RET Proto-Oncogene Genotyping Using Unlabeled Probes, the Masking Technique, and Amplicon High-Resolution Melting Analysis

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**Single bp mutations in the *RET* proto-oncogene can
cause multiple endocrine neoplasia type 2 syn-
dromes. The conventional approach for genotyping
RET mutations is sequencing the exons. A closed-tube
RET genotyping assay using a saturating DNA dye,
unlabeled probes, and amplicon high-resolution
melting analysis was developed. The method required
two sequential polymerase chain reaction stages, a
primary and secondary assay. The primary assay an-
alyzed *RET* exons 10, 11, 13, 14, and 16 with a total of
seven reactions using eight unlabeled probes. The
primary assay genotyped wild-type exons, a common
exon 13 polymorphism, and an exon 16 mutation,
whereas other *RET* sequence variation was detected.
The primary unlabeled probe data limited the possi-
ble genotypes for the detected *RET* sequence varia-
tion, which permitted genotyping in a secondary as-
say with only two to five reactions. Six probes were
designed with the masking technique and masked
selected sequence variations to allow unambiguous
analysis of other mutations elsewhere under the
probe. After this two-stage *RET* genotyping assay,
less than 0.2% of exons tested would require sequencing
for genotype. A blinded study generated from five
wild type and 29 available *RET* sequence variation
samples was 100% concordant with sequencing. Am-
plicon high-resolution melting analysis with unla-
beled probes and the masking technique is a fast,
accurate method for genotyping the >50 *RET* se-
quence variations.** *(J Mol Diagn 2007, 9:184–196; DOI:
10.2353/jmoldx.2007.060091)*

Multiple endocrine neoplasia type 2 (MEN2) syndromes,
MEN2A, MEN2B, and familial medullary thyroid carci-
noma (FMTC), are caused by germline *RET* mutations in
exons 10, 11, and 13 through 16. MEN2 syndromes are
autosomal dominant disorders that lead to a high lifetime
risk of thyroid cancer. Genetic testing for *RET* mutations
can identify patients at risk of thyroid cancer before can-
cer progression, when prophylactic thyroidectomy can
increase survival rate.

There have been many assays developed to detect
and/or genotype *RET* mutations, such as single-stranded
conformation polymorphism analysis, denaturing gradi-
ent gel electrophoresis, temperature gradient capillary
electrophoresis, restriction enzyme digestion of polymer-
ase chain reaction (PCR) products, pyrosequencing,
fluorescently labeled hybridization probes, and microar-
rays.[1–10] However, most of these methods require addi-
tional post-PCR processing of the amplicon to detect
mutations. Some of these methods have reported 95% or
less sensitivity for mutation detection or have been tested
with only a small number of *RET* mutation samples. The
methods that analyze limited regions of *RET* sequence
may target only common mutations and miss rare muta-
tions. Furthermore, the presence of nonpathogenic poly-
morphisms may also lead to aberrant results.[9,11] Be-
cause of these limitations, *RET* exon sequencing remains
the gold standard.

Sequencing is a time-consuming and expensive open-
tube assay that requires additional processing of PCR
products. In contrast, mutation scanning by high-resolu-
tion melting analysis is a rapid and cost-effective closed-
tube assay that does not require post-PCR processing of
the amplicon.[12] By using a saturating double-stranded
DNA-binding dye, LCGreen Plus, amplicon high-resolu-
tion melting analysis can detect sequence variation within
a PCR amplicon.[12–14] A systematic study of high-resolu-
tion melting detection of heterozygous point mutations
within a PCR amplicon found a sensitivity and specificity
of 100% for amplicons <400 bp in size.[14] Recently, we

demonstrated mutation scanning of *RET* exons by high-resolution melting analysis.[15] Although this assay detected 100% of the mutations in a blinded study, complete genotyping was difficult because some unique mutations had similar melting curves. In these cases, sequencing was required after the initial mutation scanning assay. Alternatively, the addition of hybridization probes may allow the majority of *RET* mutations to be genotyped without sequencing.

Fluorescently labeled hybridization probe analysis is a rapid, closed-tube assay that can genotype sequence variation by probe/allele melting temperatures (T_m) but requires expensive oligonucleotide modifications.[5] Unlabeled probes can also genotype mutations by T_m in a closed-tube assay, using LCGreen Plus.[16] Because the detection is with a double-stranded DNA-binding dye, unlabeled probe and amplicon data can be analyzed from one melting curve. Amplicon mutation scanning can be complemented with unlabeled probe genotyping analysis, as recently demonstrated by Zhou et al.[17] However, all *RET* mutations within an exon cannot be genotyped with one probe, because unique mutations under the probe can have similar or identical melting temperatures.[18]

A two-stage *RET* genotyping assay was developed for complete closed-tube genotyping, which was more cost effective and less time consuming than sequencing. The first PCR stage (primary assay) used amplicon melting to scan for any sequence variation within the *RET* exons, whereas the unlabeled probe analysis located the region of variation and identified possible genotypes. A second PCR stage (secondary assay) with a limited number of mutation-specific probes was required for unambiguous genotyping of the >50 *RET* sequence variations. Mutation identification was simplified by using probes designed with the masking technique.[18] The masking technique simplifies probe melting interpretation when the presence of multiple possible sequence variations under the probe complicates analysis. Selected sequence variation can be masked by incorporating mismatches (deletion, unmatched nucleotide, or universal base) into hybridization probes at the polymorphic location. This creates an artificial mismatch with all possible alleles at the masked polymorphic location. Both the wild-type and masked variant alleles have a single mismatch with the probe and a similar T_m, whereas mutant alleles have an additional mismatch elsewhere under the probe and are identified by a lower T_m than alleles that vary only at the masked position.

Materials and Methods

Samples

The deidentified genomic DNA samples of wild-type *RET* genotype or with *RET* sequence variation used in this report were described previously.[15,18] *RET* sequence variations that alter RET protein function to cause MEN2 syndromes are mutations, whereas *RET* sequence changes that do not cause MEN2 syndromes are poly-morphisms (>1% population frequency).[2,19–23] Rare *RET* sequence variations of unknown significance or that do not cause MEN2 syndromes are "sequence variants." In this report, *RET* sequence variation is listed by the codon number, the wild-type codon DNA sequence followed by the codon sequence change, with the mutant nucleotide in bold [eg, 618 (TGC→T**A**C)], whereas polymorphism or sequence variant nucleotide change is underlined. All of the *RET* sequence variation samples tested had a single nucleotide change and were heterozygous unless otherwise stated. *RET* mutant genotypes as well as polymorphism and sequence variant genotypes are listed in Table 1.[3,20,24]

Primers and Probes

Oligos were synthesized at Integrated DNA Technology (Coralville, IA). Primers were designed using Primer 3.[25] Primers and probes were chosen to detect all known *RET* mutations while excluding common polymorphisms from analysis (Table 2).[2,19,20] The unlabeled probes had either a 3′-phosphate or the 3′-amino modifier (Integrated DNA Technology) incorporated to prevent extension during PCR. Probes were designed to analyze the mutation hotspots within each exon.

Four types of unlabeled probes were used in the *RET* genotyping assay (Table 2): wild-type (WT) probes, mutation-specific (MS) probes, masking probes that masked a single polymorphic nucleotide, and location (L) probes that masked three polymorphic nucleotides (one codon).[18] For example, the exon 13 masking probe (Mask 1bp 769) had a single nucleotide deletion at the polymorphic nucleotide position (the "T" position in WT13 probe; Table 2). The exon 10 and 11 location probes had a three-nucleotide (codon) deletion from the wild-type probe sequence, masking one pathogenic codon to identify the codon position of a mutation. The mutation-specific probes for exon 10 and 11 had the same sequence change at both pathogenic codon positions. For example, in exon 10, the MS 618/620 T**A**C probe has the "T**A**C" sequence at both codons 618 and 620 as displayed in Table 2.

PCR

Sample DNA was amplified by asymmetric rapid-cycle PCR as described previously,[18] except that 55 cycles of PCR were used with primer concentrations given in Table 2. The exon 14 reaction included two probes: 0.5 μmol/L WT14A probe and 0.25 μmol/L WT14B probe. A wild-type control was run with each probe for the primary and secondary assay reactions. For exon 13, the WT13 primary assay reaction used two additional controls: heterozygous and homozygous codon 769 samples. The exon 10 primary assay reactions used a 620(TGC→**A**GC) mutant sample as a positive control.

High-Resolution Melting

Analysis was performed on a high-resolution melting instrument, the HR-1 (Idaho Technology, Salt Lake City,

Table 1.   *RET* Proto-Oncogene Sequence Variation

RET exon	Codon	Codon sequence change*	Amino acid change	References
10	603	AAA → **C**AA	K603Q	20
	609	TGC → A**G**C†	C609S	3
		TGC → **C**GC	C609R	3, 20, 24
		TGC → **G**GC	C609G	3, 20
		● TGC → **TA**C	C609Y	3, 20, 24
		● TGC → **TC**C	C609S	3, 20
		TGC → **TT**C	C609F	3
		TGC → T**G**G†	C609W	3, 20, 38
	611	TGC → **A**GC	C611S	3, 20
		● TGC → **C**GC	C611R	3, 20
		TGC → **G**GC	C611G	3, 20, 24
		● TGC → **TA**C	C611Y	3, 20, 24
		TGC → T**C**C†	C611S	3
		● TGC → **TT**C	C611F	3, 20
		TGC → TG**G**	C611W	3, 20, 24
	618	TGC → **A**GC	C618S	3, 20, 24
		● TGC → **C**GC	C618R	3, 20, 24
		● TGC → **G**GC	C618G	3, 20, 24
		● TGC → **TA**C	C618Y	3, 20, 24
		● TGC → **TC**C	C618S	3, 20, 24
		● TGC → **TT**C	C618F	3, 20, 24
		TGC → TG**G**	C618W	3
	620	● TGC → **A**GC	C620S	3, 20
		TGC → **C**GC	C620R	3, 20, 24
		TGC → **G**GC	C620G	3, 20, 24
		● TGC → **TA**C	C620Y	3, 20, 24
		● TGC → **TC**C	C620S	3, 20, 24
		● TGC → **TT**C	C620F	3, 20, 24
		● TGC → TG**G**	C620W	3, 20
11	630	TGC → **C**GC	C630R	3
		TGC → **TA**C	C630Y	3, 20
		TGC → **TT**C	C630F	3, 20, 24
		TGC → **TC**C	C630S	3, 20
	631	● GAC → GA**T**†	D631D	3
		GAC → **TA**C†	D631Y	3
		GAC → GA**A**†	D631E	3, 34
		GAC → G**T**C†	D631V	3, 34
	634	● TGC → **A**GC	C634S	3, 20, 24
		● TGC → **C**GC	C634R	3, 20, 24
		● TGC → **G**GC	C634G	3, 20, 24
		● TGC → **TA**C	C634Y	3, 20, 24
		● TGC → **TC**C	C634S	3, 20, 24
		● TGC → **TT**C	C634F	3, 20, 24
		● TGC → TG**G**	C634W	3, 20, 24
	640	GCC → G**G**C	A640G	20
13	768	● GAG → GA**C**	E768D	3, 20, 24
		GAG → GA**T**	E768D	3, 20
	769	● CTT → CT**G**‡	L769L	2, 19, 20
	778	GTC → **A**TC	V778I	20
	781	CAG → C**G**G	Q781R	20
	790	TTG → TT**T**	L790F	20, 24, 28
		TTG → TT**C**	L790F	20, 24, 28
	791	TAT → T**T**T	Y791F	24, 27, 28
14	804	● GTG → **A**TG	V804M	3, 20, 24
		● GTG → **T**TG	V804L	3, 20, 24
		GTG → **C**TG	V804L	3
	806	TAC → T**GC**§	Y806C	24, 35
	844	CGG → C**T**G	R844L	20, 24
	852	ATC → AT**G**†	I852M	20, 29
16	912	CGG → C**C**G	R912P	30
	918	● ATG → AC**G**	M918T	3, 20, 24
	922	TCC → TA**C**†	S922Y	20, 31

*Sequence variation is heterozygous unless otherwise stated in the text. Codon DNA sequence is listed with the nucleotide of change in bold for mutations and underlined for polymorphisms/sequence variants. ●, available *RET* sequence variation samples.

†Rare sequence variants of unknown significance or not causative of MEN2 syndromes; see references. Three variants in codons 609 or 611 are within *RET* pathogenic codons yet are not associated with MEN2 syndromes. Variants at codons 922 and 852 were previously found in MTC patients, yet may not be causative of MEN2 syndromes.

‡Benign polymorphism, 0.26 allelic frequency.

§806 is only pathogenic with 804 mutation on same allele.

Table 2. Primers and Probes

RET exon	Primers*	Amplicon (bp)	Codon of variation†	Probes‡	Probe sequence§
10	0.275 μmol/L, 1F:9R GGGCAGCATTGTTGGGGGAC TGGTGGTCCCGGCCGCCA	146	609	WT10A	GGCTATGGCACC**TGC**AAC**TGC**TTCCCTGAG
			611	L10A 611mask¶	GGCTATGGCACC**TGC**AAC---TTCCCTGAG
				MS 609/611 TAC‖	GGCTATGGCACC**TAC**AAC**TAC**TTCCCTGAG
			618	WT10B	GGAGAAG**TGC**TTC**TGC**GAGCCCGAAGACATC
			620	L10B 620mask¶	GGAGAAG**TGC**TTC---GAGCCCGAAGACATC
				MS 618/620 TAC‖	GGAGAAG**TAC**TTC**TAC**GAGCCCGAAGACATC
11	0.55 μmol/L, 5F:1R TGCCAAGCCTCACACCAC GACAGCAGCACCGAGAC	109	630	WT11	TGCGATCACCGTGCG**GCA**CAGCTCG̲TC**GCA**C
			631	L11 634mask¶	TGCGATCACCGTGCG---CAGCTCG̲TC**GCA**C
			634	MS 630/634 TAC‖	TGCGATCACCGTGCG**GTA**CAGCTCG̲TC**GTA**C
13	1.1 μmol/L, 1F:9R CTGCATTTCAGAGAACGCCTC GAACAGGGCTGTATGGAGC	187		WT13	CCCGAGTGA**G**CT̲TCGAGACCTGCTGTCAGA
			768	Mask 1bp 769**	CCCGAGTGA**G**CT-CGAGACCTGCTGTCAGA
			769	MS 768 GAC + mask**	CCCGAGTGA**C**CT-CGAGACCTGCTGTCAGA
				MS 768 GAT + mask**	CCCGAGTGA**T**CT-CGAGACCTGCTGTCAGA
14	0.55 μmol/L, 1F:5R CAGGGCCCCCTCTCTCCGC TCTCGGCCAGATACTGCAT	235		WT14A	CTCCTCCTCATC**G**TGGAGTACGCCAAA
			804	MS 804 TTG	CTCCTCCTCATC**T**TGGAGTACGCCAAA
			806	MS 804 ATG	CTCCTCCTCATC**A**TGGAGTACGCCAAA
				MS 804 CTG	CTCCTCCTCATC**C**TGGAGTACGCCAAA
			844	WT14B	GAGC**G**GGCCCTCACCATGGGCGACCTCAT**C**TCA
			852	MS 844 CTG	GAGC**T**GGCCCTCACCATGGGCGACCTCAT**C**TCA
				MS 852 ATG	GAGC**G**GGCCCTCACCATGGGCGACCTCAT**G**TCA
16	0.55 μmol/L, 1F:9R ATAGGGCCTGGGCTTCTC ACACATCACTTTGCGTGGTG	154	918 922	MS 918 ACG††	TCCAGTTAAATGGA**C**GGCAATTGAAT**C**CCT

*Final total primer concentration and forward (F) to reverse (R) primer ratio is displayed above the primer sequences. Primer sequences are listed 5′ to 3′, with the forward primer above the reverse primer.

†Codon of variation under each probe set. The underlined codons contain a polymorphism or sequence variant, whereas the other codons contain mutations.

‡Mask, masking deletion of one or three nucleotides. Wild-type probe sequence (WT exon #) is used in the primary assay for each exon, except for exon 16. If two wild-type probes are used for one exon, they are labeled as A and B probes.

§Probe sequences are listed 5′ to 3′. *RET* exons 10, 13, 14, and 16 have forward probes, whereas exon 11 has reverse probes. The known mutation locations are highlighted in bold, and the possible polymorphism or sequence variant locations are underlined.

¶Location (L) probes have a three nucleotide masking deletion (−−−) of one pathogenic codon from the wild-type probe sequence.

‖Example for the MS probes used for genotyping the mutations in exons 10 and 11. The same mutation-specific sequence is at both pathogenic codon positions within the probe. These probes have the mutation-specific sequence in the name, with the changed sequence in bold. There are seven possible mutant sequence changes from the 'TGC' wild-type cysteine codon sequence to another amino acid, therefore seven mutation-specific probes (**A**GC, **C**GC, **G**GC, **TA**C, **TC**C, **TT**C, **TG****G**) for a total of 21 MS probes for exon 10 and 11 mutations. The MS probe sets used to genotype each mutation are diagrammed in the figures and Table 3 as well as in Supplemental Tables 1 to 5.

**Exon 13 used masking probes in the primary and secondary assays. The 'Mask 1bp 769' primary probe and the exon 13 mutation-specific probes have a single nucleotide deletion (−) at the polymorphism position within codon 769 (deletion of the underlined 'T' in the wild-type probe sequence).

††Exon 16 used a codon 918(ATG→A**C**G) mutation-specific probe for the primary assay.

UT). The LightCycler capillaries were transferred into the HR-1 and heated at 0.3°C/second. In the primary assay, amplicon and unlabeled probe melting data for the *RET* exons were acquired between 60°C and 95°C. All amplicon and probe data were analyzed in the primary assay for each exon, except for exon 14. Because all reported exon 14 mutations were under the unlabeled probes, only probe data were analyzed. For the secondary assay, only unlabeled probe melting was analyzed with data acquired between 60°C and 89°C.

The high-resolution melting data were analyzed with custom software written in LabView (National Instruments, Austin, TX) as described previously.[12,15] In brief, the melting curve data for the amplicon was normalized, temperature-shifted, and then converted to a derivative plot or fluorescence difference plot. Depending on the data plot used for amplicon analysis, sequence variation was detected by a visual shift from the wild-type controls due to a unique melting curve shape, a broader derivative melting peak, a uniquely shaped derivative melting peak (shoulder or two peaks), a shift in melting temperature, and/or fluorescence difference. The amplicon melting curve, derivative plot, and fluorescence difference plot could all be used to detect *RET* sequence variation. The unlabeled probe melting data were directly converted to a derivative plot. Exon 14 probe data were normalized, and the exponential background was removed before analysis.[26]

T_ms for the unlabeled probe data were estimated at one-half the area of the derivative melting curve peaks. ΔT_ms for heterozygous samples were defined as the wild-type allele T_m minus the variant allele T_m (mutation, polymorphism, or sequence variant allele). Note that the ΔT_m value will be negative when the variant allele T_m is higher than the wild-type allele T_m (eg, with a complementary mutation-specific probe). When probe data for heterozygous samples resulted in shoulder or plateau shaped peaks, the peak area was divided into ¼, ½, and ¼ regions with the wild-type and variant allele T_ms taken at the boundaries of these regions. When only a single peak was apparent because of homozygous sequence variation (no wild-type allele) or the variant allele T_m was nearly

identical to the wild-type allele T_m (no distinguishable wild-type allele), then the wild-type control sample T_m was used for the wild-type allele T_m in the ΔT_m calculation. Wild-type samples also resulted in a single peak, so the sample's single allele T_m was subtracted from the wild-type control T_m to calculate ΔT_m.

The primary probe ΔT_m values for each *RET* sequence variation determined the mutation-specific probe set used in the secondary genotyping assay. In cases in which the *RET* mutations were not available (Table 1), predicted ΔT_ms were estimated using nearest-neighbor parameters as previously described.[13] The empirically determined and predicted ΔT_m values for *RET* sequence variations with the primary probes established the ΔT_m ranges expected to group mutations for mutation-specific probe selection. Supplemental Tables 1 through 5 (*see http://jmd.amjpathol.org*) display the ΔT_m ranges and mutation grouping with the primary probes, as well as the ΔT_m values for each available *RET* sequence variation with the primary probes and the selected secondary probes. The "T_m guidelines" were placed on a derivative plot at temperatures calculated by subtracting the primary assay ΔT_m range values from the control wild-type allele T_m, as shown for exons 10 and 11 primary probe data. These T_m guidelines are convenient for quick, visual grouping of mutant samples to select the secondary assay mutation-specific probes. However, calculating the ΔT_m value for each sample can better control for systematic T_m shifts caused by sample-specific differences (altered salt concentration, different sample purification methods, or temperature inaccuracy).

Blinded Study

Twenty-nine variant *RET* samples (the available mutations, polymorphisms, and sequence variants as listed in Table 1) and five wild-type samples were used to generate the blinded study. Ten samples each for exons 13, 14, and 16; 20 samples for exon 11; and 28 samples for exon 10 were analyzed (Supplemental Table 6; *http://jmd.amjpathol.org*). For each exon, the number of wild-type and variant samples was unknown. For the blinded study amplicon analysis, the derivative melting curve data were used to visually identify sequence variation by unique derivative melting peak shape and T_m shift from a wild-type control, although *RET* sequence variation was detectable in all three data plots: melting curve, derivative melting curve, and fluorescence difference. The amplicon derivative melting data from the primary assays using the WT10A and WT13 probes were used to scan for mutations in exons 10 and 13, respectively.

The ΔT_m values were calculated from the probe data for each sample in the primary assay (and secondary assay, if needed). The ΔT_m data charts were used to determine genotype or to select mutation-specific probes for the secondary assay (Supplemental Tables 1 through 5; *http://jmd.amjpathol.org*). Table 3 (a simplified version of Supplemental Tables 1 through 5; *http://jmd.amjpathol.org*) presents the testing schematic for the *RET* genotyping assay and can also be used to select the

secondary probe sets. The ability of the T_m guidelines to select mutation-specific probes for exons 10 and 11 was compared with using the ΔT_m calculated for each sample. After the primary assay, samples were labeled as wild-type, exon 13 polymorphism, exon 16 codon 918 mutation, or other *RET* sequence variations. The primary probe ΔT_m for the detected sequence variations determined the set of mutation-specific probes used in the secondary genotyping assay, where the *RET* sequence variations were genotyped.
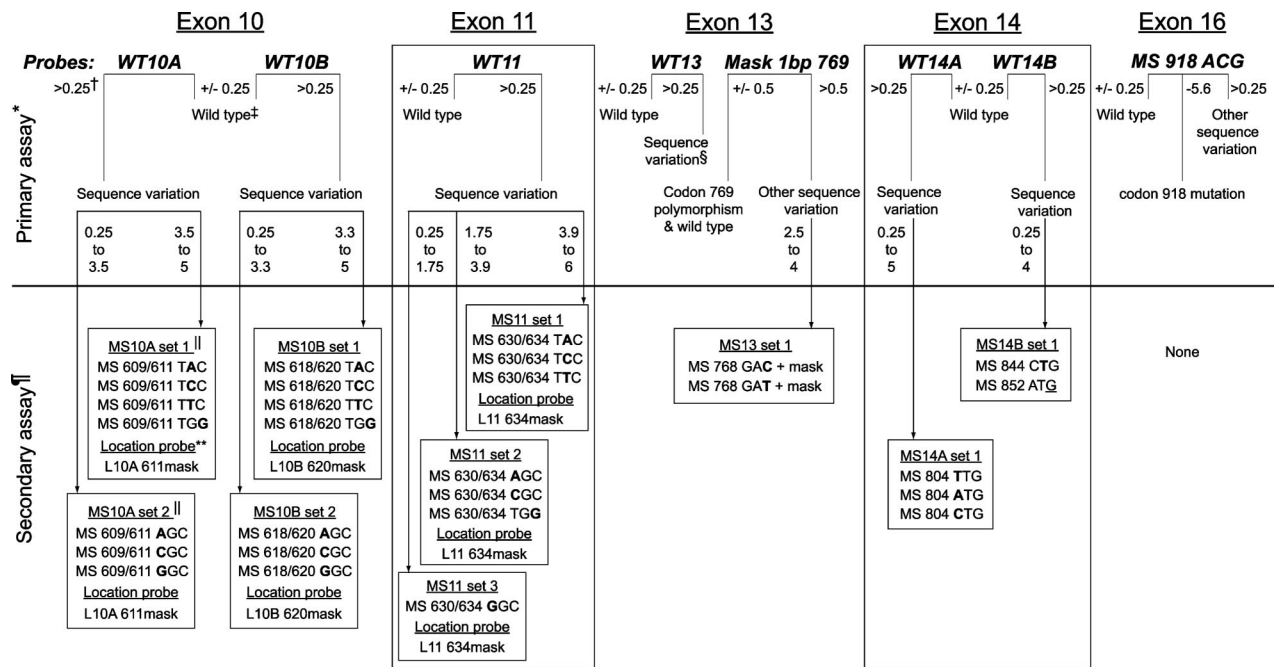
Results

Assay Design

Genotyping of *RET* exons 10, 11, 13, 14, and 16 was achieved in a two-stage, closed-tube protocol consisting of primary and secondary assays (Table 3). Because a double-stranded DNA dye was used, both unlabeled probe and amplicon melting data were generated in each reaction. The primary assay used eight probes in seven PCR reactions to scan for sequence variation and identify some genotypes (wild-type exons, the exon 13 codon 769 polymorphism, and the exon 16 codon 918 mutation). Only the exons that have sequence variation detected but not genotyped by the primary probe data were tested in the secondary assay with one selected probe set. Samples with rare sequence variation detected by the amplicon data only (wild-type sequence under the probe) or rare sequence variation not genotyped by the mutation-specific probes in the secondary assay required sequencing to determine genotype (~0.2% of exons tested).

Exons 10 and 11: Multiple Mutation Hotspots

The majority of mutations for MEN2A and FMTC are in exons 10 (codons 609, 611, 618, and 620) and 11 (codons 630 and 634) and are single nucleotide changes from the wild-type "TGC" codon DNA sequence for cysteine. There are >40 sequence variations reported in exons 10 and 11 (Table 1).[3,20]

The primary assay for *RET* exon 10 used two wild-type probes in two separate reactions. The WT10A probe covers codons 609 and 611, whereas the WT10B probe covers codons 618 and 620 (Figure 1a). All codon 618 and 620 mutant samples have wild-type sequence under the WT10A probe and have the same T_m as the control wild-type samples (Figure 1b). Each of the five codon 609 and 611 mutant samples had a mutant allele that melted at a lower T_m than the wild-type allele. Conversely, with the WT10B probe, codon 609 and 611 mutant samples had the same T_m as a wild-type sample, whereas each of the 10 codon 618 and 620 mutant samples had a mutant allele that melted at a lower T_m than the wild-type allele (Figure 1e). All *RET* sequence variations in exon 10 were detected by amplicon high-resolution melting analysis using normalized melting curves, derivative plots, or fluorescence difference data (Figure 1, c, d, f, and g; data not shown). These data demonstrate how mutations not

Table 3.   Testing Schematic for the *RET* Genotyping Assay

	Exon 10		Exon 11	Exon 13		Exon 14		Exon 16
Probes:	**WT10A** **WT10B**		**WT11**	**WT13** **Mask 1bp 769**		**WT14A** **WT14B**		**MS 918 ACG**

Primary assay*

- Exon 10 WT10A: >0.25† ; +/- 0.25 ; >0.25 — Wild type‡ — Sequence variation: 0.25 to 3.5 / 3.5 to 5
- Exon 10 WT10B: +/- 0.25 ; >0.25 — Sequence variation: 0.25 to 3.3 / 3.3 to 5
- Exon 11 WT11: +/- 0.25 ; >0.25 — Wild type — Sequence variation: 0.25 to 1.75 / 1.75 to 3.9 / 3.9 to 6
- Exon 13 WT13: +/- 0.25 ; >0.25 — Wild type ; Sequence variation§ ; Codon 769 polymorphism & wild type
- Exon 13 Mask 1bp 769: +/- 0.5 ; >0.5 — Other sequence variation: 2.5 to 4
- Exon 14 WT14A: >0.25 — Wild type — Sequence variation: 0.25 to 5
- Exon 14 WT14B: +/- 0.25 ; >0.25 — Sequence variation: 0.25 to 4
- Exon 16 MS 918 ACG: +/- 0.25 ; -5.6 ; >0.25 — Wild type ; codon 918 mutation ; Other sequence variation

Secondary assay¶

Exon 10 WT10A:
- MS10A set 1 ‖ : MS 609/611 **TAC**, MS 609/611 **TCC**, MS 609/611 **TTC**, MS 609/611 **TGG**. Location probe**: L10A 611mask
- MS10A set 2 ‖ : MS 609/611 **AGC**, MS 609/611 **CGC**, MS 609/611 **GGC**. Location probe: L10A 611mask

Exon 10 WT10B:
- MS10B set 1: MS 618/620 **TAC**, MS 618/620 **TCC**, MS 618/620 **TTC**, MS 618/620 **TGG**. Location probe: L10B 620mask
- MS10B set 2: MS 618/620 **AGC**, MS 618/620 **CGC**, MS 618/620 **GGC**. Location probe: L10B 620mask

Exon 11 WT11:
- MS11 set 1: MS 630/634 **TAC**, MS 630/634 **TCC**, MS 630/634 **TTC**. Location probe: L11 634mask
- MS11 set 2: MS 630/634 **AGC**, MS 630/634 **CGC**, MS 630/634 **TGG**. Location probe: L11 634mask
- MS11 set 3: MS 630/634 **GGC**. Location probe: L11 634mask

Exon 13 WT13:
- MS13 set 1: MS 768 **GAC** + mask, MS 768 **GAT** + mask

Exon 14 WT14B:
- MS14B set 1: MS 844 **CTG**, MS 852 AT**G**

Exon 14 WT14A:
- MS14A set 1: MS 804 **TTG**, MS 804 **ATG**, MS 804 **CTG**

Exon 16: None

*Eight probes were used in seven PCR reactions (exon 14 probes are multiplexed) in the primary assay for five *RET* exons. The primary probes are listed for each *RET* exon: WT, wild type; MS, mutation-specific. The primary assay uses probe and amplicon data to genotype wild type, exon 13 codon 769(CTT→CT**G**) polymorphism and exon 16 codon 918(ATG→A**C**G) mutation. All other sequence variations detected with a primary probe will be tested with a secondary probe set selected by the ΔT_m from the primary assay data (~7% of tested exons). There are two exceptions where samples will instead be sequenced (rare): 1) Exon 16 probe detected sequence variation other than the codon 918 mutation, or 2) the primary probe detected sequence variation has a ΔT_m outside the ranges given for secondary probe selection. This could indicate two or more sequence variations detected by the primary probe or novel sequence variation was detected.

†The ΔT_m values (°C) listed for the primary assay are used to determine sample genotype or to select the one probe set used in the secondary assay. Wild-type samples were within 0.25°C of the control wild type.

‡Wild-type sequence under the probe. Analyze amplicon data to call wild-type genotype or to detect rare sequence variation within the amplicon but not under the probe (amplicon melting curve is shifted even though probe data indicated the wild type). Any rare sequence variation detected by only the amplicon melting data would be sequenced for genotype (<0.2% of tested exons).

§Sequence variation due to mutations or the common codon 769 polymorphism. The Mask 1bp 769 probe differentiates the polymorphism from the other sequence variation detected by the WT13 probe.

¶Test only the exon(s) where sequence variation was detected with the primary assay probe.

‖Only one secondary assay probe set will be used to genotype sequence variation detected by the primary assay probe. Probe set was selected by the ΔT_m of a sample with the primary probe, either visually using T_m guidelines (Figure 2a, Supplemental Figures 1a and 2a; *http://jmd.amjpathol.org*) or by calculated ΔT_m values (Supplemental Tables 1–5; *http://jmd.amjpathol.org*). Only sequence the sample if sequence variation is not genotyped by the selected probe set (0.2% of tested exons), for example, the exon 11 (GAC→GA**T**) polymorphism.

**The location (L) probes: L10A 611mask, L10B 620mask, and L11 634mask, are used with any sequence variation detected with the primary assay probes WT10A, WT10B, and WT11, respectively.

under the unlabeled probes can still be detected using the amplicon melting data (eg, compare blue mutation traces in Figure 1, b versus c).

The wild-type probes used in the primary assay identified wild-type sequence under each probe, as indicated by a sample T_m within ±0.25°C of the control wild-type allele T_m (Table 3; Supplemental Table 1; *http://jmd.amjpathol.org*). Samples with *RET* sequence variation had an allele with a lower T_m than the wild-type allele and a ΔT_m value of >0.25°C. To limit the number of mutation-specific probes required for genotyping in the secondary assay, the ΔT_m ranges were used to group *RET* sequence variation with similar ΔT_m values. The ΔT_m ranges were used to generate the T_m guidelines for quick, visual selection of secondary assay probes. For example, the WT10B probe data in Figure 2a shows 13 samples visually divided by T_m guidelines into a wild-type and two mutation groups. The first mutation group, consisting of TAC, TCC, TTC, and TG**G** mutations at either codon 618

or codon 620, had ΔT_m values within the 3.3°C to 5°C ΔT_m range (Figure 2a; Table 3; Supplemental Table 1; *http://jmd.amjpathol.org*). Samples in this group required the four probes in MS10B probe set 1 for the secondary genotyping assay (Figure 2, b–e). The second mutation group, consisting of **A**GC, **C**GC, and **G**GC mutations at either codon 618 or codon 620, had ΔT_m values within the 0.25°C to 3.3°C ΔT_m range. This mutation group required the three probes in MS10B probe set 2 for the secondary assay (Figure 2, f–h). A similar method was used for the WT10A probe data with the codon 609 and 611 mutations (Table 3; Supplemental Figure 1; Supplemental Table 2; *http://jmd.amjpathol.org*). The exon 10 primary assay used the ΔT_m values from two exon 10 wild-type probes to limit the possible genotypes of an unknown mutation from 28 to ≤8 genotypes.

To reduce the number of probes needed to genotype mutations in the secondary assay, each exon 10 mutation-specific probe had the same sequence change at

a.
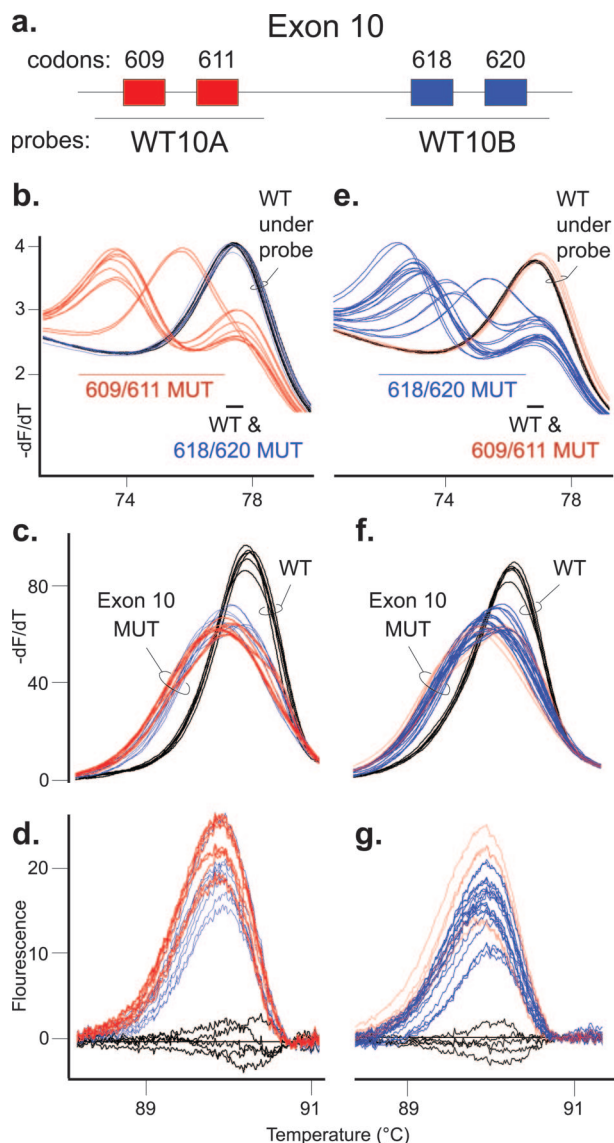
Figure 1. Primary assay for detection of sequence variation in *RET* exon 10. **a:** Diagram of *RET* exon 10 with codons 609 and 611 boxed in red and codons 618 and 620 boxed in blue. The primary assay for exon 10 used two unlabeled probes of WT sequence. As displayed, the WT10A probe detects codon 609 and 611 mutations, whereas the WT10B probe detects codon 618 and 620 mutations. In each graph, three wild-type controls in duplicate (WT, black traces) are shown with two codon 609 and three codon 611 mutant samples in duplicate (red traces) as well as five codon 618 and five codon 620 mutations (blue traces). The three left panels are unlabeled probe and amplicon high-resolution melting data from the WT10A probe reaction, and the three right panels are data from the WT10B probe reaction. For each probe, thick trace lines were used with mutations detected by the probe data, and thin trace lines were used with mutations detected by amplicon data only. **b:** Derivative melting curve plot for the WT10A probe data. The alleles are divided into two T_m ranges, and predicted codon mutant alleles in each range (609/611 MUT or 618/620 MUT), along with the WT allele, are listed. **c:** Amplicon derivative melting curve data from the WT10A probe primary assay with the WT and exon 10 mutation (exon 10 MUT) samples labeled. **d:** Difference plot of same amplicon data. **e:** Derivative melting curve plot for the WT10B probe data. The alleles are divided into two T_m ranges, and predicted codon mutant alleles in each range, along with the wild-type allele, are listed. **f:** Amplicon derivative melting curve data from the WT10B probe primary assay with wild-type and exon 10 mutant samples labeled. **g:** Difference plot of same amplicon data.

both pathogenic codon positions (Table 2). A mutant allele that was complementary at one pathogenic codon resulted in only one mismatch with the probe and a 2°C

to 5°C higher T_m than the wild-type allele with two mismatches (Figure 2, b–h). If the mutation was at a different position, the mutant allele had three mismatches with the probe and melted at a lower T_m than wild type (eg, T**GG** mutant in Figure 2, b–d, pink traces). If the mutation was at the same position as the probe sequence change but was not complementary to the probe, then the mutant allele T_m was close to the wild-type allele T_m because both alleles had two mismatches with the probe. These mutant alleles were "masked" from analysis with a derivative melting curve that was similar, if not identical, to the wild-type allele melting curve (eg, masked **A**GC and **G**GC mutant alleles in Figure 2g, red and gray traces).[18] Occasionally, a mutant allele that should appear masked melted higher than expected with a noncomplementary mutation-specific probe. For example, the codon 618(TGC→T**CC**) and 620(TGC→T**CC**) mutations were more stable (ΔT_m −1.7°C to −2.0°C) than the wild-type allele with the MS 618/620 T**AC** and T**TC** probes (Figure 2, b and d, orange traces). Even so, both heterozygous T**CC** mutations were easily identified by the higher T_m with the complementary MS 618/620 T**CC** probe (ΔT_m −4.7°C) (Figure 2c, orange traces).

The same mutant sequence change at either pathogenic codon under the probe yielded very similar derivative melting curves for the primary probe data, regardless of mutation location (Figures 1 and 2a; Supplemental Figure 1a; *http://jmd.amjpathol.org*).[15] For example, the codon 618(TGC→T**AC**) and 620(TGC→T**AC**) mutations were indistinguishable from each other, by either the primary unlabeled probe or amplicon melting data. In addition, the secondary assay mutation-specific probes were designed to only determine mutation sequence, not to identify the codon position of the mutation. A location probe was included in the secondary assay for any detected exon 10 sequence variation to identify the codon of mutation. The "L10B 620 mask" location probe, designed with a masking, three nucleotide (codon) deletion of the wild-type probe sequence over the codon 620 position (Table 2), was used to differentiate the codon 618 and 620 mutations. The codon 620 mutant alleles were masked by a similar T_m as the wild-type allele, whereas the codon 618 mutant alleles had a lower T_m than wild-type (Figure 2i). The location probes allowed the exon 10 mutation-specific probes to be designed with the same sequence change at both analyzed codons, thereby reducing the number of mutation-specific probes required in the secondary assay by one-half. Any exon 10 mutation detected by the primary assay was genotyped with only one location probe and three to four mutation-specific probes.

Exon 11 was analyzed by similar methods as exon 10 (Table 3; Supplemental Figure 2; Supplemental Table 3; *http://jmd.amjpathol.org*), and all of the tested *RET* sequence variations within exon 11 were detected by amplicon high-resolution melting analysis (data not shown). Exon 11 used one wild-type probe for the primary assay over pathogenic codons 630 and 634. Each of the eight exon 11 sequence variations tested had an allele that melted at a lower T_m than wild-type (Supplemental Figure 2a; *http://jmd.amjpathol.org*). These samples were di-

vided into three mutation groups by primary probe ΔT_m values to select the mutation-specific probes for the secondary assay. The mutation-specific probes had the same sequence change at both codon 630 and 634
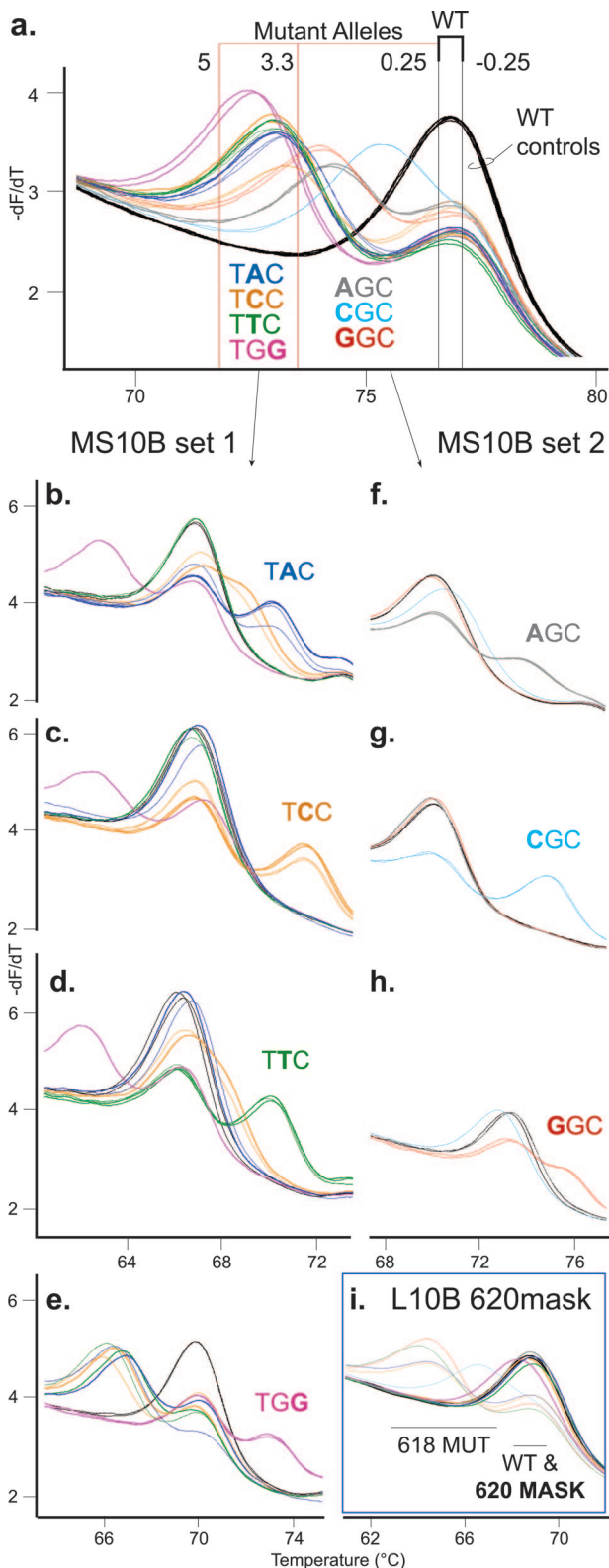
positions (Table 2). In the secondary assay, mutations at either codon 630 or codon 634 were genotyped with one location probe and one to three mutation-specific probes (Supplemental Figure 2, b–i; *http://jmd.amjpathol.org*). Although the codon 631(GAC→GA<u>T</u>) sample was detected in the primary assay, this sequence variant was outside of the pathogenic codons analyzed in the secondary assay and melted at a lower T_m than wild type due to three mismatches with each mutation-specific probe (Supplemental Figure 2, e–g). The rare codon 631 sequence variant was not genotyped by the selected mutation-specific probes and would require sequencing for identification.

Exon 13: Common Polymorphism near Mutations

Exon 13 contains seven reported pathogenic mutations (Table 1) and a common polymorphism of 0.26 allelic frequency at codon 769(CTT→CT<u>G</u>).[2,19,20] High-resolution amplicon melting revealed two distinct melting domains in the wild-type sequence (Figure 3a). Both heterozygous and homozygous polymorphisms were distinguished from the codon 768(GAG→GA**C**) mutation by amplicon melting alone (Figure 3, a and b). However, only one of seven reported mutations in exon 13 was available for study.[15,20,27,28] Five rare mutations in exon 13 are within the amplicon but not under the exon 13 primary probes. Because these other mutations may be difficult to distinguish from the common polymorphism by amplicon melting analysis data, it is important to identify the presence of the codon 769 polymorphism by using the probe data. Two unlabeled probes, WT13 and Mask 1bp 769, in two separate reactions were included in the primary assay to identify the common polymorphism and clearly detect the two reported mutations under the probe (Tables 2 and 3; Supplemental Table 4; *http://jmd.amjpathol.org*). One probe is wild type and detects any sequence variation under the probe (Figure 3c). The

Figure 2. Genotyping mutations in *RET* exon 10 at codons 618 and 620. **a:** Derivative melting curve plot for the primary WT10B probe data from Figure 1e. Three wild-type controls in duplicate (WT, black traces) are shown with 10 heterozygous mutant samples in duplicate, five at codon 618 (thin traces) and five at codon 620 (thick traces). The colors used for mutant codon DNA sequences and traces are consistent throughout the figure. Graph traces for both the codon 618 and 620 mutation sequences are gray for **A**GC, light blue for **C**GC, red for **G**GC, dark blue for T**A**C, orange for T**C**C, green for T**T**C, and pink for TG**G**. The ΔT_m range values used to generate the T_m guidelines are listed next to each T_m guideline. Wild-type allele T_ms are within the black T_m guidelines, ±0.25°C of control wild-type sample. The mutant alleles detected by the primary probe were divided into two ΔT_m ranges by the red T_m guidelines. The mutation sequences that are predicted to be grouped into each ΔT_m range are listed below the traces. The secondary assay data for the MS probe sets, MS10B set 1 or MS10B set 2, are displayed for the two mutation groups. **b–e:** The mutation group with ΔT_m values of 3.3°C to 5°C was tested with the four probes in MS10B set 1: MS 618/620 T**A**C (**b**), MS 618/620 T**C**C (**c**), MS 618/620 T**T**C (**d**), and MS 618/620 TG**G** (**e**). **f–h:** The mutation group with ΔT_m values of 0.25°C to 3.3°C was tested with the three probes in MS10B set 2: MS 618/620 **A**GC (**f**), MS 618/620 **C**GC (**g**), and MS 618/620 **G**GC (**h**). On each mutation-specific probe graph (**b–h**), the codon mutation DNA sequence that complements the MS probe is listed. **i:** All mutant alleles (primary WT10B probe data, ΔT_m of 0.25°C to 5°C) will also use the location probe L10B 620mask to locate codon position of the detected mutations. Two derivative melting temperature ranges are indicated, with the codon 618 mutant alleles (618 MUT) labeled as well as the WT allele and masked codon 620 mutant alleles (620 MASK).
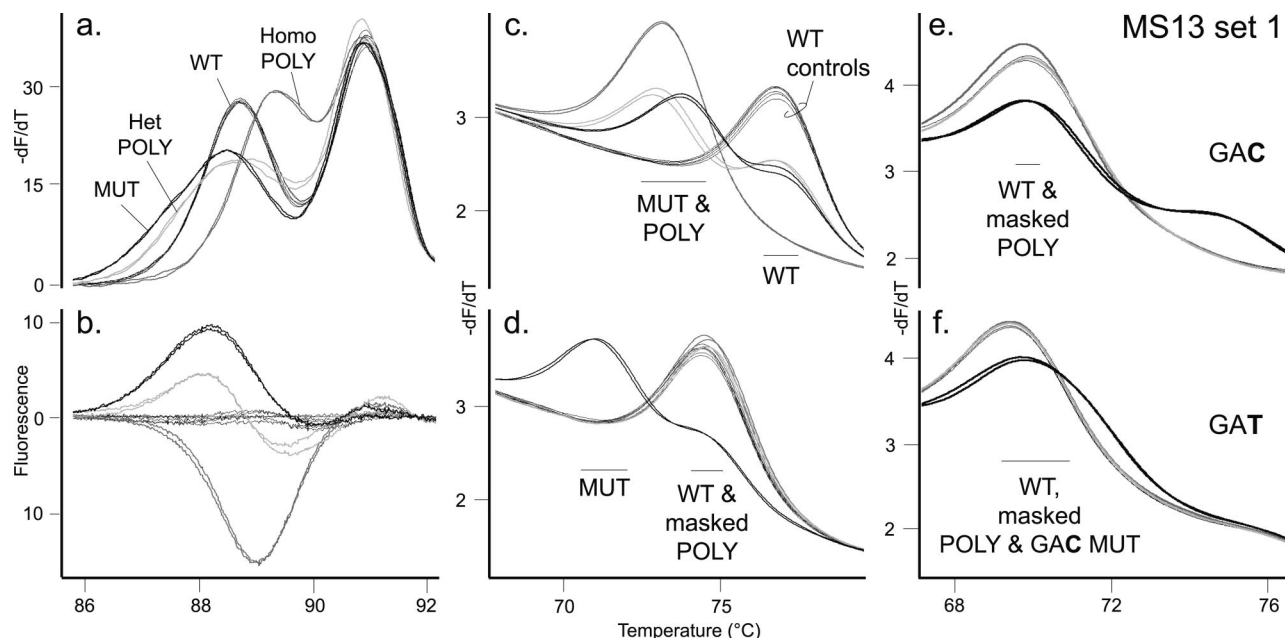
Figure 3. Genotyping sequence variation in *RET* exon 13. **a:** Amplicon derivative melting curve data generated in the WT13 probe primary assay. Two wild-type controls in duplicate (WT, thin black traces) are shown with a heterozygous codon 768(GAG→GA**C**) mutation sample in duplicate (MUT, thick black traces) as well as a heterozygous codon 769(CTT→CT**G**) polymorphism sample (Het POLY, light gray traces) and homozygous codon 769 polymorphism sample (Homo POLY, gray traces) in duplicate. The colors and trace thickness used for each genotype are consistent throughout the figure. **b:** Difference plot of the same amplicon data. **c:** Derivative melting curve plot for the primary WT13 probe data. Two T_m ranges are indicated, with the mutant (MUT) and polymorphism (POLY) alleles labeled as the WT allele. **d:** Derivative melting curve plot for primary "Mask 1bp 769" probe data (the codon 769 polymorphism masking probe). Two derivative melting temperature ranges are indicated, with the mutant allele (MUT) labeled as well as the WT allele and masked polymorphism allele (masked POLY). **e** and **f:** The sample genotypes were tested with the two probes in MS13 set 1: MS 768 GA**C** + mask (**e**) and MS 768 GA**T** + mask (**f**). These two mutation-specific probes also have the codon 769 polymorphism position masked by deletion. The mutant allele complementary to the mutation-specific probe is labeled (GA**C** or GA**T**), as well as the WT allele, masked polymorphism allele (masked POLY), and masked codon 768(GAG→GA**C**) mutant allele ("masked GA**C** MUT" in **f**). The codon 768 (GAG→GA**T**) mutation was not available for testing.

second probe masks the common codon 769 polymorphism with a single nucleotide deletion of the wild-type probe sequence at the polymorphism position.[18] With this masking probe, the polymorphism allele has nearly identical T_m as the wild-type allele, whereas the 768(GAG→GA**C**) mutant allele melts ~3.6°C lower than the wild-type allele (Figure 3d). If a mutation under the probe was detected, the secondary assay identified the known mutant genotypes at codon 768 (Figure 3, e and f). The mutation-specific probes were also designed with the single nucleotide deletion to mask the codon 769 polymorphism so that the mutations would be clearly genotyped irrespective of whether the polymorphism was present. Note that the mutant 768(GAG→GA**C**) allele was masked by the MS 768 GA**T** + mask probe because the sequence change in the probe was at the same position as the mutation but not complementary (Figure 3f). This mutant had the same number and position of mismatches with the probe as the wild-type allele and had a similar, but not identical, T_m as wild type.

Exon 14: Multiplexed Probe Analysis

For exon 14 in the primary assay, two wild-type probes were multiplexed in one reaction to detect all reported exon 14 mutations while eliminating analysis of the codon 836 (p.S836S) polymorphism (Figure 4, a and b; Table 3; Supplemental Table 5; *http://jmd.amjpathol.org*). The WT14A probe detects codon 804 and 806 mutations

between 65°C and 76°C, whereas the WT14B probe detects sequence variation at codons 844 and 852 between 76°C and 85°C. For sequence variation detected by the primary WT14A probe, the secondary genotyping assay used three mutation-specific probes (MS14A set 1; Figure 4, c–e). For example, the codon 804(GTG→**A**TG) mutant allele was genotyped by a higher T_m than the wild-type allele with the complementary MS 804 **A**TG probe (Figure 4c) and masked by both noncomplementary MS 804 **T**TG and **C**TG probes (Figure 4, d and e). All of the tested codon 804 mutant alleles expected to be masked by noncomplementary, mutation-specific probes had identical T_m as the wild-type allele. The two mutation-specific probes, MS 844 CTG and MS 852 AT**G**, were designed to detect the known sequence variation under the primary WT14B probe (data not shown).[29]

Exon 16: Mutation-Specific Primary Probe

Since ~95% of MEN2B cases are caused by the *RET* exon 16 codon 918(ATG→A**C**G) mutation, a mutation-specific probe was used in the primary assay for exon 16. The codon 918 mutant allele had a higher T_m than the wild-type allele (ΔT_m −5.6°C; Figure 4f), and the amplicon data demonstrated a shift from the wild-type control (data not shown). The sequence variant at codon 922, which was under the probe, and the rare codon 912 mutation, which was within the amplicon, were unavailable for testing.[20,30,31]
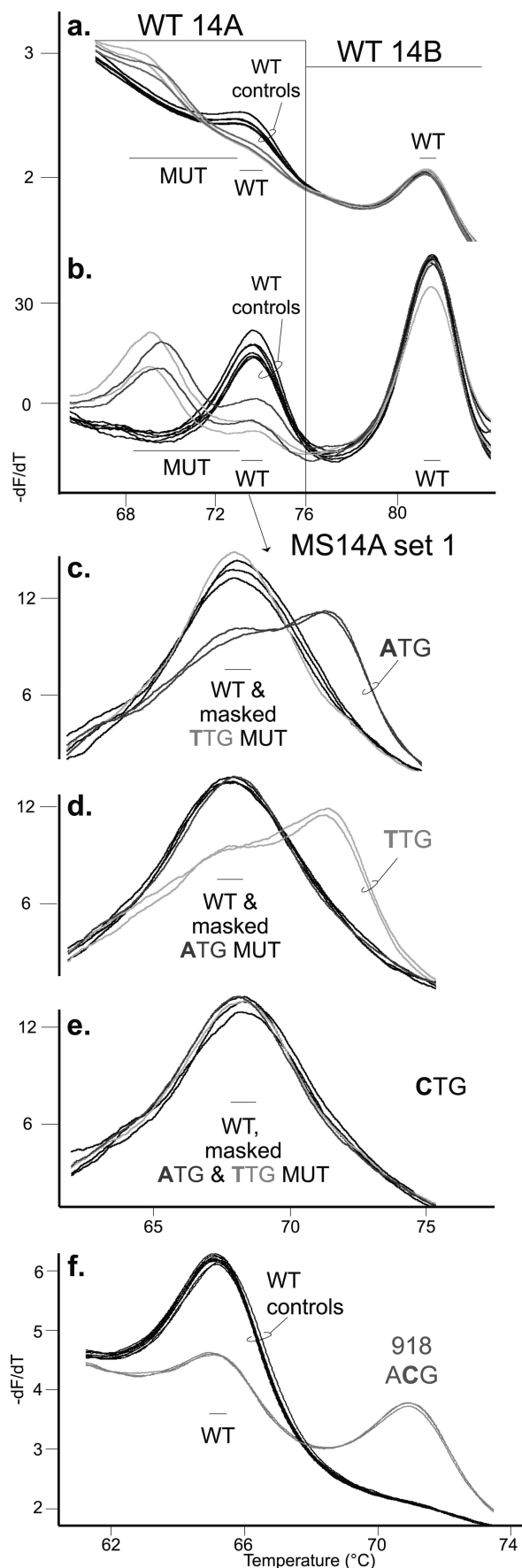
Blinded Study

A blinded combination of five wild-type samples and 29 available *RET* sequence variation samples were tested by exon in the *RET* genotyping assay (Supplemental Table 6; *http://jmd.amjpathol.org*). All 59 *RET* sequence variations were distinguished from the 19 wild-type exons genotyped in the primary assay. In addition, the heterozygous and homozygous exon 13 polymorphism samples, as well as the exon 16 codon 918(ATG→A**C**G) mutation, were genotyped in the primary assay. The probe and amplicon data results were always consistent. Identical results were obtained for exons 10 and 11 whether the secondary assay mutation-specific probe sets were selected by the T_m guidelines (eg, Figure 2a) or by calculating the ΔT_m for each sample and then using the ΔT_m data charts (Supplemental Tables 1 through 3; *http://jmd.amjpathol.org*). For each mutation, only two to five probes were needed for genotyping, and no sequencing was performed. The secondary assay genotyped all of the *RET* mutations detected in the primary assay. Although the exon 11 codon 631 sequence variant samples were detected in the primary assay, the mutation-specific probes in the secondary assay were not designed to genotype this variant, and only these samples required sequencing for genotype.

Discussion

Although sequencing is the standard for *RET* genotyping, mutation scanning is more cost effective because most exons tested are wild type. Patients with apparent sporadic medullary thyroid carcinoma are tested for *RET* mutations, whereas only 10 to 25% actually have a germline *RET* mutation. Furthermore, because MEN2 is a rare autosomal dominant disorder, MEN2 patients with a *RET* mutation in one exon will be wild type (or carry polymorphisms) in the other *RET* exons.[21,28] Relatives of MEN2 patients are commonly tested for *RET* mutations and may have a *RET* mutation in one exon or be wild type in all exons tested. Assuming 33% of patients tested for MEN2 mutations actually have a *RET* germline mutation in only

Figure 4. Genotyping mutations within exon 14 and exon 16. **a:** Derivative melting curve plot of the primary assay probe data generated by two wild-type exon 14 probes in one reaction. The WT14A probe data are in the temperature range of 65°C to 76°C, and the WT14B probe data are in the temperature range of 76°C to 85°C. Three wild-type controls in duplicate (black traces) are shown with two heterozygous mutation samples, at codon 804(GTG→**A**TG) (dark gray traces) and codon 804(GTG→**T**TG) (light gray traces), in duplicate. The codon 804(GTG→**C**TG) mutation was not available. The colors used for each genotype are consistent throughout the figure. Derivative melting temperature ranges are indicated, with the mutant alleles (MUT) labeled as well as the WT allele. **b:** Same data as in **a** with additional normalization and exponential background removal as described in Materials and Methods. **c–e:** Graphs were also analyzed as in **b**. Mutant samples detected by the WT14A probe were tested in the secondary assay with the three mutation-specific probes in MS14A set 1: MS 804 **A**TG (**c**), MS 804 **T**TG (**d**), and MS 804 **C**TG (**e**). The complementary codon mutation DNA sequence was labeled as well as the WT allele and masked mutant alleles (masked "codon DNA sequence" MUT). **f:** Derivative melting curve plot for the exon 16 codon 918 mutation-specific probe (MS 918 A**C**G) primary assay data. Three wild-type controls in duplicate (black traces) are shown with a heterozygous mutation sample 918(ATG→A**C**G) in duplicate (gray traces). The mutant allele (918 A**C**G) is labeled as well as the WT allele.

one of the five exons tested, and then 93% of exons tested will be wild type or nonpathogenic polymorphism genotype.

Mutation scanning by amplicon high-resolution melting analysis can detect 100% of *RET* sequence variation.[15] Although high-resolution amplicon melting can distinguish unique heterozygotes,[33] many *RET* genotypes could not be distinguished because the different sequence variations had similar amplicon melting curves.[15] The addition of unlabeled probes allows analysis of both amplicon and probe from the same melting curve data.[17] However, some *RET* mutations were still not distinguished when unlabeled probes of wild-type sequence were included in the reaction. For example, the *RET* mutation "TA**C**" can be found at codon 618 or 620. Either mutation resulted in nearly identical probe melting, because the wild-type probe covered both mutations, and the nearest neighbors were identical.

To completely genotype *RET* mutations with a minimal number of unlabeled probes and reactions, a sequential two-stage method was developed that consisted of a primary and secondary assay. The primary assay detected mutations anywhere between the primers by amplicon melting and used unlabeled probes to analyze *RET* mutation hotspots. Most probes for the primary assay were of wild-type sequence. An exception was the exon 16 mutation-specific probe that complemented the common MEN2B mutation at codon 918 (~95% of MEN2B cases).[32] In addition, a primary exon 13 probe was designed to clearly differentiate the common codon 769(CTT→CT<u>G</u>) polymorphism from the codon 768 mutations using the masking technique.[18] The majority of *RET* exons (93%) were genotyped as wild type or the common exon 13 polymorphism in the primary assay, using eight probes in seven PCR reactions. This primary assay also genotyped the exon 16 codon 918(ATG→A**C**G) mutation.

The percentage of *RET* mutations detected by the primary probes was estimated using the published codon mutation frequencies for each MEN2 syndrome[24] and the average incidence of each MEN2 syndrome: MEN2A, 75%; FMTC, 20%; and MEN2B, 5%.[32] The primary probes for exons 10, 11, 13, 14, and 16 will detect ~98% of reported MEN2 mutations. The majority of MEN2A and FMTC mutations are in exon 11 at codon 634, and the most common mutation in MEN2A patients is codon 634 (TGC→**C**GC) at ~50% frequency.[24,32] There are also rare mutations (Table 1) outside of the probe analyzed region, and these will be detected by the amplicon melting analysis only and then sequenced.[14] The secondary assay was used to genotype any *RET* sequence variation detected by the probes in the primary assay. This second stage of PCR required two to four mutation-specific probes, selected by primary assay probe data, to genotype each mutant sample. A location probe was also required for exon 10 and 11 mutations to identify the codon of the mutation, because the mutation-specific probes for exons 10 and 11 had the same mutant nucleotide change at each pathogenic codon in the probe. Less than 0.2% of *RET* exons tested would need sequencing due to rare sequence variation detected by

amplicon melting analysis only, or the primary probe-detected sequence variant was not genotyped by the selected secondary assay probes.

In general, mutation analysis with the mutation-specific probes produced one of three results. First, when the mutant sequence complemented the mutation-specific probe, then the mutant allele melted at a higher T_m than the wild-type allele (ΔT_m of $-2°C$ to $-5°C$). Second, when the mutation was at the same position but did not complement the mutation-specific probe sequence, then the mutant allele was masked by a similar stability and T_m as the wild-type allele ($T_m \pm 0.5°C$ of the wild-type allele T_m).[18] In this case, both the noncomplementary mutant and wild-type alleles have a mismatch with the probe at the same position, but the exact nucleotides forming the mismatch are different. How close the masked allele T_m is to the wild-type allele T_m depends on the mismatched sequence, number of other mismatches with the probe, and the surrounding complementary nucleotides. For example, the A::G mismatched mutant allele melted 2°C higher than the A::C mismatched wild-type allele (Figure 2b, T**C**C mutant and MS 618/620 TA**C** probe), whereas the two mismatches C::T and C::C had identical T_ms (Figure 2c, TA**C** mutant and MS 618/620 T**C**C probe). This result held true whether these mutant codon sequence changes were at *RET* codon 618, 620, or 609 (611 unavailable for testing). Third, when the mutation was at a different position than the mutant-specific probe sequence change, the mutant allele had an additional mismatch with the probe and melted at a lower T_m than the wild-type allele (ΔT_m 0.5°C to 5°C). If this last result was obtained with all mutation-specific probes within the selected probe set for the secondary assay, then the sequence variation was not at a known mutation location under the probe and was not genotyped. Any sample not genotyped in the secondary assay would be sequenced (eg, the exon 11 codon 631 sequence variants).

Six probes were designed using the masking technique to simplify analysis of complicated regions. The masking technique uses artificial mismatches at selected sequence variation positions to allow clear analysis of other mutations elsewhere under the probe.[18] The exon 13 masking probe (Mask 1bp 769) in the primary assay and the two exon 13 mutation-specific probes had a single nucleotide deletion at the common polymorphism position of codon 769 (CTT→CT<u>G</u>). This allowed clear detection and genotyping of the two mutations at codon 768 (GAG→GA**C** and GA**T**). The three location probes for exons 10 and 11 each used a three-nucleotide (codon) deletion to mask one pathogenic codon position. Location probes were used to identify the mutation codon position under the probe and to decrease the number of mutation-specific probes required in the secondary assay by one-half.

It is possible that known *RET* mutations are *in cis* or *in trans* with other sequence variation of known or unknown significance, such as in exon 14 (codons 804 and 806 mutations) or in exon 13 (codon 791 mutation with the codon 769 polymorphism).[27,34,35] For probe data, if the two nucleotide changes are on the same allele, a T_m lower than either mutation alone would be expected. If

the nucleotide changes are on opposite alleles, both alleles would have a lower T_m than wild type, and the sample may appear to be homozygous for mutation. In both of these cases, the samples would be sequenced, because either the variant allele was not within the ΔT_m ranges predicted for *RET* mutations or the secondary test would not identify the genotype. If the second nucleotide change is not under the probe, but within the amplicon, a lower T_m shift or a unique amplicon derivative curve shape from the *RET* mutation genotyped from probe data would be expected, and the sample would be sequenced. This situation is probable for exon 13 (eg, codon 791 mutation with the codon 769 polymorphism).[27] When the exon 13 amplicon data are analyzed for rare mutations not analyzed by the exon 13 primary probes, the presence of the common codon 769(CTT→CT<u>G</u>) polymorphism, as determined by the probe data, needs to be taken into consideration. If the sample is wild-type sequence as determined by the WT13 probe data, the sample should only be sequenced for rare mutations if the amplicon trace is shifted from the wild-type control. If the WT13 and Mask 1bp 769 probe data indicate the common codon 769 polymorphism (heterozygous or homozygous), the sample should only be sequenced if the amplicon curve is shifted from the codon 769 polymorphism control melting curves. Although extremely rare, *RET* mutations can be homozygous. If so, both alleles would complement the correct mutation-specific probe.

Table 1 lists the reported MEN2 mutations, polymorphisms, and sequence variants in exons 10, 11, 13, 14, and 16 that are analyzed by the *RET* genotyping assay. The extremely rare MEN2 mutations in exon 15, exon 8, and the recently reported exon 5[36] were not included and would have to genotyped by sequencing these exons. The exon 11 codon 631 sequence variants listed in Table 1 are expected to have the same result as the tested codon 631(GAC→GA<u>T</u>) sample: detection in the primary assay, not genotyped in the secondary assay, and then sequenced for genotype. Two common *RET* polymorphisms found in patients as well as the general population were not analyzed because they are not causative of the MEN2 syndromes: exon 14 p.S836S (3 to 10% allelic frequency) and exon 11 p.G691S (17 to 28% allelic frequency).[21,22] The exon 11 codon 691 polymorphism was excluded by primer selection, whereas the exon 14 polymorphism was eliminated from analysis by probe selection. Two probes were used in one reaction to detect all of the known exon 14 mutations surrounding the 836 polymorphism but without detecting the polymorphism. The exon 14 amplicon melting data for the codon 836 polymorphism was similar in T_m and derivative melting curve shape to a mutation and could not be distinguished.[15] Therefore the amplicon data for exon 14 was not analyzed, because the codon 836 polymorphism would then be detected but not distinguished from mutations, creating unnecessary sequencing steps.

There are other reported sequence variations within the *RET* proto-oncogene that are causative of Hirschsprung disease (HSCR) instead of MEN2 syndromes,[20] and there is always the possibility of novel mutations.

Novel or HSCR mutations detected with the primary assay probe would not be genotyped with the MEN2 mutation-specific probes in the secondary assay and would be sequenced. Novel or HSCR mutations detected by the amplicon melting data only would also be sequenced for genotype. These occurrences should be extremely uncommon, because MEN2 and HSCR have different clinical presentations.[32,37] Even so, there are reports of exon 10 codon 618(TGC→**C**GC) and codon 620(TGC→**C**GC) mutations causing both MEN2 and HSCR. In addition, the three sequence variants in exon 10 codon 609(TGC→**A**GC and TG**G**) and codon 611(TGC→T**C**C) are associated with HSCR, not MEN2.[3,19,20,38,39] With the developed *RET* genotyping assay, any sequence variation within the targeted exons will be genotyped, and appropriate treatment can be determined based on the genotype.

In summary, the *RET* genotyping assay has two PCR stages: a primary and secondary assay. The primary assay was designed so that wild-type exons are genotyped, polymorphisms are identified (or eliminated from analysis), rare mutations are detected by amplicon melting data, and common mutations are detected by both unlabeled probe and amplicon melting data, all without sequencing. The secondary assay genotypes *RET* mutations with a limited number of mutation-specific probes. This two-stage assay detected and genotyped all of the tested *RET* wild-type, mutation, polymorphism, and sequence variant samples in a blinded study with 100% accuracy in less than one-half the time required for sequencing the five *RET* exons. The *RET* genotyping assay can be used in its entirety for unknown or suspected *RET* mutations, by individual exon, or by using one mutation-specific probe (and location probe, if needed) when the *RET* mutation within the family is known.

Acknowledgments

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