Technical Advance

Rapid Diagnosis of MEN2B Using Unlabeled Probe Melting Analysis and the LightCycler 480 Instrument

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Multiple endocrine neoplasia type 2B (MEN2B) is an autosomal dominant, inherited cancer syndrome. MEN2B patients have a high risk of developing medullary thyroid carcinoma, and prophylactic thyroidectomy is recommended by 6 months of age. Genetic testing can identify MEN2B patients before cancer progression. Two *RET* proto-oncogene mutations, in exon 15 at codon 883 (GCT>TTT) and in exon 16 at codon 918 (ATG>ACG), account for more than 98% of MEN2B cases. An assay using unlabeled probes and the LightCycler 480 instrument was developed to genotype these two common MEN2B *RET* mutations. Asymmetric polymerase chain reaction was used to increase ssDNA products followed by melting analysis of the unlabeled probe/ssDNA amplicon duplex. The available samples were either patient DNA of known *RET* genotype or artificial templates. Analysis of the codon 883 heterozygous mutation demonstrated a ΔT_m of $5.70 \pm 0.11^\circ\text{C}$, while the codon 918 heterozygous mutation generated a ΔT_m of $-5.72 \pm 0.11^\circ\text{C}$. Samples with the targeted *RET* mutation genotypes were accurately detected and easily distinguishable from five other reported sequence changes using these probes. Thus, MEN2B diagnosis using unlabeled probes and the LightCycler 480 is a rapid, closed-tube method that is less time consuming and less expensive than sequencing. This assay demonstrates 100% specificity and sensitivity for the identification of *RET* mutations causative of MEN2B. (J Mol Diagn 2008, 10:123–128; DOI: 10.2353/jmoldx.2008.070111)

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominant, inherited disorder consisting of three syndromes: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC). “Gain of function” mutations in the *RET* proto-oncogene cause the MEN2 syndromes.

MEN2 *RET* mutations are almost exclusively heterozygous, with only a few reports of rare homozygous mutations.¹ MEN2 syndromes result in a high lifetime risk of developing medullary thyroid carcinoma (MTC). MTC can metastasize early resulting in poor prognosis, since MTC responds poorly to chemotherapy. The only known cure is thyroidectomy before MTC metastasis, and is usually performed between 6 months and 10 years of age, depending on the *RET* mutation.² Genetic diagnostic testing can identify MEN2 patients before cancer progression, significantly improving patient survival.

FMTC families present the clinical manifestation of MTC only. In addition to MTC, MEN2A families can develop pheochromocytomas and hyperparathyroidism. MEN2B patients can also develop pheochromocytomas and (rarely) hyperparathyroidism, but these patients have some additional distinguishing physical symptoms as well, such as ganglioneuromas of the digestive tract, mucosal neuromas of the lips and tongue, corneal nerve thickening, marfanoid habitus, and skeletal abnormalities.^{2,3} The MEN2B patients have a very high risk of developing MTC and prophylactic thyroidectomy is recommended by 6 months of age.² This report demonstrates a *RET* genotyping assay that can identify patients with MEN2B in a cost effective, rapid manner compared to sequencing or restriction digestion assays.

An unlabeled probe genotyping assay uses asymmetric polymerase chain reaction (PCR) to generate ssDNA product.⁴ The unlabeled probe is 3' blocked to prevent probe extension during PCR. After PCR, the unlabeled probe and ssDNA target are annealed and subjected to high-resolution melting analysis. The detection is by a saturating dsDNA dye, LCGreen^{Plus+}. Since a dsDNA dye is used, both the amplicon data as well as the unlabeled probe data can be analyzed, if necessary.^{5,6} The closed-tube unlabeled probe genotyping assay reduces contamination and hands-on time compared to other

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genotyping assays that require post-PCR processing. Unlabeled probe assays have proven to be rapid, cost effective, and accurate for genotyping.^{4–8} The newly available LightCycler 480 (LC480) instrument, with its 96- or 384-well format, has great potential as a platform for high-throughput and rapid unlabeled probe assays. A recent report by Herrmann et al⁹ compares the LC480 and other commercially available instruments for amplicon melting analysis.

RET mutations within exons 15 and 16 can cause MEN2B. The MEN2B *RET* mutation for exon 15 is a deletion/insertion mutation at codon 883(GCT>TTT) of genotype c.2647_2648delinsTT.³ The MEN2B *RET* mutation for exon 16 is a missense mutation at codon 918(ATG>ACG) of genotype c.2753T>C. Approximately 5% of MEN2B patients have the codon 883(GCT>TTT) mutation, while ~94% of MEN2B patients have the codon 918(ATG>ACG) mutation.^{2,10} A genotyping assay targeting these two *RET* mutations will identify >98% of MEN2B patients. In addition, there are other *RET* sequence changes within the region of the two targeted MEN2B *RET* mutations that instead cause FMTC or Hirschsprung disease (HSCR). HSCR can be caused by “loss of function” mutations within the *RET* proto-oncogene that result in the congenital absence of ganglia in portions of the digestive tract and can lead to potentially lethal intestinal blockage. The MEN2B *RET* genotyping assay distinguishes the two targeted MEN2B *RET* mutations from other reported sequence variations under the analysis probes.

Materials and Methods

DNA Samples

In this report, *RET* sequence variation was listed by the codon number, the wild-type codon DNA sequence followed by the codon sequence change. The nucleotide changes are in bold for the targeted *RET* mutations, 883(GCT>TTT) and 918(ATG>ACG), while the nontargeted *RET* nucleotide changes will be underlined; for example, 883(GCT>ACT). Normal samples have the *RET* consensus, wild-type sequence (under the probe) that is not associated with disease. De-identified normal samples and the heterozygous *RET* exon 16 codon 918(ATG>ACG) mutation sample were obtained from the Coriell Institute (Camden, NJ) as described previously.¹¹ Coriell samples GM16658 (*RET* exon 15 and 16 normal controls) and GM11629 (heterozygous for the exon 16, codon 918 mutation) were used for the data in the figures and table. Coriell samples were purified using a Qiagen QIAquick kit (Qiagen, Valencia, CA); while 12 additional de-identified normal samples were purified using MagNa PURE (Roche Diagnostics Corp, Indianapolis IN). All sample genotypes were confirmed by sequence analysis (GenBank *RET* genomic reference NC_000010.9).

PCR Primers

The PCR amplification oligos were synthesized at Integrated DNA Technologies, Inc. (IDT, Coralville, IA). Exon

15 primer sequences were 5'-CTGGGAGCCCCGCCT-CAT-3' for the reverse and 5'-CACACACCACCCCTCT-GCTG-3' for forward. Exon 16 primer sequences were 5'-ACACATCACTTTGCGTGGTG-3' for the reverse and 5'-TCTCCTTTACCCCTCCTTCCT-3' for forward.

Unlabeled Probes

The unlabeled probes were oligos that incorporated a 3' amino modifier (3 AmM) to prevent DNA polymerase extension during PCR.^{4,12} The unlabeled probes were synthesized by IDT. The exon 15 probe had *RET* wild-type sequence 5'-CATCCTGGTAGCTGAGGGGCGGAA-GATGAAGATT/3AmM/-3' (34 nucleotides). The exon 16 probe had the codon 918(ATG>ACG) mutation-specific sequence 5'-GGTCGGATTCCAGTTAAATGGACGGCAAT/3AmM/-3' (29 nucleotides).

Artificial Templates

The thirteen artificial templates of the unavailable *RET* sequence variations used oligos that were 3' blocked with an amino modifier (synthesized at IDT) to prevent amplification during PCR.¹² These oligos complement the analysis probe sequences but include the mutation sequence change(s), and therefore functioned as a mutant allele template for hybridization of the analysis probe. The oligos were 34 nucleotides for exon 15 and 29 nucleotides for exon 16. The 10 μ mol/L oligo stock was added at 1 to 9 μ L of either water or a normal genomic DNA sample (10–50 ng/ μ L) to generate the artificial templates for the homozygous mutation and heterozygous samples, respectively. The heterozygous exon 15 codon 883(GCT>TTT) mutation, all homozygous sequence variation samples, and all of the heterozygous non-MEN2B *RET* sequence variations were artificial templates.

MEN2B RET Genotyping Assay

A final PCR reaction volume of 10 μ L contained either 2 μ L of sample genomic DNA (20–100 ng total) or 2 μ L of an artificial template. The asymmetric PCR reaction contained final concentrations of 1X FastStart master hybridization mix (LightCycler FastStart DNA Master Hybridization Probe Kit from Roche), 2 mmol/L additional of MgCl₂, 0.01 U/ μ L AmpErase uracil *N*-glycosylase (Applied Biosystems, Foster City, CA) and 1X LCGreen^{Plus+} (Idaho Technology, Salt Lake City, UT). The two targeted MEN2B *RET* mutations were tested in separate reactions, with 0.5 μ mol/L final concentration of either the exon 15 or exon 16 unlabeled probe. The exon 15 reaction used 0.5 μ mol/L final concentration for the reverse primer and 0.1 μ mol/L for the forward primer. The exon 16 reaction used 0.54 μ mol/L for the reverse primer and 0.06 μ mol/L for the forward primer. Reactions were overlaid with 13 μ L of mineral oil to minimize a rise in background fluorescence, improving data interpretation. The plate was sealed with LC480 sealing foil (Roche) and spun at 1000 RPM for 1 minute before loading into the LC480 instrument (Roche).

Table 1. MEN2B *RET* Genotyping Assay Precision

Exon	Genotype	Allele* or ΔT_m	Within run [†]		Between run [‡]	
			Ave [§]	SD	Ave	SD
15	Codon 883(GCT>TTT) heterozygous	WT	73.80	0.06	73.71	0.12
		MUT	68.05	0.09	68.01	0.07
		ΔT_m	5.75	0.08	5.70	0.11
		WT	73.81	0.03	73.62	0.14
16	Normal control	WT	65.93	0.01	65.85	0.15
	Codon 918(ATG>ACG) heterozygous	MUT	71.71	0.03	71.58	0.10
		ΔT_m	-5.79	0.03	-5.72	0.11
		WT	65.97	0.00	65.85	0.20
	Normal control	WT				

*WT, wild-type allele; MUT, mutant allele; ΔT_m = wild-type allele T_m - mutant allele T_m .[†]Within run samples were in triplicate.[‡]Between run samples were tested in five different runs.[§]Ave, average (°C). For WT and MUT alleles, the average T_m is listed. The ΔT_m was calculated individually per each heterozygous sample (ΔT_m = wild-type allele T_m - mutant allele T_m), then average ΔT_m and SD were calculated. SD, 1 standard deviation (°C).

Thermocycling was performed on a LC480 instrument with the following parameters: initial uracil *N*-glycosylase step (50°C for 3 minutes) and polymerase activation (95°C for 7 minutes), followed by 55 PCR cycles (denaturation at 95°C for 10 seconds, annealing at 64°C for 10 seconds, and extension at 72°C for 10 seconds). After PCR, probe/ssDNA amplicon duplexes were generated by heating samples to 95°C for 30 seconds, then cooling to 50°C for 30 seconds. The melting data were collected between 53°C and 95°C at 30 acquisitions per °C, using the “melting curves” analysis mode. Then samples were cooled to 50°C for 10 seconds. The LC480 run was finished in approximately 1 hour and 45 minutes. All LC480 default values for rates were used: 2.2°C/s for cooling or 4.4°C/s for heating. Reaction volume was set at 10 μ l (even though 13 μ l of oil overlay was added). The LCGreen dye detection format used the 450 excitation filter and 500 emission filter combination.

Data Analysis

The unlabeled probe data were analyzed between 56°C and 77°C. The melting data were directly converted to a derivative plot using the LC480 software module, *T_m* Calling. This automatic *T_m* Calling function was designed for fluorescently labeled hybridization probes only and did not reliably call the melting temperatures for the unlabeled probe assay. Therefore, derivative melting peak *T_m*s were called using the “manual *T_m* method” within the *T_m* Calling software. The *T_m* for the unlabeled probes was visually assigned as the temperature at the approximate (1/2) area of the derivative melting curve peak. ΔT_m s for heterozygous samples were defined by calculating the wild-type allele *T_m* minus the mutant allele *T_m*. The average allele *T_m*s and ΔT_m values for the normal samples and the targeted MEN2B *RET* mutation samples are given in Table 1, as well as the standard deviation. Note that since exon 16 uses a mutation-specific probe, the 918(ATG>ACG) mutant allele *T_m* is higher than the wild-type allele *T_m*, resulting in a negative ΔT_m value.

Results

The exon 15 reaction used an unlabeled probe of wild-type *RET* sequence to detect the targeted MEN2B *RET* mutation: 883(GCT>TTT). Fifteen normal samples were tested and had a wild-type allele *T_m* of 73.74 $\pm$ 0.11°C (average $\pm$ 1 SD). Since the targeted mutation sample was not available, a codon 883(GCT>TTT) artificial template was used and had a ΔT_m value of 5.70 $\pm$ 0.11°C (Table 1, “between run” value). Occasionally, the exon 15 no template control had primer-dimer peak at ~84°C which was confirmed by gel, but it did not interfere with the unlabeled probe data between 56°C and 77°C. Example data for a normal sample, the heterozygous artificial template for a codon 883(GCT>TTT) mutation sample, and a no template control is shown in Figure 1A. Precision values for within run and between runs are given in Table 1.

The exon 16 reaction used a codon 918(ATG>ACG) mutation-specific unlabeled probe to identify the targeted MEN2B *RET* mutation: 918(ATG>ACG). Fourteen normal samples were tested and had an average wild-type allele *T_m* of 66.03 $\pm$ 0.1°C. A patient sample was available for the targeted MEN2B *RET* mutation at codon 918(ATG>ACG), and had a ΔT_m value of -5.72 $\pm$ 0.11°C (Table 1, “between run” values). Example data for a normal sample, the heterozygous 918(ATG>ACG) mutation sample, and a no template control is shown in Figure 2A. Precision values for within run and between runs are given in Table 1.

There are five other reported *RET* sequence changes under the analysis probes which can cause FMTC or HSCR.^{13,14} Although patients with these diseases have different clinical symptoms than an MEN2B patient, heterozygous artificial templates were generated for these sequence changes to determine whether the ΔT_m values were distinguishable from the two targeted MEN2B *RET* mutations (Figures 1B and 2B). Homozygous artificial templates were also tested for all *RET* sequence variations (data not shown).

Exon 15 had three reported non-MEN2B *RET* sequence changes under the unlabeled probe (Figure 1B and data not shown). The first sequence change is a rare, single

Figure 1. MEN2B *RET* genotyping assay, exon 15 data. **A:** A typical result for the detection of the targeted codon 883(GCT>TTT) heterozygous mutation (solid gray trace) using the exon 15 unlabeled probe of wild-type sequence. The normal control sample is the solid black trace and the no template control is the gray dotted trace. Wild type and *RET* mutant allele positions are marked on the graph as well as the ΔT_m value. **B:** Detection of other reported, non-MEN2B *RET* sequence variations. A normal sample and the 883(GCT>TTT) heterozygous mutation sample are shown with the addition of two other known heterozygous, non-targeted *RET* sequence variation samples. The black dashed trace is the HSCR mutation at codon 884(GAG>GAC), and the gray dashed trace is the FMTC mutation at codon 883(GCT>ACT). The T_m positions for the mutant alleles are marked with a line of the same color and style as the sample trace. All of the exon 15 heterozygous samples were artificial templates.

family FMTC mutation at codon 883(GCT>ACT) of the genotype c.2647G>A, which overlaps the targeted mutation position.¹ This mutation is homozygous in two family members with MTC, while heterozygous family members were unaffected. The ΔT_m value for this FMTC mutation was $2.32 \pm 0.06^\circ\text{C}$. The second sequence change is at codon 886(CGG>TGG) of genotype c.2656C>T.¹⁴ It is unknown at this time if this rare, single family variant is an FMTC mutation or a benign variant found in a sporadic MTC pa-

Figure 2. MEN2B *RET* genotyping assay, exon 16 data. **A:** A typical result for the detection of the targeted codon 918(ATG>ACG) heterozygous mutation (solid gray trace) using the exon 16 unlabeled probe of 918(ATG>ACG) mutation-specific sequence. The normal sample is the solid black trace and the no template control is the gray dotted trace. Wild type and *RET* mutant allele positions are marked on the graph as well as the ΔT_m value. **B:** Detection of other reported, non-MEN2B *RET* sequence variations. A normal sample and the 918(ATG>ACG) heterozygous mutation sample are shown with the addition of two other known heterozygous, non-targeted *RET* sequence variation samples. The black dashed trace is the HSCR mutation at codon 912(CGG>CAG), and the gray dashed trace is the FMTC mutation at codon 912(CGG>CCG). The T_m positions for the mutant alleles are marked with a line of the same color and style as the sample trace. The two heterozygous codon 912 sequence variation samples were artificial templates.

tient. The third sequence change causes HSCR and is at codon 884(GAG>GAC) of genotype c.2652G>C. The ΔT_m value for 886(CGG>TGG) was 3.4°C and for the codon 884 HSCR mutation was 0.9°C .

Exon 16 had two reported non-MEN2B *RET* sequence changes under the unlabeled probe (Figure 2B). The first sequence change is a rare, single family FMTC mutation (two family members had MTC) at codon 912(CGG>CCG) of the genotype c.2735G>C.¹⁵ The ΔT_m value for this mu-

tation was $3.47 \pm 0.19^\circ\text{C}$. The other sequence change is a HSCR mutation at codon 912 (CGG>CAG) of genotype c.2735G>A,¹⁶ with a ΔT_m value of 3.0°C .

To test the accuracy of the artificial templates for predicting ΔT_m s for patient samples, the ΔT_m data for a codon 918(ATG>ACG) artificial template was compared to the available codon 918(ATG>ACG) mutation sample. The artificial template mutant allele was 0.9°C higher T_m than the patient sample mutant allele, resulting in a 0.9°C increase of ΔT_m to 6.6°C for the artificial template (data not shown).

Discussion

The MEN2B *RET* genotyping assay targets two mutations in exons 15 and 16 using unlabeled probes, detecting >98% of known MEN2B *RET* mutations. There are five other reported *RET* sequence variations under the analysis probes that could cause FMTC or HSCR. Although FMTC and HSCR patients will have different clinical symptoms than MEN2B patients, these non-MEN2B *RET* mutations were tested since they would also be detected in the MEN2B *RET* genotyping assay. The reported non-MEN2B *RET* sequence variations under the exon 16 mutation-specific probe were easily distinguished from the targeted 918(ATG>ACG) mutation by a lower T_m than the wild-type allele. The exon 15 MEN2B *RET* mutation is a dinucleotide sequence change and had a greater ΔT_m than the three other reported *RET* point mutations under the analysis probe. All tested heterozygous and homozygous non-MEN2B *RET* sequence variation samples were distinguished from the two targeted MEN2B *RET* mutations.

Artificial templates were used for the unavailable heterozygous and homozygous sequence variation to estimate the T_m and ΔT_m values. For the available exon 16 codon 918(ATG>ACG) mutation, the artificial template mutant allele was 0.9°C higher T_m than the mutant allele from the actual patient's sample. Depending on the mutant allele T_m relative to the wild-type allele T_m , this will create either a higher or lower ΔT_m than expected with a patient's sample. The difference between the artificial template and patient's sample may be due to sample salt concentration. Initial differences in sample salt concentrations (due to different DNA purification methods' elution buffers) can lead to differences in sample T_m values.¹⁷ We recommend purifying control and patient DNA by the same method to avoid this potential problem. Another possible reason for the difference in T_m s; the patient's sample was amplified with dUTPs (present in the Roche kit), while the artificial template oligos were made with dTTPs (complementary 3' blocked oligo used to create the mutant allele; not amplified during PCR). The artificial templates can only estimate the expected patient ΔT_m values, so patient samples should be used as positive controls when available.

Exon 15 has been excluded from some MEN2 assays due to the rarity of mutations within that exon. However, the exon 15 codon 883(GCT>TTT) mutation

is found in ~5% of MEN2B patients and should be included in any MEN2B-specific diagnostic assay to identify these rare individuals that will develop MTC early in life. Although this is the first report for genotyping the codon 883(GCT>TTT) mutation using unlabeled probes, the exon 16 codon 918(ATG>ACG) mutation has been included in an unlabeled probe assay on the LightCycler and HR-1 instrument.⁵ However, in the present study, the exon 16 forward primer was moved to exclude a position of nucleotide discrepancy between primers reported in the literature and some GenBank sequences for the intronic position c.2731–70, since this may be a previously unreported polymorphism. Also, Komminoth et al reported an intronic polymorphism at position c.2731–74 found in three MTC tumor DNA samples.¹⁸ Furthermore, the probe was shifted off the codon 922 position, since an MEN2B family was reported to have a codon 922 sequence variant *in cis* with the codon 918 mutation.¹⁹

The LC480 data with the new exon 16 primer and probe is similar to the previous HR-1 data for detecting and identifying the exon 16 MEN2B *RET* mutation.⁵ Both assays are closed-tube and reduce possible contamination that could occur with genotyping assays that require post-PCR processing. Although the unlabeled probe assay using the LightCycler and HR-1 melting is more cost effective for testing one patient's sample, the LC480 has the cost advantage when more than one sample is tested, since the fixed cost of the 96-well plate is divided among two or more tested samples. The MEN2B *RET* genotyping assay, using unlabeled probes and the LC480, has less hands-on time and is more cost effective than other assays commonly used to detect MEN2B *RET* mutations, such as sequencing and restriction enzyme digestion.

The *RET* exon 15 codon 883(GCT>TTT) and exon 16 codon 918(ATG>ACG) mutations were accurately genotyped. The targeted MEN2B *RET* mutations were easily distinguished from normal samples and other reported sequence variations under the analysis probes for 100% analytical specificity and sensitivity. Targeted mutation analysis of these two common MEN2B *RET* mutations has a 95% clinical sensitivity.² Approximately 5% of MEN2B patients either have no detectable *RET* mutation, even upon sequencing, or have extremely rare, double mutations in exons 14–15 (<2% of known MEN2B *RET* mutations). Therefore, if neither of the targeted MEN2B *RET* mutations are found and clinical symptoms or family history still indicate the MEN2B phenotype, then further *RET* proto-oncogene analysis is recommended. *RET* exons 14–16 should be sequenced to detect the rare double mutations causative of MEN2B or full *RET* sequencing could be done. If no mutation is found in the entire *RET* gene, affected family members can be identified by linkage analysis. The MEN2B *RET* genotyping assay using the LC480 and unlabeled probes was 100% accurate with the advantages of a closed-tube assay, hands-off operation, and is less expensive than sequencing or other genotyping alternatives.

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