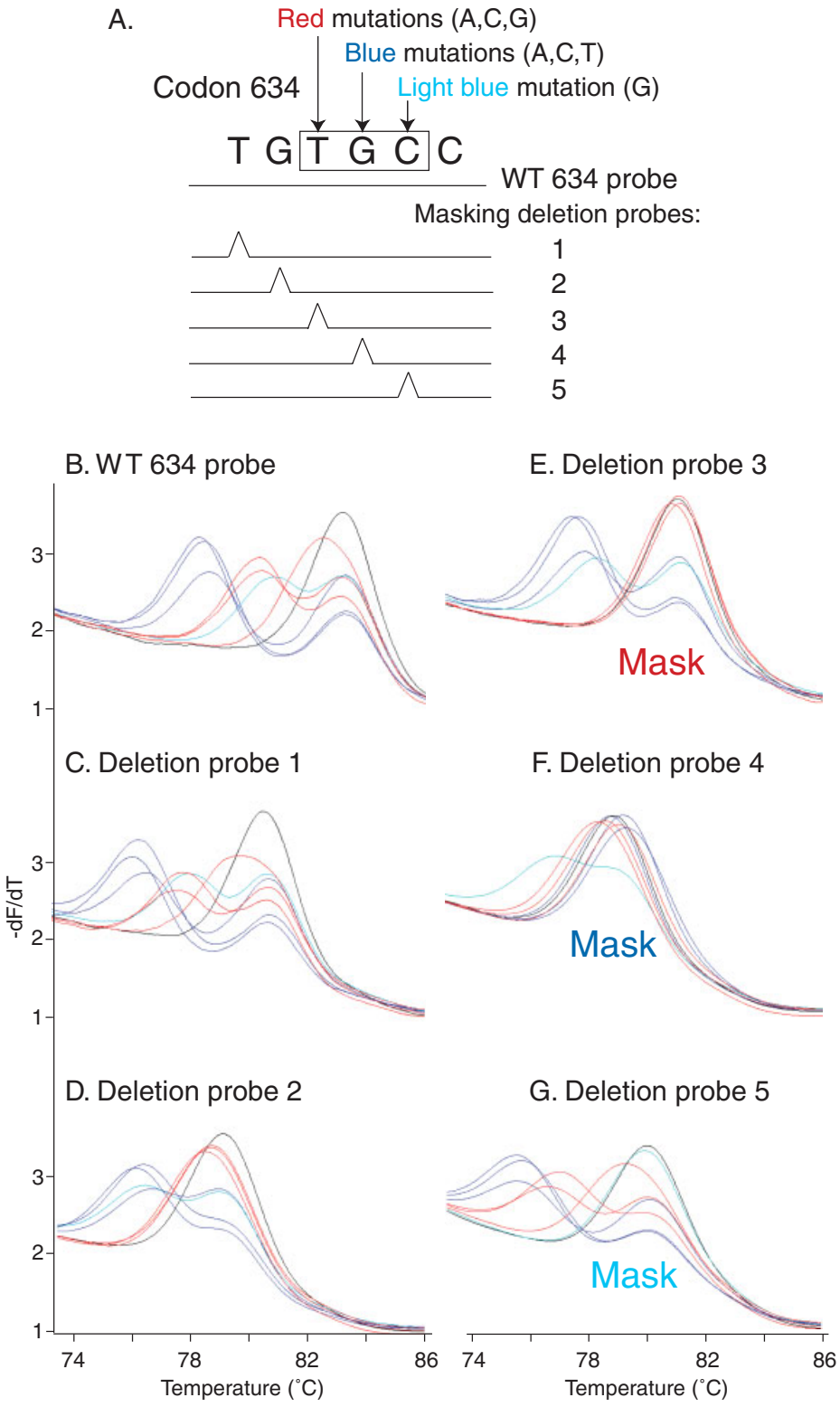
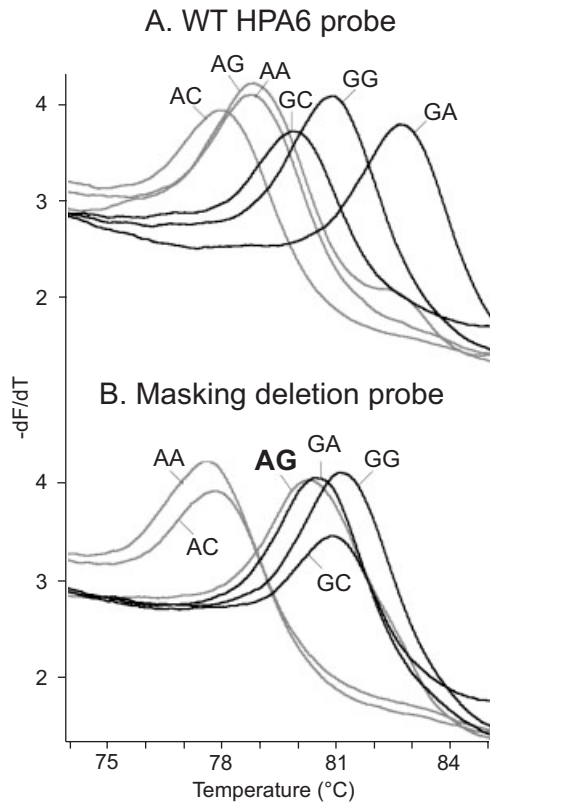


FIGURE 4. **Masking probes with deletions near or adjacent to the targeted mutation. A:** The diagram illustrates *RET* exon 11 with codon 634 (boxed) of the wild-type nucleotide sequence "TGC." Each masking probe has a 1-bp deletion of the wild-type sequence, near or within codon 634 as illustrated in the diagram. The codon 634 mutations are listed above each position within codon 634 and the melting curve traces are color coded by this mutation position. In each graph **(B–G)** red traces are for the three unique heterozygous mutations at the first position of codon 634 ("red mutations," c.1900T>**A**, **C**, and **G**), dark blue traces are for the three unique heterozygous mutations at the second position of codon 634 ("blue mutations," c.1901G>**A**, **C**, and **T**), and light blue traces are for the heterozygous mutation at the third position of codon 634 ("light blue mutation," c.1902C>**G**). The black traces are homozygous wild-type samples. B: Wild-type probe (WT 634 probe). C–G: Masking deletion probes 1–5. Mutations that should be masked by a deletion probe are noted in the panels by the word "Mask" in the mutation position color (red, dark blue, or light blue; for the first, second, and third position codon 634 mutations, respectively). The seven *RET* exon 11 heterozygous mutations tested in this figure are listed in Table 1 (the codon 634 mutations).



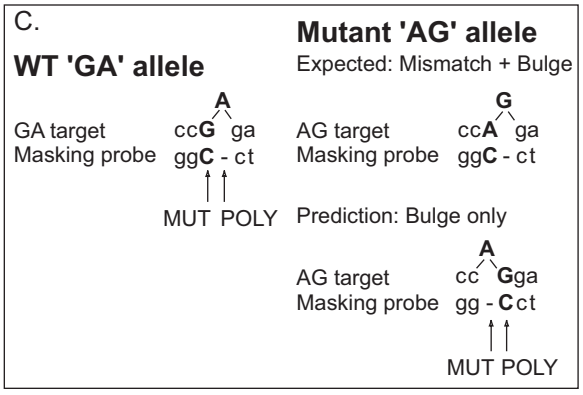


FIGURE 5. **Masking immediately adjacent sequence variation with deletion probes. Homozygous engineered templates of six different combinations of the c.1544G>A mutation and adjacent polymorphism sequences (c.1545G>A or C) were tested: GA, GC, GG, AA, AC, and AG. The three mutant sample traces are in gray (AA, AC, and AG), while the three wild-type sample traces are in black (GA, GC, and GG). A: The wild-type probe (WT HPA6 probe). B: Masking deletion probe. The mutant "AG" allele with a T_m suggesting wild-type is shown in bold type on the graph. C: The proposed duplexes of the two genotypes with very similar T_ms are displayed. The target sequences are shown above the complementary masking deletion probe sequence, with the mutation location (MUT) and polymorphism location (POLY) indicated. The dash (–) indicates the position of the probe deletion, which is located opposite the unpaired, bulged base. For the mutant "AG" allele with the masking deletion probe, the expected duplex with a mismatch and a single-base bulge at the polymorphism position is displayed above the predicted duplex, with only a single-base bulge at the mutant position. For both the wild-type "GA" allele and mutant "AG" allele, the single-base bulge "A" is surrounded by matched base pairs, resulting in similar T_ms.**

DISCUSSION

Unlabeled masking probes and high-resolution melting analysis can detect, locate, and genotype a mutation in the background of other mutations or polymorphisms. The designed masking probe has only a selected position(s) masked, allowing detection of multiple possible known or novel mutations at other positions under the probe. Masking can be achieved with universal bases, deletions, or unmatched nucleotides over a nontargeted polymorphic region, so that targeted pathogenic mutations can be clearly identified. The ability of universal bases (DNA base analogs) to increase the tolerance of probes or primers to polymorphic sequences is well known [Loakes, 2001; Szemes and Schoen, 2003]. The deletion and unmatched nucleotide probes often performed as well as the more expensive universal base probes, as judged by the difference in T_m between the wild-type and masked alleles. When unmatched nucleotide probes are used for masking, one unmatched nucleotide can be better than the other, as predicted by nearest-neighbor thermodynamics of mismatches [Breslauer et al., 1986; Peyret et al., 1999].

Hybridization probe assays have advantages over other mutation detection assays (such as allelic-specific PCR, temperature gradient capillary electrophoresis, or restriction enzyme digestion of PCR products) because they use a closed-tube, rapid assay that can detect multiple sequence variations under the probe without additional manipulation of the PCR amplicon. Detection of multiple sequence variations under the probe can be a disadvantage of some hybridization probe melting assays, if the wild-type probes cannot distinguish the possible mutations and polymorphisms [von Ahsen et al., 1999b; Warshawsky et al., 2002; Zhou et al., 2004]. If multiple unique alleles are likely and their probe T_ms are similar, it is not possible to achieve definitive genotyping without further analysis. For example, *RET* exon 13 contains a benign polymorphism and a mutation with similar T_m (Fig. 1). The masking technique provided unambiguous genotyping of the targeted mutation during melting analysis, and thus prevented the polymorphism from being interpreted as a mutation. Mismatches introduced by the masking probes cause all alleles to shift to a lower T_m, but the ΔT_m between the mutant and wild-type alleles are generally unchanged or slightly increased.

Hybridization probe melting assays can use probes that complement the targeted mutation to distinguish the mutant allele from other sequence variation [Lyon, 2001]. By using mutation-specific probes, the mutant alleles are perfectly matched, while wild-type alleles have one mismatch. Single-base polymorphisms under the probe, not at the position of the mutation, will result in two mismatches with the mutation-specific probe, as long as the mutation and polymorphism are not in *cis*. If the mutation and polymorphism are on the same allele, one mismatch results and there is a risk of misinterpreting the mutation as wild-type. Another possible disadvantage of mutation-specific probes occurs when mutations result from more than one base change at the same nucleotide position (e.g., n.G>A, T, or C). If only one mutation-specific probe is used, other mutations at the same nucleotide position will result in a single mismatch with the probe and could be interpreted as wild-type. Masking probes can decrease the number of probes needed for mutation detection and decrease the risk of aberrant results that may be generated with melting analysis using the mutation-specific probe. If masking mismatches are incorporated into mutation-specific probes, the detected mutations can be confirmed while masking other nearby mutations or polymorphisms that would complicate analysis.

Masking probes can be used to analyze one mutation at a time in complicated gene sequences, or to locate the mutation to a codon (as demonstrated for *RET* exon 10 in Fig. 3) or a single nucleotide position (as demonstrated for exon 11 in Fig. 4). When mutations in one codon are masked with a 3-bp deletion, mutations within the other codon are identified by a lower T_m. However, any other sequence variation not at the two pathogenic codons would be clearly identified by an additional mismatch with both masking codon deletion probes (data not shown). If single-base deletion probes are used, the exact nucleotide position of the mutation can usually be identified.

Masking probes that incorporate a deletion, universal base, or unmatched nucleotide can be used when at least one matched base pair is between the sequence variation and the targeted mutation. Aberrant results were sometimes obtained when immediately adjacent sequence variation was masked with deletion probes. For example, when the deleted base was immediately adjacent to mutations at the first position of codon 634 (Fig. 4), the mutant and wild-type alleles had similar stability. Using an unmatched "T" or "G" nucleotide for masking instead of the deletion allowed us to make a clear distinction between the mutant and wild-type alleles (data not shown). An additional limitation of using deletion probes to mask immediately adjacent sequence variation is illustrated in Figure 5C. Single-base deletion probes create a single-base bulge in the target DNA strand, usually at the position opposite the deleted base, but the bulge can be at alternate positions depending on the nearest neighbors [Zhu and Wartell, 1999]. An immediately adjacent mutation would be expected to result in further destabilization (a mismatch next to a single-base bulge). However, if the mismatched nucleotide in the probe can complement the otherwise bulged base in the target, then the position of the bulge will "shift," resulting in a single-base bulge surrounded by matched pairs. In this case both the wild-type and mutant duplexes have a similar stability (both single-base bulges). Such a situation cannot be avoided when the possible nucleotides for the mutation and the adjacent polymorphism are the same (e.g., both n.G>A). In other cases one can usually avoid this complication by choosing a probe sequence (wild-type or mutant nucleotide) that will not complement the possible adjacent bulged nucleotides. Alternatively, a masking probe with an unmatched nucleotide or universal base at the polymorphism location can be used.

The masking technique should also be useful with other probe designs, including Hybprobes®, Eclipse™ probes, SimpleProbes®, and Taqman™ probes [Bernard et al., 1998; Lyon, 2001; Lyon et al., 1998; Schutz et al., 2000; Szemes and Schoen, 2003; Teupser et al., 2001; Warshawsky et al., 2002]. Artificial mismatches in probes at nonpolymorphic locations have been used to increase discrimination between mutant and wild-type alleles [Guo et al., 1997; Lyon, 2001; Teupser et al., 2001], indicating that these probes should tolerate mismatches incorporated for masking. The masking probe length may have to be increased if the incorporated mismatches reduce the hybridization temperature of the probe to a degree that compromises detection.

Microarrays could also benefit from the masking technique [Erali et al., 2003]. Masking probes could be designed to tolerate only benign polymorphisms, allowing detection of wild-type and mutant alleles. Mismatches have been introduced at nonpolymorphic locations in microarray probes to increase probe specificity to the target [Lee et al., 2004], and a systematic analysis of the hybridization properties of mismatched microarray probes was recently reported [Karaman et al., 2005]. These reports suggest that microarray probes should tolerate masking mismatches over selected sequence variation for specific analyses of neighboring sequences.

The masking technique allows targeted mutations to be genotyped within a complicated background of multiple possible sequence variations. Regions that were previously difficult to analyze with probes because of surrounding polymorphisms or several possible mutations can now be analyzed using the masking technique. The use of masking probes reduces the need for sequencing in many genotyping assays.

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