RET Mutation Scanning
Update: Exon 15

To the Editor:

Mutation scanning by high-resolution melting analysis has high diagnostic sensitivity and specificity (1, 2). Our 2006 Clinical Chemistry report demonstrated that mutation scanning of the RET protooncogene (ret protooncogene) detects mutations causative of multiple endocrine neoplasia type 2 (3). To illustrate mutation-scanning data for exon 15, we used cell lines that had the wild-type sequence or were heterozygous for a common, nonpathogenic exon 15 polymorphism (c.2712C>G, p.S904S), which occurs at an allele frequency of 11%–27% (4, 5), but samples with other exon 15 sequence variants were not available at that time. We have revisited this mutation-scanning assay for exon 15 because a sample with a novel exon 15 sequence variant (c.2673G>A, p.S891S) (5) became available for testing. Additionally, the published exon 15 forward primer had inadvertently been designed to anneal over a nonpathogenic polymorphism within intron 14, c.2608-24G>A (rs24772737). The variant allele for this polymorphism occurs at a frequency of 6%–28%, depending on the population (4, 5). Because this polymorphism is common and closer to the 5′ end of the exon 15 forward primer, we used samples of known genotypes to test the function of this published primer for amplification efficiency, allele dropout, and mutation-scanning results for RET exon 15.

The protocol for RET mutation scanning by high-resolution melting analysis was described previously (3). In brief, samples were amplified on the LightCycler® 1.5 system (Roche); the amplicons were then analyzed by high-resolution melting with the HR-1™ instrument (Idaho Technology). The exon 15 reverse and forward primers were 15R1 (5′-CTGGGAGCCCCGCTTCA-3′) and 15F1 (5′-GCCTGAC(G)ACTCGTGCTATT-3′), respectively. The position of intron 14 polymorphism c.2608-24G>A in 15F1 is marked as (G). Another forward primer, 15F4 (5′-CAACACCACCCCTGCTGCA-3′), which does not anneal over the c.2608-24G>A polymorphism, was used with reverse primer 15R1 under the same experimental conditions. All de-identified samples were sequenced for RET exon 15, which includes the c.2608-24G>A polymorphism region [forward primer 15FSeq (5′-TGCTGTCACACCAGGCTG-3′) and reverse primer 15RSeq (5′-TGCCCCATGTTGCACTCGT-3′)] were used for amplification and sequencing]. Fig. 1 shows the primer positions and the locations of sequence variants. Of note, the 15F4 and 15FSeq primers are not recommended for mutation scanning, because their use would detect 2 common polymorphisms, complicating the assay results.

To test amplification efficiency, we compared quantification cycle (Cq) values for samples homozygous for intron 14 polymorphism c.2608-24G>A with samples that had the wild-type sequence at this polymorphism position. Because samples can have inherent differences, such as DNA concentration, that affect Cq values, we also amplified the samples with a different forward primer (15F) that is unaffected by the c.2608-24G>A polymorphism. The samples homozygous for the c.2608-24G>A polymorphism amplified with primer 15F1 had a delay of approximately 1 cycle compared with the wild-type samples or when the 15F4 primer was used. Samples that were heterozygous for both polymorphisms (i.e., intron 14 c.2608-24G>A and exon 15 c.2712C>G) had similar Cqs with the two exon 15 forward primers 15F1 and 15F4.

Because the 15F1 primer has the wild-type G nucleotide at position c.2608-24, amplicons produced from any sample with that primer, including samples heterozygous or homozygous for the c.2608-24G>A polymorphism under the 15F1 primer, will also have the wild-type G nucleotide at that position (confirmed by sequencing; data not shown). Therefore, use of the 15F1 primer actually masks the presence of the c.2608-24G>A polymorphism, thereby avoiding this complication in analyzing melting-curve data. Samples homozygous wild type for the exon 15 polymorphism had identical melting curves, regardless of the presence or absence of intron 14 polymorphism c.2608-24G>A (Fig. 1). Samples homozygous for exon 15 polymorphism c.2712C>G were indistinguishable from the wild-type samples in this assay. Samples heterozygous for both polymorphisms (c.2712C>G and c.2608-24G>A) had the same melting curves as samples heterozygous for only the c.2712C>G polymorphism, indicating there was no allele dropout due to intron 14 polymorphism c.2608-24G>A. In addition, the new sample that was heterozygous for both the novel c.2673G>A variant and exon 15 polymorphism c.2712C>G was distinguished from the other genotypes (Fig. 1).

Although the 15F1 primer for the RET mutation-scanning assay was inadvertently designed to anneal over intron 14 polymorphism c.2608-24G>A, there were no substantial differences in amplification efficiency for samples homozygous for the c.2608-24G>A polymorphism, no allele dropout was detected with the heterozygous samples, and the RET exon 15 mutation-scanning result was as expected for each
sample. Thus, we have demonstrated that the presence of intron 14 polymorphism c.2608-24G>A in a sample does not interfere with the exon 15 mutation-scanning assay with the published 15F1 primer and that mutation scanning detects the novel exon 15 variant (c.2673G>A).

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References


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**Fig. 1.** Schematic of RET exon 15 and plots of high-resolution melting data. Schematic of RET exon 15 (white box) is shown with the positions of primers indicated (arrows). Asterisks indicate the locations of the 3 sequence polymorphisms: intron 14 polymorphism c.2608-24G>A (Intron 14 POLY), exon 15 polymorphism c.2712C>G (Exon 15 POLY), and novel variation c.2673G>A. The derivative plot of the fluorescence (F) with respect to temperature (T) and the fluorescence difference plot of the high-resolution melting data are shown in the top and bottom graphs, respectively. Dark gray traces indicate 10 samples heterozygous for the c.2712C>G polymorphism, 6 of which were also heterozygous for the c.2608-24G>A polymorphism. The 11 black traces include 7 samples that were homozygous wild type for exon 15 (3 homozygous for the c.2608-24G>A polymorphism and 4 homozygous for the exon 15 c.2712C>G polymorphism). Light gray traces are duplicates of a sample heterozygous for both exon 15 polymorphism c.2712C>G and novel variant c.2673G>A.