High Resolution Melt Parameter Considerations for Optimal Data Resolution

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Introduction

The popularity of high resolution melt (HRM) analysis has grown considerably in the last few years and HRM analysis now encompasses applications including single nucleotide polymorphism (SNP) analysis (Liew et al. 2004), mutation scanning (Taylor 2009), DNA methylation analysis (Balic et al. 2009), and species identification (Castellanos et al. 2010).

Melt curves are routinely used to analyze the purity of reaction products following real-time qPCR. In these assays, the presence of nonspecific DNA products or primer-dimers can be detected by the appearance of multiple melt peaks, reducing the need for post-run electrophoresis to verify reaction specificity. While HRM analysis builds on the fact that nucleotide sequence and sequence complementarity affect the melting temperature (T_m) and melt profile of an amplified sample, saturating dye technology and software capable of performing melt profile normalization are required to discriminate the fine melt profile differences generated from small sequence variations such as SNPs.

Increased interest by the research community in simplifying the analysis of SNPs led to the development of HRM technology. In 2001, Venter et al. analyzed the occurrence of various SNP classes in the human genome (Table 1).

Table 1. SNP classification and frequency.

SNP Class	Base Change	Frequency in the Human Genome (Venter et al. 2001)
I	C>T, T>C, G>A, A>G	64%
II	C>A, A>C, G>T, T>G	20%
III	C>G, G>C	9%
IV	A>T, T>A	7%

The class IV SNPs are both the rarest and most difficult to identify because they differ by the transposition of A and T bases, causing the smallest melt curve temperature shift compared to class I through III SNPs. In the literature, it has been reported that a class IV SNP introduces less than a 0.2°C T_m shift and, consequently, requires data collected at <0.02°C temperature increments on a system with <0.02°C uniformity variability (Tesoriero 2008). However, successful discrimination of sequence variants, including the most difficult-to-detect class IV SNPs, is not solely dependent on the temperature increments from which melt data are collected. Several factors influence DNA melting behavior, including reaction mix composition, amplicon size, GC content, and DNA concentration.

In this work we discuss the analysis steps performed by HRM software to identify thermal profile differences and we examine the effect of assay optimization techniques, temperature increments, and instrument selection on the ability to distinguish different genotypes using HRM analysis. For a more detailed discussion of recommendations for HRM assay development, please refer to Taylor et al. 2010.

Methods

Data were generated using the CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Inc.) and the Rotor-Gene Q system (QIAGEN) and analyzed using Precision Melt Analysis™ software (Bio-Rad) and Rotor-Gene Q system software (QIAGEN), respectively. All reactions were performed on a CFX96 real-time PCR detection system, except where indicated.

For HRM reaction optimization, the following parameters were varied: number of copies of template (500–50,000) and annealing temperature (58–62°C). A single primer concentration was selected (500 nM). Each 20 µl reaction mix included a derivative of the pUC19 vector with either a wild-type (T) or a class IV SNP (A) at nucleotide position 444 in the ampicillin resistance gene *bla*, or a 1:1 mixture of the two. For the optimized reactions, plasmid template was present at a concentration of 50,000 copies per well.

The CFX96 system amplification protocol was 98°C for 30 sec, followed by 30 cycles of 96°C for 2 sec, 60°C for 5 sec, and a plate read. Following denaturation at 95°C for 30 sec and cooling to 70°C for 30 sec, a melt curve was generated by heating from 70 to 90°C with either 0.2 or 0.5°C increments, 10 sec dwell time, and a plate read at each temperature.

The Rotor-Gene Q system amplification protocol was 98°C for 30 sec, followed by 35 cycles of 96°C for 5 sec, 60°C for 20 sec, and a plate read. The same melt curve parameters were used as described for the CFX96 system.

Results and Discussion

Software Discrimination of High Resolution Melt Profiles

In HRM analysis, differences in T_m and normalized curve shape are used together to discriminate even the most difficult-to-detect sequence variations. Appropriate assay design and optimization are essential for the success of this discrimination. To understand how reaction conditions affect sequence discrimination, it is valuable to discuss how the software is performing profile identification, discrimination, and clustering.

In HRM analysis, the dissociation curves plotted following a melt protocol are normalized and similar melt profiles clustered together to identify like sequences. To do this, the software plots fluorescence signal intensity on the y-axis and data for the various temperature increments on the x-axis (Figure 1A). Data are then baselined using values both before and after the melt phase and rescaled (normalized) so that each profile ranges from 0 (baseline noise) to 1 (maximum signal) fluorescence intensity (Figure 1B). To identify and cluster unique thermal profiles, the software then completes a signal-to-noise ratio difference analysis of each sample versus a reference sample at each data collection temperature (Figure 1C). For each temperature point, the average value of a reference curve is calculated and subsequently subtracted from each sample's normalized relative fluorescence unit (RFU) value to generate a difference plot (Figure 1D). Consequently, unlike traditional melt curve analysis where differences in T_m and, thus, sequence are identified by a shift in melt peak along the x-axis, in HRM analysis, a vertical shift in fluorescence intensity on the y-axis, following reference signal subtraction, is used to identify sequence differences.

Good reaction design and optimization can increase the amplitude of the profile difference at a given temperature and make sequence discrimination easier. The larger the difference in fluorescence readings at a specific temperature, the higher the confidence in sample clustering. Factors that impact this difference include a variety of experimental parameters not directly related to instrument settings, including amplicon length, background noise from non-target sequence, and dye selection.

Reaction Optimization

Successful HRM analysis is highly dependent on the quality of the amplicons being compared. A single DNA species of the expected size, as evidenced by a single thermal melt peak and one band on an electrophoretic gel, is key. Reaction optimization typically involves the use of a thermal gradient to quickly determine the annealing temperature that yields a reaction efficiency of 90–105%, has the earliest

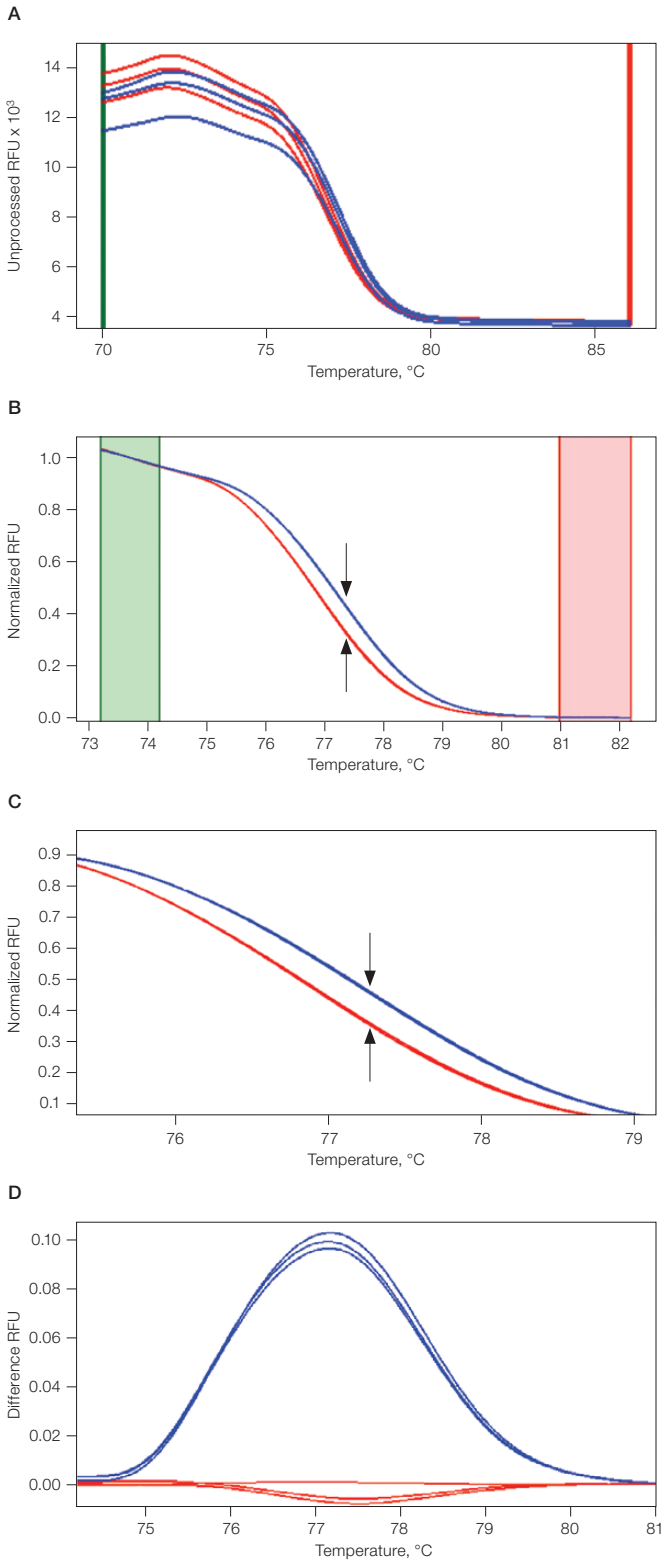

Fig. 1. Melt curves of wild-type and mutant samples. A, melt profile of wild-type (■) and mutant (■) sequences before normalization; **B**, same profile after normalization; **C**, close-up of profile in panel B with an individual temperature point indicated by arrows; **D**, difference curve derived from profile in panel B. RFU, relative fluorescence units.

quantification cycle (Cq), and produces a single product (Taylor et al. 2010). Identification of robust amplification conditions for each amplicon using the thermal gradient assists generation of reliable and repeatable HRM data and maximal thermal profile differences (Leong et al. 2010). Primer and template concentrations can also be adjusted if further optimization is required.

Maximization of Fluorescence Signal Intensity Differences

When detecting potentially small thermal changes, such as class IV SNPs, a useful strategy to obtain discriminating data is to decrease the amplicon size, accentuating the subtle profile differences caused by this small sequence change. Thus, even small perturbations in sequence can result in relatively large and easily measurable differences in normalized fluorescence signal at a given temperature. Decreasing amplicon size from 300 to 50 bp had a significant impact on the accurate discrimination and identification of genotypes (Figure 2). Reducing amplicon size increased the difference in signal at a given temperature (maximal at the T_m) between two sequences that differed at only one nucleotide position. This increase in signal intensity greatly enhances the ability to distinguish between input sequences in subsequent melt curve analyses.

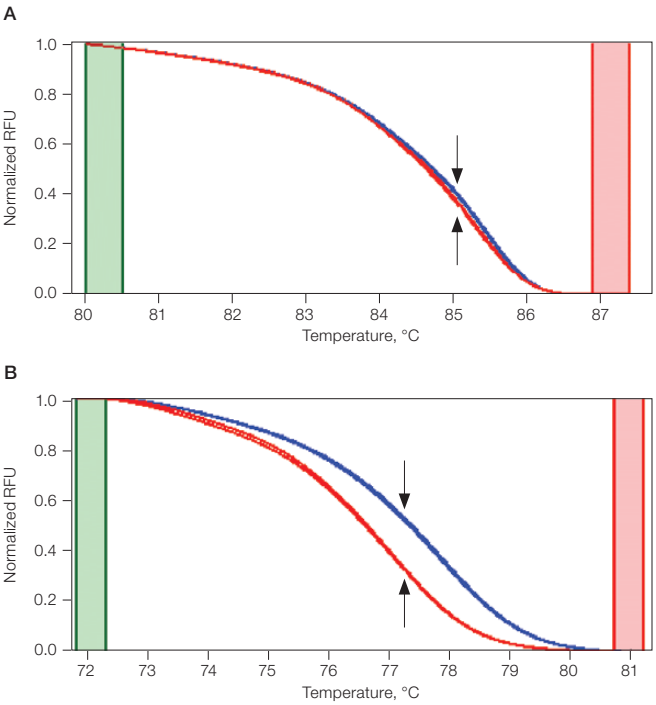

Fig. 2. Decreasing amplicon size accentuated differences in fluorescence signal intensity. Comparison of normalized HRM curves from 300 bp (**A**) and 50 bp (**B**) amplicons containing a class IV SNP. Decreasing the size of the amplicon made differences in template composition more readily apparent. The difference in fluorescence at T_m is highlighted by arrows. RFU, relative fluorescence units.

Impact of Melt Curve Temperature Increments on Data Quality

As can be seen in Figure 3, the difference RFU traces for the three genotypes (A/A, T/T, and T/A) cluster into visually distinct groups when data were collected for the 50 bp amplicon using either 0.2 or 0.5°C increments. The difference RFU values (y-axis) are approximately equivalent regardless of the increment size used (grey rectangles). Collecting data at narrower temperature increments provided no increase in data quality for this experimental model of class IV SNP genotypes.

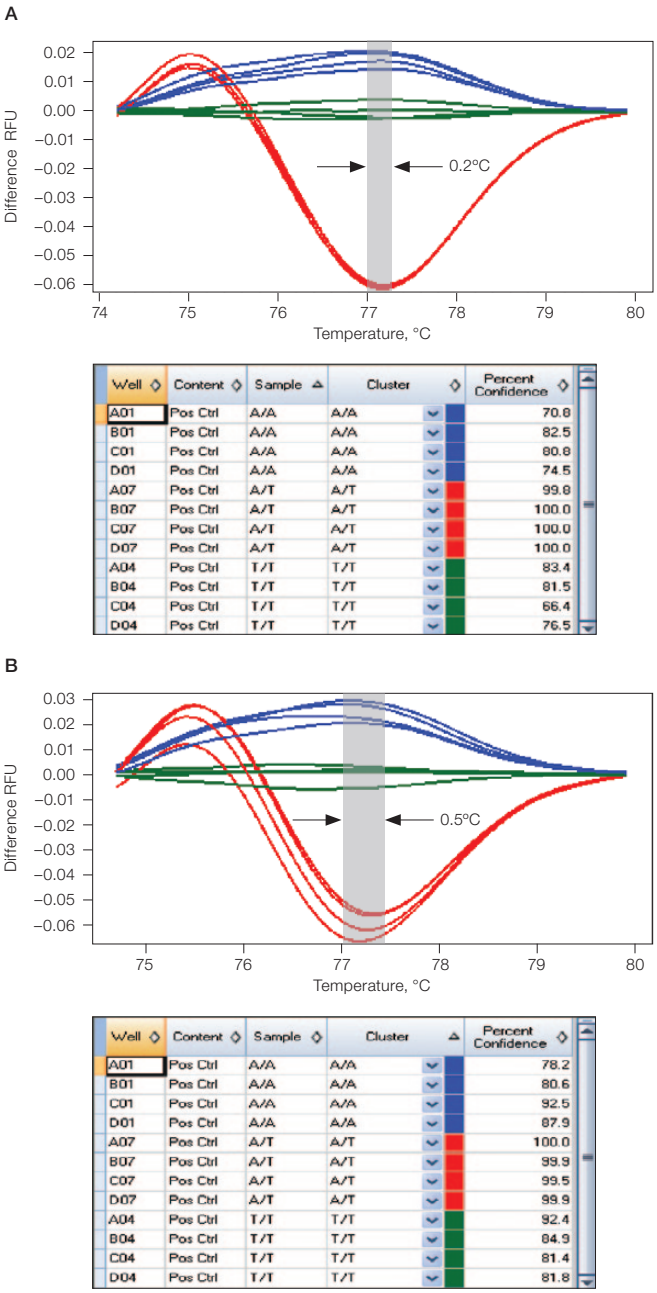

Fig. 3. Impact of temperature increments on melt profiles. HRM data for a 50 bp amplicon containing a class IV SNP were collected at 0.2°C (**A**) and 0.5°C (**B**) temperature increments. The normalized difference curve (top panel) and percentage confidence reported by Precision Melt Analysis software (bottom panel) for the different genotypes demonstrate that a relatively broad range of temperature increments can be used to successfully discriminate class IV A to T SNPs. RFU, relative fluorescence units.

Precision Melt Analysis software was able to predict the specific cluster that samples belonged to with a comparable, high degree of confidence. This finding illustrates that the extent of vertical shift in fluorescence intensity (difference RFU) at a given temperature, in combination with the differences in the shape of normalized melt curves, can be used to readily distinguish between amplicons of differing sequence in a way that is not solely dependent on the temperature increments used to collect these data.

Impact of Instrument on Data Quality

Prior to reaction condition optimization (Figure 4, A and B top panels), it was difficult to distinguish between the three cluster types with any precision or consistency. Accurate clustering and genotype identification were only achievable on the two different platforms examined after reaction conditions were appropriately optimized for each instrument (Figure 4, A and B bottom panels). For the difference curves, the reference cluster chosen was the one that resulted in the most distinct visual difference between the various sequence types analyzed. The optimized reactions produced curves that clustered into discrete groups with minimal trace overlap.

These results illustrate that appropriate assay design and optimization can be more important for successful HRM analysis than the specific instrument used for data collection. They also rebut the claim that the thermal characteristics of block-based instruments, including the CFX96 and CFX384™ systems, preclude their use for discrimination of difficult-to-detect small thermal profile differences, including class IV SNPs.

Conclusions

HRM analysis is a flexible technique that can be applied to a range of experimental questions and applications. Our results demonstrate that, when using well optimized reactions as described above, data collection at temperature intervals less than 0.2˚C are not required to obtain confident HRM results. While selection of an instrument with excellent thermal control and uniformity is important, maximizing the vertical fluorescence signal difference at any single temperature is essential for accurate discrimination of small thermal profile differences and the generation of robust, repeatable, and informative HRM data.

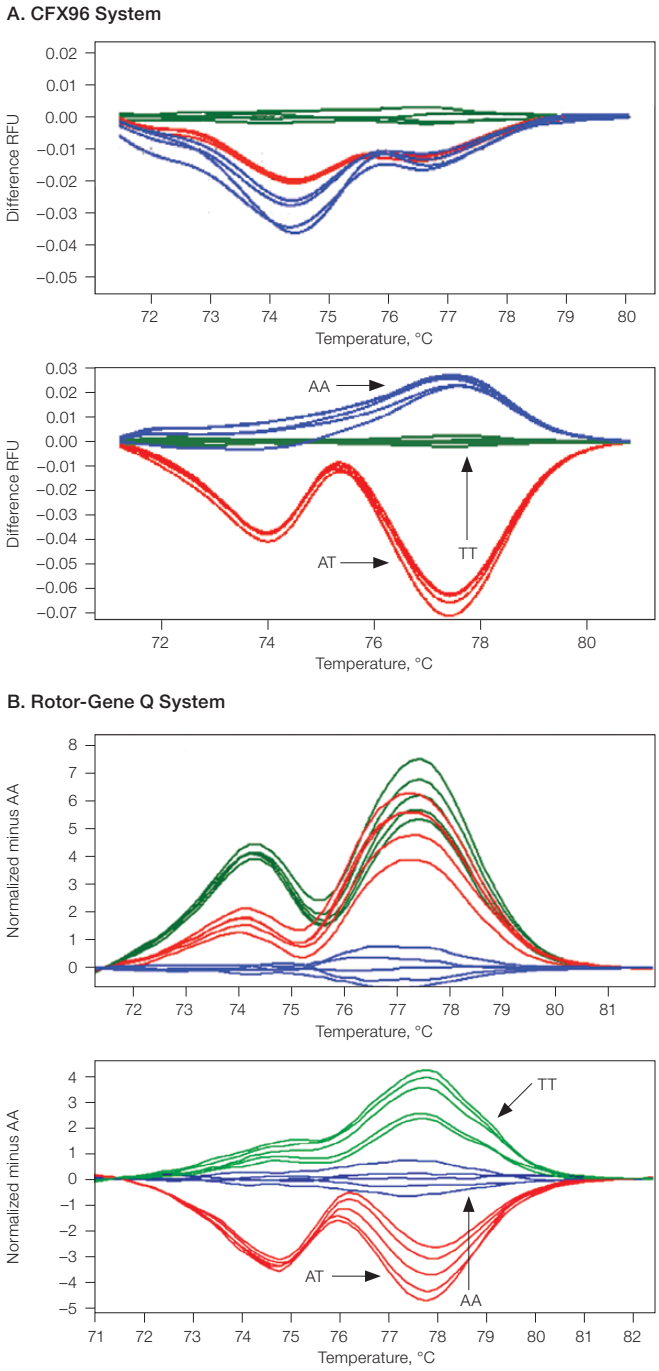

Fig. 4. Platform comparison. Comparison of HRM data for a class IV SNP containing 50 bp amplicon on the CFX96 system (**A**) and the Rotor-Gene Q system (**B**) before (top panel) and after (bottom panel) optimization. The comparable level of visible distinction between the genotypes analyzed illustrates platform independence in data generation. 0.2˚C temperature increments were used for data collection. RFU, relative fluorescence units.

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