Letters to the Editor

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 RET (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, non-pathogenic 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 (rs2472737). 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’-CTGG GAGCCCCCGCCTCAT-3’) and 15F1 (5’-GCTGAC(G)ACTCAT GCTT-3’), respectively. The position of intron 14 polymorphism c.2608-24G>A in 15F1 is marked as (G). Another forward primer, 15F4 (5’-CACAACC ACCCTCTGCTG-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’-TGCTGACACC
AGGCTG-3’) and reverse primer 15RSeq (5’-TGCCCATGGTG ACCTG-3’) were used for amplification and sequencing].

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 (15F4) 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 crine neoplasia type 2 (6).
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).

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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. Light 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 of which were homozygous for the c.2608-24G>A polymorphism) and 4 samples homozygous for the exon 15 c.2712C>G polymorphism. Dark gray traces are duplicates of a sample heterozygous for both exon 15 polymorphism c.2712C>G and novel variant c.2673G>A.
Limitations of Automated Remnant Lipoprotein Cholesterol Assay for Diagnostic Use

To the Editor:

I wish to comment on the limitations of an automated remnant lipoprotein cholesterol (RemL-C)1 assay reported in Clinical Chemistry (1). Remnants are lipoprotein particles produced after newly formed triglyceride-rich lipoproteins (TRLs) of either hepatic or intestinal origin enter the plasma and undergo lipolysis via the action of lipoprotein lipase in the capillary bed. During this process, these lipoproteins lose triglyceride and pick up cholesteryl ester and apolipoprotein E (apoE) from other lipoproteins through the action of cholesteryl ester transfer protein. The development of a clinical diagnostic method for measuring remnant lipoprotein cholesterol has been hampered by difficulties with isolation. Moreover, the characteristics of remnant lipoproteins have not been clearly defined. The most consistent definition of remnant lipoproteins has been proposed as an apoE-rich lipoprotein fraction within TRLs, which increases in the postprandial state. The original immunoseparation method for the measurement of remnant-like lipoprotein particle cholesterol (RLP-C) was developed by Nakajima and colleagues in Japan and satisfied these criteria. Normal ranges in the US for this analyte were developed using samples from the Framingham Offspring Study (2). The separation of newly formed TRLs from remnant lipoproteins is crucial because the later lipoproteins are atherogenic and are important for the assessment of coronary heart disease (CHD) risk, especially in women.

My colleagues and I previously reported that an increased RLP-C, in contrast to triglyceride, was a significant independent predictor of prospective CHD in female participants in the Framingham Offspring Study, after adjustment for other standard risk factors including LDL and HDL cholesterol concentrations (3). In addition, in this study triglyceride values correlated with RLP-C (r = 0.79, P < 0.001), but the correlation was not strong enough to affect the clinical utility of the RLP-C assay. We also recently measured remnant lipoprotein cholesterol with the RemL-C assay as described (1) in samples from 3201 male and female participants in cycle 6 of the Framingham Offspring Study. We noted a highly significant correlation between these values and triglyceride concentrations (r = 0.93, P < 0.0001). Moreover, values obtained with this assay were not independent predictors of CHD in either a case-control or prospective fashion. These data suggest that this recently developed automated assay for remnant lipoprotein cholesterol does not accurately measure the cholesterol content of remnant lipoproteins, but rather total TRLs. This issue has taken on increasing importance since prospective studies have reported that postprandial triglyceride (TG) concentrations are associated with significantly...