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# Identification of ryanodine receptor 1 single-nucleotide polymorphisms by high-resolution melting using the LightCycler 480 System

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**Abstract**

High-resolution melting (HRM) allows single-nucleotide polymorphism (SNP) detection/typing using inexpensive generic heteroduplex-detecting double-stranded DNA (dsDNA) binding dyes. Until recently HRM has been a post-PCR process. With the LightCycler 480 System, however, the entire mutation screening process, including post-PCR analysis, can be performed using a single instrument. HRM assays were developed to allow screening of the ryanodine receptor gene (*RYR1*) for potential mutations causing malignant hyperthermia (MH) and/or central core disease (CCD) using the LightCycler 480 System. The assays were validated using engineered plasmids and/or genomic DNA samples that are either homozygous wild type or heterozygous for one of three SNPs that lead to the RyR1 amino acid substitutions T4826I, H4833Y, and/or R4861H. The HRM analyses were conducted using two different heteroduplex-detecting dsDNA binding dyes: LightCycler 480 HRM dye and LCGreen Plus. Heterozygous samples for each of the HRM assays were readily distinguished from homozygous samples with both dyes. By using engineered plasmids, it was shown that even homozygous sequence variations can be identified by using either small amplicons or the addition of exogenous DNA after PCR. Thus, the LightCycler 480 System provides a novel, integrated, real-time PCR/HRM platform that allows high throughput, inexpensive SNP detection, and genotyping based on high-resolution amplicon melting.


*Keywords:* High-resolution melting; SNP identification; LightCycler 480; Ryanodine receptor 1; Malignant hyperthermia; Central core disease

Genetic testing has an important role in many diagnostic laboratories and can provide dramatic prognostic and clinical benefits. Many genetic tests are available to detect and/or type single-nucleotide polymorphisms (SNPs).[1] Most of these techniques, however, require an additional separation step that makes them less favorable for high-throughput assays. Examples of such methods are single-strand conformation polymorphism [1], denaturing gradient gel electrophoresis [2], restriction endonuclease analysis, and DNA sequencing. Homogeneous, closed-tube methods for SNP detection/typing that do not require separation steps are available and are based on either allele-specific PCR using SYBR Green I [3,4] or expensive fluorescently labeled probes [5,6] or primers [7]. SNP genotyping based on allele-specific PCR requires three primers, two of which need to be allele specific. Thus, different mutations require different allele-specific primers. When using labeled probes for SNP detection/typing, only SNPs that lie under the probe can be detected. Consequently, multiple relatively costly probes are needed to cover all potential SNPs. In addition, the use of probes often requires extensive optimization. These conditions limit the usefulness of these methods for screening purposes. If PCR is performed with a 5′-labeled primer as described by Gundry and coworkers [7], high-resolution amplicon melting allows genotyping and mutation scanning without probes. However, this method requires at least one expensive labeled oligonucleotide.

High-resolution melting (HRM) was introduced as a homogeneous closed-tube system that allows mutation scanning and genotyping without the need for costly labeled

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[1] *Abbreviations used:* SNP, single-nucleotide polymorphism; HRM, high-resolution melting; dsDNA, double-stranded DNA; $T_m$, melting temperature; MH, malignant hyperthermia; CCD, central core disease; *RYR1,* ryanodine receptor gene; cDNA, complementary DNA.

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oligonucleotides. It relies on a new generation of generic heteroduplex-detecting double-stranded DNA (dsDNA) binding dyes. Heteroduplex products are identified by the presence of a second low-temperature melting transition [8]. The LightCycler 480 HRM dye is a recently introduced member of this new family. Unlike SYBR Green I, the generic heteroduplex-detecting dsDNA dyes can be used at saturating concentrations without inhibiting or adversely affecting the PCR. The reason why this new family of dyes can detect heteroduplexes, whereas SYBR Green I cannot, is not entirely clear, but dye redistribution during melting is thought to be one reason [8].

The LightCycler 480 System provides a unique format in which the entire experiment, including real-time and post-PCR analysis, can be done on one instrument in a 96- or 384-well format and can be completed within 1 h. Different sequence variants can be identified based on differences in melting curves using the LightCycler 480 Gene Scanning Software. Heterozygous samples are best distinguished from homozygous samples by an altered shape in the melting curve. These differences are best visualized using difference plots because slight differences in curve shape and melting temperature ($T_m$) become obvious. A more detailed description can be found elsewhere [8]. Different homozygous samples, on the other hand, are best distinguished by a change in $T_m$. Smaller amplicons have been found to improve discrimination between genotypes [7].

In this study, inexpensive and high-throughput HRM assays were developed and analyzed using the LightCycler 480 System to allow screening of the gene that encodes the ryanodine receptor skeletal muscle calcium release channel (RyR1) for mutations associated with malignant hyperthermia (MH, MIM no. 145600) and/or central core disease (CCD, MIM no. 117000). The coding region of the ryanodine receptor gene (*RYR1,* MIM no. 180901, NM_000540) is more than 15,000 bp in size; thus, there is a constant search for more distinctive, faster, and cheaper screening methodologies. Both MH and CCD are associated with defects in the *RYR1* gene on chromosome 19q13.1, which is the primary locus of MH in humans (MHS1) [9]. Until recently, approximately 50% of MH had been linked to this locus [10]. Preliminary analyses based on sequencing the entire *RYR1* complementary DNA (cDNA) suggest that the linkage to the MHS1 locus might be as high as 70% [9]. Genomic DNA samples of known *RYR1* genotypes with either the wild-type sequence or a mutation associated with MH and/or CCD were used to validate the HRM assays. The SNPs investigated in this study led to the RyR1 amino acid substitutions T4826I (linked to MH), H4833Y (linked to MH), and/or R4861H (linked to CCD). Nearly all mutations associated with MH and/or CCD occur in the heterozygous state. Nevertheless, homozygous missense mutations have been reported on rare occasions [11,12]. Therefore, four different homozygous *RYR1* genotypes were studied using engineered plasmids to show that even homozygous sequence variations can be identified using HRM on the LightCycler 480 System. HRM analyses were conducted with both the LightCycler 480 HRM dye and LCGreen Plus.

## Materials and methods

### *DNA samples*

Human genomic DNA was prepared from whole blood samples using the Wizard Genomic DNA Kit (Promega) or the MagNA Pure LC DNA Isolation Kit I (Roche) according to the manufacturer's standard protocol. Informed consent was obtained from participating subjects, and the study was carried out after ethical approval was obtained from the Whanganui–Manawatu human ethics committee. To validate the HRM assays, 3 homozygous wild-type and 3 heterozygous mutant genomic DNA samples of known genotypes were screened for the R4861H *RYR1* mutation. For each of the T4826I and H4833Y *RYR1* mutations, 10 homozygous wild-type and 10 heterozygous genomic DNA samples of known genotype were screened. Engineered plasmids were created by cloning wild-type genomic DNA flanking the *RYR1* 4861 wild-type sequence into the vector pGEM–T Easy (Promega). SNPs representing C, T, or A sequence variants at the defined position were introduced using QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer's standard protocol. The engineered plasmids were used to address the possibility of discrimination between different types of homozygotes. DNA concentrations were determined by $A_{260}$.

### *PCR and HRM conditions*

Primers were designed using the LightCycler Probe Design Software 2.0. Primer sequences used in PCR are listed in Table 1. Amplicon lengths were kept relatively short (61–81 bp) to improve discrimination between genotypes. Real-time PCR cycling and HRM analysis of the engineered plasmids and genomic DNA samples were carried out on the LightCycler 480 System (Roche). Experiments were conducted with both the LightCycler 480 HRM dye (Roche) and LCGreen Plus (IT Biochem).

The reaction mixture for HRM using the LightCycler 480 HRM dye consisted of 0.2 to 0.3 μM of each primer,

Table 1
Primer sequences, primer concentrations, and amplicon sizes

| Target | Primers (5′–3′) | Primer concentrations (μM) | Amplicon size (bp) |
|---|---|---|---|
| 4826 | ACTTCTTCTTTGCTGCC | 0.3 | 77 |
| | GGTGACAGAGGACAGGAT | 0.3 | |
| 4833 | TCTCCTGGACATCGCC | 0.3 | 78 |
| | CACACCTGTTTCCCATTG | 0.3 | |
| 4861 | CCGTGGTGGCCTTCAA | 0.2 | 81 |
| | GGTTCATCCTCATCCTCG | 0.2 | |
| 4861 | GGTGGTCGTCTACCTGT | 0.2 | 61 |
| | GGTTCATCCTCATCCTCG | 0.2 | |

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1 × LightCycler 480 HRM dye, and 3 mM $MgCl_2$. The reaction mixture for HRM using LCGreen Plus consisted of 0.2 to 0.3 μM of each primer, 1 × LC480 Probe Master Mix, and 1 × LCGreen Plus. DNA templates were used at approximately $10^4$ copies for engineered plasmid constructs or at 10 to 150 ng for genomic DNA samples.

Assays were carried out in a 96-well format in 10-μl volumes and were performed using the following touchdown PCR cycling and HRM conditions. The PCR was initiated with a 10-min hold at 95 °C, followed by 40 cycles of 95 °C for 10 s, a touchdown cycling step (decreasing 0.5 °C/cycle) annealing ranging from 62 to 56 °C for 10 s, and 72 °C for 4 s. After amplification, the samples were heated to 95 °C for 1 min and then cooled to 40 °C for 1 min to encourage heteroduplex formation. HRM curve data were obtained by melting over the desired range (76–92 °C unless otherwise stated) at a rate of 25 acquisitions per 1 °C.

## Results

Amplicon melting analyses in the presence of the heteroduplex detecting the dsDNA binding dye LightCycler 480 HRM dye or LCGreen Plus were used to detect SNPs using the LightCycler 480 System. Amplicons were 61 to 81 bp in length to allow definitive discrimination and identification of homozygous sequence variations. Fig. 1 shows the difference plots produced by the HRM analysis, which followed the real-time PCR amplification of 81-bp amplicons from genomic DNA flanking the 4861 position using either the LightCycler 480 HRM dye or LCGreen Plus. HRM analysis with either dye allows clear discrimination between the homozygous and heterozygous genomic DNA samples based on differences in melting curve shapes. All samples were of known genotypes and were grouped correctly by the LightCycler 480 Gene Scanning Software.

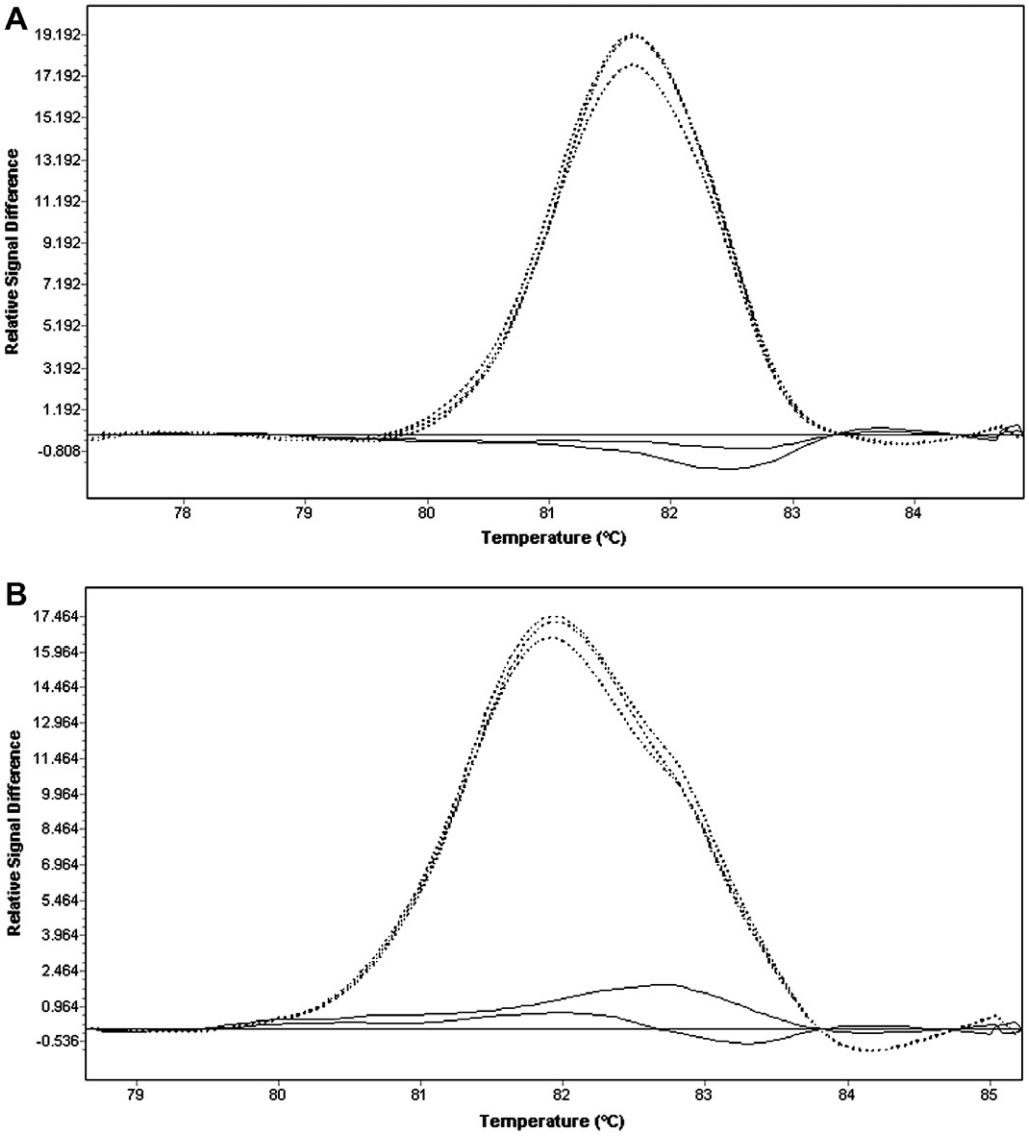


Fig. 1. Difference plots of HRM analyses of the 4861 81-bp amplicons. Here 3 heterozygous samples (dotted lines) and 3 homozygous samples (solid lines) were analyzed using the LightCycler 480 HRM dye (A) or LCGreen Plus (B).

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Two other HRM assays were designed and allowed screening of the *RYR1* gene for the T4826I and H4833Y *RYR1* mutations. Each of the assays was validated by screening 10 homozygous wild-type and 10 heterozygous genomic DNA samples of known genotypes for the SNPs causing the T4826I and H4833Y amino acid substitutions. Unambiguous differences were visible in the shapes of the melting curves for heteroduplexes and homoduplexes. The difference plots shown in Figs. 2 and 3 clearly separate homozygous genomic DNA samples from heterozygous ones for the 4826 and 4833 HRM assays, respectively. All samples were grouped correctly by the LightCycler 480 Gene Scanning Software with both the LightCycler 480 HRM dye and LCGreen Plus. Both homozygous and heterozygous samples analyzed for the 4833 SNP by HRM using LCGreen Plus show an increase in variability between melting curves (Fig. 3B). The HRM assay performed with the LightCycler 480 HRM dye shows no such variability (Fig. 3A). The real-time PCR, which precedes the HRM analysis, revealed that although the amplification curves of both assays look similar and up to standard, the crossing points of the reactions using the LC480 Probe Master Mix with LCGreen Plus were delayed by at least three cycles compared with the LightCycler 480 HRM dye. This trend could be detected in all experiments. In addition, the LightCycler 480 HRM dye generates a fluorescence signal that is at least eight times stronger than that with LCGreen Plus.

Engineered plasmids were used to study homozygote discrimination. Four plasmids (identical except for a G, C, T, or A at the specified position) containing the sequence flanking the 4861 SNP were used alone to simulate homozygous genotypes or in binary combinations to simulate heterozygous genotypes. HRM analyses of 81- and 61-bp amplicons were conducted to determine the effect of ampli-

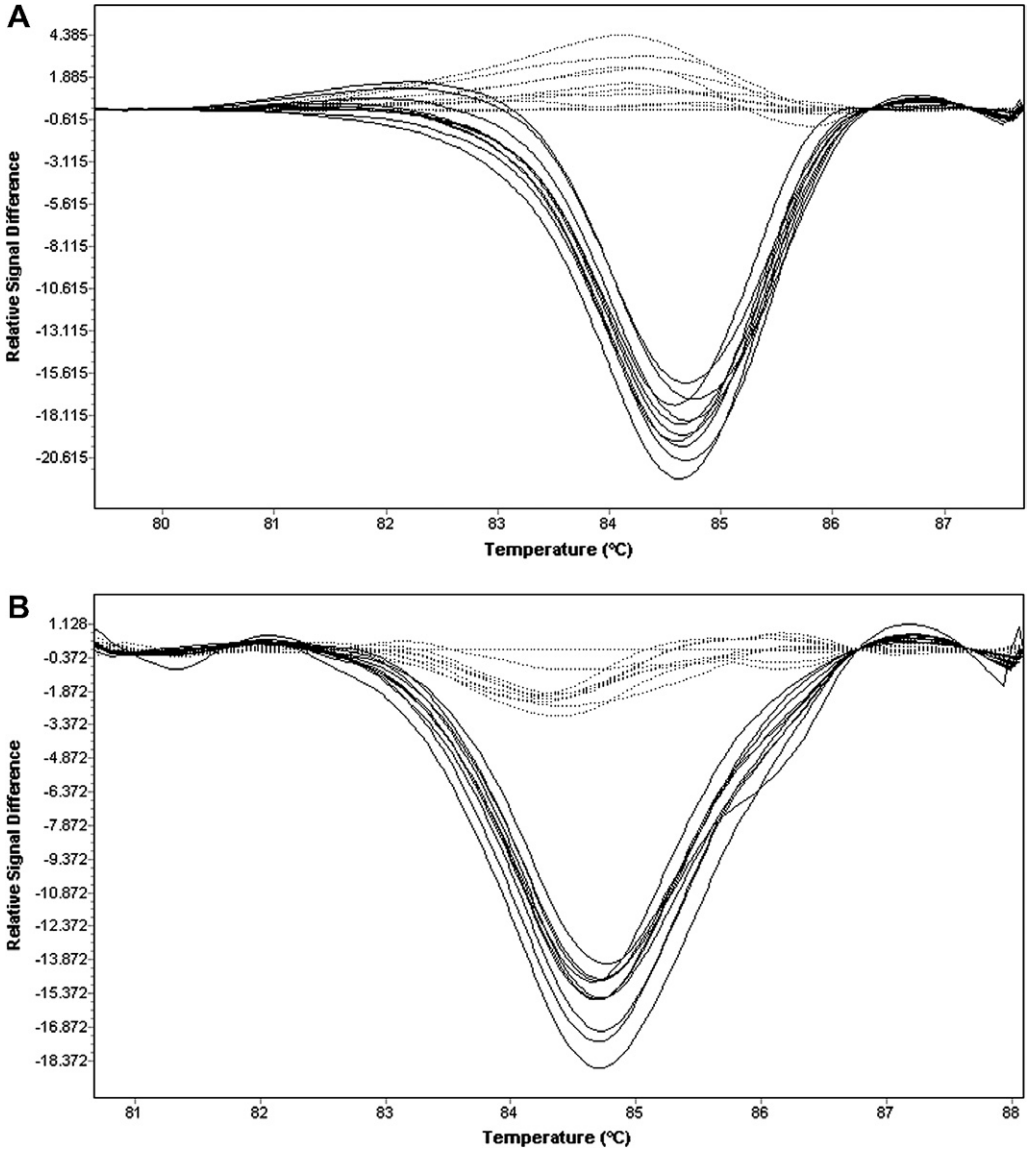


Fig. 2. Difference plots of HRM analyses of the 4826 77-bp amplicons. Here 10 heterozygous samples (dotted lines) and 10 homozygous samples (solid lines) were analyzed using the LightCycler 480 HRM dye (A) or LCGreen Plus (B).

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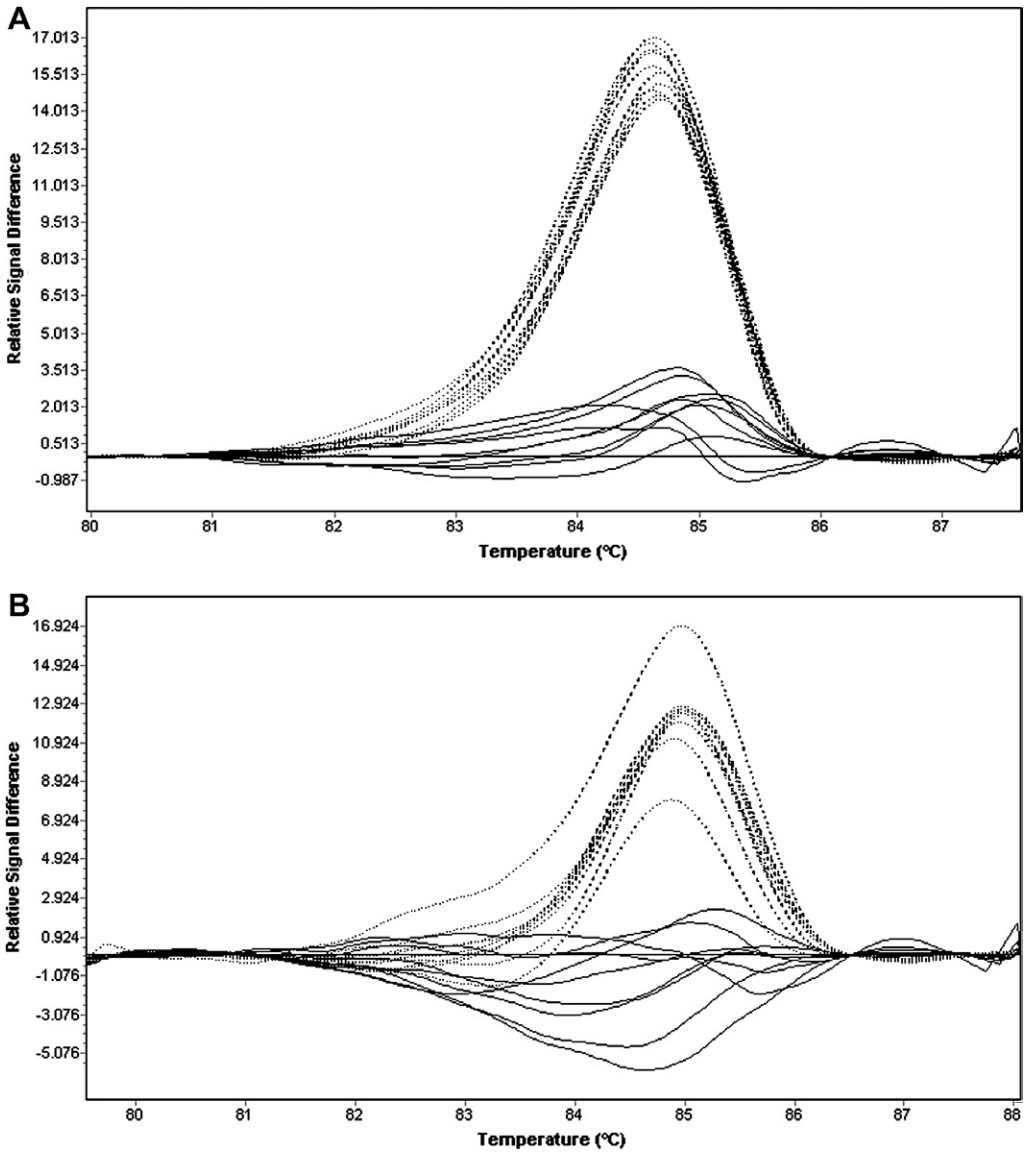


Fig. 3. Difference plots of HRM analyses of the 4833 78-bp amplicons. Here 10 heterozygous samples (dotted lines) and 10 homozygous samples (solid lines) were analyzed using the LightCycler 480 HRM dye (A) or LCGreen Plus (B).

con length on genotype differentiation. The difference plot in the HRM assay for the 81-bp amplicon causing the 4861 SNP using the LC480 HRM dye is shown in Fig. 4. Heterozygotes were easily distinguished from homozygotes based on shape of the melting curves. Difference plot analysis also allows discrimination between different heterozygotes. Homozygote discrimination is based on differences in $T_m$. These differences are best detected by normalized melting curves without temperature shifting and not by the temperature-shifted difference curves [7]. Thus, for the detection of homozygote variants, amplicon melting data should be analyzed both with and without temperature shifting. As shown in Fig. 4B, no differentiation is possible between homozygous A and T based on HRM analysis of the 81-bp amplicon. The $T_m$ values of the homozygous A and T variants differ by only approximately 0.1 °C (Fig. 4B).

Complete genotyping of all the 4861 SNPs in 81-bp amplicons with HRM was possible by adding exogenous wild-type DNA amplicons (in a 1:1 ratio) to unknown homozygous samples. If unknown samples are wild type, their melting curves do not change after the addition of exogenous wild-type DNA amplicons. If the unknown samples are homozygous mutants, heteroduplexes are produced and samples can be correctly identified as homozygous mutant. Fig. 5 shows the result of adding 81-bp amplicons containing the flanking wild-type 4861 sequence to the homozygous samples. Heteroduplexes were formed when homozygous mutants were present. The shapes of the melting curves that were generated by the addition of exogenous DNA to homozygous mutants correlated with those of the original heterozygotes and, therefore, allowed SNP genotyping.

HRM analyses using smaller 61-bp amplicons allowed discrimination between different heterozygous and different

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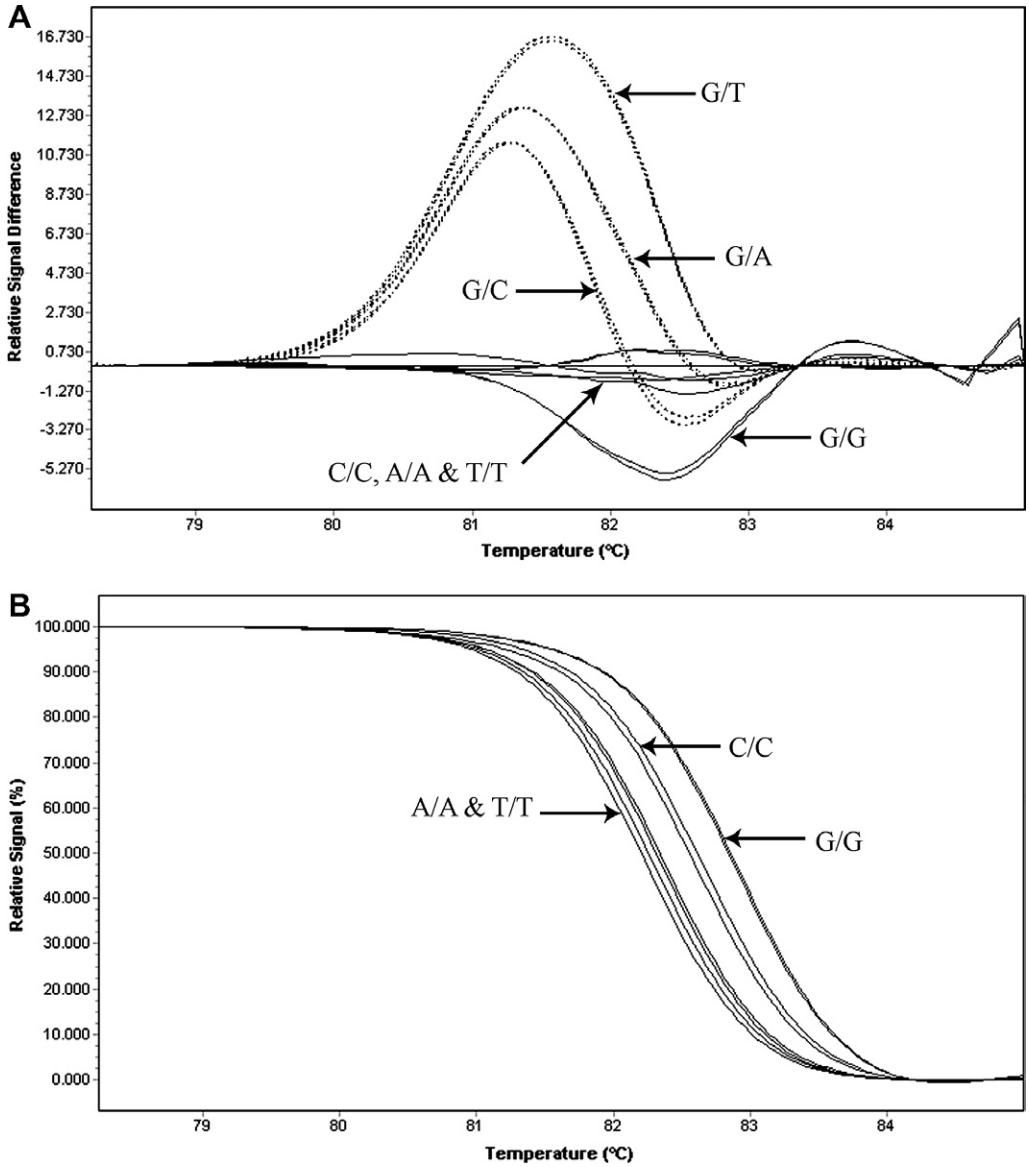


Fig. 4. HRM analysis of possible SNP genotypes at the 4861 position using the LightCycler 480 HRM dye (81-bp amplicons). Here 2 samples of each genotype were analyzed and included four homozygotes (solid lines) and three heterozygotes (dotted lines). (A) Difference plot of the HRM analyses. (B) Normalized HRM curves of the wild-type samples. $T_m$ values of homozygote variants: 82.96 and 82.94 °C for G/G, 82.67 and 82.60 °C for C/C, 82.22 and 82.28 °C for T/T, and 82.34 and 82.37 °C for A/A.

homozygous samples without the addition of exogenous DNA. Heterozygous SNP variants were readily identified using difference plots (Fig. 6A). Homozygous SNP variants at the 4861 position were identified using non-temperature-shifted normalization curves (Fig. 6B). The $T_m$ difference between the homozygous A and T variants was approximately 0.2 °C and proved to be sufficient for discrimination between the two. Occasionally, homozygote SNP identification may also be possible by using difference plots (Fig. 6A). Because these smaller (61-bp) amplicons have lower $T_m$ values, the melting range was adjusted to 69 to 92 °C.

## Discussion

HRM has been introduced as a homogeneous closed-tube post-PCR method for genotyping and mutation scanning that does not need costly labeled oligonucleotides [8]. Instead, it relies on new generation generic heteroduplex-detecting dsDNA binding dyes. Using this new technique, SNPs have been genotyped in products as large as 544 bp [7]. HRM SNP detection and/or genotyping, however, is strongly sequence dependent, and often short amplicons and/or unlabeled oligonucleotide probes are necessary or preferred [13–15]. This study focused on using HRM analysis of relatively small amplicons for SNP detection and identification without the use of unlabeled probes. By using only two standard unlabeled primers, the robustness of the assay increases significantly because optimization typically is not needed. Hence, all assays described in this article could be conducted using identical PCR and HRM conditions, making it ideal for high-throughput screening purposes. In addition, the LightCycler 480 Sys-

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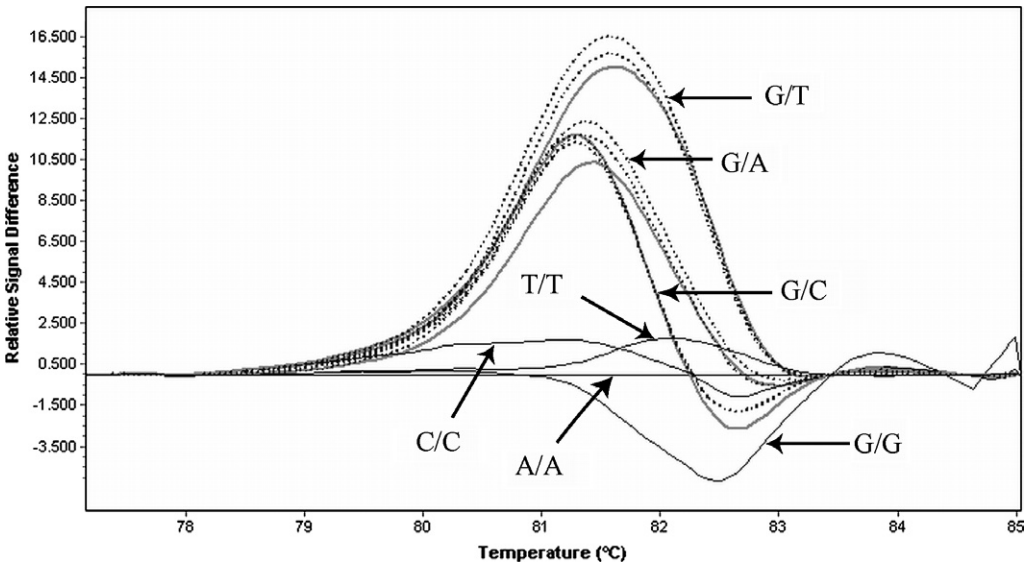


Fig. 5. HRM analysis of possible SNP genotypes at the 4861 position by adding wild-type DNA amplicons using the LightCycler 480 HRM dye (81-bp amplicons). Unknown homozygous genotypes (black solid lines) were mixed with wild-type amplicons after PCR, creating heterozygotes (G/T, G/C, and G/A, thick gray lines) that have melting curves similar to those of the original heterozygotes (black dotted lines).

tem provides a unique format in which the entire experiment, including real-time PCR and post-PCR HRM analysis, can be done in a 96- or 384-well format and completed within 1 h.

In this study, HRM assays were developed and analyzed using the LightCycler 480 System. The assays screened 61- to 81-bp RYR1 amplicons for mutations associated with MH (T4826I and H4833Y) and/or CCD (R4861H). HRM analyses were conducted using two different heteroduplex-detecting dsDNA binding dyes: LightCycler 480 HRM dye and LCGreen Plus.

When the purpose of the analysis is to scan for heterozygotes, using normalized and temperature-shifted difference plots is a convenient way of viewing HRM data because slight differences in curve shape become obvious. All assays that were developed in this study allowed unambiguous discrimination between heterozygous and homozygous samples. The use of the LightCycler 480 HRM dye has some advantages over the use of the LC480 Probe Master Mix with LCGreen Plus. The fluorescence signal generated by the LightCycler 480 HRM dye is at least eight times as strong, and PCR crossing points are lowered by at least three cycles. The latter of the two can be crucial for accurate mutation scanning and/or genotyping because it has been suggested that the validity of HRM analysis of samples with late or poor amplification is questionable [16]. The real-time PCR preceding the HRM analysis, therefore, can provide a useful quality control measure. Thus, the late PCR crossing points are likely to be the cause of the increase in variability between the melting plots shown in Figs. 3A and 3B.

Engineered plasmids, which contain the genomic DNA sequence flanking the 4861 SNP, were used to show that all four possible homozygous genotypes at one position could readily be distinguished from each other by HRM analysis. This is an important element for *RYR1* screening for possible MH and/or CCD mutations because homozygous missense mutations have been reported on rare occasions [11,12]. First, differentiation between genotypes of 81-bp amplicons was possible by spiking unknown samples with exogenous DNA after PCR (Fig. 5). Spiking samples after PCR has the advantage that only homozygous samples need to be retested because heterozygous samples can already be identified based on difference plot analysis. In addition, this technique eliminates strict monitoring of DNA concentrations and differences in amplification efficiencies between samples and spike because exogenous DNA is added after the PCR. Second, SNP genotyping without the addition of exogenous DNA was possible by using 61-bp amplicons that maximize differences in $T_m$ and, therefore, improve discrimination between genotypes (Fig. 6). When looking at differences in $T_m$, however, one should acknowledge the possible effects that ionic strength, product concentrations, and differences in PCR amplifications can have on the $T_m$ between different samples [7].

Studies with genomic DNA samples and engineered plasmids suggest that both SNP detection and genotyping of all possible base combinations at one position by HRM analysis of relatively small amplicons (61–81 bp) is possible using the LightCycler 480 System. Depending on the sequence that is studied, HRM assays on larger amplicons might need to be used in conjunction with a sequencing method to determine the precise mutation. Nevertheless, HRM is inexpensive, has the potential for high throughput, and can greatly benefit mutation screening and genotyping of clinical samples for many genetic disorders, including MH and CCD.

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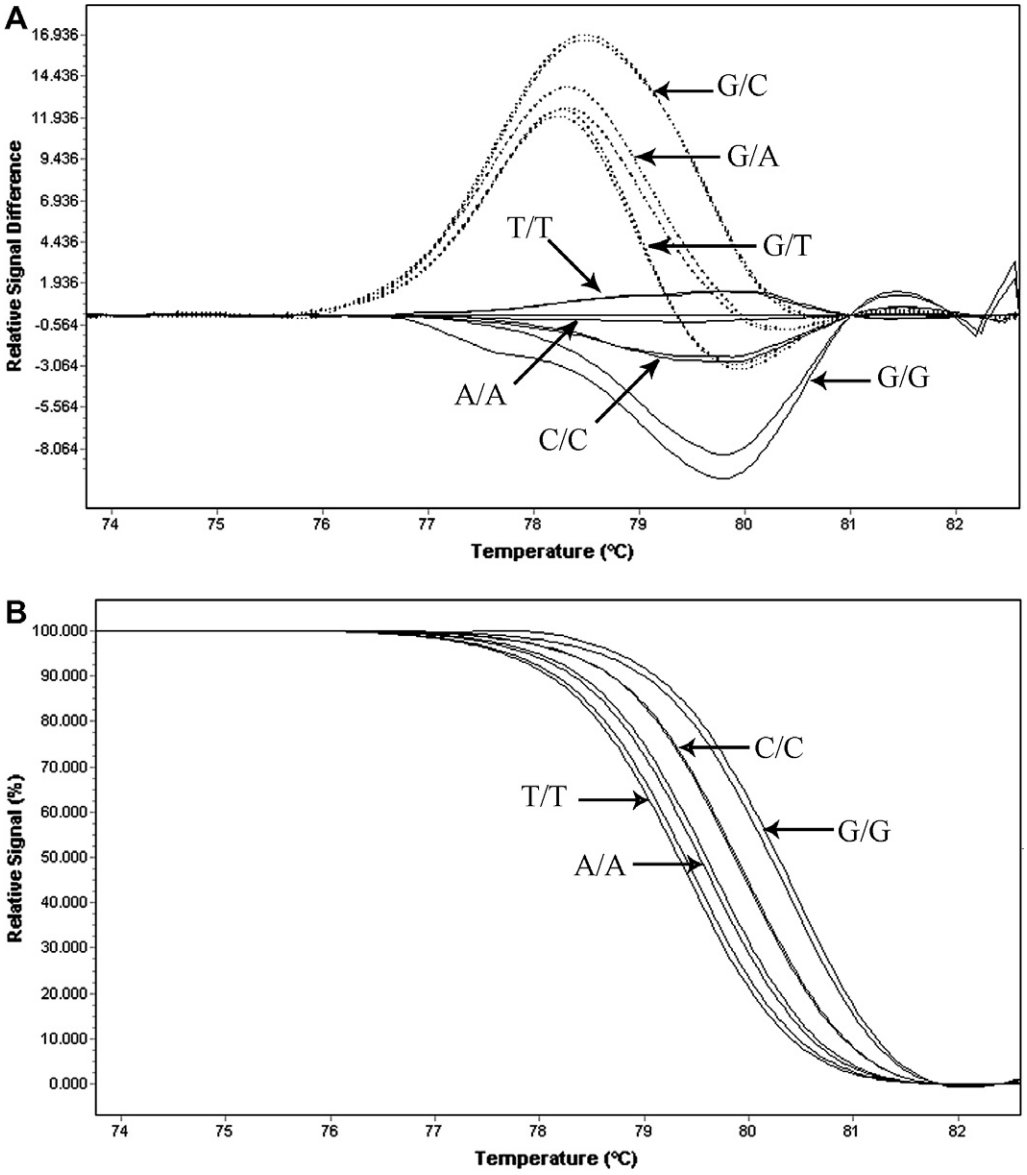


Fig. 6. HRM analysis of possible SNP genotypes at the 4861 position using the LightCycler 480 HRM dye (61-bp amplicons). Here 2 samples of each genotype were analyzed and included four homozygotes (solid lines) and three heterozygotes (dotted lines). (A) Difference plot of the HRM analyses. (B) Normalized HRM curves of the wild-type samples. $T_m$ values of homozygote variants: 80.50 and 80.39 °C for G/G, 79.97 and 79.95 °C for C/C, 79.37 and 79.43 °C for T/T, and 79.58 and 79.64 °C for A/A.

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